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The structure of the core part of *Proteus penneri* strain 16 lipopolysaccharide

Evgeny Vinogradov [a,*,1], Zigmunt Sidorczyk [b]

[a] *Carlsberg Laboratory, Department of Chemistry. Gamle Carlsberg Vej 10, DK-2500 Valby, Copenhagen, Denmark*

[b] *Institute of Microbiology and Immunology, University of Lodz, 90-237 Lodz, Banacha 12/16, Poland*

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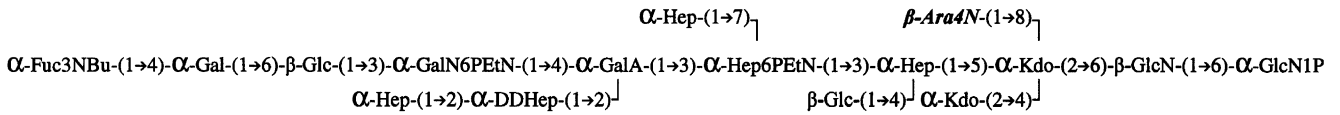
Abstract

The structure of the carbohydrate backbone of the lipid A-core region of the lipopolysaccharide (LPS) from *Proteus penneri* strain 16 was determined using NMR and chemical analysis of the core oligosaccharide, obtained by mild acid hydrolysis of the LPS, and of the products of alkaline deacylation of the LPS:



Incomplete substitution is indicated by bold italics. All sugars are in the pyranose form, α-Hep is the residue of L-*glycero*-α-D-*manno*-Hep, α-DD-Hep is the residue of D-*glycero*-α-D-*manno*-Hep, Bu is the (*R*)-3-hydroxybutyryl residue.

Keywords: LPS; *Proteus*; *Proteus penneri*; Core

1. Introduction

Bacteria of the genus *Proteus* are important opportunistic pathogens causing serious nosocomial wound and urinary tract infections [1]. The genus *Proteus* consists of four species: *P. vulgaris*, *P. mirabilis*, *P. myxofaciens* and *P. penneri*. Lipopolysaccharide (LPS) is the major surface antigen of Gram-negative bacteria. The structures of many O-specific polysaccharides from *Proteus* have been determined [2]. The structures of the LPS core from several serotypes of *P. vulgaris* and *P. mirabilis* have also been analyzed [3–10], but no data existed until now about the *P. penneri* core. In this work we present a complete structure of the core part of the LPS from *P. penneri* strain 16. The structure of the O-specific polysaccharide part of this LPS was determined earlier [11].

2. Experimental

Bacterial strain, isolation and degradation of lipopolysaccharide.—*P. penneri* strain 16 was

Abbreviations: Ara4N, 4-amino-4-deoxy-L-arabinose; Fuc3NBu, 3-(3-hydroxybutyramido)-3,6-dideoxygalactose; Fuc3N, 3-amino-3,6-dideoxygalactose; GalA, galacturonic acid; DD-Hep, D-*glycero*-D-*manno*-heptose; Hep, L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; LPS, lipopolysaccharide; P, phosphate; PEtN, phosphoethanolamine.

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

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

[1] Present address: Institute of Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, Ont., Canada K1A 0R6.

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kindly provided by Professor Don J. Brenner (Center for Diseases Control, Atlanta, USA). Bacteria were cultivated in dry meat infusion broth (Warsaw Serum Vaccines Laboratory) with 1% D-glucose added. LPS was isolated from dry bacterial cells by extraction with hot aq phenol [12] and purified by treatment with cold aq 50% trichloroacetic acid followed by dialysis of the supernatant.

NMR spectroscopy and general methods.—^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-600 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 DQCOSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), ROESY (mixing time 250 ms), HSQC, HMQC-TOCSY (mixing time 50 ms). NOESY spectra were recorded for oligosaccharides **1** and **2**, ROESY for compounds **3** and **4**. Gas–liquid chromatography (GLC) and gas–liquid chromatography–mass spectrometry (GLC–MS), methylation and monosaccharide analysis, including determination of absolute configurations, were performed as previously described [13]. The absolute configuration of (*R*)-3-hydroxybutyric acid was determined in the hydrolyzate of the core using the D-β-hydroxybutyrate dehydrogenase enzymatic test (Sigma).

Preparation of oligosaccharide **1**.—The LPS (200 mg) was hydrolyzed with 2% AcOH (100 °C, 5 h). The resulting precipitate was removed by centrifugation, and the supernatant separated on a Sephadex G50 (S) gel (Pharmacia) column (2.5 × 80) using pyridine–AcOH buffer (pH 4 and 10 mL in 1 L water) with monitoring by a Waters differential refractometer. The core fractions were further separated on a TSK-DEAE column (1.5 × 20 cm) in water–1 M NaCl gradient to give neutral (**1**, 20 mg) and acidic (5 mg) core fractions.

