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Note

Structure of the O-specific polysaccharide isolated from the lipopolysaccharide of *Citrobacter gillenii* serotype O12a,12b strain PCM 1544

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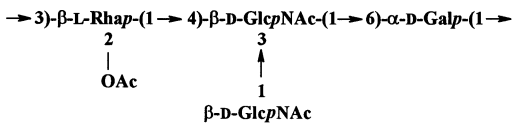
Abstract

A neutral O-specific polysaccharide was isolated from the lipopolysaccharide of *Citrobacter gillenii* strain PCM 1544, representing serotype O12a,12b. The polysaccharide was studied by sugar and methylation analyses and Smith degradation along with ^{1}H and ^{13}C NMR spectroscopy, including a ROESY experiment. The following structure of the tetrasaccharide repeating unit was established, in which substitution with terminal GlcNAc is ~60%.



Keywords: Citrobacter; Enterobacteria; O-antigen; Lipopolysaccharide; Structure

Gram-negative bacteria of the genus *Citrobacter* of the family *Enterobacteriaceae* are widely distributed in nature and found in soil, water, sewage and food, as well as in human and animal intestine.[1] *Citrobacter* strains may cause opportunistic infections, including urinary and respiratory tract infections, especially in immunocompromised host,[2] and are also associated with meningitis, brain abscesses, and neonatal sepsis.[3,4] At present, strains of the genus *Citrobacter* are divided into 11 species (Ref. 5 and refs. cited therein) and 42 O-serogroups.[6,7] Some *Citrobacter* strains are serologically related to other bacteria, including *Escherichia coli*,[8,9] *Salmonella*,[10] *Hafnia alvei*,[11,12] and other bacteria.[6,10,13]

Comparative studies on the chemical composition of the cell wall O-antigenic lipopolysaccharides (LPS) of *Citrobacter* strains

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from different O-serogroups enabled their classification into 20 chemotypes.[13] Aiming at creation of the molecular basis for classification of *Citrobacter* strains and substantiation of their serological cross-reactivity, structures of the O-specific polysaccharide chains of LPS of more than 20 serologically different *Citrobacter* strains have been established.[9,14–16] A number of the polysaccharides were found to contain uncommon components, such as 4-deoxy-D-*arabino*-hexose,[14,17] 3-amino-3,6-dideoxy-D-galactose,[11,14,18] 4-amino-4,6-dideoxy-D-mannose (perosamine),[9] neuraminic acid,[19] and glycerol phosphate.[14]

The aim of this study was to elucidate the structure of the O-antigen of *Citrobacter gillenii* (genotype 10^5) strain PCM 1544, which is a representative of serotype O12.

On mild acid hydrolysis, LPS of *C. gillenii* PCM 1544 released the carbohydrate portion, which was fractionated by GPC on Sephadex G-50 to give four fractions. A high-molecular-mass fraction P1 (41.3%) corresponded to the O-specific polysaccharide. Fraction P2 (3.7%) represented the core oligosaccharide substituted with a short O-chain polysaccharide, and fraction P3 (36.4%) the core oligosaccharide. The last fraction was 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) released from LPS.

Chemical and enzymatic analyses of the O-specific polysaccharide revealed rhamnose, D-galactose, D-glucosamine, and *O*-acetyl groups as the main components in the molar ratios of 0.8:1.0:1.9:0.8, respectively. The L configuration of rhamnose was determined by the glycosylation effect values in the ^{13}C NMR spectra.[20]

Methylation analysis was performed using three different hydrolysis conditions (Table 1) necessary to achieve the optimal release of partially methylated derivatives of either rhamnose or GlcN. The polysaccharide was found to contain 3-substituted rhamnose, 6-substituted galactose, terminal GlcN, 4-substituted GlcN, and 3,4-disubstituted GlcN.

