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Structure determination of the O-specific polysaccharides from *Citrobacter* O4- and O27-lipopolysaccharides by methylation analysis and one- and two-dimensional ^{1}H-NMR spectroscopy

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Using sugar and methylation analyses, and one- and two-dimensional ^{1}H-NMR spectroscopy at 500 MHz it was established that poly-β-1,2-4-deoxy-D-arabinohexopyranose occurs as O-specific chains of lipopolysaccharides in *Citrobacter* serotypes O4, O27 and O36. Strong serological cross-reactivity between these serotypes is in full agreement with the chemical identity of their O-specific polysaccharides.

Structure determination; O-specific polysaccharide; Methylation analysis; ^{1}H-NMR; *(Citrobacter freundii)*

1. INTRODUCTION

Lipopolysaccharides of *Citrobacter freundii* O4, O27 and O36 serotypes are associated with the chemotype CC-G and contain a characteristic sugar component, 4-deoxy-D-arabinohexose [1,2]. In a previous study on the lipopolysaccharides of the CC-G chemotype [3], their strong serological cross-reactivity and a 4-deoxy sugar content of up to 90% in their O-specific polysaccharides were observed. Thus O4-, O27- and O36-specific polysaccharides were inferred to have identical structures. Here, it was ascertained with the aid of methylation analysis and ^{1}H-NMR spectroscopy that *Citrobacter* O4- and O27-specific polysaccharides are linear homopolymers of (β1→2)-linked 4-deoxy-D-arabinohexose like the O36-specific polysaccharide described earlier [4].

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Abbreviations: COSY, two-dimensional scalar shift-correlated NMR spectrum; 1D and 2D, one- and two-dimensional; NOE, nuclear Overhauser effect

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

C. freundii strains 52/57 (O4) (serotypes are given in parentheses), 91/57 1560 (O27) and 114/66 (O36) were kindly provided by Dr J. Sourek from the Czechoslovakian National Collection of Type Cultures, Prague; strain 1488 (O4) derived from the collection of the Institute of Immunology and Experimental Therapy, Wrocław and strain neg^{s}Tc^s from the Institute of Microbiology, Wrocław University.

2.2. *Preparative and analytical procedures*

Preparations of lipopolysaccharides, O-specific polysaccharides and antisera, and sugar and methylation analyses were carried out as in [4].

For NMR measurements, the samples were repeatedly exchanged with D_2O, with intermediate lyophilization, and then dissolved in 0.3 ml D_2O containing a trace of acetone, which was used as

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internal reference (δ 2.225). ^{1}H-NMR spectra were measured at 500 MHz on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer. Bruker standard software was used. The spectral width was 2 kHz for all spectra. The NOE difference spectrum was recorded as in [5]. The data size of the time domain 2D scalar shift-correlated spectrum (COSY) was 1K points in t_2 and 256 points in t_1. Prior to Fourier transformation the matrix was multiplied in t_1 and t_2 with the sine-bell window function [6] and zero-filled in t_1. Digital resolution in the symmetrized [7] 512 × 512 magnitude mode matrix was 3.9 Hz per point.

2.3. *Serological procedure*

Passive hemagglutination inhibition test was performed according to [8].

3. RESULTS AND DISCUSSION

3.1. *Isolation and characterization of the specific polysaccharides*

The specific polysaccharides prepared after mild acid hydrolysis of the lipopolysaccharides of *Citrobacter* serotypes O4, O27 and O36 were purified by gel filtration on a Biogel P-10 column. Their content in lipopolysaccharides varied from one serotype to another: lipopolysaccharide O4 (strain 1488) contained 42% of O-specific material whereas lipopolysaccharide O27 only 15%. 4-Deoxy-D-arabinohexose was present as the main component (70–95%) in all these polysaccharides. Additionally, some of the following sugars, being of core origin, D-galactose, D-glucose, L-glycero-D-mannoheptose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine, were found in low percentages ($\leqslant$3%) in the preparations examined.

