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The structure of the carbohydrate backbone of the core–lipid A region of the lipopolysaccharide from *Proteus mirabilis* serotype O28

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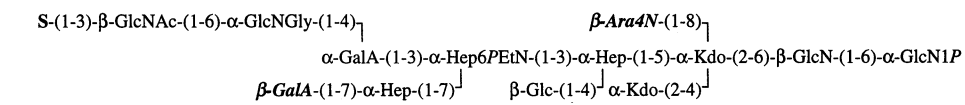
Abstract

The following structure of the lipid A–core region of the lipopolysaccharide (LPS) from *Proteus mirabilis* serotype O28 was determined using NMR, MS, and chemical analysis of the core oligosaccharide, obtained by mild acid hydrolysis of LPS, and of the products of alkaline deacylation of the LPS:



where S = β-GalALys (amide of β-D-galactopyranosyluronic acid with the α-amino group of L-lysine) or β-GalALys4*P*EtN are present in the ratio of ~1:1. β-GalA and Ara4N (indicated by bold italics) are present in non stoichometric amount. All sugars are present in the pyranose form and all except L-Ara4N have the D configuration.

Keywords: LPS; *Proteus mirabilis*; Core; O-Antigen; Structure; NMR

1. Introduction

Proteus mirabilis is an important opportunistic pathogen causing nosocomial and urinary tract infections [1]. *P. mirabilis* infections are difficult to treat and often even fatal, as these bacilli are resistant towards a wide spectrum of antibiotics [2]. Lipopolysaccharides (LPS) have been identified as an important virulence factor of *P. mirabilis* [3]. Structural analysis of the core part of *Proteus* LPS have showed that it has conserved inner part but contains a variable di- to tetrasaccharide region attached to O-4 of the α-GalA residue. The structure of the outer region is not related to the structure of the polysaccharide part in all analysed serotypes [4–10] and thus may represent another important antigenic deter-

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; *P*EtN, 2-aminoethylphosphate; Ara4N, 4-amino-4-deoxyarabinose; GalALys, amide of D-galacturonic acid with NH_2-2 of L-lysine.

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minant. Here we report the structure of the core part of the *P. mirabilis* serotype O28 LPS; the structure of its polysaccharide part have been reported previously [11,12].

2. Experimental

Bacterial strains and lipopolysaccharide isolation.—*P. mirabilis* O28 originated from the strains collection of the Institute of Microbiology and Immunology, University of Łódź, Poland. Bacteria were cultivated in dry meat infusion broth (Warsaw Serum Vaccines Laboratory) with 1% D-glucose added. Lipopolysaccharide was obtained by the procedure of Westphal [13] and purified by DNase and RNase (Sigma Chemical Co., St. Louis, MO, USA) treatment and repeated runs in a preparative ultracentrifuge.

NMR spectroscopy and general methods.—^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 500 spectrometer in D_2O at 25 °C with acetone standard (2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C) using standard pulse sequences gCOSY, TOCSY (mixing time 120 ms), NOESY (mixing time 250 ms), gHSQC, gHMBC (optimized for 5 Hz long-range coupling constant), gHSQC-TOCSY (mixing time 80 ms). ^{31}P and ^{1}H–^{31}P HMQC (optimized for 7 Hz P–H coupling constant) spectra were recorded on a Varian Inova 400 spectrometer. Spectra were assigned with the help of the PRONTO program [14]. Electrospray mass spectra were obtained on a Micromass Quattro spectrometer in negative mode, with direct injection in 50% MeCN with 0.2% HCO_2H at flow rate 15 μL/min. GLC, GLC–MS, methylation, monosaccharide, and amino acid analysis were performed as described previously [15].

*Preparation of oligosaccharide **1**.*—LPS (200 mg) was hydrolysed with 2% AcOH (100 °C, 4 h). The resulting precipitate was removed by centrifugation, and the supernatant separated on a Sephadex G50 SF gel (Pharmacia) column (2.5 × 80) using pyridine–AcOH buffer (4 mL of pyridine and 10 mL of AcOH in 1 L of water) with monitoring by a Waters differential refractometer. The core fraction (40 mg) was further separated by HPLC on an anion-exchange column (Hamilton PRP 100) in a gradient of 0–1 M NaCl to give a neutral Fraction **1** (20 mg) and acidic fraction (5 mg).

