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The structure of the core part of *Proteus mirabilis* O27 lipopolysaccharide with a new type of glycosidic linkage

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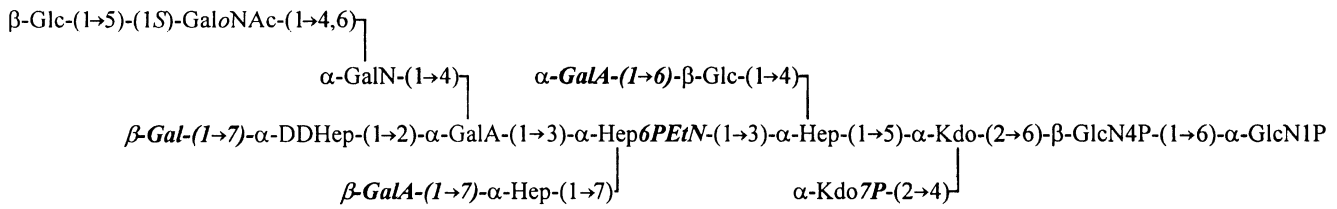
Abstract

The structural assignment of the intact lipopolysaccharide core from *Proteus mirabilis* O27 has been completed based on a combination of chemical degradation studies, NMR spectroscopy and ES-MS spectroscopy. The overall core structure is as follows:



where all sugars are in the pyranose form except the *N*-acetylglycosamine residue, Hep refers to L-*glycero*-α-D-*manno*-heptopyranose and α-DDHep to D-*glycero*-α-D-*manno*-heptopyranose. Bold italics indicate non-stoichiometric substituents. A new type of glycosidic linkage has been discovered wherein a GalNAc residue is linked as an open form acetal to the 4- and 6-positions of a 2-amino-2-deoxygalactopyranose residue. This structural element is abbreviated Gal*o*NAc-4,6-, where the '*o*' indicates the open form of the sugar residue.

Keywords: Lipopolysaccharide; Heptose; Kdo; Core; *Proteus mirablis*

1. Introduction

Lipopolysaccharides (LPSs) are components of the outer membrane of Gram-negative bacteria and play an important role as bacterial virulence factors [1]. LPSs comprise three regions: the O-antigenic polysaccharide, lipid A, and the core, a non-repetitive oligosaccharide linking the O-antigenic polysaccharide to lipid A. Normally, the core oligosaccharides have complex structures relatively conserved for each type of bacteria, but the biological functions of the particular core structures are unknown. Due to the similar structures of core oligosaccharides in groups of bacteria, cross-protective antibodies raised towards core fragments can be obtained [2]. Gram-negative bacteria of the genus *Proteus* are important pathogens that cause wound and urinary tract infections. However, limited information about the complete core structure from these species is presently available, therefore structural studies of such strains form the basis of

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the present work. The core fraction of *Proteus* LPS has previously been mainly investigated by chemical methods [3–8] and in one case the structure of the core from a rough mutant has been analyzed by NMR [9]. According to the data obtained, *Proteus* cores were classified into three chemotypes [10]. In the present work chemical degradation and spectroscopic data of the LPS core from the strain *Proteus mirabilis* O27 are presented and analyzed, leading to a complete structural proposal for this core-type. Preliminary data from this work have been published recently [11] and the structure of the O-specific polysaccharide from the LPS of this serotype has been previously determined [12].

2. Results

Complete O,N-deacylation of the *P. mirabilis* O27 LPS by treatment with 4 M NaOH and separation of the products by gel-chromatography and HPAEC (Fig. 1) gave four major products (oligosaccharides **1**–**4**). For all oligosaccharides complete sets of NMR spectra [^{1}H (Fig. 2), DQF COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY, gradient-enhanced HMBC, ^{31}P and 1D and 2D ^{1}H-^{31}P HMQC] were recorded and interpreted using the Pronto program [13]. The results are presented in Table 1 (^{1}H NMR data), Table 2 (^{13}C NMR data) and Table 3 (NOE data). The identity of monosaccharides was determined on the basis of their vicinal coupling constants (all data not presented are in agreement with expected values), intraresidual NOEs and ^{13}C chemical shifts. The discrimination between LD- and DD-heptoses presents a special problem. However, analysis of the ^{13}C spectral data for these monosaccharides as described in Ref. [14] suggests that they can be distinguished on the basis of the chemical shift of the C-6 signals. These resonate at about 70 ppm in the spectrum of a non-substituted α-LD heptoses and at about 72 ppm in the spectrum of the α-DD isomer. This observation is also true in the spectra of the analyzed substances, except in two cases. In one case the C-6 of the LD-heptose unit F signal is observed at ~73 ppm in the products **2**, **3**, and **4** due to phosphorylation, which is absent in compound **1**. In the other, a high-field shift of C-6 of the DD-heptose unit T (~69 ppm) in **1** is due to the β-effect from the glycosylation with a β-Gal residue at C-7. Since the heptose residue T in compounds **2**, **3**, and **4** without this β-Gal substituent has the C-6 signals resonating at ~73 ppm, it must therefore have the D-*glycero*-D-*manno*-heptose configuration. On the other hand, all other heptose residues have C-6 signals at ~70 ppm and must thus possess the L-*glycero*-D-*manno*-configuration. The structure of residue H, 4-deoxy-β-L-*threo*-hex-4-enopyranosyl, in compounds **1**–**4**, is a result of the alkaline elimination of the substituents from O-4 in the original galacturonic acid. Both Kdo residues possess anomeric α configurations as seen from the position of the signals of their H-3 protons [15]. All sugars were present in pyranosidic form, as characteristic low-field signals of furanosidic carbon atoms (below 80 ppm) were absent. The monosaccharide composition of the oligosaccharides was confirmed by conventional monosaccharide analysis (GLC of alditol acetates or methyl glycoside acetates, data not shown).

