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Note

Structure of a D-*glycero*-D-*manno*-heptan from the lipopolysaccharide of *Helicobacter pylori*

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Abstract

A lipopolysaccharide (LPS) was isolated by hot phenol–water extraction from *Helicobacter pylori* strain D4 and found to contain no fucosylated poly-*N*-acetyllactosamine chain typical of most *H. pylori* strains studied but a homopolymer of D-*glycero*-D-*manno*-heptose (DD-Hep). The heptan attached to a core oligosaccharide was released by mild acid degradation of the LPS, and the following structure of the trisaccharide-repeating unit was established by chemical methods and ^{1}H and ^{13}C NMR spectroscopy:

→2)-D-α-D-Hep*p*-(1→3)-D-α-D-Hep*p*-(1→3)-D-α-D-Hep*p*-(1→

^{1}H NMR spectroscopy performed on small amounts of the intact LPS revealed the presence of the same polysaccharide in LPS of *H. pylori* strains D2 and D5, but not strain D10.

Keywords: *Helicobacter pylori*; Lipopolysaccharide; Polysaccharide structure; D-*glycero*-D-*manno*-Heptan

Helicobacter pylori is a prevalent pathogen of humans,[1] and infection is associated with the development of gastritis, gastric and duodenal ulcers, and gastric cancer.[2] As the lipopolysaccharide (LPS) of *H. pylori* interacts with the bacterial microenvironment and the infected host, attention is focusing on its structure and contribution to pathogenesis.[3]

The low phosphorylation and unusual acylation pattern of the lipid A moiety[4] have been suggested to contribute to the low endotoxic activity and low immunological response observed with *H. pylori* LPS.[3,5] Structural studies on the LPS of certain *H. pylori* strains have shown that the O-chain polysaccharide exhibits mimicry of Lewis^{x} and/or Lewis^{y} blood group antigens by expression of the corresponding determinants in the O-chain or located at the nonreducing end of the O-chain polysaccharide.[6–14] Typically, the *O*-polysaccharide is an α-L-fucosylated poly(*N*-acetyl-β-

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lactosamine) chain,[8–14] which may be additionally decorated with α-D-glucose or α-D-galactose residues.[7,14] Furthermore, Lewis[a], Lewis[b], and H type I antigenic determinants have been found in the LPS of some other *H. pylori* strains.[6,13] Although Lewis and related blood group antigens are present in the human gastric mucosa,[15] the pathogenic relevance of Lewis antigen mimicry by *H. pylori* remains largely unclear. Recently, a different polysaccharide type, which contains no typical *H. pylori* O-chain components, but D- and L-rhamnose, and a new branched sugar, 3-*C*-methyl-D-mannose, has been reported in the LPS of three *H. pylori* strains.[16]

A peculiar feature of the LPS of some *H. pylori* strains is the presence of an additional D-*glycero*-D-*manno*-heptan chain,[8,9,12] which was postulated to intervene between the O-chain and the LPS core.[8,9] In the present paper we report on the structure of the D-*glycero*-D-*manno*-heptan present in the LPS of some *H. pylori* strains, which are non-typeable with anti-Lewis antibodies.

Mild acid degradation of the LPS from *H. pylori* strain D4 resulted in one oligosaccharide and two polysaccharide fractions, which were separated by GPC on Sephadex G-50. Sugar analysis of the oligosaccharide fraction revealed typical components of *H. pylori* LPS core, namely, Glc, Gal, D-*glycero*-D-*manno*-heptose (DD-Hep), and L-*glycero*-D-*manno*-heptose (LD-Hep),[10,12] together with minor amounts of Fuc, Man, and GlcNAc. The negative mode electrospray ionisation mass spectrum showed a doubly charged peak for the pseudomolecular ion $[M-2H]^{2-}$ at m/z 620.8, which corresponds to a Hex_2Hep_3-anhKdo*P*Etn oligosaccharide (where anhKdo is an anhydro form of 3-deoxy-D-*manno*-oct-2-ulosonic acid and *P*Etn is 2-aminoethyl phosphate) with a calculated molecular mass of 1243.4 Da. The structure of a core oligosaccharide of the same composition from the LPS of *H. pylori* AF1 has been reported.[12]