*Preparation of oligosaccharides **2** and **3**.*—LPS (200 mg) was heated at 100 °C for 4 h in 4 M NaOH (4 mL) with a small amount of $NaBH_4$, cooled, and 2 M HCl (7.5 mL) was added. The precipitate was removed by centrifugation and the substance desalted on a Sephadex G50 column. The oligosaccharide fraction was separated on a Carbopac PA100 (250 × 9 mm) column using a gradient of 20–80% of 1 M NaOAc in 0.1 M NaOH at 3 mL/min over 1 h to give, after desalting, 5 mg each of the oligosaccharides **2** and **3** and many minor products.

3. Results

Mild acid hydrolysis of *P. mirabilis* O28 LPS with 2% acetic acid followed by the separation of products by gel and anion-exchange chromatography gave core oligosaccharide **1** and an acidic core fraction. Complete O,N-deacylation of LPS by treatment with 4 M sodium hydroxide and separation of the products by gel chromatography and HPAEC gave major oligosaccharides **2** and **3** and several minor products that were not analysed.

The monosaccharide composition of all oligosaccharides was determined by conventional monosaccharide analysis (GLC of alditol acetates or methyl glycoside acetates). *P. mirabilis* O28 LPS had the following chemical composition (%, combined data based on GLC determination of neutral and amino sugars, thiobarbituric acid assay for Kdo, amino acid analysis, and color reactions for GalA and phosphate): Gal 2.9, Glc 2.9, Hep 6.7, Kdo 3.5, GalA 16.0, GlcN 15.3, P 2.0; core oligosaccharide **1**: Gal 1.4, Glc 9.6, Hep 20.8, GalA 11.5, GlcN 6.2, Gly 1.6, Lys 2.7, EtN 1.7, P 1.9. Absolute configurations (GLC of the acetates of 2-butylglycosides) were determined for D-GalA, LD-Hep and D-GlcN in oligosaccharide **1**. The L configuration of Ara4N was determined previously [16].

Interpretation of NMR spectra of all oligosaccharides (Tables 1–3, Figs. 1–3) led to the structures on Scheme 1. NMR data for

oligosaccharide **3** are not shown, because it was found to be identical with a previously described product from *P. mirabilis* O3 (compound **1a** in Ref. [8]) by NMR, MS and HPAEC. Product **1** had different Kdo variants at the reducing end, and contained two major structures, **1a** and **1b**, in roughly equal amount, differing by the absence or presence of the *P*EtN residue on O-4 of the terminal GalA residue S, respectively. The identity of the monosaccharides was determined on the basis of the vicinal coupling constants (determined from COSY and TOCSY spectra), which were in agreement with the expected values, the intraresidual NOEs, and ^{13}C NMR chemical shifts. The monosaccharide sequence was assigned on the basis of the interresidual NOEs (Table 3) and long range C–H correlations (HMBC spectra, where for all residues correlations from H-1 to the carbon atom over glycoside bonds were observed) and confirmed by ^{13}C NMR spectral data. Localization of the amide-linked components was performed using HMBC data (Fig. 2). A correlation between proton M2 and glycine carbonyl shows that the amino group of GlcN is acylated with glycine. Proton L2 correlates with the acetyl carbonyl group, thus GlcN L is N-acetylated. H-2 of the lysine residue in both **1a** and **1b** gives correlations to C-6 of GalA S, thus phosphorylated and non-phosphorylated GalA forms amides with lysine. HMBC spectra also allowed assignment for signals of C-6 carbon atoms of residues of H and K.

Oligosaccharide **1a** contained an ethanolamine phosphate substituent at O-6 of heptose residue F; and product **1b** contained one more ethanolamine phosphate substituent at O-4 of GalALys residue S. $^{1}H-^{31}P$ correlations were observed from the ^{31}P signal at 0.5 ppm to H-4 and H-5 of the GalA S, and from ^{31}P signal at 0.8 ppm to the proton F6 (Fig. 3).