The monosaccharide sequence was assigned on the basis of the inter-residual NOEs and confirmed by ^{13}C NMR spectral data (Tables 2 and 3). The Kdo-α-(1→4)-Kdo linkage

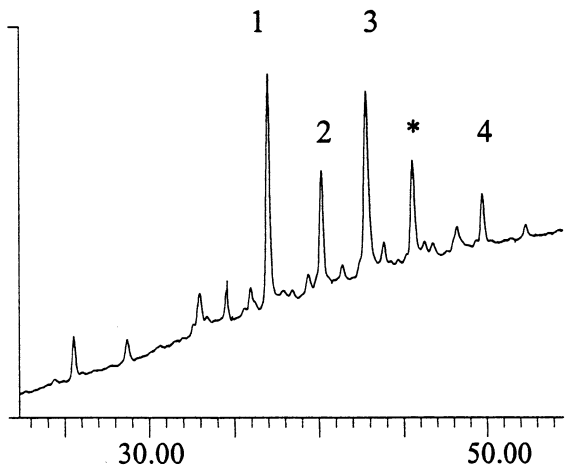


Fig. 1. Separation of the products from alkaline deacylation of *P. mirabilis* O27 LPS on a Carbopac PA100 preparative column. Numbers on peaks correspond to the substance numbers in the text. The peak marked with an asterisk contains a mixture of two compounds, the structure of which was not determined.