A minor polysaccharide fraction that eluted from Sephadex G-50 first, consisted mainly of a (1→3)-linked galactan (sugar and methylation analysis data). Sugar analysis of the major polysaccharide fraction that eluted next, revealed mainly DD-Hep together with smaller amounts of Rha, Fuc, Man, Glc, Gal, GlcN, and LD-Hep (the ratios were 1:0.03:0.02:0.02:0.4:0.07:0.16:0.04). The absolute configuration of DD-Hep was confirmed by GLC analysis of the (+)-octyl glycosides.[17] Methylation analysis of the heptan demonstrated the presence of 3-substituted and 2-substituted heptopyranose residues in the ratio ~2:1.

The ^{1}H NMR spectrum of the heptan (Fig. 1) showed three major signals in the anomeric region (δ 5.39, 5.12, and 5.03) and signals for other ring protons in the region δ 3.72–4.22. In addition, there were a number of minor signals, including those for ethanolamine (δ 3.29, CH_2N), GlcNAc (δ 2.05, s, NAc), Fuc (δ 1.17, d, H-6; correlating in the COSY spectrum with δ 4.3, H-5), and Rha (δ 1.31, d, H-6; correlating in the COSY spectrum with δ 3.8, H-5). The ^{13}C NMR spectrum showed the major signals for three anomeric carbons at δ 103.7, 103.6, and 101.9, three HO*C*H_2–C groups at δ 63.7 and 63.6 (2C), and 15 other carbons in the region δ 80.1–68.3. These and methylation analysis data indicated that the heptan has a trisaccharide repeating unit.

The ^{1}H and ^{13}C NMR spectra of the heptan were assigned using two-dimensional homo- and heteronuclear correlated experiments COSY, TOCSY, and H-detected ^{1}H,^{13}C HMQC (Table 1). The DD-Hep residues were denoted as **A**, **B**, and **C** according to decreasing ^{1}H NMR chemical shifts of the signals from the anomeric protons. All three showed non-resolved $J_{H\text{-}1,H\text{-}2}$ couplings, intraresidue H-1,H-2 correlations in a NOESY experiment with no correlations between H-1 and H-3/H-5, and δ at high numerical values (Fig. 2); hence, all DD-Hep residues are α-linked.

Downfield displacements of the signals for C-2 of unit **A** and C-3 of units **B** and **C** to δ 81.1–79.8 demonstrated the glycosylation pattern of the heptan. The monosaccharide sequence was determined using a NOESY experiment (Fig. 2), which showed interresidue correlations between the following transglycosidic protons: **A** H-1/**B** H-3 at δ 5.39/3.97, **B** H-1/**C** H-3 at δ 5.11/3.88, and **C** H-1/**A** H-2 at δ 5.02/4.06. These data show that the heptan from the LPS of *H. pylori* D4 has the following structure:

→2)-D-α-D-Hep*p*-(1→3)-D-α-D-Hep*p*-(1→3)
A B
-D-α-D-Hep*p*-(1→
C

This structure is different from that reported for a D-*glycero*-D-*manno*-heptan present in the LPS of *H. pylori* serogroups O:3 and O:6, which suggested it to be an α-(1→3)-linked homopolymer.[8]

The failure to detect, in the methylation analysis, a penta-methylated DD-Hep derivative indicated that the polysaccharide chain is terminated with another sugar residue or an oligosaccharide built up of minor components, such as Fuc, Rha, Gal or/and GlcNAc. As it is evident from the ^{1}H NMR spectrum, by comparing anomeric and CH_3 signals, that the O-chain is short, for example in the order of some ten repeating units, it should be possible to see terminal sugars. Therefore, the heptan may intervene between the different LPS regions, e.g., between the core oligosaccharide and an O-antigen oligosaccharide or polysaccharide in *H. pylori*.