Table 1
^{1}H NMR data (ppm)

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
A, **2**	5.72	3.44	3.91	3.58	4.10	3.83	4.26		
B, **2**	4.83	3.11	3.63	3.46	3.62	3.50	3.60		
C, **2**		1.94	2.17	4.16	4.26	3.64	4.06	3.71	3.90
D, **2**		1.78	2.16	4.07	4.05	3.66	3.98	3.73	3.96
E, **1**	5.17	3.99	4.00	4.27		4.15	3.72		
	5.09	3.96	3.95						
E, **2**	5.28	4.10	4.15	4.49	4.17	4.11	3.79	3.91	
F, **1**	5.35	4.16	4.05			4.72			
F, **2**	5.23	4.39	4.15	4.02	3.85	4.75	3.72	3.87	
G, **1**	4.92	3.97	3.82	3.87	3.73				
G, **2**	4.89	3.95	3.86	3.85	3.70	4.03	3.73	3.73	
H, **1**	5.41	3.86	4.11	4.33	4.44				
H, **2**	5.40	3.87	4.37	5.82					
I, **1**	4.56	3.30	3.52	3.37	3.40	3.71	3.87		
I, **2**	4.56	3.29	3.50	3.36	3.41	3.74	3.86		
K, **1**	4.44	3.56	3.72	4.22	4.15				
L, **1**	4.55	3.91	3.88	3.56	3.53	3.80	3.94		
M, **1**	4.93	3.89	3.74	3.47	4.30	3.80	4.09		
S, **1a**	4.55	3.55	3.73	4.21	4.23				
S, **1b**	4.58	3.60	3.79	4.78	4.30				
Z, **1**	5.00	3.75	4.16	3.71	3.82	4.10			
Z, **2**	5.02	3.73	4.18	3.69	3.78	4.12			
Lys, **1a**		4.27	1.84	1.43	1.68	3.00			
Lys, **1b**		4.15	1.76	1.43	1.68	3.00			
Gly		3.80							
		4.00							
EtN	4.13	3.27							
NAc		2.04							

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
A, **2**	92.7	55.3	70.7	70.8	74.0	70.6		
B, **2**	100.6	57.0	73.7	71.3	75.8	62.9		
C, **2**			35.5	71.5	70.7	73.7	69.1	72.0
D, **2**			35.5	67.5	67.8	73.5	71.6	64.4
E, **1**	99.6	70.0	75.3	74.9		69.1	63.9	
E, **2**	100.4	71.7	77.6	73.7	71.5	70.2	65.2	
F, **1**	102.3	70.5	79.0			72.7	67.4	
F, **2**	103.4	70.0	80.6	66.4	73.2	73.9	68.5	
G, **1**	101.4	70.8	71.8	67.2	73.1			
G, **2**	101.2	71.3	71.7	67.6	73.2	70.5	64.3	
H, **1**	101.6	69.4	69.6	80.9	72.3	175.4		
H, **2**	101.2	71.6	67.1	108.9				
I, **1**	103.6	75.0	76.6	70.7	77.2	62.4		
I, **2**	102.9	75.1	76.7	71.2	77.5	62.6		
K, **1**	103.6	71.5	73.8	71.2	76.3	175.4		
L, **1**	102.4	56.0	83.0	69.8	76.6	62.0		
M, **1**	99.9	54.7	69.0	70.7	71.8	69.3		
S, **1a**	103.6	71.5	73.1	70.1	75.4	170.9		
S, **1b**	103.6	71.5	73.1	75.2	75.5	169.9		
Z, **1**	99.5	69.0	66.6	52.9	59.2			
Z, **2**	99.8	69.4	66.8	53.1	59.3			
Lys, **1a**	179.4	55.6	31.7	22.9	27.1	40.3		
Lys, **1b**	179.2	55.1	31.7	22.9	27.1	40.3		
Gly	168.5	41.7						
EtN	63.0	41.1						
NAc	176.0	23.1						

Negative-mode ESMS of the oligosaccharides **1a**,**b** contained $[M-2H]^{2-}$ and $[M-3H]^{3-}$ peaks, corresponding to molecular mass of 2292.1 and 2414.8 (calculated for **1a** $C_{82}H_{136}N_7O_{65}P_1 = 2291.2$; for **1b** $C_{84}H_{142}N_8O_{68}P_2 = 2414.3$ for anhydro Kdo). The acidic core fraction gave peaks corresponding to the masses of 2160.6 and 2283.1, thus it differed from compounds **1a**,**b** by the absence of an Ara4N residue. Mass spectra of compounds **2** and **3** in agreement with the proposed structures contained single peaks at 2011.0 and 2187.0, respectively (calculated for **2**, $C_{68}H_{116}N_4O_{60}P_2 = 2011.8$; for **3**, $C_{74}H_{124}N_4O_{66}P_2 = 2188.0$).