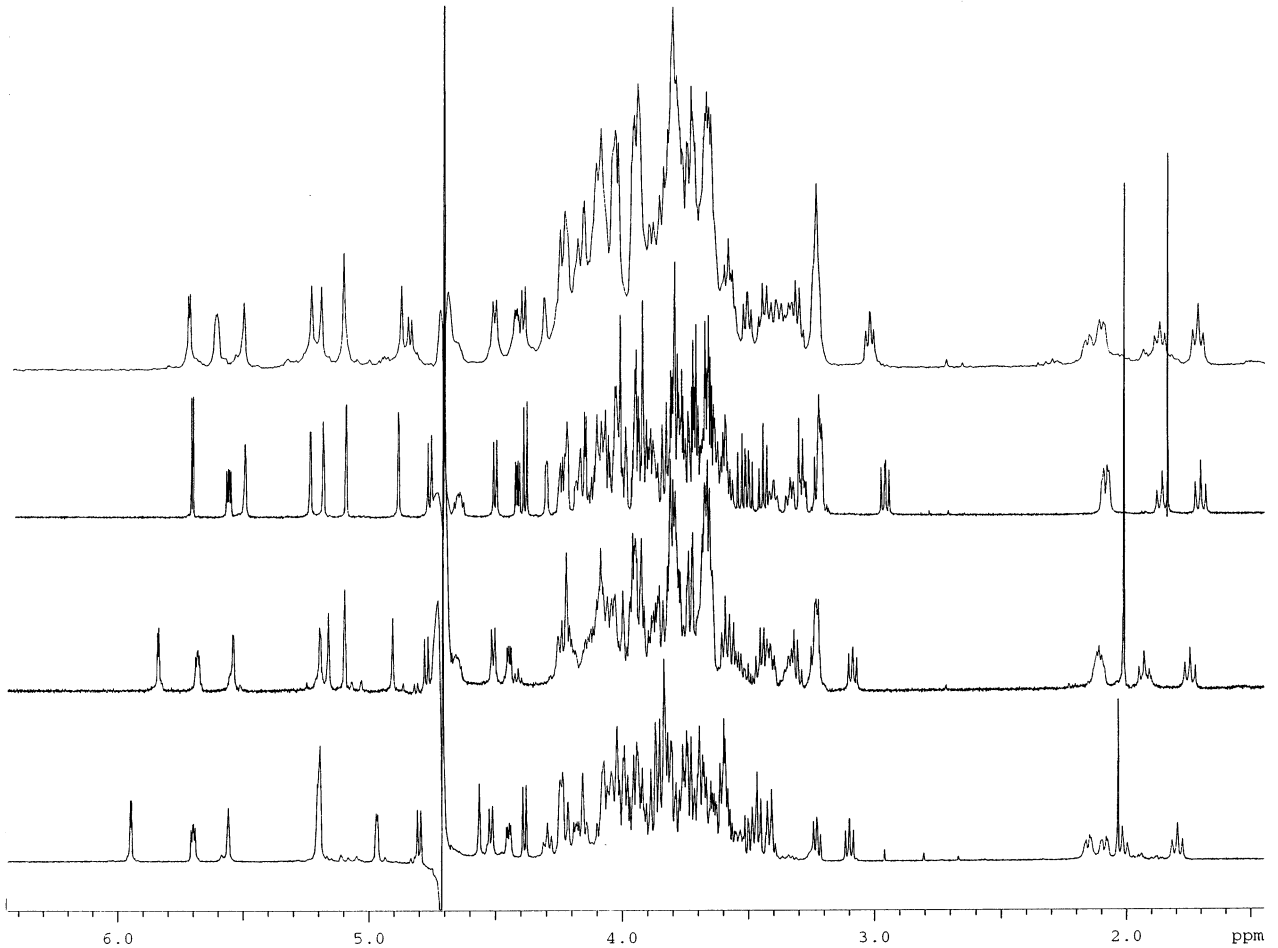


Fig. 2. ^{1}H NMR spectra of oligosaccharides **1**–**4** (from bottom to top, respectively). The sharp signal at about 2 ppm arises from traces of acetate present in the samples.

(residues C and D, see Scheme 1 for identity) was confirmed by the observation of strong NOEs between protons C3 and D6, which at the same time also confirms the identical absolute configuration of both residues and the α-configuration of residue D [16]. NOEs of equal intensity were observed between I1-E4 and I1-E6, but the carbon chemical shifts are only compatible with the attachment of Glc residue I to O-4 of Hep residue E. The NOE between F1 and E2,3 suggests an F-3E linkage, which is furthermore confirmed by the position of corresponding carbon signals.

The total mass of the oligosaccharides **1**–**3** was confirmed by electrospray (positive mode) mass spectroscopy: peaks $[M+H]^+$ were detected for product **1** at m/z 2176.1, calculated 2175.8; for **2** at m/z 2153.1, calculated 2152.6, and for **3** at m/z 2328.6, calculated 2328.7. Oligosaccharide **4** did not produce any ions in either positive or negative mode.

In order to determine the nature of alkali labile components of the core, the LPS was hydrolyzed with 2% AcOH and the products were separated by gel chromatography. The core fraction was then separated by ion-exchange chromatography. The biosynthetic heterogeneity, increased by the different Kdo transformation products at the reducing end, precluded the isolation of individual oligosaccharides. However, the major product obtained, **5**, was analyzed by NMR (Tables 1–3). NMR analysis of **5** allowed the identification of all the corresponding residues found in the oligosaccharides **1**–**4**, as well as an additional trisaccharide fragment **6** S-L-M, Scheme 2, attached to O-4 of galacturonic acid residue H. The structure of this trisaccharide fragment is described in a preliminary account of this work [11].

Table 1
^{1}H NMR chemical shift data (ppm from acetone at 2.225 ppm at 25 °C)