Small amounts (~3 mg) of the nondegraded LPS from three more *H. pylori* strains were analysed by ^{1}H NMR spectroscopy. The major sugar signals in the spectrum of the LPS from *H. pylori* strains D2 and D5 were essentially the same as in the spectrum of the heptan from *H. pylori* strain D4, and no significant signals belonging to another polysaccharide were present. Sugar and methylation analyses of the carbohydrate material derived by mild acid degradation of these LPS confi-

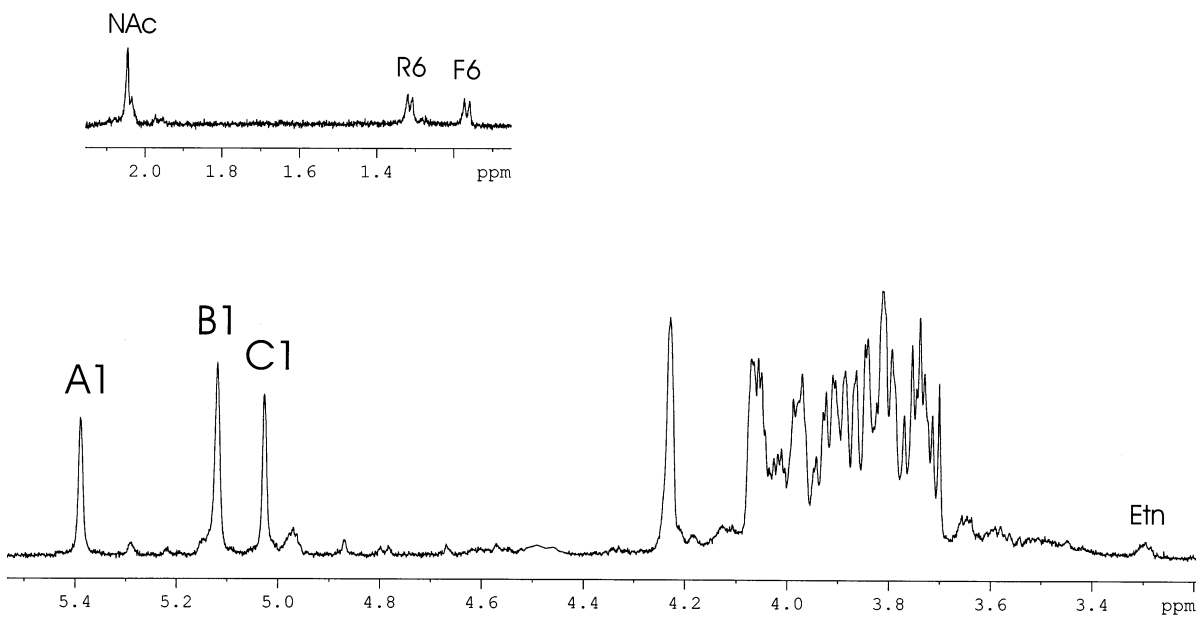

Fig. 1. ^{1}H NMR spectrum of the heptan from *H. pylori* strain D4 LPS. Sugar residues are denoted by letters as follows: **A**–**C**, heptose residues; **F**, fucose; **R**, rhamnose. NAc and Etn stand for the *N*-acetyl group of GlcNAc and the NCH_2 group of ethanolamine phosphate, respectively.

