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Structure of a new 2-deoxy-2-[(*R*)-3-hydroxybutyramido]-D-glucose-containing O-specific polysaccharide from the lipopolysaccharide of *Citrobacter gillenii* PCM 1542

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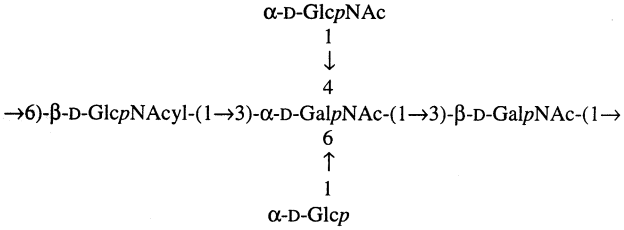
Abstract

The O-specific polysaccharide of *Citrobacter gillenii* PCM 1542 from serotype O-12a,12b is composed of one residue each of D-glucose, D-GlcNAc, 2-deoxy-2-[(*R*)-3-hydroxybutyramido]-D-glucose (D-GlcNAcyl) and two GalNAc residues. On the basis of sugar and methylation analyses of the intact and Smith degraded polysaccharides, along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, the following structure of the branched pentasaccharide repeating unit of the O-specific polysaccharide was established:



This structure differs significantly from that of the O-specific polysaccharide of *C. gillenii* PCM 1544 from the same serotype O-12a,12b, which has been established earlier (Kübler-Kiełb, J. et al. *Carbohydr. Res.* **2001**, *331*, 331–336). Serological studies confirmed that the two O-antigens are not related and suggested that strains PCM 1542 and 1544 should be classified into different O-serogroups.

Keywords: *Citrobacter gillenii*; Enterobacteria; O-antigen; Lipopolysaccharide; Bacterial polysaccharide structure; Serological classification; ^{1}H,^{13}C NMR

1. Introduction

Strains of the genus *Citrobacter* from the family *Enterobacteriaceae* are widespread in the environment, including soil, sewage and food, and are normal inhabitants of animal and human intestine.[1] *Citrobacter* is an opportunistic human pathogen causing urinary and respiratory tract infections;[2] incidents of meningitis, brain abscesses, and neonatal sepsis caused by *Citrobacter* have also been reported.[3,4]

At present, the genus *Citrobacter* that was originally described as *Citrobacter freundii* (or Bethesda–Ballerup group), is divided into 11 species.[5] *Citrobacter* is an

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antigenically heterogenous group of bacteria. The first O-antigen-based classification scheme of *Citrobacter* strains was established by West and Edwards in 1954[6] and has been significantly revised recently.[7,8] Strains of *Citrobacter* were classified into 42 O-serogroups.[9,10] O-antigens of a number of *Citrobacter* strains are serologically and structurally related to other enteric bacteria, such as *Escherichia coli*,[11,12] *Hafnia alvei*,[13–15] *Salmonella*,[16] and others.[9,16,17]

O-antigens of *Citrobacter* strains belong to at least 20 chemotypes.[17] Structures of more than 20 O-specific polysaccharides (O-antigens) from the lipopolysaccharides of various *Citrobacter* serogroups have been established, and the chemical basis of the serological cross-reactivity of some *Citrobacter* strains with the other bacterial genera was elucidated (Refs. 15 and 18 and references cited therein). The most *Citrobacter* O-antigens studied are built up of mono- to heptasaccharide repeating units containing neutral sugars and amino sugars; *N*-acetylneuraminic acid[19] and glycerol phosphate[20] were found in one strain each. Strains of some serogroups, e.g., O-27, O-4, and O-36, differ in the lipopolysaccharide core structures, whereas their O-specific polysaccharides are structurally identical.[21]

Now we report on the structure of the O-specific polysaccharide of *Citrobacter gillenii* PCM 1542 (former *C. freundii* PCM 1542), which belongs to the same serotype O-12a,12b as *C. gillenii* strain PCM 1544 investigated by us earlier.[18] The finding that the chemical composition of the two polysaccharides is different prompted us to elucidate also the structure of the O-antigen of *C. gillenii* PCM 1542.