Substance	H-1	H-2(3a)	H-3(3e)	H-4	H-5	H-6	H-7(6′)	H-8(7′)	H-8′
Unit A									
1	5.71	3.42	3.89	3.60	4.09	3.77	4.23		
2	5.73	3.46	3.92	3.63	4.11	3.80	4.28		
3	5.61	3.34	3.88	3.58	4.13	3.74	4.29		
4	5.66	3.44	3.89	3.64	4.14	3.89	4.29		
Unit B									
1	4.81	3.10	3.86	3.82	3.73	3.48	3.71		
2	4.82	3.13	3.88	3.83	3.68	3.48	3.71		
3	4.81	3.01	3.80	3.69	3.68	3.45	3.66		
4	4.90	3.08	3.86	3.78	3.73	3.46	3.70		
Unit C									
1		2.016	2.15	4.07	4.25	3.68	3.82	3.57	3.85
2		1.968	2.16	4.15	4.27	3.70	3.84	3.60	3.92
3		1.904	2.13	4.14	4.27	3.72	3.86	3.63	3.90
4		1.922	2.21	4.15	4.27	3.70	3.89	3.63	3.92
Unit D									
1		1.79	2.09	4.03	4.05	3.56	3.92	3.66	3.93
2		1.80	2.16	4.07	4.05	3.65		3.72	3.95
3		1.75	2.13	4.07	4.04	3.65	3.97	3.75	3.95
4		1.77	2.15	4.15	4.09	3.81	4.28	3.99	3.99
Unit E									
1	5.20	4.08	4.05	4.30	4.15	4.04	3.90	3.76	
2	5.25	4.08	4.13	4.28	4.19	4.10	3.92	3.78	
3	5.28	4.07	4.14	4.28	4.21	4.08	3.93	3.79	
4	5.29	4.09	4.16	4.30	4.23	4.09	3.94	3.80	
Unit F									
1	5.21	4.16	3.99	3.96	3.69	4.01		3.62	
2	5.22	4.30	4.14	3.99	3.83	4.70	3.84	3.72	
3	5.23	4.35	4.11	3.98	3.82	4.70	3.86	3.72	
4	5.25	4.37	4.13	3.99	3.84	4.70	3.87	3.74	
				4.23		4.10			
5				4.20		4.03			
Unit G									
2	4.96	3.98	3.86	3.85	3.70	4.02			
3	4.94	3.97	3.84	3.85	3.76	4.23	4.07	3.94	
4	4.93	3.99	3.86	3.86	3.77	4.24	4.09	3.94	
	4.30	3.90	3.82	3.80	3.61				
5	4.87	3.92	3.77	3.80	3.60				
Unit H									
1	5.57	3.95	4.45	5.96					
2	5.59	4.00	4.50	5.89					
3	5.55	3.99	4.47	5.76					
4	5.56	4.00	4.48	5.77					
5	5.59	4.01	4.17	4.43	4.43				
Unit I									
1	4.52	3.23	3.47	3.41	3.54	3.84	3.75		
2	4.56	3.29	3.50	3.36	3.39	3.85	3.72		
3	4.55	3.28	3.50	3.34	3.38	3.83	3.71		
4	4.56	3.29	3.50	3.36	3.40	3.84	3.71		
5	4.49	3.25	3.45	3.32	3.34	3.82	3.67		
Unit K									
3	4.43	3.55	3.71	4.20	4.06				
4	4.44	3.56	3.72	4.21	4.07				
5	4.42	3.56	3.69	4.21	4.20				

Table 1 (Continued)

Substance	H-1	H-2(3a)	H-3(3e)	H-4	H-5	H-6	H-7(6′)	H-8(7′)	H-8′
Unit L									
5	4.84	4.27	4.19	3.36	4.02	3.76	3.67		
6	4.87	4.34	4.26	3.42	4.08	3.81	3.73		
$J_{n,n+1}$, Hz	5.5	1.5	10	2	$J_{5,6'}$ 6	$J_{6,6'}$ 12	$J_{5,6}$ 7		
Unit T									
1	5.20	3.99	3.83	3.86	3.76	4.18	4.00	3.82	
2	5.14	4.00	3.83	3.78	3.86	4.01	3.78	3.72	
3	5.15	4.00	3.82	3.76	3.84	4.00	3.78	3.71	
4	5.16	4.02	3.83	3.77	3.85	4.01	3.79	3.72	
5	5.07	3.92	3.80	3.69	3.89				
Unit S									
5	4.52	3.28	3.44	3.32	3.41	3.89	3.62		
6	4.58	3.40	3.49	3.39	3.42	3.94	3.69		
Unit M									
5	5.22	3.58	4.15	4.20	4.39	4.07	3.97		
6	5.09	3.93	4.39	4.38	4.00	4.16	4.03		
Unit R									
1	4.98	3.80	3.94	4.24	4.57				
Unit N									
1	4.39	3.50	3.61	3.87	3.65	3.75	3.70		
EtN									
2	4.16	3.28							
3	4.14	3.27							
4	4.16	3.83							
5	4.14	3.25							
Diaminobutane	3.21 3.43	1.67	1.60	3.01					

Taken together, the data presented allow for a proposal of the overall core structure of *P. mirabilis* O27 LPS as presented in Scheme 1 (bold italics indicate non-stoichiometric substituents).