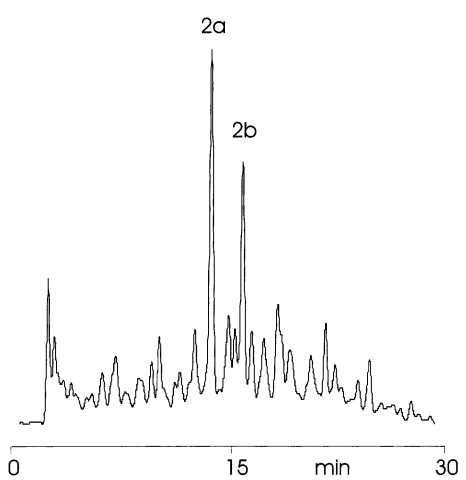


Fig. 1. HPAEC separation of the products of alkaline deacylation of *P. penneri* 16 LPS.

Preparation of oligosaccharide **2**.—LPS (200 mg) was heated for 4 h at 100 °C in 4 M NaOH (2 mL), cooled, and 2 M HCl (3 mL) was added. The precipitate was removed by centrifugation and the substance desalted on a Sephadex G50 column. The product was separated on a Carbopac PA100 (250 × 9 mm) column in one run using a gradient of 20–80% of 1 M NaOAc in 0.1 M NaOH at 3 mL min^{-1} over 1 h. Fractions of 1 min were collected and analyzed on an analytical Carbopac PA100 (250 × 4.6 mm) column using the same gradient. Fractions containing similar peaks were combined and desalted to give compounds **2** (20 mg) as well as many minor fractions that were not analyzed.

Preparation of oligosaccharides **3** *and* **4**.—Oligosaccharide **1** (20 mg) was treated with 48% HF (1 mL, 10 °C, 24 h), evaporated in vacuum desiccator over NaOH, dissolved in water (2 mL), 5 mg of $NaNO_2$ and 30 mL of AcOH added, and after 1 h at 20 °C desalted by gel chromatography on a TSK HW40(S) gel (E. Merck) column (1.6 × 80 cm). Fractions containing oligosaccharides **3** and **4** (detected by NMR) were separated by ascending paper chromatography on Whatman No. 1 paper in 1:1:1:1 pyridine–BuOH–AcOH–water, with alkaline $AgNO_3$ detection.

3. Results and discussion

Mild acid hydrolysis of *P. penneri* strain 16 LPS and separation of the products by gel chromatography on Sephadex G50 and ion-exchange chromatography on DEAE adsorbent gave a neutral oligosaccharide **1a**, eluted with water from a DEAE column, as well as an acidic core fraction **1b**. Both oligosaccharides contained different Kdo transformation-products at the reducing end, but attempts to purify them further on a Carbopak column did not yield any more individual compounds.

Table 1
^{1}H NMR data (ppm) [a]