2. Results and discussion

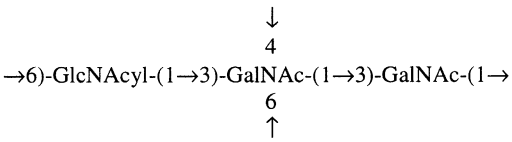
Isolation and chemical analyses of the polysaccharide.—The lipopolysaccharide was isolated from dried bacteria by phenol–water extraction in a yield of 3.4%. Hydrolysis of the lipopolysaccharide with 1% HOAc (100 °C, 1.5 h) followed by centrifugation gave a carbohydrate-containing supernatant (46% of the lipopolysaccharide weight) and a lipid A sediment. The material present in the supernatant was fractionated by GPC on Sephadex G-50 to the O-specific polysaccharide (fraction P_1, 16.5%), core oligosaccharide (fraction P_3, 58%), and a fraction containing 3-deoxyoct-2-ulosonic acid (fraction Kdo, 25.5%).

Sugar analysis of the polysaccharide using GLC–MS of the alditol acetates revealed glucose, glucosamine and galactosamine in the molar ratios 1.3:2.0:2.2 or 0.93:1.0:0.6 when 10 M HCl (80 °C, 30 min) or 2 M CF_3CO_2H (120 °C, 2 h) was used for hydrolysis of the polysaccharide, respectively.

The D configuration of glucose, galactosamine and glucosamine was determined by treatment of the polysaccharide hydrolysate with D-glucose oxidase, D-galactose oxidase, and hexokinase, respectively. The content of D-glucose and D-galactosamine was estimated as 0.94 and 1.7 μmol/mg (17 and 30%, respectively). D-Glucosamine was phosphorylated completely by hexokinase in the presence of ATP as shown by GLC–MS. The *R* configuration of 3-hydroxybutyric acid (0.8 μmol/mg) linked to a glucosamine residue (see below) was determined using (*R*)-3-hydroxybutyrate dehydrogenase.

Methylation analysis was carried out using four different hydrolysis conditions to establish the proper proportions of the polysaccharide constituents (Table 1). It was found that the repeating unit of the polysaccharide is a branched pentasaccharide containing one residue each of terminal glucose, terminal GlcNAc, 3-substituted GalNAc, 3,4,6-trisubstituted GalNAc, and 6-substituted GlcNAcyl, where Acyl is 3-hydroxybutyryl. Remarkably, derivatives from neither 3-substituted nor 3,4,6-trisubstituted GalNAc residues could be released by hydrolysis with CF_3CO_2H but by hydrolysis with HCl or solvolysis with anhyd HF. In GLC–MS, 3,4-Me_2GlcNMeAcyl derived from GlcNAcyl was identical to that from the O-specific polysaccharide of *H. alvei* ATCC 13337, which also contains 6-substituted GlcNAcyl.[22] It should be noted that the 3-hydroxybutyryl group was cleaved completely during hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 30 min) and 3,4-Me_2GlcNMeAc was detected instead of 3,4-Me_2GlcNMeAcyl. The 3-hydroxybutyryl group could be preserved and 3,4-Me_2GlcNMeAcyl identified only when solvolysis with anhyd HF was used for depolymerisation of the methylated polysaccharide.