A fraction of the core, released by mild acid hydrolysis, contained a diaminobutane residue, probably linked to a carboxyl group of a sugar acid via an amide bond as suggested by the chemical shift separation of its H-1 protons (Table 1). This core variant could be separated from the other part, which does not contain diaminobutane, by anion-exchange chromatography, and constituted about 20% of all the core material. All attempts to detect NOEs between the diaminobutane amide protons and the sugar protons (NOESY spectrum in 10% D_2O in H_2O at 25 and 10 °C) failed. The carbohydrate portion of this variant does not differ significantly from the structure of the non-substituted portion.

3. Discussion

The structural analysis of the core part of *Proteus* LPS is hampered by the presence of the residue of 4-substituted galacturonic acid (residue H), linking the inner and outer core. This residue undergoes β-elimination during the alkaline deacylation of the LPS, which results in the loss of part of the core. Analysis of the products from mild acid hydrolysis of the LPS is difficult because of problems in separating and isolating pure products. Usually, it is not possible to isolate individual products from the mixture of the many oligosaccharides produced by the different forms of Kdo derivatives at the reducing end, and also from the different biosynthetic products. In the present work, the structure of the complete core of *P. mirabilis* O27, the heterogeneity of which was previously reported [6], is determined from the combined results of alkaline deacylation, mild acid hydrolysis and

Table 2
^{13}C NMR chemical shift data (ppm from acetone at 31.5 ppm at 25 °C)

Substance	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Unit A								
1	91.9	54.2	69.4	69.6	72.7	69.4		
2	92.4	54.6	69.9	70.0	73.1	70.0		
3	91.1	54.9	70.1	70.1	72.5	69.9		
4	91.0	55.0	70.0	70.0	73.1	70.0		
Unit B								
1	99.3	55.7	71.8	74.5	74.0	62.6		
2	99.7	56.0	72.3	74.9	74.4	63.0		
3	100.0	55.9	73.1	73.6	74.5	62.8		
4	100.0	56.1	72.5	74.6	74.5	63.1		
Unit C								
1			34.4	70.8	68.6	72.4	69.4	63.7
2			34.7	70.8	70.0	72.9	69.9	64.4
3			34.7	70.5	70.0	72.5	70.0	64.0
4			34.5	70.7	70.1	72.8	70.1	64.3
Unit D								
1			34.5	66.1	66.3	72.8	70.4	63.3
2			34.9	66.6	67.0	73.0		63.6
3			34.8	66.4	66.8	72.3	70.5	63.2
4			35.0	66.3	66.4	71.6	73.9	62.1
Unit E								
1	98.7	70.8	75.2	73.0	72.1	69.1	64.0	
2	99.8	71.0	77.0	73.4	72.6	69.6	64.4	
3	99.4	70.8	76.6	72.8	72.2	69.3	64.2	
4	99.7	71.2	76.9	73.2	72.5	69.6	64.5	
Unit F								
1	101.9	69.4	80.9	65.3	71.9	68.9	63.4	
2	102.6	69.5	79.6	65.7	72.6	73.3	67.7	
3	102.6	68.8	79.8	65.4	72.1	73.1	67.6	
4	103.0	69.3	80.1	65.8	72.4	73.3	67.7	
5			77.3			73.2	67.8	
Unit G								
2	100.5	70.6	71.2	66.8	72.4	69.7		
3	100.5	70.1	70.6	66.6	72.5	67.6	72.2	
4	100.6	70.4	71.0	66.9	72.9	68.2	72.5	
Unit H								
1	98.7	74.3	64.4	110.5				
2	98.7	74.8	64.9	109.4				
3	98.2	74.6	64.6	107.1				
4	98.5	74.9	65.0	107.4				
5	97.4	71.1	67.1	79.0	71.7			
Unit I								
1	102.3	74.1	75.7	70.0	74.8	67.0		
2	102.9	74.4	76.0	70.5	76.9	61.8		
3	102.1	74.1	75.6	70.2	76.6	61.4		
4	102.4	74.4	75.9	70.5	76.9	61.8		
5	103.0	74.2	75.9	70.1	76.5	61.8		
Unit K								
3	102.6	70.8	73.0	70.6	75.7			
4	102.9	71.1	73.4	70.9	76.0			
5	102.9	70.7	72.7	70.2	75.0			
Unit T								
1	98.7	70.1	70.6	66.2	72.1	67.5	72.0	
2	99.1	70.3	71.0	67.7	73.7	72.8	62.6	
3	99.1	69.9	70.7	67.4	73.3	72.5	62.2	
4	99.5	70.3	71.0	67.7	73.7	72.8	62.6	
5	97.2	70.3	70.9	67.8	73.6			

Table 2 (Continued)

