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Note

The structure of the O-specific polysaccharide of *Citrobacter freundii* O32, a partially *O*-acetylated homopolymer of 3,6-dideoxy-3-(L-glyceroylamino)-α-D-galactopyranose

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A number of O-specific polysaccharides of *Citrobacter*, a serologically highly heterogeneous genus of the family Enterobacteriaceae, have been structurally elucidated (ref. 1 and refs. cited therein). The structure of the O-antigen of a strain of *Citrobacter freundii* O32 was reported [2], but later this polysaccharide was found to belong to a different unknown contaminating microorganism (authors' unpublished data). We report now the structure of the true O-antigen of *C. freundii* O32.

Lipopolysaccharide was isolated from dried bacterial cells by extraction with aqueous phenol [3] and degraded with dilute acetic acid. After removal of a lipid precipitate, a water-soluble portion was separated by gel chromatography on Sephadex G-50 to give O-specific polysaccharide (PS-I).

The ^{13}C NMR spectrum of PS-I (Fig. 1a) contained a number of signals with different integral intensities, most likely, due to nonstoichiometric *O*-acetylation (a signal for Me of an *O*-acetyl group was present at 21.4 ppm). In fact, the spectrum of the polysaccharide *O*-deacetylated with aqueous ammonia (PS-II) was typical of

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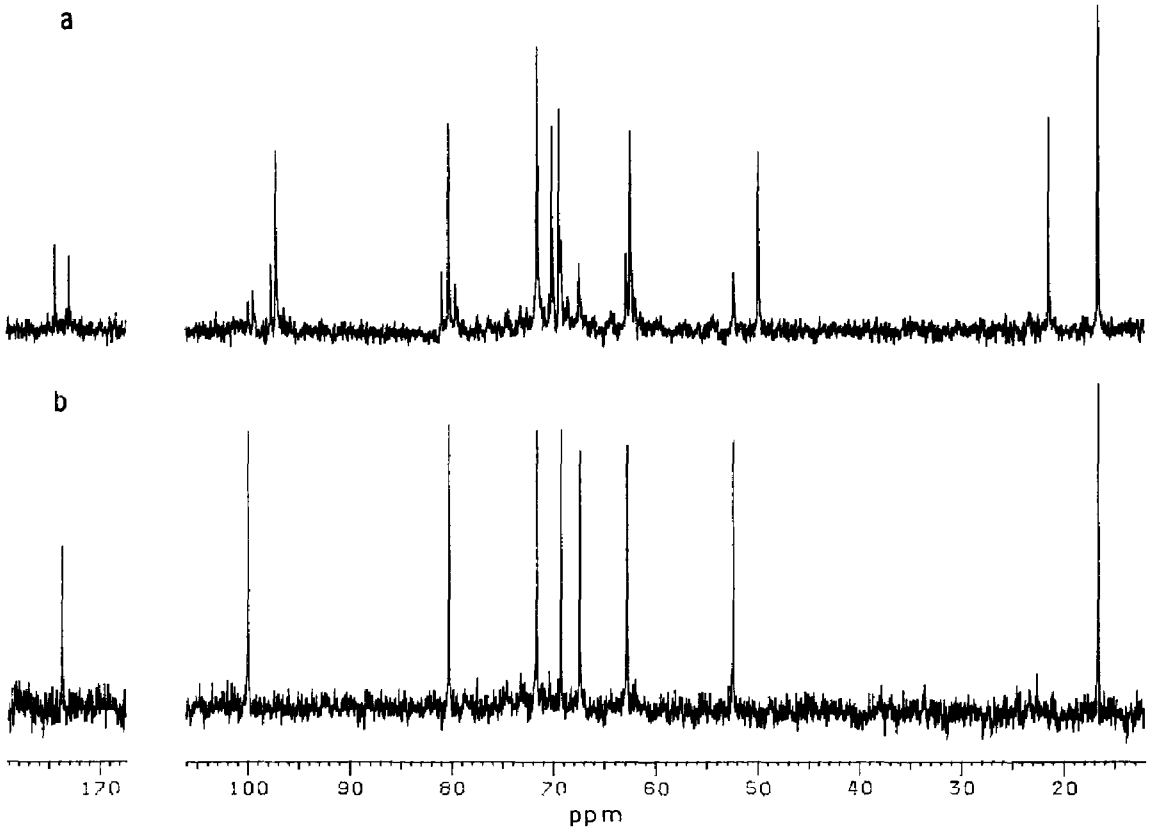


Fig. 1. 75-MHz ^{13}C NMR spectra of (a) O-specific polysaccharide of *C. freundii* O32 (PS-I) and (b) *O*-deacetylated polysaccharide (PS-II)

a regular polymer (Fig. 1b). It contained nine signals including the signals for one anomeric carbon at 99.9 ppm, one methyl group at 16.6 ppm, one hydroxymethyl group at 62.7 ppm, one carbon bearing nitrogen at 52.3 ppm, and one carbonyl group at 173.6 ppm. Thus, PS-II has a monosaccharide repeating unit.