The polysaccharide was oxidised with $NaIO_4$, then reduced with $NaBH_4$, and the oxidised material was hydrolysed with aq 2% HOAc (100 °C, 2 h). The products were fractionated by GPC on BioGel P-2 to give Smith-degraded products eluted with the volume characteristic for octa- to decasaccharides. Prolonged hydrolysis with 2% HOAc (100 °C, 4 h) gave a smaller oligosaccharide. Methylation analysis of $NaIO_4$-oxidised polysaccharide, Smith-degraded polysaccharide, and the oligosaccharide (Table 1) showed that both terminal sugars (GlcNAc and Glc) and 6-substituted GlcNAcyl were oxidised completely. Hydrolysis of the oxidised polysaccharide for a shorter time cleaved completely only the linkage of the oxidised terminal sugar attached at position 6 of 3,4,6-trisubstituted GalNAc. In contrast, hydrolysis for a longer time resulted in nearly complete cleavage of all oxidised sugars. Similar results of Smith degradation have been described for other polysaccharides containing a 3,4-disubstituted *N*-acetylamino sugar.[18,23] The Smith degradation data showed the presence in the polysaccharide of the following sequence:



NMR spectroscopic studies and structure of the polysaccharide.—The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for five anomeric carbons in the region δ 93.9–104.4, five CH_2O groups (C-6), of which three are nonsubstituted (δ 61.7–62.1) and the other two substituted (δ 67.3 and 68.8, data of attached-proton test[24]), four nitrogen-bearing carbons (C-2) at δ 49.5–56.5, other sugar ring carbons in the region δ 64.4–77.0, one *N*-(3-hydroxybutyryl) (NAcyl) and three *N*-acetyl (NAc) groups (CH_3 at δ 23.0–23.6, CO at δ 175.0–175.2, C-2 and C-3 of NAcyl at δ 45.8 and 66.0, respectively).

The ^1H NMR spectrum of the polysaccharide contained signals for five anomeric protons in the region δ 4.50–5.08, H-2 and H-4 of NAcyl at δ 2.51 (d, $J_{2,3} \sim 6$ Hz) and 1.25 (d, $J_{3,4} \sim 6$ Hz), respectively, and three *N*-acetyl groups at δ 2.00, 2.08, and 2.14 (all s). These data confirmed the presence of a pentasaccharide repeating units containing four amino sugars, three of which are *N*-acetylated and one is *N*-acylated by a 3-hydroxybutyryl group.

The ^1H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, NOESY, and ^1H,^{13}C HSQC experiments (Tables 2 and 3). Spin systems of the sugars having the *gluco* (Glc and GlcN) and *galacto* (GalN) configurations were distinguished by typical coupling constant values,[25] estimated from the 2D NMR spectra, and the amino sugars were identified by correlation in the HSQC spectrum of protons at nitrogen-bearing carbons (H-2) to the corresponding carbons (C-2). As judged by relatively large $J_{1,2}$ coupling constant values of <4 Hz for the anomeric protons at δ 4.89–5.08, Glc and one residue each of GlcN and GalN are α-linked, whereas two remaining amino sugar residues are β-linked (δ 4.50 and 4.58, $J_{1,2} > 6$ Hz).[25]

In the ^{13}C NMR spectrum of the polysaccharide, the signals for C-3 of β-GalN, C-3,4,6 of α-GalN, and C-6 of β-GlcN were shifted downfield by 4.5–6.7 ppm, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides.[26] The chemical shifts for C-2,3,4,6 of α-Glc and α-GlcN were close to those of the nonsubstituted sugars.[26] These data defined the glycosylation pattern of the polysaccharide.

The sequence of the monosaccharides in the repeating unit was determined by a NOESY experiment. In addition to intraresidue cross-peaks (H-1,H-2 for the α-linked sugars, H-1,H-3 and H-1,H-5 for the β-linked sugars), the following interresidue cross-peaks between the anomeric and linkage protons were observed: β-GlcN H-1,α-GalN H-3; α-GalN H-1,β-GalN H-3 and H-4; β-GalN H-1,β-GlcN H-6a,6b; α-GlcN H-1,α-