Periodate oxidation of the polysaccharide destroyed galactose and a part of the GlcN residues, whereas rhamnose was stable. Fractionation of the Smith degraded products on BioGel P-2 gave two oligosaccharides, OS-I (37.6%) and OS-II (23.6%), both containing rhamnose and GlcN. Methylation analysis (Table 1) showed that OS-II is a disaccharide composed of terminal rhamnose and 4-substituted GlcN, whereas OS-I, having a higher molecular mass, contained terminal rhamnose and 3,4-disubstituted GlcN. Formation of OS-I, together with the expected OS-II, could be accounted for by the ability of the oxidised terminal GlcN to sustain hydrolysis with 2% AcOH (100 °C, 1 h) following Smith degradation. A similar behaviour in these conditions was reported for a degraded lateral GlcNAc residue attached at position 3 of a 3,4-disubstituted GalNAc residue, which could not be

Table 1
Methylation analysis data. Retention time of the alditol acetates is related to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (2,3,4,6-Me_4Glc) [a]

Partially methylated sugar	Relative retention time	GLC detector response related to 2,3,4-Me_3Rha or 2,4-Me_2Rha								
		O-specific polysaccharide			$NaIO_4$ oxidised polysaccharide		OS-I		OS-II	
		CF_3CO_2H	HCl	H_2SO_4	CF_3CO_2H	HCl	CF_3CO_2H	HCl	CF_3CO_2H	HCl
2,3,4-Me_3Rha	0.68						1.0	1.0	1.0	1.0
2,4-Me_2Rha	0.92	1.0	1.0	1.0	1.0	1.0				
2,3,4-Me_3Gal	1.36	1.2	1.4	2.0						
3,4,6-Me_3GlcN	1.68	0.9	1.1	1.8						
3,6-Me_2GlcN	1.84	0.5	0.5	1.2	0.5	1.0			2.4	3.5
6-MeGlcN	2.00	0.9	0.9	2.0	0.9	1.8	1.8	2.6		

[a] For conditions of hydrolysis with CF_3CO_2H, HCl, and H_2SO_4 see Section 1.

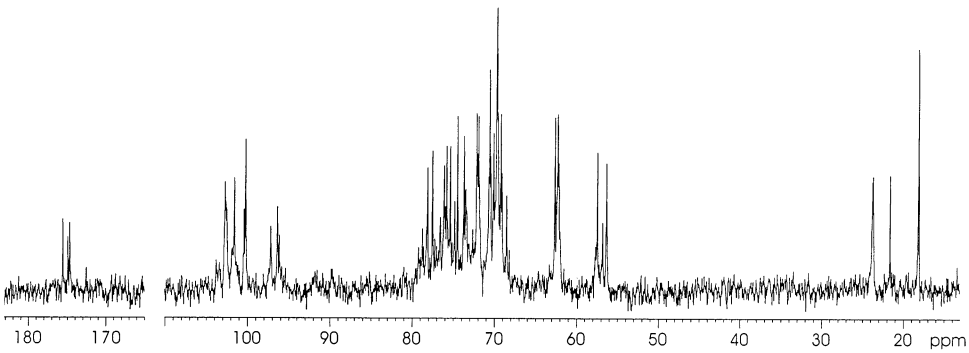

Fig. 1. 125 MHz ^{13}C NMR spectrum of the polysaccharide.

Table 2
500 MHz ^{1}H NMR chemical shifts (δ in ppm). Additional chemical shifts for NAc groups are δ 2.01–2.08

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b			
O-Deacetylated polysaccharide										
Repeating unit **1**										
→3)-β-L-Rha*p*-(1→(**A**)	4.90	4.26	3.64	3.48	3.48	1.32				
→4)-β-D-Glc*p*NAc-(1→(**B**)	4.52	3.70	3.70	3.69	3.53	3.93	3.75			
→6)-α-D-Gal*p*-(1→(**C**)	5.10	3.85	3.93	3.97	4.30	3.98	3.72			
Repeating unit **2**										
→3)-β-L-Rha*p*-(1→(**A**)	4.89	4.41	3.64	3.48	3.48	1.32				
→3,4)-β-D-Glc*p*NAc-(1→(**B**)	4.46	3.80	4.03	3.70	3.53	3.84	3.82			
→6)-α-D-Gal*p*-(1→(**C**)	5.10	3.86	3.93	3.97	4.22	3.98	3.69			
β-D-Glc*p*NAc-(1→(**D**)	4.57	3.59	3.59	3.37	3.39	3.95	3.71			
	H-1a	H-1b	H-2	H-3a	H-3b	H-4a	H-4b	H-5	H-6a	H-6b
Oligosaccharide-glycerol **3** (OS-II)										
β-L-Rha*p*-(1→	4.87		4.05	3.53		3.34		3.37	1.40	
→4)-β-D-Glc*p*NAc-(1→	4.51		3.70	3.68		3.68		3.50	3.92	3.83
→1)-Gro	3.92	3.60	3.84	3.49	3.52					
Oligosaccharide-glycerol **4** (OS-I)										
β-D-Glc*p*NAc-(1→ [a]	4.85		4.21	3.59	3.49	3.70 [b]	3.60 [b]	3.78	3.73 [b]	3.73 [b]
β-L-Rha*p*-(1→	4.82		4.12	3.42		3.37		3.37	1.31	
→3,4)-β-D-Glc*p*NAc-(1→	4.54		3.80	3.96		3.75		3.53	3.86	3.93
→1)-Gro	3.92	3.60	3.84	3.60	3.54					