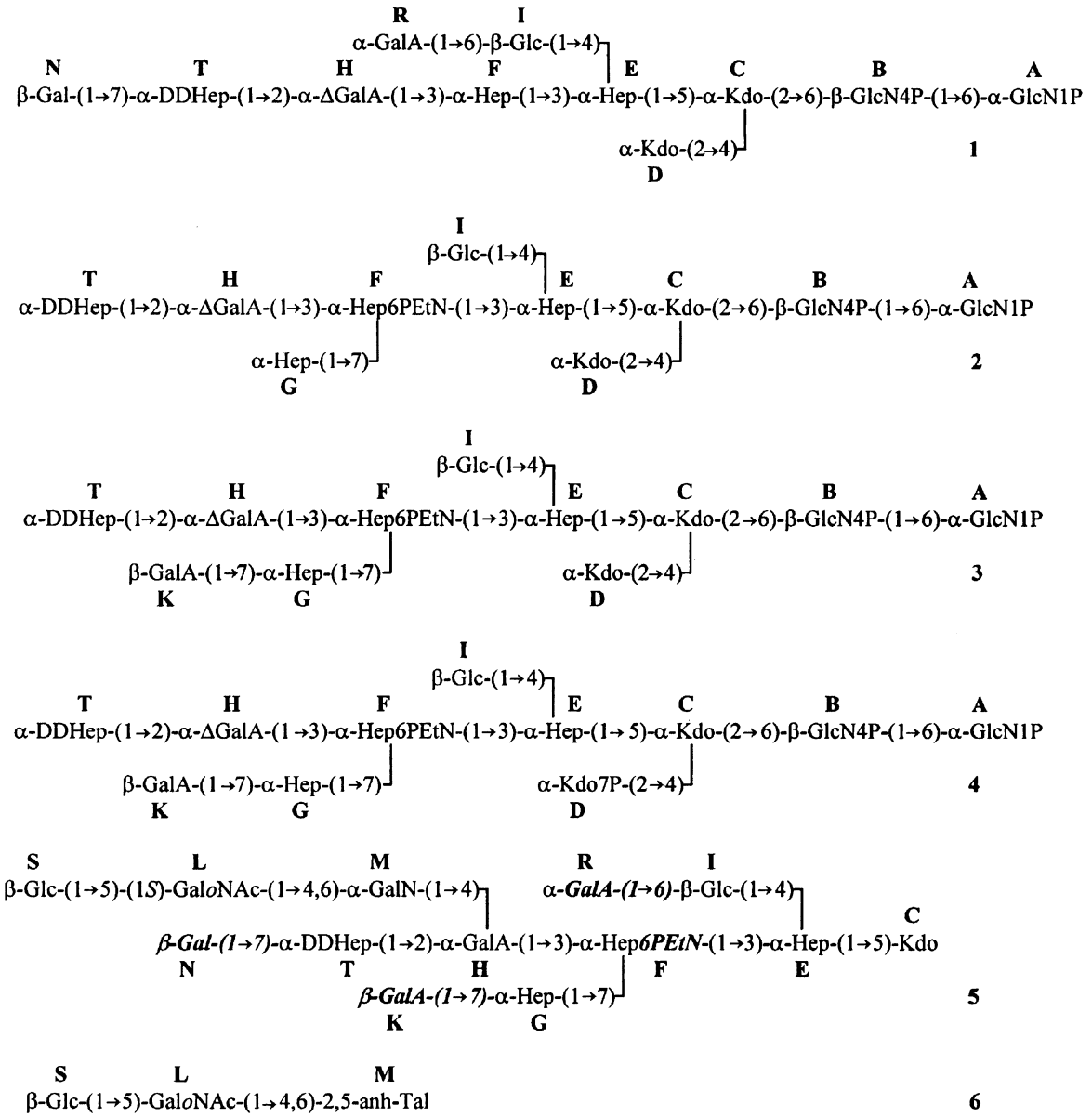
Substance	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Unit L								
5	100.6	52.3	67.8	69.2	78.4	62.2		
7	98.8	52.5	67.9	69.4	78.5	62.0		
Unit M								
5	96.9	51.2	65.2	75.0	64.1	69.0		
7	90.4	84.0	73.8	76.6	73.4	67.4		
Unit S								
5	102.9	73.7	75.9	70.1	76.0	61.7		
7	102.8	73.9	76.2	70.4	76.2	61.7		
Unit R								
1	98.0	68.0	69.2	70.6	71.2			
Unit N								
1	103.2	71.0	72.8	68.8	75.3	61.1		
EtN								
2	62.6	40.6						
3	62.3	40.5						
4	62.6	40.7						
5	62.6	40.8						
Diaminobutane	38.8	24.3	25.8	39.5				