Substance, unit	H-1	H-2(3a)	H-3(3e)	H-4	H-5	H-6(5′)	H-7(6′)	H-8(7′)	H-8′
2, unit A	5.39	2.70	3.62	3.46	4.05	4.23	3.80		
2, unit B	4.43	2.74	3.32	3.44	3.56	3.56	3.50		
1a, unit C				4.47	4.11	4.14	4.12	3.80	3.67
1a′, unit C				4.63	4.26	4.12	3.94	3.80	3.67
2a, unit C		1.90	2.18	4.18	4.24	3.67	4.00	3.79	3.79
2b, unit C		1.90	2.18	4.18	4.23	3.62	3.85	3.93	3.57
2, unit D		1.78	2.18	4.07	4.05	3.67	4.00	3.95	3.75
1a, unit E	5.13	3.97	3.99	4.26	3.70	4.11	3.72	3.65	
1a′, unit E	5.04	3.95	3.96	4.21	3.59	4.09	3.72	3.65	
2, unit E	5.27	4.08	4.16	4.31	4.22	4.11	3.95	3.79	
1a, unit F	5.30	4.10	4.00		3.73	4.70	3.91	3.84	
1a′, unit F	5.27	4.09	4.00		3.76	4.70	3.91	3.84	
2, unit F	5.29	4.31	4.13	4.05	3.81	4.70	3.86	3.72	
1a, unit G	4.91	3.95	3.78	3.84	3.59	4.02	3.72	3.67	
1a′, unit G	4.91	3.95	3.81	3.83	3.63	4.02	3.72	3.67	
2a or **2b**, unit G	4.88	3.95	3.86	3.85	3.70	4.01	3.70	3.75	
2b or **2a**, unit G	4.91	3.97	3.87	3.86	3.71	4.01	3.70	3.75	
1a, unit H	5.44	4.05	4.20	4.46	4.43				
2, unit H	5.52	3.96	4.53	5.77					
1a, unit I	4.53	3.28	3.48	3.34	3.36	3.72	3.85		
1a′, unit I	4.54	3.26	3.48	3.34	3.36	3.72	3.85		
2, unit I	4.57	3.29	3.52	3.37	3.39	3.73	3.85		
1a, unit L	4.69	3.37	3.50	3.50	3.68	3.95	3.76		
3, unit L	4.61	3.37	3.50	3.50	3.66	3.95	3.78		
4α, unit L	5.23	3.53	3.70	3.51	4.00				
4β, unit L	4.66	3.24	3.49	3.50	3.63	3.78	3.96		
1a, unit X	5.04	4.02	3.81	3.65	3.71	3.98	3.71	3.71	
2, unit X	5.07	4.04	3.84	3.87	3.68	4.00	3.72	3.72	
1a, unit T	5.22	3.93	3.98	3.76	3.98	3.98	3.79	3.66	
2, unit T	5.29	4.02	3.96	3.79	3.87	4.01	3.79	3.70	
1a, unit Y	5.02	3.95	3.95	4.05	3.96	3.80	3.80		
3, unit Y	5.05	3.97	3.97	4.06	4.02	3.83			
4, unit Y	5.04	3.95	3.99	4.07	4.04	3.84	3.81		
1a, unit M	5.24	3.69	4.24	4.37	4.65	3.98	3.98		
3, unit M	5.07	3.91	4.40	3.38	4.13	3.76	3.81		
1a, unit P	4.88	3.82	4.19	3.74	4.48	1.13			
3, unit P	4.90	3.83	4.22	3.76	4.52	1.15			
4, unit P	4.90	3.84	4.22	3.76	4.52	1.15			
1a, unit Z	4.97	3.71	4.13	3.67	4.07	3.75			
2a, unit Z	4.94	3.80	3.98	3.15	3.94	3.55			
EtN, **1a**	4.14	3.27							
EtN, **2**	4.01	2.99							
Bu, **1a**		2.44	4.19	1.19					
Bu, **3**		2.48	4.21	1.23					
Bu, **4**		2.46	4.21	1.23					

[a] Only data for major two variants of Kdo in **1a** are presented.

Complete O,N-deacylation of the LPS by treatment with 4 M NaOH (100 °C, 18 h) and the separation of the products by gel chromatography and HPAEC (Fig. 1) gave products **2a** and **2b**.

The core fraction was dephosphorylated with 48% HF and deaminated with nitrous acid. The resulting mixture was separated using gel and paper chromatography. Two major products **3** and **4** were thus obtained.

Compound **3** was the expected deamination product, whereas compound **4** was a side-product formed by the hydrolysis of the glycosidic linkage of glucose unit L during dephosphorylation or as a by-product of deamination [14].

For oligosaccharides **1a**, **2a**,**b**, **3** and **4**, NMR spectra were recorded and interpreted using the PRONTO program [15] (Tables 1–3, Fig. 2). The identity of monosaccharides was determined on the basis of vicinal coupling constants, intraresidual NOEs and chemical shifts of the carbon signals. In the spectra of oligosaccharide **1a** several variants of the residues C,E,F, and G could be traced due to the presence of different Kdo degradation products (probably 4,7-anhydro derivatives; no special efforts were made to understand the structure of Kdo in **1a**, as all necessary structural information about the Kdo region was obtained from the structure of oligosaccharides **2a**,**b**) at the reducing end. The absence of proton signals of H-3 of Kdo in the spectra of oligosaccharide **1a** may be explained by deu-