Table 1
Chemical shifts (δ) of the signals in the ^{1}H and ^{13}C NMR spectra of the D-*glycero*-D-*manno*-heptan from *H. pylori* D4 LPS

Sugar residue	^{1}H/^{13}C							
	1	2	3	4	5	6	7 (7a)	7b
→2)-α-Hep*p*-(1→	5.39	4.06	3.97	3.82	3.83	4.06	3.73	3.82
A	101.9	80.1	71.9	69.5	75.4	73.3	63.6	
→3)-α-Hep*p*-(1→	5.11	4.22	3.97	3.90	3.88	4.04	3.75	3.84
B	103.6	71.1	80.0	68.3	75.0	73.6	63.6	
→3)-α-Hep*p*-(1→	5.02	4.22	3.88	3.91	3.79	4.01	3.72	3.79
C	103.7	71.0	79.8	68.7	75.3	73.8	63.7	

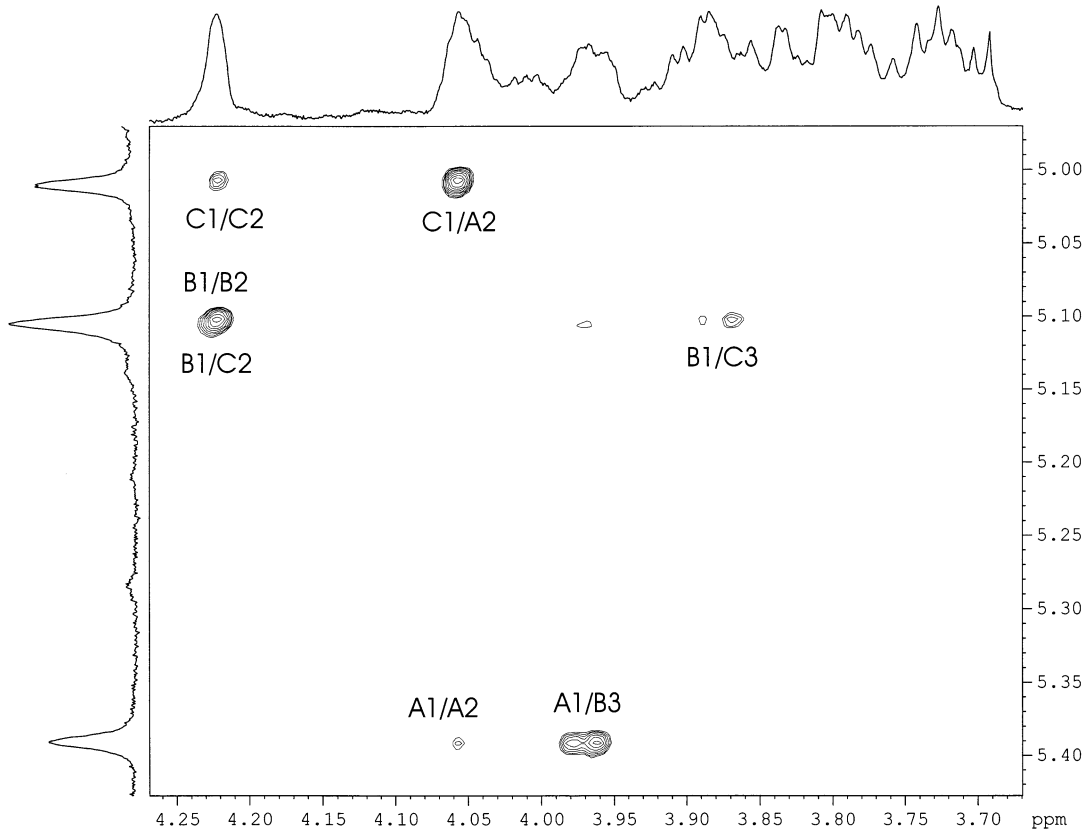

Fig. 2. Part of a NOESY spectrum of the heptan from *H. pylori* strain D4 LPS. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Sugar residues are denoted as shown in Table 1.

rmed the presence of a heptan having the same structure as shown above. In contrast, the ^{1}H NMR spectrum of the LPS from *H. pylori* D10 contained no signals from the heptan, but a single signal for an anomeric proton at δ 5.40 (d, $J_{1,2} \sim 3$ Hz). This belonged to an α-(1 $\rightarrow$ 4)-linked glucan (bacterial glycogen), as followed from sugar and methylation analyses data. A similar glucan has been identified in the LPS preparation from *H. pylori* 007.[12] Therefore, ^{1}H NMR spectroscopy applied to small amounts of LPS is a useful tool for screening *H. pylori* strains for the presence of various polysaccharide types.