deamination of the LPS. This structure includes an unusual component with a new type of glycosidic linkage — the open-chain *N*-acetylgalactosamine residue linked as cyclic acetal to positions 4 and 6 of the galactosamine residue. As previously shown, this component is also present in a core structure of another *Proteus* strain, OX2 [11]. This type of linkage between sugar residues — open-chain cyclic acetal linkage — has not been previously reported in natural products and has not even been discussed as a theoretical possibility [17]. Cyclic acetals built up of non-sugar oxo-compounds, such as pyruvic acid, are common components of bacterial polysaccharides [18,19]. The chemical stability of the acetal linkage formed by an *N*-acetylgalactosamine residue does not differ substantially from that of a normal glycosidic linkage. It survives hydrolysis with acetic acid and methylation analysis, but can be cleaved under the hydrolytic conditions normally used in monosaccharide analysis (2 M HCl, 100 °C). It may therefore be more widely occurring in natural poly- or oligosaccharides but not detected, since conventional analysis of *Proteus* serotype O27 core (monosaccharide analysis, methylation, mass spectrometry) would lead to the identification of terminal glucose, terminal *N*-acetylgalactosamine and 4,6-disubstituted galactosamine instead of the structural fragment described here. We propose to use symbol '*o*' (for 'open') to identify this type of sugar form in abbreviated formulas, thus the formula of the disaccharide fragment could be written as (1*S*)-Gal*o*NAc-(1→4,6)-α-GalN.

The new component may be specifically recognized by anti-LPS antibodies. In this respect

Table 3
Inter-residual NOE data from compounds **1**–**6** (s, strong; m, medium; w, weak)

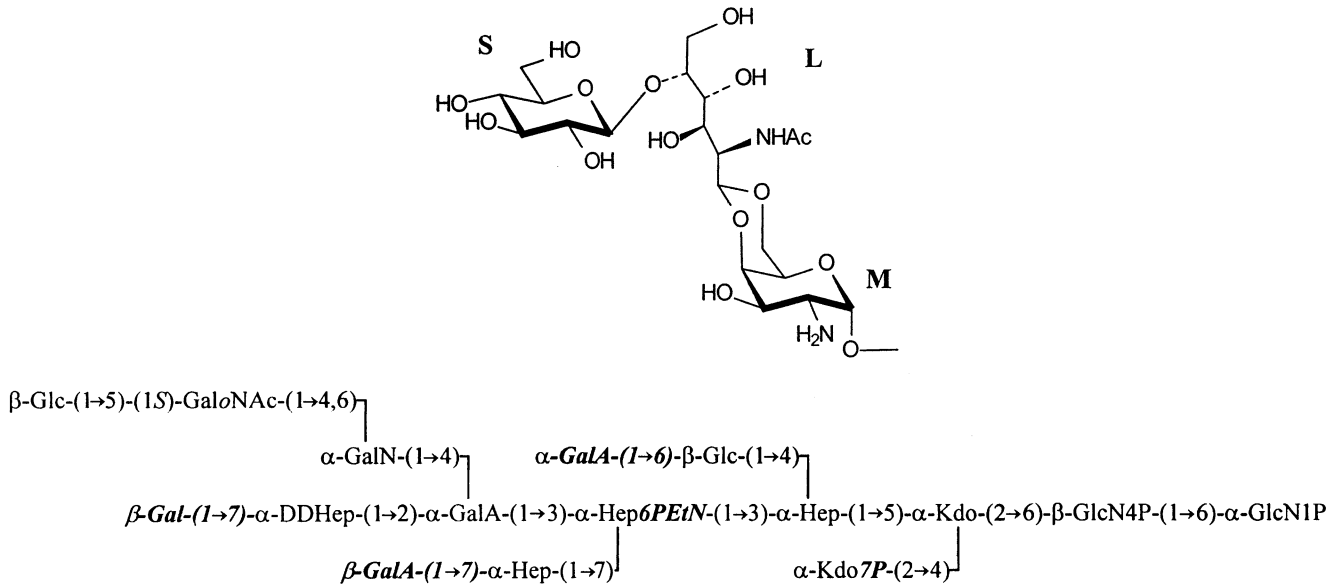
Compound	From proton	To protons
1–**4**	B1	A6s, A6′s, A5w
1–**4**	C3a,e	D6s, D8w, E5w
1–**4**	C3	G1w
1–**4**	E1	C5s, C7s, C6w
1–**5**	F1	E2s, E3s
2–**5**	G1	F7s, F7′s, F5w, F6m, E2w
1–**5**	H1	F2m, F3s, T1s
1–**5**	I1	E4s, E6s
5	M1	H4s, H5m
1–**5**	T1	H1s, H2s
3–**5**	K1	G7s, G7′s, G6m
1	R1	I6s, I6′s, I5m
1,**5**	N1	T7w, T7′w
5,**6**	S1	L5s
5,**6**	L1	M4s, M6s, M5w



Scheme 1. All sugars are in pyranose form except where indicated. α-Hep refers to L-*glycero*-α-D-*manno*-heptopyranose, α-DDHep to D-*glycero*-α-D-*manno*-heptopyranose. ΔGalA is 4-deoxy-β-L-*threo*-hex-4-enopyranosyl residue. Bold italics indicate non-stoichiometric substituents.

it would be interesting to trace its participation in the Weil–Felix test, which is based on cross reaction of some *Proteus* LPSs with antisera to *Rickettsia* [20–22].

