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Note

The structure of the O-specific polysaccharide of the lipopolysaccharide from *Chromobacterium violaceum* NCTC 9694

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Chromobacterium violaceum [1,2] is a Gram-negative microorganism inhabiting soil and water. Despite being generally considered non-pathogenic, several cases of infections of humans [3–8] and animals [9,10] have been reported, including urinary tract infections, diarrhoea, and systemic infections with abscesses in multiple organs. Most cases have occurred in tropical and subtropical climates, and were associated with a high mortality rate.

In an early investigation [11], the presence of D-*glycero*-D-*galacto*-heptose, together with L-rhamnose and D-glucosamine, in the ''specific polysaccharide'' of *C. violaceum* was reported. Later [12], the lipopolysaccharide (LPS) was isolated and its lipid A moiety structurally characterised. Studies concerning the relative amount of 4-amino-4-deoxy-L-arabinose in lipid A have also been undertaken [13]. No investigations on the structure of the core region and the O-specific antigen have so far been published. We now report the structure of the latter, proving that D-*glycero*-D-*galacto*-heptose is a constituent of this LPS region.

Isolated LPS of *C. violaceum* strain NCTC 9694 was hydrolysed and the O-antigenic polysaccharide was separated by gel-permeation chromatography on Sephadex G-50 and lyophilised (8% of the LPS, by mass). Monosaccharide analysis of the polysaccharide

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Table 1
^{13}C NMR data [a] (in ppm, 90.6 MHz, ^2H_2O) of the isolated polysaccharide from LPS of *C. violaceum* NCTC 9694

C-1	C-2	C-3	C-4	C-5	C-6	C-7
Unit **A** (α-rhamnose)						
100.2	77.4	71.0	73.3	71.0	18.0	
Unit **B** (β-heptose)						
105.0	72.1	74.8	76.3	74.6	70.2	64.5 [b]
Unit **C** (α-glucosamine)						
99.7	54.3	81.0	69.7	73.1	61.7	
Unit **D** (α-heptose)						
98.9	69.6	70.2	77.9	71.6	70.0	64.3 [b]

[a] Other signals: *N*-acetyl, 23.4, 175.5 ppm.
[b] Assignment interchangeable.

identified rhamnose, glucosamine, and heptose in molar ratios of ~1:1:2. The heptose alditol possessed a retention time identical to that obtained for L-*glycero*-D-*manno*- and D-*glycero*-D-*galacto*-heptose which on reduction yield the same alditol. The absolute configurations of the components were shown by gas–liquid chromatography (GLC) of the acetylated (*R*)-2-butyl (heptose and glucosamine) and (*R*)-2-octyl (rhamnose) glycosides to be L-rhamnose, D-glucosamine, and D,D-heptose, indicating the presence of D-*glycero*-D-*galacto*-heptose in the O-antigenic polysaccharide. Methylation analysis yielded 1,2,5-tri-*O*-acetyl-6-deoxy-3,4-di-*O*-methyl-L-(1-^{2}H)mannitol, 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-(1-^{2}H)glucitol, and 1,4,5-tri-*O*-acetyl-2,3,6,7-tetra-*O*-methyl-D-*glycero*-D-*galacto*-(1-^{2}H)heptitol; thus, rhamnose was substituted at O-2, glucosamine at O-3, and both heptose residues presumably at O-4.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using ^{1}H,^{1}H-correlation spectroscopy (COSY), COSY with one-step (RCT) and two-step coherence transfer (RCT2), and ^{1}H,^{13}C-heteronuclear COSY methods (Table 1 and Table 2). The spectra corresponded to a regular polymer with a tetrasaccharide repeating unit; the total number of signals corresponded to the presence of two hexoses and two heptoses. One of the hexoses is an *N*-acetylated amino sugar (^{13}C NMR, C-2 54.3 ppm, *N*-acetyl group signals at 23.4 and 175.5 ppm; ^{1}H NMR, *N*-acetyl group at 1.98 ppm) while the second hexose is a 6-deoxy sugar (^{13}C NMR, C-6 18 ppm; ^{1}H NMR, H-6 1.25 ppm). The vicinal proton coupling constants of H-2–H-5 of both heptose residues established their *galacto* configuration; the D configuration at C-6 was confirmed by the large coupling constant of 9 Hz between H-5 and H-6 (in L-*glycero*-D-*manno*-heptose, $J_{5,6}$ is ~1 Hz). One heptose residue was linked in α ($J_{1,2}$ 3.5 Hz), the other in β ($J_{1,2}$ 8 Hz), and glucosamine in α configuration ($J_{1,2}$ 3.5 Hz, C-2 at 54.3 ppm). The linkage of the rhamnose residue was α, as indicated by the low-field position of its H-1 signal (5.29 ppm).