Hydrolysis of PS-II with 2 M CF_3COOH followed by GLC–MS analysis of the derived alditol acetates revealed two derivatives of a 3-amino-3,6-dideoxyhexose in the ratio 1.77 : 1. The main compound with a smaller retention time in GLC was indistinguishable from 3-acetamido-1,2,4,5-tetra-*O*-acetyl-3,6-dideoxygalactitol. The second compound was identified as the fully *O*-acetylated derivative of 3,6-dideoxy-3-glyceroylaminogalactitol, which has been described formerly [4] in a study of the cell-wall antigen of *Eubacterium saburreum* L135. Since *N*-acetyl was absent from PS-II, the *N*-acetyl derivative of 3-amino-3,6-dideoxygalactose was derived by partial *N*-deacylation of the *N*-glyceroyl derivative during hydrolysis of the polysaccharide followed by *N*-acetylation. 3,6-Dideoxy-3-glyceroylaminogalactose is thus the repeating unit of PS-II.

The D configuration of 3-amino-3,6-dideoxygalactose was determined by GLC of acetylated (−)-2-octyl glycosides [5]. The L configuration of glyceric acid, isolated

from the hydrolysate by paper electrophoresis, was determined on the basis of its negative optical rotation value. The same two components have been identified [4] in the cell-wall antigen of *E. saburreum* L135.

Smaller amounts of rhamnose, glucose, galactose, and heptose in the ratios 0.30 : 1.00 : 0.11 : 0.56 as well as 2-amino-2-deoxyhexoses were also present in the hydrolysate of PS-II and are considered to originate from the core lipopolysaccharide to which the O-specific polysaccharide is attached.

Methylation of PS-II followed by hydrolysis with 2 M CF_3COOH, sodium borohydride reduction, and acetylation resulted again in two different *N*-acyl derivatives, in this case the *N*-glyceroyl derivative being predominant (the ratio 0.18 : 1). Fragmentation pathways in the mass spectra of these derivatives followed those described [4,6]. It was found that in both compounds the two hydroxyl groups of the sugar residue were methylated, while in the *N*-glyceroyl derivative one of the hydroxyl groups of the glyceric acid residue is methylated and the other is acetylated. This acid residue serves thus as the site of attachment of the neighboring glycosyl residue in PS-II, the sugar moiety being unsubstituted. At this stage the exact position of the glycosidic linkage remains unknown.

The ^1H NMR spectrum of PS-II was completely assigned using 2D shift-correlated spectroscopy (COSY) and sequential, selective spin-decoupling, and, with the ^1H NMR spectrum assigned, the ^{13}C NMR spectrum was interpreted with the aid of 2D heteronuclear COSY (Table 1). A downfield displacement of the signal for C-2 of the glyceric acid residue to 80.2 ppm and an upfield shift of the signal for its C-3 to 62.8 ppm, as compared with their position at 73.6 and 64.7 ppm, respectively, in the spectrum of unsubstituted glyceric acid [4] (Table 1), showed that in

Table 1
^{13}C NMR chemical shifts (δ in ppm)

Unit	C-1	C-2	C-3	C-4	C-5	C-6
O-Deacetylated polysaccharide of *C. freundii* 032 (PS-II) [a]						
α-D-Fuc*p*3N-(1 →	99.9	67.3	52.3	71.6	69.2	16.6
	(99.9)	(67.4)	(52.4)	(71.6)	(69.2)	(16.6)
L-Glyceric acid	173.6	80.2	62.8			
	(173.6)	(80.2)	(62.8)			
O-Specific polysaccharide of *C. freundii* O32 (PS-I) [b]						
α-D-Fuc*p*3N-(1 →	97.2	70.1	49.9	71.5	69.4	16.6
L-Glyceric acid	172.9 [c]	80.2	62.5			
OAc	174.3 [c]	21.4				
O-Specific polysaccharide of *Pseudomonas syringae* pv. *tomato* [d]						
α-D-Fuc*p*3N-(1 →	97.5	67.2	52.6	71.8	68.3	16.5

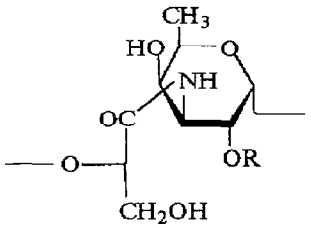
[a] Data from ref 4 are given in parentheses.
[b] Data for the *O*-acetylated repeating unit.
[c] Assignment may be interchanged.
[d] Data from ref 7.