Table 1
Methylation analysis data. GLC 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 2,3,4,6-Me_4Glc, 4,6-Me_2GalNMeAc, or 6-MeGalNMeAc						
		O-specific polysaccharide				$NaIO_4$ oxidised polysaccharide	Smith degradation products	
							Polysaccharide	Oligosaccharide
		A	B	C	D	B	B	B
2,3,4,6-Me_4Glc	1.00	1.0	1.0	1.0	1.0			
3,4,6-Me_3GlcNMeAc	1.69	1.4	1.4	1.1	1.3			
3,4,6-Me_3GalNMeAc	1.78						0.5	2.0
4,6-Me_2GalNMeAc	1.97		1.6[c]	1.6[c]	0.9	1.0	1.9	1.0
3,4-Me_2GlcNMeAc	1.98	0.8						
6-MeGalNMeAc	2.02						1.0	0.2
GalNMeAc	2.26		0.6	0.8	0.5	1.0		
3,4-Me_2GlcNMeAcyl[b]	2.44				0.6			

[a] Hydrolysis conditions: A, 2 M CF_3CO_2H, 120 °C, 2 h; B, 10 M HCl, 80 °C, 30 min; C, 10 M HCl, 100 °C, 30 min; D, anhyd HF, 20 °C, 3 h, followed by 1% HOAc, 100 °C, 30 min.

[b] Acyl stands for 3-methoxybutyryl.

[c] Value for a mixture of 4,6-Me_2GalNMeAc and 3,4-Me_2GlcNMeAc.