To establish the sequence of monosaccharides, one-dimensional nuclear Overhauser effect (NOE) experiments were performed with sequential pre-irradiation of all anomeric protons (Table 3, Fig. 1). The expected intraresidual NOE as well as changes in the intensity of some signals (H-3 in case of α-sugars) due to spin diffusion were observed. The following interresidue NOE signals were observed: between H-1 of the rhamnose residue (unit **A**, see formula below) and H-4 of the β-heptose residue **B** and H-1 of the α-heptose residue **D**,

Table 2
^1H NMR data [a] (in ppm, 360 MHz, ^2H_2O) and coupling constants (in Hz) of the isolated polysaccharide from LPS of C. violaceum NCTC 9694

H-1 $J_{1,2}$	H-2 $J_{2,3}$	H-3 $J_{3,4}$	H-4 $J_{4,5}$	H-5 $J_{5,6}$	H-6	H-7a	H-7b
Unit **A** (α-rhamnose)							
5.29	4.08	3.87	3.45	3.75	1.25		
br s	2.5	9	9	6.5			
Unit **B** (β-heptose)							
4.44	3.50	3.74	4.16	3.53	3.77	3.60	3.76
7.5	10	2	<1	9			
Unit **C** (α-glucosamine)							
5.03	4.09	3.86	3.59	4.20	3.74 [b], 3.7 [c]		
3.5	10	10	9				
Unit **D** (α-heptose)							
5.01	3.84	3.95	4.17	4.03	3.69	3.60	3.76
3.5	10.5	3.5	<1	9			

[a] Other signals: *N*-acetyl, 1.98 ppm.
[b] H-6a.
[c] H-6b.

proving the sequence **D**-(1-2)-**A**-(1-4)-**B**; between **B**1 and **C**3, due to the glycosylation at O-3 of the glucosamine **C** by β-heptose (**B**); between **D**1 and **A**1, **A**2, and **B**4, again indicating the presence of the fragment **D**-(1-2)-**A**-(1-4)-**B**, in which the protons **D**1 and **B**4 are in close spatial contact over one sugar residue **A**. Thus, the structure of the repeating unit of *C. violaceum* NCTC 9694 O-antigenic polysaccharide was established as

$$\underset{\mathbf{A}}{2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 4)}\text{-}\underset{\mathbf{B}}{\beta\text{-Hep}p\text{-}(1\rightarrow 3)}\text{-}\underset{\mathbf{C}}{\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)}\text{-}\underset{\mathbf{D}}{\alpha\text{-Hep}p\text{-}(1\rightarrow}$$

Heptoses are typical constituents of LPS, in most cases occurring in the core region in the L-*glycero*-D-*manno* or D-*glycero*-D-*manno* configuration [14]. However, L-*glycero*-D-*manno*-heptose has also been identified as a constituent of the O-specific polysaccharide of strain L of *Pseudomonas cepacia* [15], and D-*glycero*-D-*manno*-heptose is a constituent of the O-3 [16] and O-21 [17] antigens of *Vibrio cholerae*. In addition, D-*glycero*-D-*altro*-heptose has been found in the O-23 and O-36 antigens of *Campylobacter jejuni* [18]. So