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

Substance	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
2, unit A	94.7	55.9	73.8	70.3	71.9	69.4		
2, unit B	103.9	56.8	76.3	70.3	74.8	62.0		
1a, unit C				78.2	85.2	77.1	82.4	63.0
1a′, unit C				75.9	80.1	76.1	82.5	63.0
2a, unit C			34.7	70.7	69.4	72.3	68.3	70.5
2b, unit C			34.7	70.7	69.4	72.9	70.0	64.3
2, unit D			34.8	66.6	67.0	72.3	70.9	63.2
1a, unit E	99.2	70.6	74.9	74.1	71.8	68.8	63.2	
1a′, unit E	97.6	70.5	75.7	74.0	71.9	68.9	63.2	
2, unit E	99.4	71.0	76.4	72.9	72.3	69.4	64.4	
1a, unit F	101.7	70.3	79.3		72.7	72.4	66.1	
2, unit F	102.7	69.5	79.5	65.7	72.7	73.1	67.7	
1a, unit G	100.0	70.5	71.4	66.7	72.0	69.3	63.5	
2, unit G	100.3	70.4	70.9	66.7	72.2	69.6	63.6	
1a, unit H	98.7	72.5	67.3	78.2	71.4			
2, unit H	98.7	76.0	64.7	107.6				
1a, unit I	103.1	74.5	76.0	70.2	76.6	61.9		
2, unit I	102.0	74.3	75.7	70.4	76.6	61.6		
1a, unit L	104.0	75.6	76.0	69.7	74.9	66.4		
3, unit L	104.3	74.3	77.1	71.0	75.8	67.4		
4β, unit L	96.5	74.5	76.2	69.9	74.7	66.4		
1a, unit X	102.7	70.6	70.9	66.8	72.9	70.1	63.9	
2, unit X	102.4	70.4	70.9	66.6	72.7	69.6	63.5	
1a, unit T	96.0	80.0	70.3	68.4	74.7	72.6	62.9	
2, unit T	97.6	79.3	70.4	67.8	73.9	72.6	62.6	
1a, unit Y	98.8	69.1	69.6	78.6	72.0	60.8		
3, unit Y	99.8	70.4	70.4	79.6	72.9	61.7		
4, unit Y	98.7	68.8	69.5	78.4	71.8	60.7		
1a, unit M	96.0	50.0	77.8	67.6	70.2	65.2		
3, unit M	91.2	83.1	83.1	73.3	82.6	62.0		
1a, unit P	100.3	66.8	51.5	71.0	67.8	15.7		
3, unit P	101.2	67.0	52.4	71.9	68.8	16.7		
4, unit P	101.1	66.7	51.4	70.9	67.7	16.0		
1a, unit Z	98.9	68.4	66.0	52.3	58.6			
2a, unit Z	99.5	68.7	69.2	50.8	62.8			
1a, EtN	65.5	40.5						
2, EtN	66.1	41.1						
1a, Bu		44.2	65.5	22.2				
3, Bu		46.2	66.6	23.0				
4, Bu		45.1	65.5	22.2				

Table 3
Inter-residual NOE data [a]

Substance	From proton	To protons
2	B1	A6s, A6′s
2	C3e	D6s, D8w, E5m
2	C3a	D6s, D8w, E5m, G1w
2	D3e	E1w
1a	E1	C4s, C5s
2	E1	C5s, C7s, D3ew
1a, **2**	F1	E2s, E3s
1a, **2**	G1	F7s, F7′s, F6w
1a, **2**	H1	T1s, F3s, F2m
1a, **2**	I1	E4s, E6s
1a, **2**	T1	H1s, H2s, X5m
1a	M1	H4s, H5w
1a, **3**, **4**	Y1	L6s, L6′s
1a, **3**	L1	M3
1a, **3**, **4**	P1	Y3s
1a, **2**	X1	T1s, T2s
1a, **2a**	Z1	C7w, C8s

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

terium exchange of H-3 through enol tautomerization. Five heptose residues were identified in **1** and **2a**,**b**, among them four had the L-*glycero*-D-*manno* and one (residue T) the D-*glycero*-D-*manno* configuration, distinguished on the basis of the position of their C-6 signals [4,5,16]. The following observations were used for the assignment of heptose signals: correlations between H-1 and H-2,3,4,5,6 for the residues G,X,T and between H-1 and H-2,3,5 for the residues F and E are visible in TOCSY spectra (Fig. 1 does not contain all these correlations because the spectrum was cut at high level to reduce noise); HMQC-TOCSY contained correlations between H-2 and H-3, H-3 and H-5, H-6 and H-7 (no correlation between H-6 or H-7 with other protons was observed); correlations of H-4 to H-3 and H-5 were observed in some cases; E4 and E6 protons can be found from the NOE correlation to I1; F6 is separated from other protons because of phosphorylation and gives a COSY correlation to F7 and NOE to F5 and G1; NOE correlation between T1 and X5 protons, characteristic for an α-(1→2) linkage, was observed. Assignment of hexose signals is straightforward except for the Gal residue Y, which has overlapped or nearly overlapped protons 2,3,4,5. Its configuration and substitution were inferred from the data for the oligosaccharides **3** and **4**. Characteristic signals of H-6 of 3-amino-3-deoxyfucose at 1.15 ppm and of 3-hydroxybutyrate at 1.19 (H-4) and 2.44 ppm (H-2) were present. The substitution of N-3 of the 3-aminofucose residue by 3-hydroxybutyrate follows from the results of deamination of oligosaccharide **1a**, which degrades galactosamine residue M and leaves intact both the 3-aminofucose and hydroxybutyrate residues.

The monosaccharide sequence for all oligosaccharides was assigned on the basis of the inter-residual NOEs and confirmed by ^{13}C NMR spectral data (Tables 2 and 3). In cases where NOE from H-1 of one sugar residue to several protons of another residue was observed (F1E2,3; I1F4,6; H1F2,3), the substitution position was determined on the basis of carbon chemical shifts. α-Kdo-(2→4)-α-Kdo linkage, as well as the α-D configuration of Kdo residues, was confirmed by the observation of a strong NOE between protons C3 and D6 [17].