Oligosaccharides, obtained after AcOH hydrolysis of the LPS, according to the mass spectra did not contain the structure without β-GalA K, corresponding to the oligosaccharide **2**. Although the true reason for this is not known, one can speculate that the structure without residue K may be present in the core connected to the O-specific chain, which is absent in the product **1**. It could be also not visible in the mass spectrum of the core fraction because of its low content and poor ionization due to the absence of the negatively charged residue of galacturonic acid.

A chemotyping scheme for *Proteus* LPS core parts, proposed earlier [17,18], was based

Table 3
Interresidual NOE data [a]

Compound	From proton	To protons
2, **3**	B1	A6w, A6′w
2, **3**	C3a,e	D6s, D8w
2, **3**	E1	C5s, C7s, C6w
1–**3**	F1	E2w E3s
1–**3**	G1	F7m, F7′m
1–**3**	H1	F2m, F3s
1–**3**	I1	E4s, E6s
3	K1	G7m, G7′m
1	M1	H4s, H5m
1	L1	M6s, M6′s
1	S1	L3m

[a] s, strong; m, medium; w, weak.

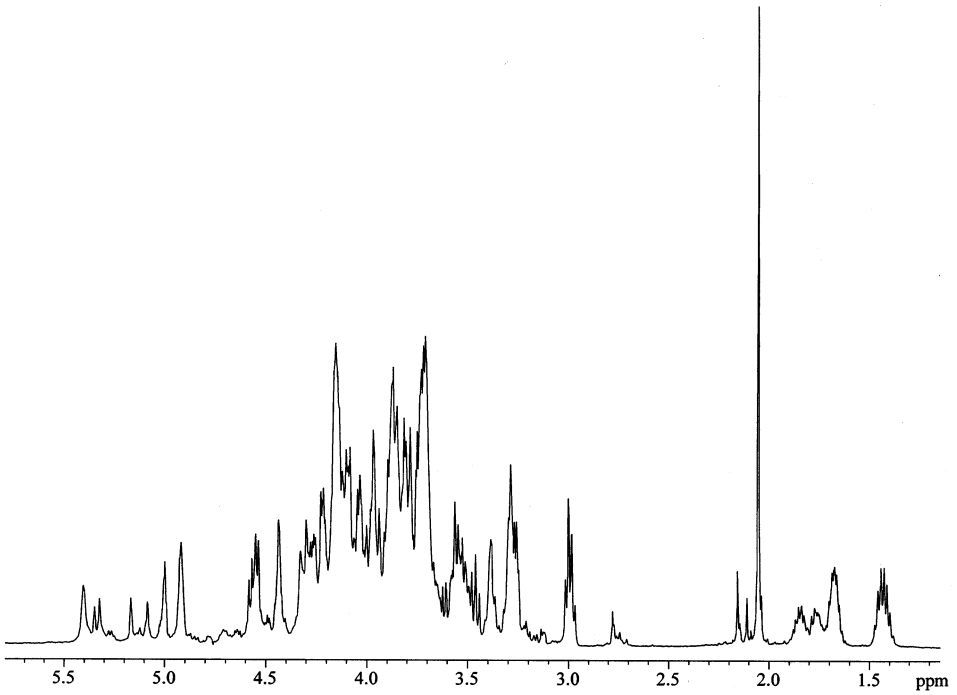


Fig. 1. ^{1}H NMR spectrum of the core fraction **1** obtained by gel and anion-exchange chromatography of *P. mirabilis* O28 LPS hydrolysate.