[a] The oxidised residue (see R in Fig. 3).
[b] Tentative assignment.

eliminated without cleavage of the glycosidic linkages of non-degraded sugars.[21]

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for *N*-acetyl and *O*-acetyl groups (CH_3 at δ 23.6–23.7 and 21.6 ppm, respectively; CO at δ 174.8, 175.0, and 175.7 ppm), and a number of sugar carbon signals having different integral intensities. After O-deacetylation with aqueous ammonia, the ^{13}C NMR spectrum showed the same level of heterogeneity.

The ^{1}H and ^{13}C NMR spectra of the O-deacetylated polysaccharide were assigned using COSY, TOCSY, and ^{1}H and ^{13}C HSQC experiments (Tables 2 and 3). Repeating units of two types were revealed. One is a trisaccharide including one residue each of rhamnose (unit **A**), GlcNAc (unit **B**), and galactose (unit **C**), and the other is a tetrasaccharide containing one GlcNAc residue (unit **D**) more.

A ROESY experiment (Fig. 2) showed H-1,H-3 and H-1,H-5 cross-peaks for units **A**, **B**, and **D**, and thus demonstrated their β configuration, whereas the absence of such correlations for unit **C** indicated its α configuration. The glycosylation pattern and sugar sequence in both repeating units were determined using the same ROESY experiment, which showed

correlations between all pairs of the transglycosidic protons (Fig. 2). The H-1 signal of unit **C** gave an additional interresidue cross-peak with H-2 of unit **A**, which is typical of the D-Gal*p*-(α1→3)-L-Rha*p* disaccharide.[22]

Table 3
125 MHz ^{13}C NMR chemical shifts (δ in ppm). Additional chemical shifts for NAc groups are δ 23.2–23.8 (CH_3) and 175.0–176.2 (CO)

	C-1	C-2	C-3	C-4	C-5	C-6
O-Deacetylated polysaccharide						
Repeating unit **1**						
→3)-β-L-Rha*p*-(1→(**A**)	101.7	68.7	79.2	71.7	73.4	18.1
→4)-β-D-Glc*p*NAc-(1→(**B**)	102.6	56.9	74.8	78.3	75.9	62.0
→6)-α-D-Gal*p*-(1→(**C**)	97.2	69.5	70.5	70.1	70.1	69.3
Repeating unit **2**						
→3)-β-L-Rha*p*-(1→(**A**)	102.4	68.1	79.0	71.7	73.4	18.1
→3,4)-β-D-Glc*p*NAc-(1→ (**B**)	102.7	56.4	78.2	77.0	75.6	62.0
→6)-α-D-Gal*p*-(1→(**C**)	97.2	69.5	70.6	69.8	69.9	68.9
β-D-Glc*p*NAc-(1→(**D**)	101.9	57.4	74.4	71.7	77.2	62.4
Oligosaccharide-glycerol **3** (OS-II)						
β-L-Rha*p*-(1→	102.2	72.1	74.1	73.4	73.7	18.0
→4)-β-D-Glc*p*NAc-(1→	103.1	57.1	74.9	78.2	76.0	62.2
→1)-Gro	72.2	71.9	63.8			
Oligosaccharide-glycerol **4** (OS-I)						
β-D-Glc*p*NAc-(1→ [a]	103.9	55.9	61.4	62.6	82.3	62.6
β-L-Rha*p*-(1→	102.1	71.1	74.0	72.8	73.3	17.7
→3,4)-β-D-Glc*p*NAc-(1→	102.2	56.4	78.4	76.35	75.4	61.7
→1)-Gro	71.9	71.4	63.4			

[a] The oxidised residue (see R in Fig. 3).

