*Carbohydrate Research*, 179 (1988) 349–357
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands



# THE STRUCTURE OF THE O-SPECIFIC POLYSACCHARIDE CHAIN FROM *Citrobacter* O23-LIPOPOLYSACCHARIDE*

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(Received November 2nd, 1987; accepted for publication, December 24th, 1987)

ABSTRACT

The structure of *Citrobacter* O23-specific polysaccharide has been shown by sugar and methylation analyses of the native and chemically degraded polysaccharide and by $^1$H- and $^{13}$C-n.m.r. spectroscopy to consist of the tetrasaccharide repeating-units:

$$\rightarrow 4)\text{-}\alpha\text{-D-Man-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man-}(1\rightarrow 2)\text{-}\beta\text{-D-Man-}(1\rightarrow 3)\text{-}\alpha\text{-D-GalNAc-}(1\rightarrow,$$

80% of which are substituted by *O*-acetyl groups.

## INTRODUCTION

The first structural studies of *Citrobacter* O-antigens were carried out[1] on strain 396. Recently, the structures of the O-specific polysaccharides from the *Citrobacter* CC-G chemotype, comprising serotypes O36, O4, O27, and strain PCM 1487, have been established[2–4]. The O36-, O27-, and O4-specific polysaccharides are linear homopolymers of (1→2)-linked 4-deoxy-β-D-*arabino*-hexose[2,3] and the O-specific chain of PCM 1487 lipopolysaccharide (LPS) contains 4-deoxy-D-*arabino*-hexose, 2-acetamido-2-deoxyglucose, and 2-acetamido-2-deoxygalactose residues[4].

According to Keleti *et al.*[5], *Citrobacter* serotype O23 (strain 87/57) belongs to the CC-H chemotype, and its lipopolysaccharide contains mannose, 2-acetamido-2-deoxygalactose, 4-deoxy-D-*arabino*-hexose, glucose, galactose, 2-amino-2-deoxy-

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0008-6215/88/$ 03.50 

glucose, heptose, and 3-deoxy-D-*manno*-2-octulosonic acid (KDO). Our immunochemical studies[6] revealed that *Citrobacter* O23 LPS does not contain 4-deoxy-D-*arabino*-hexose although the same *Citrobacter* strain (87/57) was used in these experiments.

This finding prompted a study of the structure of the O23-specific polysaccharide.

## RESULTS AND DISCUSSION

*Preparation of the O-specific polysaccharide and its chemical characterisation.* — Hydrolysis of the pure LPS (2.4% of the dry bacteria) isolated from *Citrobacter* strain 87/57 followed by fractionation of the products on Sephadex G-50 gave the high-molecular-weight fraction corresponding to the O-specific polysaccharide (0.3% of the dry bacteria), which had $[\alpha]_{578}^{28.4}$ +110° (water). The main sugar constituents of the polysaccharide were mannose (65%) and 2-acetamido-2-deoxygalactose (19%) in the molar ratio 3:1. Both sugars were D since hexokinase converted the mannose into mannose 6-phosphate and the 2-amino-2-deoxygalactose was a substrate for D-galactose oxidase. The O-specific polysaccharide also contained non-stoichiometric amounts of glucose (6.8%) and *O*-acetyl groups (0.22 μmol/mg), *N*-acetyl groups ($^{13}$C-n.m.r. spectroscopy), but no free amino groups. The glucose could have originated from the core region of the LPS.

*N.m.r. data.* — The native polysaccharide gave complex $^{1}$H- and $^{13}$C-n.m.r. spectra, suggestive of heterogeneity. This complexity disappeared after *O*-deacetylation and the $^{1}$H-n.m.r. spectrum then contained four signals of equal intensity for anomeric protons. The $^{13}$C-n.m.r. data (Table I) confirmed the presence of four sugars in the repeating unit. The proton-coupled spectrum revealed a $^{1}J_{C,H}$ value of 161 Hz for one anomeric carbon and values of ~171 Hz (Table I) for the

TABLE I

$^{13}$C-N.M.R. DATA[a]

| *Compound* | *Sugar unit* | *Chemical shifts (p.p.m.)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* |
| *O*-Deacetylated polysaccharide | →4)-α-Man-(1→ | 102.0 (170) | 71.2[b] | 70.4[c] | 73.7 | 72.2[b] | 61.1[d] |
| | →2)-α-Man-(1→ | 99.7 (171) | 78.2 | 70.2[c] | 67.0 | 73.7 | 61.1[d] |
| | →2)-β-Man-(1→ | 101.5 (161) | 77.0 | 71.7[b] | 66.3 | 76.6 | 61.1[d] |
| | →3)-α-GalNAc-(1→ | 98.4 (173) | 49.2 | 75.9 | 68.6 | 71.7[b] | 60.1[d] |
| Oligosaccharide D | α-Man-(1→ | 102.1 (170) | 70.2 | 69.8[d] | 66.5 | 73.0[e] | 60.6[f] |
| | →2)-α-Man-(1→ | 99.4 (173) | 78.1 | 69.6[d] | 66.5 | 73.5[e] | 60.6[f] |
| | →2)-β-Man-(1→ | 100.4 (159) | 75.2 | 71.5 | 66.4 | 76.5 | 60.1[f] |

[a]Chemical shifts relative to 1,4-dioxane (66.5 p.p.m. relative to $Me_4Si$). Spectra were measured on solutions in $D_2O$ at 27°. Values of $^{1}J_{C-1,H-1}$ are given in Hz in parentheses. [b–f]Assignments may be interchanged.