The ^{31}P NMR spectrum of the oligosaccharide **1a** showed one peak for phosphodiester groups at 0.25 ppm, giving correlations to protons F6, M6, and EtN1,2. Oligosaccharides **2a**,**b** gave phosphate signals of equal intensity at 2.5 (A1) and 0.7 ppm (F6). Thus GlcN B in **2a**,**b** was not phosphorylated, which also follows from the upfield position of its C-4 signal at 70.4 ppm.

The spectra of the products **2a** and **2b** showed that they differ by the 4-amino-4-deoxyarabinose residue Z. These oligosaccharides were identical to the previously described products of the alkaline deacylation of the LPS from *P. vulgaris* OX2 [4], except for the absence of phosphate group at B4 in **2a**,**b**.

The monosaccharide composition was confirmed by chemical analysis (GLC of alditol acetates). The absolute D configurations of Glc, Gal, GlcA, and Fuc3N (comparing with Fuc3N from the O-chain of *P. penneri* 16 LPS) were determined using GLC of acetylated 2-(*S*)-butyl glycosides. The absolute configuration of (*R*)-3-hydroxybutyric acid was determined in the hydrolyzate of the core using the D-β-hydroxybutyrate dehydrogenase enzymatic test (Sigma). For additional confirmation of the absolute configurations of

monosaccharides, ^{13}C NMR spectra for all disaccharide pairs (except for Kdo, Ara4N, and GlcN A and B residues) were calculated [18] assuming the same or different absolute configurations of the components, and compared with experimental data. The results of the calculation confirm that all analyzed monosaccharide residues have the same absolute configuration.

Compounds **1a**,**b** and **2a**,**b** were analyzed by electrospray mass spectrometry in pure form and in the mixtures obtained after mild acid and strong alkaline hydrolysis of LPS. The spectra of mixtures (Figs. 3 and 4) show that they contain two major products each, corresponding to the structures **1a**,**b** (**1a** with anhydro-Kdo, $C_{92}H_{159}O_{76}N_5P_2$, $M = 2613.2$; **1b** with anhydro-Kdo, $C_{87}H_{150}O_{73}N_4P_2$, $M = 2482.1$) and **2a**,**b** (**2a**, $C_{82}H_{140}O_{72}N_4P_2$, $M = 2395.9$; **2b**, $C_{77}H_{131}O_{69}N_3P_2$, $M = 2264.8$); pure substances gave corresponding single peaks. Thus compound **1b** differs from **1a** only by the absence of an Ara4N residue. ESMS spectra of the oligosaccharides **3** and **4** contained $[M + Na]^+$ peaks at m/z 758 and 596, in agreement with proposed structures.

Variants of the LPS with Ara4N and without this residue, as can be assessed from the data of mass spectrometry and HPAEC of the products of alkaline deacylation of LPS, are present in roughly equal amounts.

Combined together, these data allow the structure of the core–lipid A region of the LPS, presented in Scheme 1, to be proposed.

The 3-deoxy-3-(3-hydroxybutyramido)-fucose residue is present in both the O-specific polysaccharide and the core part of analyzed LPS with different anomeric configurations. Although this residue can be the attachment point of the polysaccharide, no direct evidence for this was obtained.