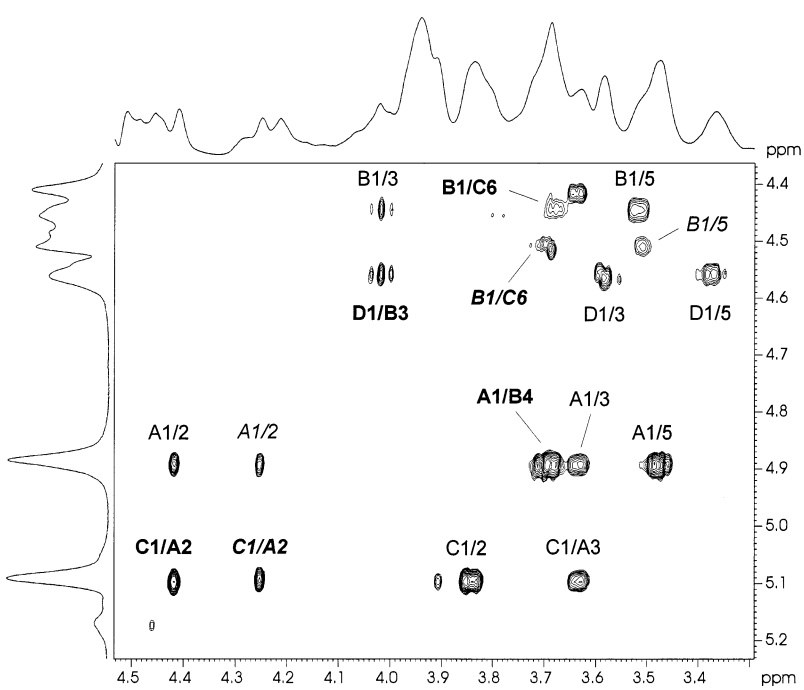

Fig. 2. Part of a 500 MHz ROESY spectrum of the O-deacetylated polysaccharide. The corresponding parts of the ^1H NMR spectrum are displayed along the axes. Arabic numerals refer to protons in sugar residues denoted as shown in Table 2. Designations for the repeating unit **1** are italicised; cross-peaks between the transglycosidic protons are shown in bold.

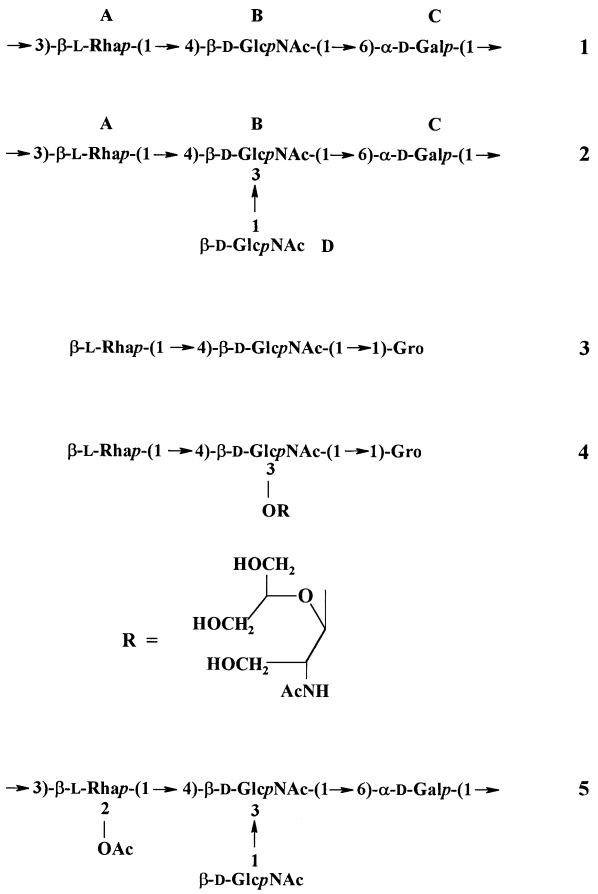

Fig. 3. Structures of the repeating units **1** and **2** of the O-deacetylated polysaccharide, the oligosaccharide-glycerols **3** and **4** derived by Smith degradation, and the O-specific polysaccharide **5**. In **5**, the degree of substitution with terminal GlcNAc is ~60%

The ^{13}C NMR chemical shift data (Table 3), compared to published data for the corresponding monosaccharides,[23] confirmed the anomeric configurations and the modes of sugar substitution. The latter followed from downfield displacements of the signals for the linkage carbons and were in agreement with methylation data (see above).