Table 3
NOE signals observed on pre-irradiation of anomeric protons

Irradiation of H-1 of unit	NOE observed on unit, proton number			
	A	**B**	**C**	**D**
A (α-rhamnose)	2	3,4,5		1,2
B (β-heptose)		2,3	3	
C (α-glucosamine)			2,3	4,3
D (α-heptose)	1,2	4		2,3

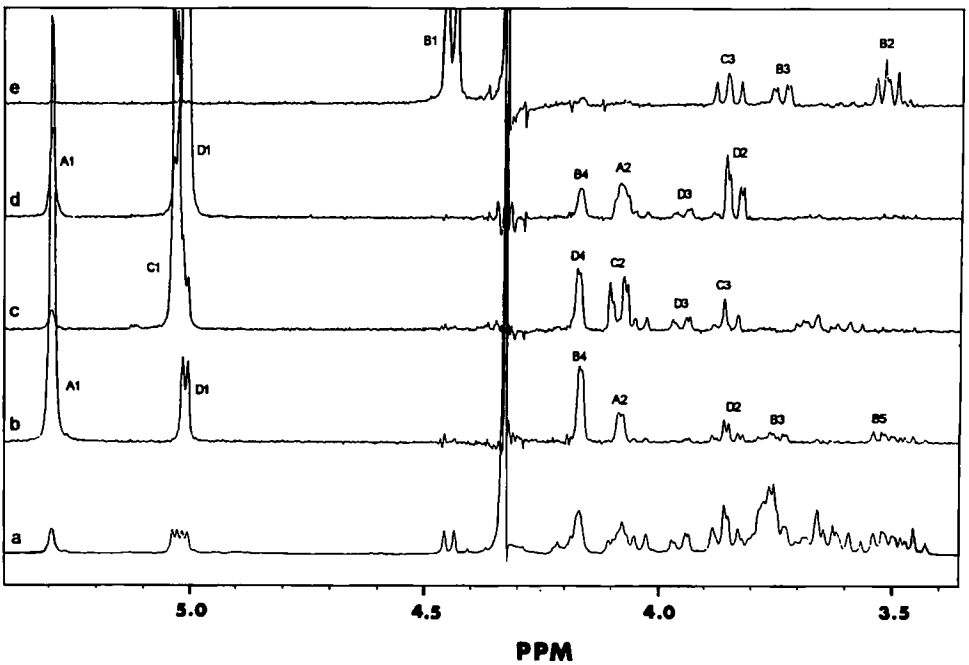

Fig. 1. a, One-dimensional ^1H NMR spectrum (360 MHz, ^2H_2O) of the O-specific polysaccharide. b–e, One-dimensional NOE spectra with pre-irradiation of H-1 of residue **A** (b), **C** (c), **D** (d), and **B** (e).

far, D-*glycero*-D-*galacto*-heptose has been identified only in LPS of the Gram-negative bacterium *C. violaceum*; it is, however, a typical component of the cell-wall antigenic polysaccharides of the Gram-positive microorganism *Eubacterium saburreum* [19–21]. Since D-*glycero*-D-*galacto*-heptose gives upon alditol acetate preparation the same product as L-*glycero*-D-*manno*-heptose, it cannot be excluded that this sugar is also present in other LPS which were compositionally analysed by GLC of alditol acetates.

1. Experimental

Bacteria and bacterial LPS.—*Chromobacterium violaceum* was grown in a fermenter (14 L), and the cells were killed with phenol and centrifuged. The bacteria were washed successively with EtOH, acetone (twice), and ether, then dried. The LPS was isolated (3%) from dry bacteria by a modified phenol–$CHCl_3$–light petroleum method [22].

General methods.—The conditions for NMR spectroscopy, GLC, GLC–mass spectrometry (MS), gel-permeation chromatography, monosaccharide analysis, and determination of the absolute configuration were as described [23]. All NMR spectra were measured in D_2O at 323 K.

Methylation analysis.—Methylation of the polysaccharide (1 mg) was performed according to Ciukanu and Kerek [24]. The methylated product was isolated by CH_2Cl_2–water extraction, then hydrolysed (2 M CF_3CO_2H, 100°C, 1 h), and the sugars were converted into alditol acetates and analysed by GLC–MS.

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