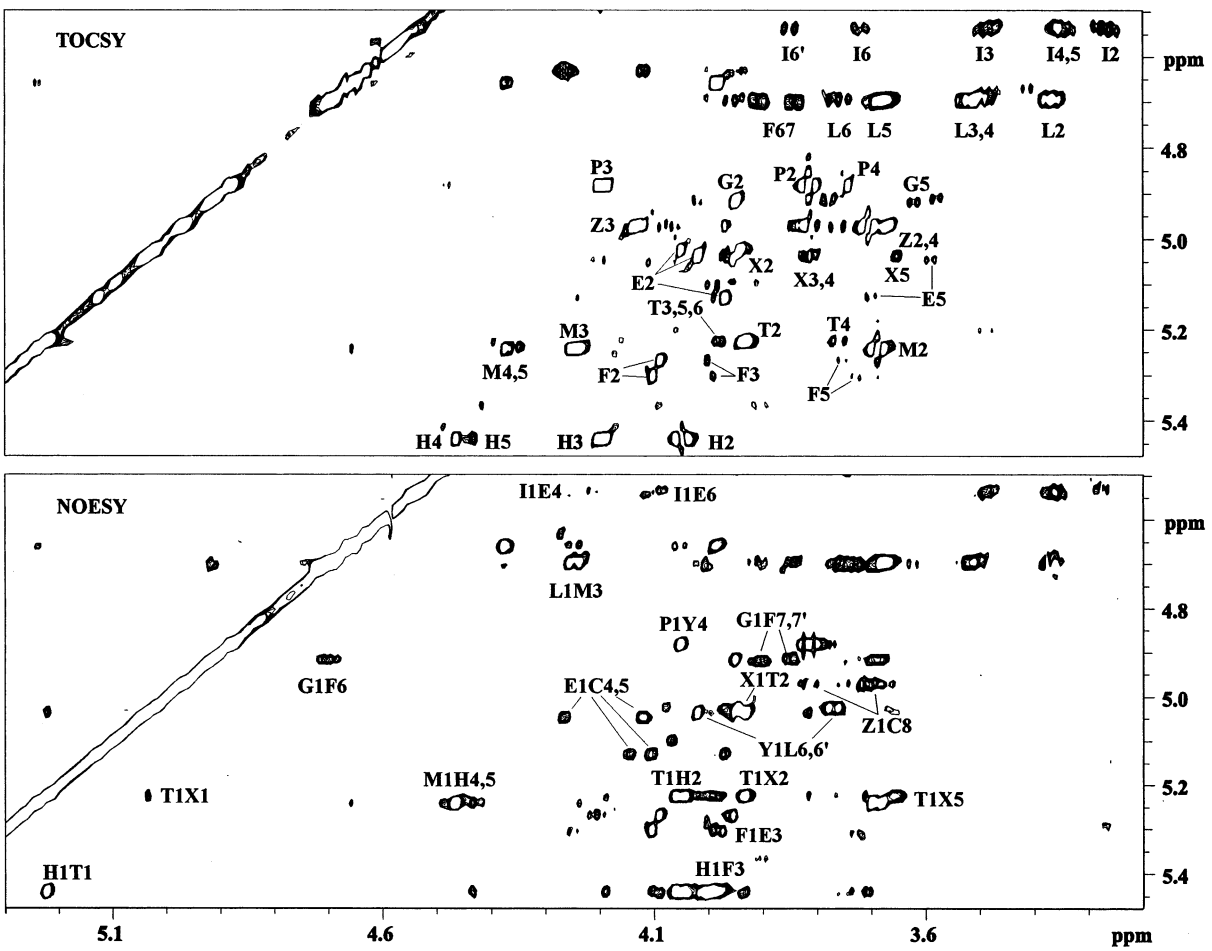


Fig. 2. Parts of TOCSY (top) and NOESY (bottom) spectra of the oligosaccharide **1a**. Lettering indicates residue and proton number; on TOCSY part single numbers mean correlation between H-1 and corresponding proton of the same residue.