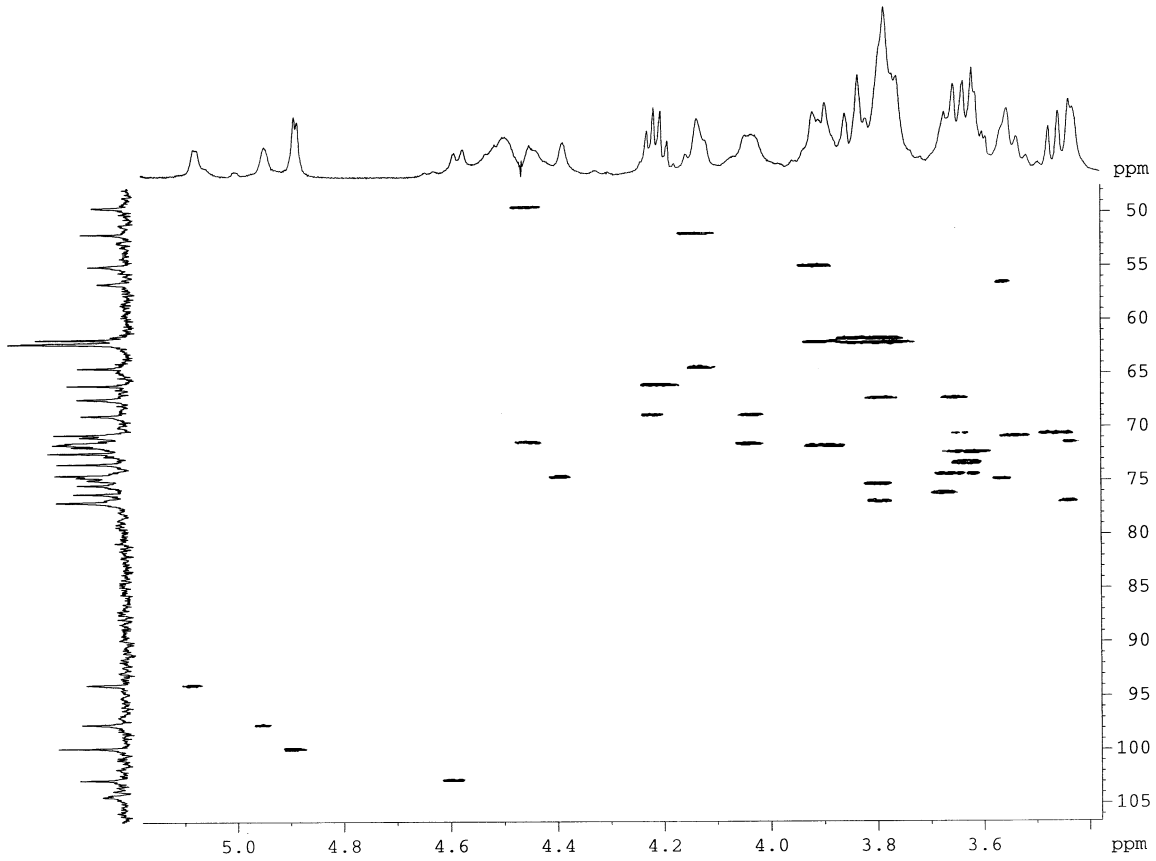


Fig. 1. Part of a $^{1}H,^{13}C$ HSQC spectrum of the O-specific polysaccharide of *C. gillenii* PCM 1542. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are shown along the vertical and horizontal axis, respectively.

Table 2
500-MHz ^{1}H NMR data of the O-polysaccharide (δ in ppm). The chemical shifts for NAc are δ 2.00–2.14, for H-2, H-3, and H-4 of NAcyl δ 2.51, 4.21, and 1.25, respectively

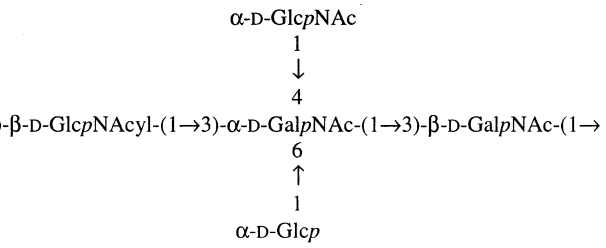
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→6)-β-D-Glc*p*NAcyl-(1→	4.50	3.57	3.56	3.44	3.44	4.22	4.04
→3,4,6)-α-D-Gal*p*NAc-(1→	5.08	4.46	3.81	4.40	4.04	3.79	3.65
→3)-β-D-Gal*p*NAc-(1→	4.58	4.14	3.80	4.14	3.68	3.78	3.82
α-D-Glc*p*NAc-(1→	4.95	3.91	3.90	3.54	4.45	3.78	3.91
α-D-Glc*p*-(1→	4.89	3.62	3.66	3.46	3.63	3.79	3.85

Table 3
125-MHz ^{13}C NMR data of the O-polysaccharide (δ in ppm). The chemical shifts for Me and CO of NAc and NAcyl are δ 23.0–23.6 and 175.0–175.2, for C-2 and C-3 of NAcyl δ 45.8 and 66.0, respectively

	C-1	C-2	C-3	C-4	C-5	C-6
→6)-β-D-Glc*p*NAcyl-(1→	104.4	56.5	74.9	71.4	77.0	68.8
→3,4,6)-α-D-Gal*p*NAc-(1→	93.9	49.5	75.3	74.6	71.6	67.3
→3)-β-D-Gal*p*NAc-(1→	102.8	51.9	76.9	64.4	76.1	62.1
α-D-Glc*p*NAc-(1→	97.6	54.9	71.8	70.9	71.4	62.0
α-D-Glc*p*-(1→	99.9	72.3	74.4	70.6	73.4	61.7

GalN H-4; and α-Glc H-1,α-GalN H-6a,6b at δ 4.50/3.81; 5.08/3.80 and 4.14; 4.58/4.04 and 4.22; 4.95/4.40, and 4.89/3.65 and 3.79, respectively.

Therefore, the NMR spectroscopic data were in agreement with methylation analysis and Smith degradation data and showed that the pentasaccharide repeating unit of the O-specific polysaccharide of *C. gillenii* PCM 1542 has the following structure:



As most other *Citrobacter* O-antigens, the O-specific polysaccharide of strain PCM 1542 is neutral. It contains an *N*-linked 3-hydroxybutyryl group, which is also a constituent of the O-specific polysaccharide of *C. freundii* O-41[13] and some other bacteria, e.g., *H. alvei*.[27]

Serological study.—The structure of the O-specific polysaccharide of *C. gillenii* PCM 1542 differs dramatically from that of *C. gillenii* PCM 1544 from the same serotype O-12a,12b,[8,17] which has been established earlier.[18] Therefore, we tested lipopolysaccharides of both strains in double immunodiffusion test with anti-*C. gillenii* PCM 1544 serum. The precipitation was observed only with the homologous lipopolysaccharide, whereas the lipopolysaccharide of strain PCM 1542 showed no reactivity. These finding suggested that the O-antigens of *C. gillenii* PCM 1542 and 1544, which belong to different chemotypes, are not related also serologically and should be classified in different O-serogroups.