1. Experimental

Bacterial strains and cultivation.—*H. pylori* strains D2, D4, D5, and D10 were isolated from biopsies at the Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark. Serological analysis by both Western blotting and an enzyme-linked immunosorbent assay with monoclonal antibodies against a variety of Lewis antigens (anti-Le^x-Le^y-sialyl-Le^x, -Le^a-Le^b, or -H type I) or against blood group determinants (anti-A, -B, or -AB), as described previously,[12] showed that strains were non-typeable. The bacterial strains were grown on blood agar to produce biomass as described previously.[18]

Isolation and degradation of lipopolysaccharides.—LPS (12 mg) of strain D4 was isolated by extraction of biomass with hot aq phenol[19] in a yield of 3.6% (dry weight), and degraded with 0.1 M AcONa buffer, pH 4.2, for 2 h at 100 °C. The water-soluble carbohydrate portion was fractionated by GPC on a column (70 $\times$ 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate, pH 4.5, as eluent and a flow rate of 0.5 mL/min. Monitoring was performed with a Waters differential refractometer and 10-mL fractions were collected. The yield of the two polysaccharide fractions was 1 mg each ($\sim$ 8% of the LPS weight), and that of the core oligosaccharide was 3 mg (20%).

LPS of strains D2, D5, and D10 were isolated in small amounts (3 mg each) by the phenol–water procedure.[19] After ^{1}H NMR spectroscopic analysis, they were degraded as above, and the carbohydrate material was studied by sugar and methylation analysis without fractionation by GPC.

Sugar and methylation analyses.—Hydrolysis was performed with 2 M trifluoroacetic acid (120 °C, 2 h). Monosaccharides were identified by GLC of the alditol acetates[20] using a Hewlett–Packard 5880 instrument equipped with a DB-5 fused-silica capillary column (25 m × 0.25 mm) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. The absolute configuration of DD-Hep was established by GLC of the acetylated (+)-2-octyl glycosides,[17] using for comparison a sample derived from the synthetic authentic monosaccharide.

Methylation was performed using methyl iodide in dimethyl sulphoxide in the presence of sodium methylsulphinylmethanide.[21] Hydrolysis was performed as for sugar analysis, and subsequently partially methylated monosaccharides were reduced with $NaBD_4$, converted to the alditol acetates, and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer, using the same chromatographic conditions as in GLC.

NMR spectroscopy and MS.—NMR spectra of the heptan from *H. pylori* D4 (1 mg) in 600 μL of 99.96% D_2O were recorded at 50 °C using a Bruker DRX-500 instrument. Chemical shifts are reported in ppm relative to internal acetone (δ_H 2.225, δ_C 31.45) as a reference. Standard Bruker software (XWINNMR 1.2) was used to acquire and process NMR data. A mixing time of 100 and 150 ms was used in TOCSY and NOESY experiments, respectively. NMR spectra of the whole LPS from *H. pylori* D2, D5, and D10 were recorded on a Varian Inova 600 instrument at 25 °C.

Electrospray ionisation MS was performed in the negative mode using a VG Quattro triple quadrupole mass spectrometer (Micromass, Altrincham, UK) with MeCN as the mobile phase at a flow rate of 10 μL/min. Samples were dissolved in 50% aq MeCN at a concentration of about 50 pmol/μL, and 10 μL was injected via a syringe pump into the electrospray source.

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