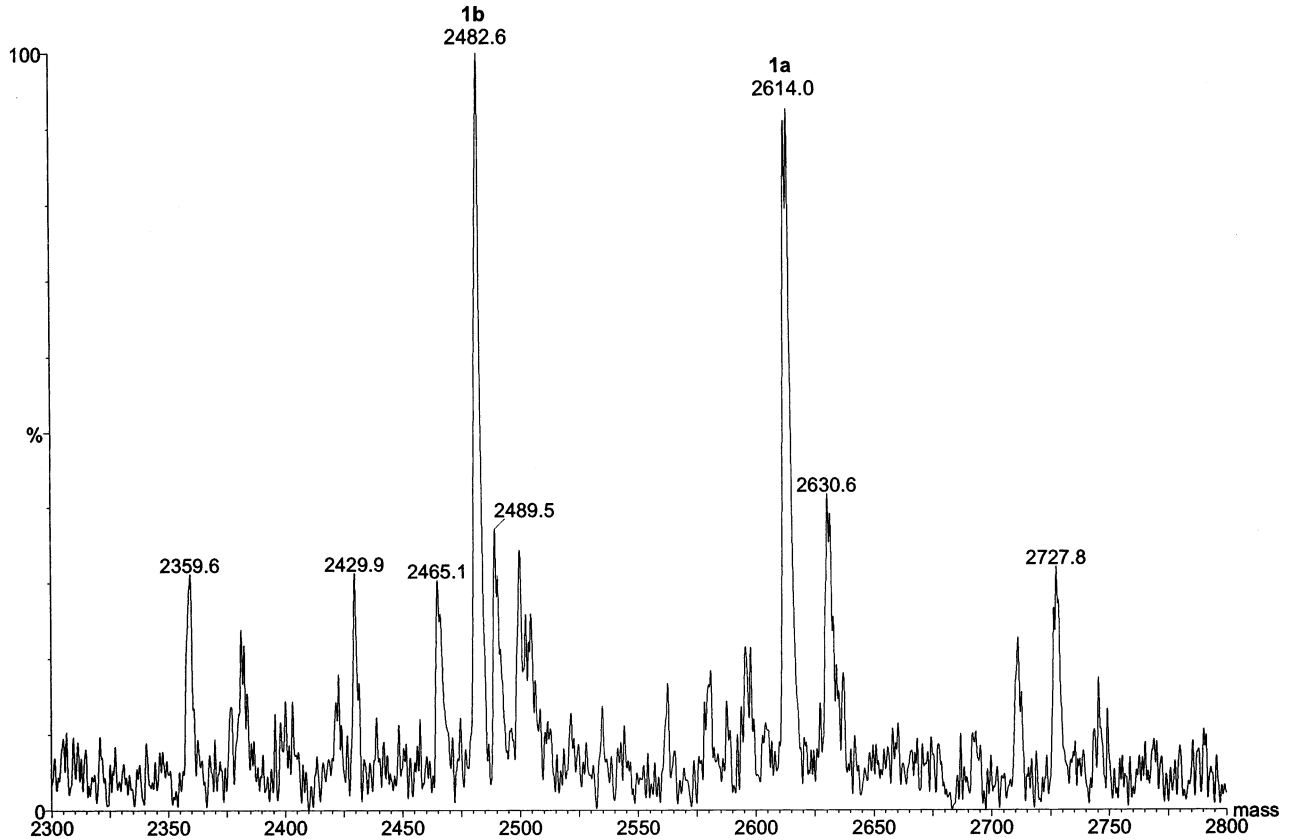


Fig. 3. Transformed negative-mode ESMS spectrum of the core fraction from *P. penneri* 16 LPS.

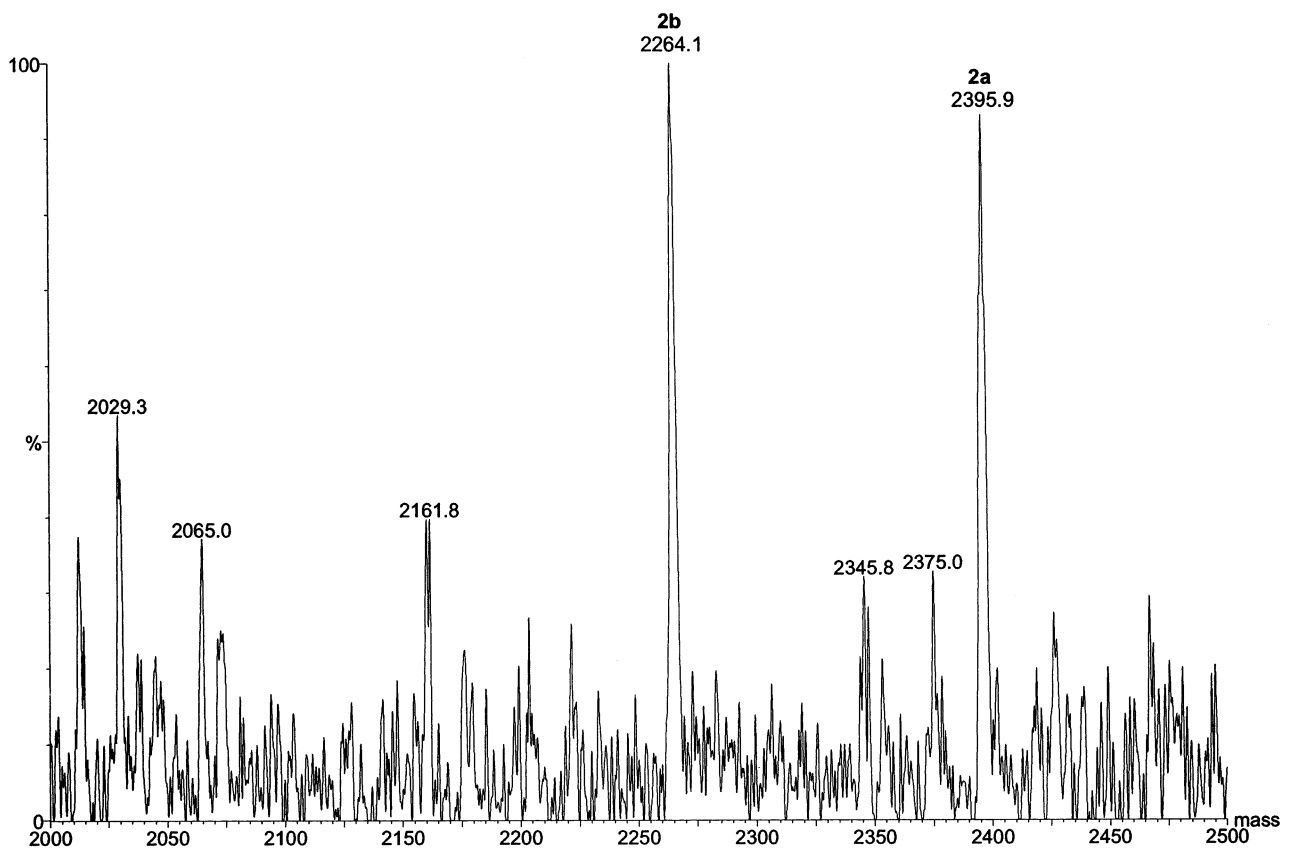
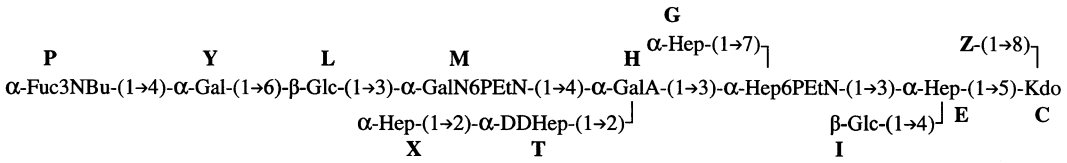
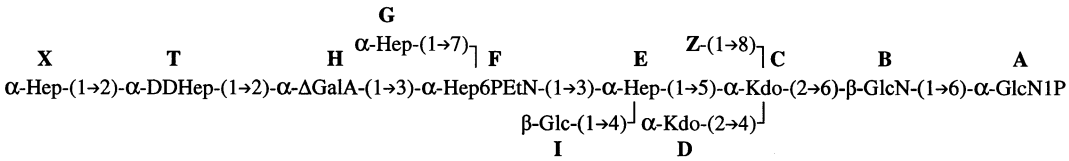