On the basis of these data, the structures **1** and **2** could be established for the two repeating units of the O-deacetylated polysaccharide (Fig. 3). They are present in the ratio ~4:6, which followed from the relative intensities of the ^{13}C NMR signals, in particular, of the signals for C-1 of unit **A** at 101.7 and 102.4 ppm in repeating units **1** and **2**, respectively. It is not clear if repeating units of both types are present within one polysaccharide chain, and thus a partial substitution of unit **B** with unit **D** occurs, or there are two different polysaccharide chains, one consisting of repeating units **1** and the other of repeating units **2**.

The structures **3** and **4** of the Smith degradation products OS-II and OS-I, respectively, (Fig. 3) were established by similar NMR spectroscopic studies (Tables 2 and 3). In particular, the presence of the fragments of the degraded unit **D** in OS-I was confirmed by the additional signals for three $HOCH_2$–C groups at δ 61.4 and 62.6, one carbon bearing nitrogen at δ 55.9, and one *N*-acetyl group, compared to OS-II. The oligosaccharide structures were in full agreement with the proposed structures **1** and **2** of the polysaccharide.

Comparison of the ^1H NMR spectra of the O-deacetylated and initial polysaccharides showed a significant displacement of the signal for H-2 of unit **A** from δ 4.26 and 4.41 ppm in the repeating units **1** and **2** in the former to δ 5.62 and 5.65 ppm in the latter. This displacement is due to the deshielding effect of the *O*-acetyl group, and hence the rhamnose residue is O-acetylated at position 2. The ratio of intensities of the H-2 signals confirmed the presence of the repeating units **1** and **2** in the ratio ~4:6.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *C. gillenii* O12a,12b strain PCM 1544 has structure **5** shown in Fig. 3. This structure is unique among the bacterial O-antigen structures. The polysaccharide studied is neutral, which is typical of O-antigens of *Citrobacter*.[14,15]

1. Experimental

Bacterial strain and isolation of the O-specific polysaccharide.—A representative strain of *Citrobacter gillenii* of serotype O12a,12b:35,36 (*C. freundii* PCM 1544, IHE Be 73/57, Bonn 16824[6,13,24]) was obtained from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Cultivation of bacteria and isolation of LPS in a yield of 2.3% by phenol–water extraction[25] were performed as described.[26] Mild acid hydrolysis of LPS with 1% HOAc (100 °C, 75 min) followed by fractionation of a water-soluble portion

(58% of LPS weight) by GPC on a column (2 × 100 cm) of Sephadex G-50 resulted in the O-specific polysaccharide in a yield of 0.85% of dry bacterial mass weight.

General methods.—Sugar analysis, determination of the absolute configurations of the monosaccharides, O-deacetylation, and Smith degradation were performed as described earlier.[26,27] *O*-Acetyl groups were assayed by the method of Hestrin.[28]

Methylation analysis was performed by the procedure of Gunnarsson,[29] and the methylated product was hydrolysed with 10 M HCl (80 °C, 30 min), 2 M CF_3CO_2H (120 °C, 2 h), or 0.25 M H_2SO_4 in 95% HOAc (80 °C, 18 h). Partially methylated sugars were converted into the alditol acetates and analysed by GLC–MS on a Hewlett–Packard 5971A system using a HP-1 glass capillary column (0.22 mm × 12 m) and a temperature program of 150–270 °C at 8 °C min^{-1}.

^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer for solutions in D_2O at 42 and 30 °C for the polysaccharides and oligosaccharides, respectively. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45 ppm) as reference. A mixing time of 100 and 200 ms was used in TOCSY and ROESY experiments, respectively.

Acknowledgements

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