The polysaccharides inhibited strongly the passive hemagglutination of their homologous systems (lipopolysaccharide – anti-strain serum) and, distinctly enough, the heterologous ones (table 1). The minor deviations in inhibitory activities observed between the polysaccharides of this group of *Citrobacter* were probably due to their heterogeneity. Analysis of the lipopolysaccharides of the above-mentioned serotypes by SDS-polyacrylamide gel electrophoresis proved that they are highly heterogeneous with respect to the degree of polymerization of the O-specific chains and to the pattern of their core regions (unpublished).

3.2. *Methylation analysis*

Permethylated polysaccharides of the three examined serotypes afforded mainly 4-deoxy-3,6-di-*O*-methyl-D-arabinohexopyranose and therefore 4-deoxy sugar residues appeared to be involved in their O-specific chains by a 1,2-linkage. Trace amounts of 4-deoxy-2,3,6-tri-*O*-methyl-D-arabinohexopyranose derived from terminal 4-deoxy sugar residues.

3.3. *^{1}H-NMR spectroscopy*

The 1D ^{1}H-NMR spectra of the polysaccharides isolated from lipopolysaccharides O4 (strains 52/57 and 1488), O27 (strain 91/57 1560) and O36

Table 1

Passive hemagglutination inhibition of *Citrobacter* O-specific polysaccharides in homologous and heterologous systems

Inhibitor		Test system			
Derived from strain	Serotype	52/57 LPS anti-52/57 serum	91/57 1560 LPS anti-91/57 1560 serum	114/66 LPS anti-114/66 serum	neg^{s}Tc^s LPS anti-Tc^s serum
52/57	O4	3.9	31.2	62.5	7.8
1488	O4	15.6	31.2	31.2	15.6
91/57 1560	O27	15.6	15.6	62.5	31.2
114/66	O36	7.8	15.6	15.6	7.8
neg^{s}Tc^s	O36	7.8	31.2	7.8	7.8

LPS, lipopolysaccharide; amounts given in μg

(strain 114/66) are almost identical (fig.1a–d), except for minor signals for terminal residues and impurities. Analysis of the COSY spectrum (fig.1f), performed in much the same way as for the O-specific polysaccharide from strain PCM 1487 [9], has shown the constituent sugar in these polysaccharides to be 4-deoxy-D-*arabino*-hexopyranose. The downfield shifts of the H-1, -2 and -3 resonances vs those for the polymer investigated in [9] (0.41, 0.34 and 0.12 ppm, respectively) are readily explainable in terms of glycosylation-induced shifts and indicate 2-glycosylation ([10] and references therein). Other resonances differ by $\leqslant$0.02 ppm. The H-1/H coupling remained unresolved in an extremely strongly resolution-enhanced spectrum (not shown), hence $^3J_{1,2} \leqslant$ 1H which points to the anomeric β-configuration (see discussion in [9]). Decisive proof of this configuration was furnished by the NOE difference spectrum (fig.1e). NOE can be observed as the intensity change of a proton signal, if another proton, located nearby in space, is being irradiated at its resonance frequency. Such intensity changes are especially clearly visible in difference spectra, since signals of interacting protons are the only ones left after a spectrum recorded with irradiation has been subtracted from a control unaltered spectrum. Upon irradiation of H-1, NOE signals of three protons were observed: (i) a strong one for H-2, resulting from both the inter-residue interaction over the glycosidic bond and the intraresidue interaction of H-1 with the *gauche* H-2 [10]; (ii) a weaker one for H-3, produced by inter-residue interaction simultaneously with that mentioned in item (i) for H-2; NOEs of this type occur for equatorial protons located vicinally to the glycosidic bond [10,11]; (iii) a strong one for H-5, corresponding to an intraresidue 1,5-synaxial interaction and thus conclusive of the β-configuration. To the best of our knowledge NOE has not yet been applied to determine anomeric configurations.