Fig. 4. Transformed negative-mode ESMS spectrum of the oligosaccharide fraction, obtained after alkaline deacylation of *P. penneri* 16 LPS.



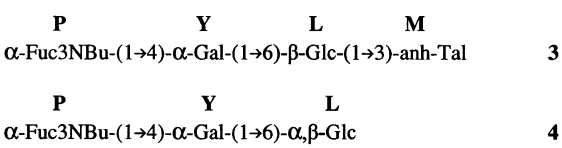
1a, Z = β-Ara4N; variants F-E-C, F'-E'-C' due to different forms of Kdo

1b, Z = H

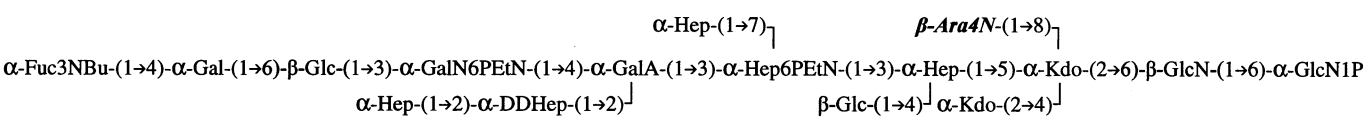


2a, Z = β-Ara4N

2b, Z = H



Overall structure



Scheme 1. Oligosaccharide structure and structural proposal for the core region of *P. penneri* 16. Incomplete substitutions are indicated by bold italics. All sugars are in pyranose form (except Kdo in **1a** and residue M in **3**). anh-Tal is the residue of 2,5-anhydro-talose, the product of the deamination of GalN; α-ΔGalA is 4-deoxy-β-L-*threo*-hex-4-enopyranosyl residue, a product of the alkaline elimination of the substituents from O-4 in the α-galacturonic acid.

The core structure of *P. penneri* 16 LPS is similar in the inner part to that of *P. vulgaris* OX2 [4], differing by the oligosaccharide substituent at O-4 of α-GalA and by the substituent at O-4 of β-GlcN in lipid A. After alkaline treatment both LPSs gave oligosaccharides differing by the phosphate at B4, absent in *P. penneri* 16 and present in the product from *P. vulgaris* OX2. The absence of a phosphate group at O-4 of glucosamine B in the alkaline-treated products may indicate that this position in LPS was substituted with a residue of the 4-aminoarabinose phosphate, as found in *Proteus* R45 strain [6], which eliminates in the conditions of alkaline deacylation.

We have analyzed LPS from two other strains of *P. penneri*. LPS from *P. penneri* strain 18 was found to be structurally identical to LPS from strain 16 in all respects except for a slightly increased degree of O-acetylation of the O-chain. LPS from *P. penneri* strain 17 gave no O-specific polysaccharide on acetic acid hydrolysis, and its core fraction was identical to the core from *P. vulgaris* OX2 LPS [4]. On alkaline deacylation it gave the same products as *P. penneri* 16, as identified by HPAEC.

The core structures of *Proteus* are different from those of *Salmonella* and *Escherichia coli* type [19] and resemble the structure of the *Klebsiella* core [20]. The common part of the carbohydrate backbone of *Klebsiella* and *Proteus* cores includes sugar residues from **A** to **I** [16,21,22], with two differences — known *Klebsiella* cores do not have an EtNP substituent at O-6 of heptose F and 4-amino-4-deoxyarabinose.

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