Most of the isolated oligosaccharides contain ethanolamine phosphate substituents at O-6 of 7-substituted heptose residues. This situation makes ethanolamine phosphate stable to alkaline conditions because of the absence of neighboring hydroxyl groups. It was previously shown that the loss of this substituent proceeds with participation of adjacent hydroxyls [23,24]. The absence of EtNP substituents on O-6 of heptose F in the oligosaccharide **1**, which is not substituted at O-7 as in all other isolated oligosaccharides, may be due to the loss of this substituent during the alkaline deacylation. The partial substitution of Kdo residue D with phosphate at O-7 may also be an experimental artifact. If the LPS contains ethanolamine phosphate at O-7 or O-8 of this residue, after alkaline treatment ethanolamine will be lost completely and the phosphate group will be distributed be-



Scheme 2. Structural diagram of the open-chain element in fragment S-L-M of compound **5** and overall *P. mirabilis* O27 core structure.

tween O-7 and O-8 and also partially lost. Compounds with a phosphate group at D8 were not isolated, but may be present among the numerous minor components.

The fraction of core with 1,4-diaminobutane substituents is a characteristic component of *Proteus* cores. Diaminobutane or spermidine substituents in similar amounts are present in the most of *Proteus* cores (unpublished data). Most probably they are present as amides of uronic acid. A similar amide of diaminopropane with galacturonic acid was found in the core part of *Achinetobacter* LPS [25].

4. Experimental

Bacterial strain and lipopolysaccharide isolation.—Bacteria were cultivated and LPS isolated as described previously [26,12].

NMR spectroscopy and general methods.—^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-600 spectrometer in D_2O (pD ~7) at 25 °C with acetone standard (2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C) using standard pulse sequences. NOESY spectra were recorded for core oligosaccharides, and ROESY (mixing time 250 ms) for compounds **1**–**3**. NMR spectra were assigned using the program Pronto [13] and NMR, GLC, GLC–MS, methylation and monosaccharide analysis were performed as previously described [25,27].

Preparation of core oligosaccharide.—The LPS (200 mg) was heated in 4 M KOH (2 mL) at 100 °C for 4 h, 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 (25 × 0.9 cm) column in one run using a gradient of 30–80% 1 M NaOAc in 0.1 M NaOH at 3 mL/min over 1 h. Fractions of 1 min were collected and analyzed on an analytical Carbopac PA100 (25 × 4.6 mm) column using the same gradient. Fractions containing similar peaks were combined and desalted to give compounds **1**–**4** (5–20 mg each) as well as many minor fractions that were not analyzed. ^{31}P spectral data in ppm relative to 85% external inorganic phosphoric acid: oligosaccharide **1**: A1 2.0, B4 3.6; oligosaccharides **2**,**3**: A1 1.9, B4 3.8, F6 0.4; oligosaccharide **4**: A1 2.7, B4 4.0, F6 0.4, D7 5.3.

Preparation of core oligosaccharide.—The LPS (200 mg) was hydrolyzed with 2% acetic acid (100 °C, 5 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–acetic acid buffer (4 and 10 mL in 1 L water) and monitoring by a Waters differential refractometer. Core fractions were further separated on a TSK-DEAE column (1.5 × 20 cm) in water–1 M NaCl gradient to give several fractions. The front fraction contained diaminobutane-substituted core; compound **5** was eluted with water as the second fraction.

Preparation of the oligosaccharide **6**.—The core oligosaccharides (20 mg) were dissolved in water (2 mL), 5 mg of $NaNO_2$ and 30 μL of AcOH added, and after 1 h at 20 °C, the mixtures were desalted by gel-filtration chromatography on a TSK HW40(S) gel (E. Merck) column (1.6 × 80 cm). Fractions containing oligosaccharide **6** (detected by NMR) were separated by ascending paper chromatography on Whatman No 1 paper in 1:1:1:1 pyridine–butanol–acetic acid–water, with alkaline silver nitrate detection. Compound **6** was eluted from paper with water.

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