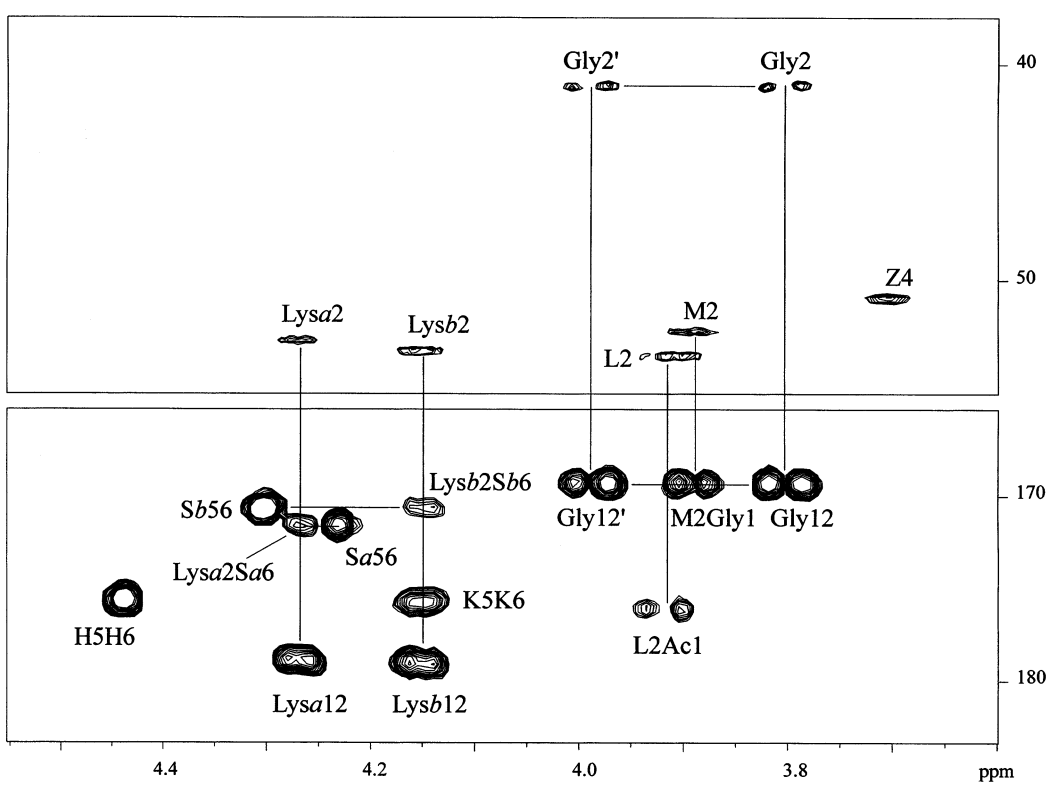
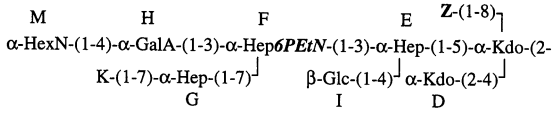


Fig. 2. Fragments of HSQC (upper) and HMBC (lower) spectra of the compound **1a**,**b**. Labels consist of residue designating letters and atom numbers, italic *a* and *b* refer to the structures **1a** and **1b**, respectively. For example, K5K6 is a cross peak between C-6 and H-5 of the residue K; Lys*a*2S*a*6 is a cross peak between H-2 of the lysine residue from structure **1a** and C-6 of the GalA residue S.

on the results of chemical analysis of the whole core oligosaccharide. On the basis of our new data concerning the structure of *Proteus* LPS core part, we suggest another typing scheme of inner core structures. It can not be based on compositional analysis of the whole core fraction. All analysed structures have the following common structure of inner part of the core:



There are three characteristic substituents (Table 4), which form most of the variety of the core structures. Some of these substituents are present in nonstoichometric amount, giving