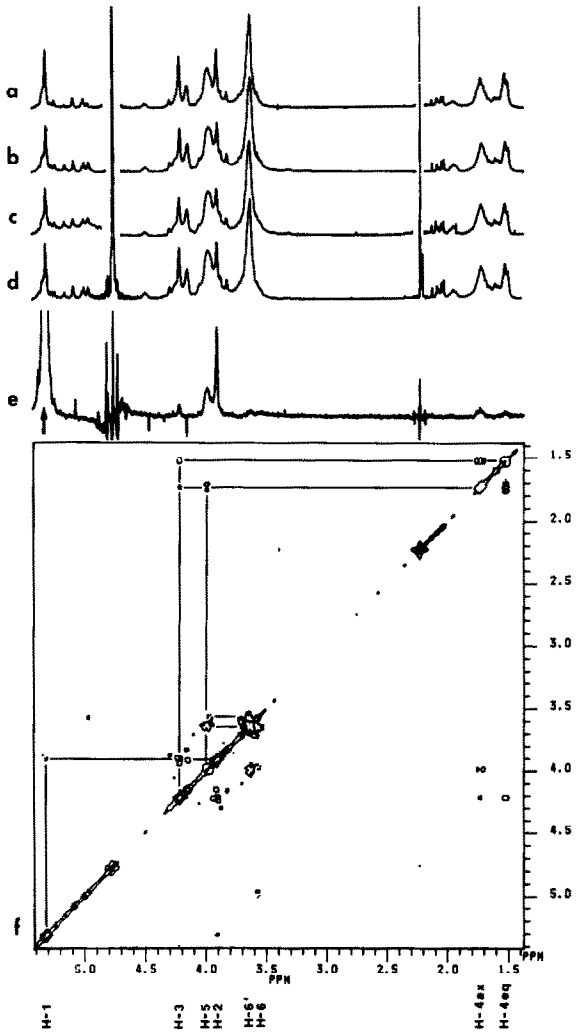

Fig.1. 500 MHz ^{1}H-NMR spectra of O-specific polysaccharides from *Citrobacter* recorded in D_2O at 303 K. Chemical shifts measured from internal acetone peak set at 2.225 ppm. (a–d) Resolution-enhanced 1D spectra of polymers from serotypes O4 (strain 52/57), O4 (strain 1488), O27 (strain 91/57 1560) and O36 (strain 114/66), respectively. The HDO and acetone peaks have been erased in a–c. (e) NOE difference spectrum; irradiation of H-1 indicated by the arrow. (f) Contour plot of the 2D COSY spectrum of the same sample as in a. Resonances lying along the diagonal in the 2D coordinate system, δ_1, δ_2 (vertical and horizontal scale, respectively) correspond to those in the 1D spectrum but are seen from above in the form of level contours. Coupled nuclei, e.g. H-1 and H-2, exhibit off-diagonal contours located in the corners of the square defined by δ_2 (H-1), δ_2 (H-2), δ_1 (H-1), δ_1 (H-2). Analogous squares occur for the H-2/H-3 coupled pair, etc. The connectivity track for the entire spin system is drawn (–) along the sides of these squares above the diagonal, and assignments are given below the δ_2 scale. The chemical shifts read from this spectrum were δ(H-1) 5.31, δ(H-2) 3.90, δ(H-3) 4.21, δ(H-4ax) 1.73, δ(H-4eq) 1.52, δ(H-5) 3.98, δ(H-6) 3.64 and δ(H-6′) 3.57.

3.4. *Conclusions*

The present results demonstrate clearly the same structure of O-specific polysaccharide chains occurring in *Citrobacter* strains of serotypes O4, O27 and O36. They are all linear homopolymers of (β1→2)-linked 4-deoxy-D-arabinohexopyranosyl residues. However, lipopolysaccharides of serotypes O4 and O36 are richer in O-specific chains as compared to the lipopolysaccharide of serotype O27 (strain 91/57 1560). Bacteria of the latter are unstable and produce lipopolysaccharide molecules mostly lacking O-specific chains; their carbohydrate moiety was reduced almost to the core region. Further structural studies on the core regions of *Citrobacter* serotypes will be needed to explain their serological diversity.

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