PS-II glycosylation occurs at position 2 of the glyceric acid residue. The chemical shifts for Fuc3N in PS-II were similar to those for the terminal Fuc3NAc in the O-antigen of *Pseudomonas syringae* pv. *tomato* [7] (Table 1), thus confirming that this residue is unsubstituted at positions 2 and 4 in PS-II.

In the ^{13}C NMR spectrum of PS-I, the ratio of integral intensities of the signals for C-6 of Fuc3N and Me of the *O*-acetyl group was 1 : 0.75 and, hence, most of the repeating units are *O*-acetylated. Nonstoichiometric *O*-acetylation is common in bacterial polysaccharides; however, partial loss of the *O*-acetyl groups during acidic degradation of the lipopolysaccharide cannot be excluded.

The main series of the signals in the spectrum belongs thus to the *O*-acetylated units, while minor series are derived from non-*O*-acetylated units and the *O*-acetylated units adjacent to the non-*O*-acetylated units. Comparison of the ^{13}C NMR spectra of PS-II and PS-I (the main series, Table 1) revealed displacements of the signals for C-1,2,3 of Fuc3N from 99.9, 67.3, and 52.3 ppm to 97.2, 70.1, and 49.9 ppm, respectively, whereas differences in the positions of the signals for C-4,5,6 of Fuc3N were insignificant. The directions and the values of these displacements are characteristic [8] for *O*-acetylation of Fuc3N at position 2.

On the basis of these data, it was concluded that PS-I and PS-II have the following structure:



PS-I R = Ac (~75%) or H (~25%)
PS-II R = H

This structure which was found to be the same in three batches of cells of *C. freundii* O32 strain 103/57 from two different sources does not fit in with the sugar composition of this O-antigen reported earlier [9]. One of the possible reasons for this discrepancy may be alteration of the strain due to inherited instability.

A polysaccharide having the same structure as PS-II and, correspondingly, similar ^{1}H and ^{13}C NMR spectra was obtained previously [4] by modification of the cell-wall antigen of *Eubacterium saburreum* L13. Like the O-antigen of *C. freundii* O32, the *E. saburreum* L13 cell-wall antigen is *O*-acetylated (the position of the *O*-acetyl group remains unknown) but differs from PS-I by the presence of β-D-fructofuranosyl groups attached to the position 2 of approximately every third Fuc3N residue. The antigens from both bacterial species are unusual polymers with monomers linked by glycosidic and amidic linkages. Another example of such a polysaccharide is the O-antigen of *Pseudomonas aeruginosa* O9, in which glycosylation occurs at the (*R*)-3-hydroxybutyryl group attached at N-7 of 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic (pseudaminic) acid [10].

1. Experimental

General methods. — Optical rotations were measured with a Jasco DIP 360 polarimeter at 25°C. Gel chromatography was performed on a column (70 × 3 cm) of Sephadex G-50 in pyridine–acetic acid buffer (pH 5.5). GLC was carried out using a Hewlett–Packard 5890 instrument equipped with a glass capillary column (25 m × 0.2 mm) of Ultra 1 stationary phase. GLC–MS was performed with a Hewlett–Packard 5971 A instrument using a glass capillary column (12 m × 0.2 mm) of HP-1 stationary phase. ^{1}H and ^{13}C NMR spectra were recorded on Bruker WM-250 and AM-300 spectrometers (Germany) in D_2O at 60°C using acetone as the internal standard (δ_H 2.225 ppm, δ_c 31.45 ppm). Standard Bruker software was used for running two-dimensional spectra.

Two batches of cells of *C. freundii* O32 strain 103/57 of the National Collection of Reference Strains (Institute of Hygiene, Prague) were prepared using the strain from the Collection of the I.I. Mechnikov Institute of Vaccines and Sera (Moscow), the third batch was obtained using the strain provided by Professor E. Romanowska (L. Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław). Growth of bacteria [1], isolation of lipopolysaccharide [3], and O-specific polysaccharide [1] were performed as described.

Chemical methods. — PS-I (50 mg) was *O*-deacetylated with aq 10% ammonia for 16 h at 20°C to give PS-II (40 mg).

PS-II (1 mg) was hydrolyzed with 2 M CF_3COOH for 1 h at 120°C, the amino sugar was conventionally converted into the alditol acetate and analyzed by GLC–MS. The hydrolysate (from 26 mg of PS-II) was treated with a KU-2 cation-exchanger (H^+ form) and separated by paper electrophoresis to give L-glyceric acid (5.3 mg); $[\alpha]_D - 5.5°$ (*c* 0.62, H_2O), cf. [11] $[\alpha]_D - 14.2°$ (H_2O) for calcium L-glycerate.

Methylation analysis of PS-II (2 mg) was performed by the published method [12].

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