TABLE II

$^1$H-N.M.R. CHEMICAL SHIFTS[a]

| *Compound* | *Sugar unit* | *Chemical shifts (p.p.m.)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *H-1* | *H-2* | *H-3* | *H-4* | *H-5* | *H-6* |
| Polysaccharide | →4)-α-Man-(1→ | 5.06 | | | | | |
| | →2)-α-Man-(1→ | 5.31 | | | | | |
| | →2)-β-Man-(1→ | 4.79 | | | | | |
| | →3)-α-GalNAc-(1→ | 5.28 | | | | | |
| *O*-Deacetylated polysaccharide | →4)-α-Man-(1→ | 5.05 | | | | | |
| | →2)-α-Man-(1→ | 5.33 | 4.11 | 4.04 | | | |
| | →2)-β-Man-(1→ | 4.80 | 3.99 | 3.74 | 3.60 | 3.41 | 3.73, 3.95 |
| | →3)-α-GalNAc-(1→ | 5.31 | 4.29 | 4.00 | 4.16 | | |
| Oligosaccharide D | α-Man-(1→ | 5.03 | 4.05 | 3.83 | 3.67 | | |
| | →2)-α-Man-(1→ | 5.40 | 4.10 | 4.02 | 3.71 | | |
| | →2)-β-Man-(1→ | 4.80 | 4.17 | 3.74 | 3.65 | 3.39 | 3.75, 3.90 |
| | →3)-2,5-Anhydrotalitol | 3.65, 3.80 | 3.97 | 4.25 | 4.37 | 4.12 | 3.71, 3.82 |
| Oligosaccharide E | α-Man-(1→ | 5.04 | 4.06 | 3.83 | | | |
| | →2)-α-Man-(1→ | 5.23 | 4.01 | 3.96 | | | |

[a]Chemical shifts are expressed in p.p.m. downfield from the signal for 3-(trimethylsilyl)propanesulfonic acid. All spectra were obtained for solutions in $D_2O$ at 27°.

others, consistent with one β and three α residues. Each of the four anomeric protons had a $J_{1,2}$ value of <4 Hz so that the 2-acetamido-2-deoxygalactose must be α and one of the mannose residues must be β.

The $^1$H- and $^{13}$C-n.m.r. data (Tables I and II) were assigned by using literature data for related structures[7–9], and by comparison with spectra of oligosaccharides *(vide infra)*.

The $^{13}$C-n.m.r. spectrum of the native polysaccharide indicated the presence of two types of acetyl groups (22.1 and 20.5 p.p.m.). The latter signal disappeared after *O*-deacetylation. Similarly, two peaks for $CH_3CO$ (2.05 and 2.18 p.p.m.) were present in the $^1$H-n.m.r. spectrum; the latter, which had a relative intensity of 0.8, disappeared on *O*-deacetylation, indicating that the polymer contained 0.8 *O*-acetyl group per repeating unit.

*Periodate oxidation.* — Almost all of the mannose was destroyed on periodate oxidation of the polysaccharide, whereas the 2-acetamido-2-deoxygalactose was resistant. The polysaccharide consumed 38 μmol of $NaIO_4$ per 10 mg and, since ~90% of the mannose was oxidised, it can be concluded that each mannose unit is substituted at C-2 and/or C-4 (substitution at C-6 would require an uptake of 2 mol of $NaIO_4$ per mol of mannose) and that the 2-acetamido-2-deoxygalactose is substituted at C-3 or C-4.

N-*Deacetylation and deamination.* — In order to obtain oligosaccharides, the

polysaccharide was subjected to hydrazinolysis followed by deamination. The degree of *N*-deacetylation (50%) was calculated from the content of free amino groups in the modified polysaccharide. A second hydrazinolysis did not increase the content of free amino groups, so that 50% are resistant. The difficulty in *N*-deacetylating polysaccharides containing 3-substituted 2-acetamido-2-deoxy-hexoses has been described[10].

The *N*-deacetylated polysaccharide was separated, using Dowex 50-X8 ($H^+$) resin, into neutral and basic fractions, each of which contained mannose and 2-amino-2-deoxygalactose in the molar ratio 3:1, indicating that the original polysaccharide was not a mixture of a mannan and a polysaccharide made up of Man and GalNAc.

Fractionation of the deaminated and borohydride-reduced material on Bio-Gel P-2 (Fig. 1) gave fractions *A*–*E*, of which *D* and *E* were eluted in the regions characteristic for tetra- and tri-saccharide, respectively, and each constituted 25% of the total material eluted. Fractions *A*–*C* were the products of the incomplete degradation of the polysaccharide.