3. Experimental

General methods.—Double immunodiffusion test[28] was carried out with anti-*C. gillenii* PCM 1544 serum obtained as described.[29] GPC was carried out on columns (2 × 100 cm) of Sephadex G-50, Sephadex G-25, and BioGel P-2 in pyridinium acetate buffer (pH 5.6) and monitored by the phenol–sulfuric acid reaction. GLC–MS was performed with a Hewlett–Packard 5971A instrument equipped with an HP-1 glass capillary column (12 m × 0.2 mm) using a temperature program of 150 → 270 °C at 8 °C/min.

NMR spectroscopy.—^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer for solutions in D_2O at 50 °C. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45 ppm) as reference. A mixing time of 150 and 200 ms was used in TOCSY and ROESY experiments, respectively.

Bacterial strain, isolation of lipopolysaccharide and O-specific polysaccharide.—A representative strain of *C. gillenii* O-12a,12b:57 (*C. freundii* PCM 1542, IHE Be 71/57, St 18597[8,9,17]) was obtained from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Bacteria were cultivated in a liquid medium,[30] and the lipopolysaccharide was isolated by phenol–water extraction.[31,32] The lipopolysaccharide was hydrolysed with 1% HOAc (100 °C, 1.5 h), and the carbohydrate portion was fractionated by GPC on Sephadex G-50 to give an O-specific polysaccharide in a yield of 7.3% of the lipopolysaccharide weight.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 30 min), monosaccharides were converted conventionally into the alditol acetates and analysed by GLC–MS. The absolute configurations of Glc, GalN, GlcN, and 3-hydroxybutyric acid were determined in the hydrolysates using D-glucose oxidase,[33] D-galactose oxidase,[34] hexokinase,[35] and (*R*)-3-hydroxybutyrate dehydrogenase,[36] respectively. The content of glucose was determined after hydrolysis with 2 M CF_3CO_2H (100 °C, 2 h), that of GalN and GlcN after hydrolysis with 4 M HCl (100 °C, 18 h), and 3-hydroxybutyric acid was quantified after hydrolysis with 4 M HCl (100 °C, 2.5 h).

Methylation analysis.—Methylation of the polysaccharides and oligosaccharide was carried out using the procedure of Gunnarsson,[37] and methylated products were hydrolysed with 10 M HCl (80 or 100 °C, 30 min) or 2 M CF_3CO_2H (120 °C, 2 h). Alternatively, the methylated polysaccharide was cleaved by solvolysis with anhyd HF (20 °C, 3 h) followed by hydrolysis with 1% HOAc (100 °C, 30 min). Partially methylated sugars were converted into the alditol acetates and analysed by GLC–MS.

Smith degradation.—The O-specific polysaccharide (11 mg) was oxidised with 0.1 M $NaIO_4$ (1.1 mL, 20 °C, 72 h), then ethylene glycol (0.05 mL) was added, the mixture was reduced with $NaBH_4$ (60 mg, overnight), neutralised with aq 50% HOAc, evaporated, and boric acid was removed by evaporation several times with MeOH, whereas the product was desalted by GPC on Sephadex G-25. The oxidised polysaccharide (6.5 mg) was hydrolysed with 2% HOAc (100 °C, 2 h) and fractionated by GPC on BioGel P2 to afford a Smith-degraded polysaccharide (2.6 mg). The degraded polysaccharide (0.8 mg) was further hydrolysed with 2% HOAc (100 °C, 4 h) to give an oligosaccharide, which was subjected to methylation analysis.

Acknowledgements

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