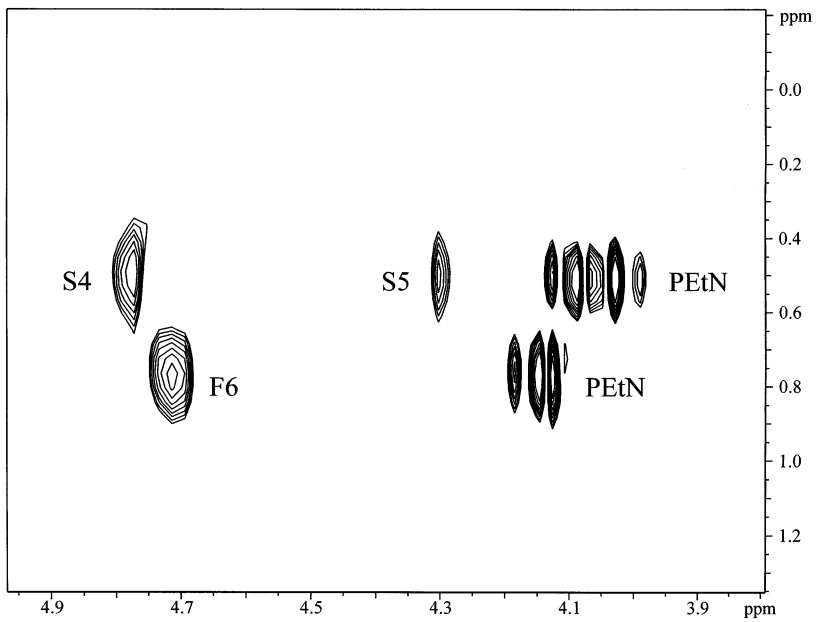
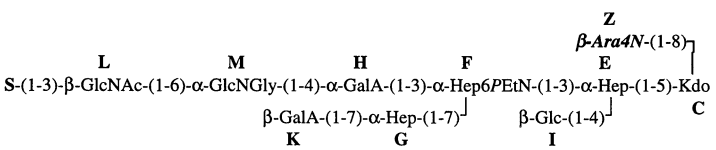
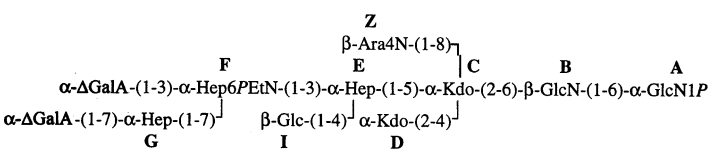


Fig. 3. $^{1}H-^{31}P$ HMQC correlation spectrum of the products **1a**,**b**.



1a S = β-GalALys

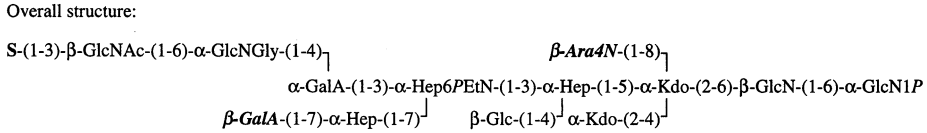
1b S = β-GalALys4*P*EtN



2 R^1 = H

3 R^1 = β-GalA

Overall structure:



where S = β-GalALys or β-GalALys4*P*EtN are present in the ratio of ~1:1.

Scheme 1. Structures of the isolated compounds and structure proposal for the *P. mirabilis* O28 LPS core. All sugars except Kdo in **1** are in the pyranose form. Bold italics indicate nonstoichometric substituents.

Table 4
Characteristic components of the inner part of *Proteus* core [a]

Group	Serotype	Ara4N (Z)	β-GalA (K)	Substituent at O-2 of α-GalA	Reference
1	*P. vulgaris* O2, O25, *P. penneri* 8, 16, 17, 18, 107	+		α-Hep-(1-2)-α-DDHep-(1-	[4,9,10]
2	*P. penneri* 7, 14, 37, 44	+	±	α-Hep-(1-2)-α-DDHep-(1-	Unpublished
3	*P. mirabilis* O3, O6, O48, O57	+	+	α-DDHep-(1-	[7,8]
4	*P. mirabilis* O28, *P. vulgaris* O8	+	+		
5	*P. mirabilis* O27	−	+	α-DDHep-(1-	[6]

[a] +, present in > 50% of the structures; −, absent; ±, present in 10–30% of the structures.

rise to heterogeneity of each core. 4-Amino-4-deoxyarabinose is present usually in > 50% amount and is absent completely only in the *P. mirabilis* O27. Ethanolamine phosphate at O-6 of heptose F can be partially present, but since all strains contain it to some extent, it was not used for the classification. Strains with α-Hep-(1-2)-α-DDHep-(1-2)- at O-2 of α-GalA H do not usually contain β-GalA K, although in *P. penneri* 7, 14, 37, and 44 it is present in small amounts. Other strains contain nearly 100% of β-GalA K. In all strains containing β-GalA K, except *P. mirabilis* O28, it is partially present in the form of amide with spermidine or putrescine; in *P. penneri* 7, 14, 37, and 44 it is completely amidated (structural analysis of these LPS will be published elsewhere). Some strains contain additional substituents, but they were not used for the classification since they occur only in single strains.

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