*Methylation analyses.* — The results of methylation analyses (Table III) showed the native and *O*-deacetylated polysaccharides each to contain 2-substituted mannose, 4-substituted mannose (molar ratio 2:1), and 3-substituted 2-acetamido-2-deoxygalactose; *D* to be Man$^{d}$-(1→2)-Man$^{c}$-(1→2)-Man$^{b}$-(1→3)-2,5-anhydrotalitol$^{a}$; and oligosaccharide *E* to be Man-(1→2)-Man-(1→2)-Mannitol, which was probably formed from *D* under the basic conditions of reduction.

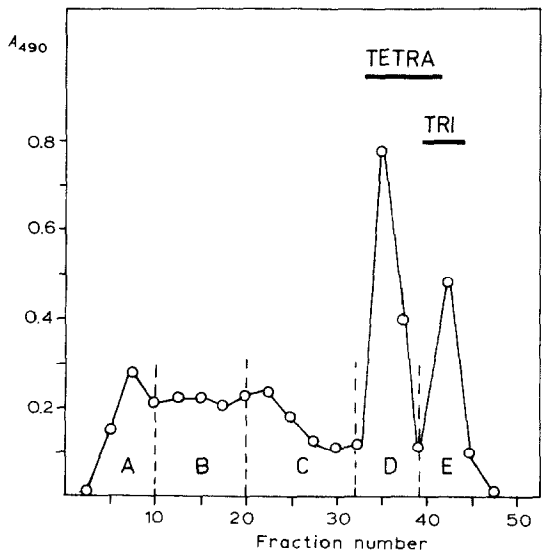


Fig. 1. Fractionation of the *N*-deacetylated and deaminated *Citrobacter* 87/57 specific polysaccharide on a column (2 × 90 cm) of Bio-Gel P-2. Elution with pyridine–AcOH buffer (pH 5.4) at 2 mL/20 min. Sugar contents were determined by the phenol–sulfuric acid method. The regions of tetra- and tri-saccharide elutions are marked.

TABLE III

METHYLATION ANALYSIS DATA FOR THE *Citrobacter* 87/57 SPECIFIC POLYSACCHARIDE (NATIVE AND *O*-DEACETYLATED) AND OLIGOSACCHARIDES D AND E

| *Methylated sugar* | *Molar ratio* | | | |
|---|---|---|---|---|
| | *Polysaccharide* | | *Oligosaccharide* | |
| | *Native* | *O-Deacetylated* | *D* | *E* |
| 1,3,4,5,6-$Me_5$-mannitol | – | – | – | 1.0 |
| 1,4,6-$Me_3$-2,5-anhydrotalitol | – | – | +[a] | – |
| 2,3,4,6-$Me_4$-Man | 0.16 | 0.25 | 1.0 | 1.0 |
| 3,4,6-$Me_3$-Man | 1.8 | 2.0 | 1.8 | 1.2 |
| 2,3,6-$Me_3$-Man | 1.0 | 1.0 | – | – |
| 2,3,6-$Me_3$-Glc | 0.31 | 0.15 | – | – |
| 4,6-$Me_2$-GalNMeAc | +[a] | + | – | – |

[a]Non-quantitative response.

These data establish the repeating unit of the specific polysaccharide to be:

→4)-Man-(1→2)-Man-(1→2)-Man-(1→3)-GalNAc-(1→.

*N.m.r. data for* D *and* E. — The $^{13}C$-n.m.r. data confirmed the presence of three sugars and a polyol in *D*, and the $^1J_{C,H}$ values for the anomeric carbons (Table I) showed two sugars to be $\alpha$ and one to be $\beta$. The $^1H$-n.m.r. data for *D* support the structure given above. COSY (Fig. 2) and relayed COSY (not shown) experiments enabled most of the proton signals to be assigned, as shown in the insert to Fig. 2. Values of chemical shifts (Table II), especially for the resonances of the anomeric protons, indicated unit *d* to be $\alpha$-mannose and unit *b* to be $\beta$-mannose. The signals of unit *c* are consistent with a 2-substituted $\alpha$-mannose. Anhydrotalitol can be assigned as unit *a*, as it contains two $CH_2OH$ groups. N.O.e. experiments showed that, on irradiation of H-1c, the signals of H-2 in →2)-$\beta$-Man and →2)-$\alpha$-Man and an unidentified signal at 3.68 p.p.m. were enhanced. On irradiation of H-1d, the signals of H-2 of →2)-$\alpha$-Man and $\alpha$-Man were enhanced, and irradiation of H-1b showed n.O.e. for H-3 of →3)-anhydrotalitol and H-2 of →2)-$\beta$-man. These results confirm the sugar sequence in *D* and show unit *b* to be $\beta$.

In the $^1H$-n.m.r. spectrum of fraction *E*, sub-spectra belonging to two sugars and a polyol could be identified. The signals from the terminal mannose had the same chemical shifts as in fraction *D*, and, as expected, signals from the 2-substituted $\beta$-mannose were missing.

Thus, the structure of the specific polysaccharide is as shown in the Abstract.

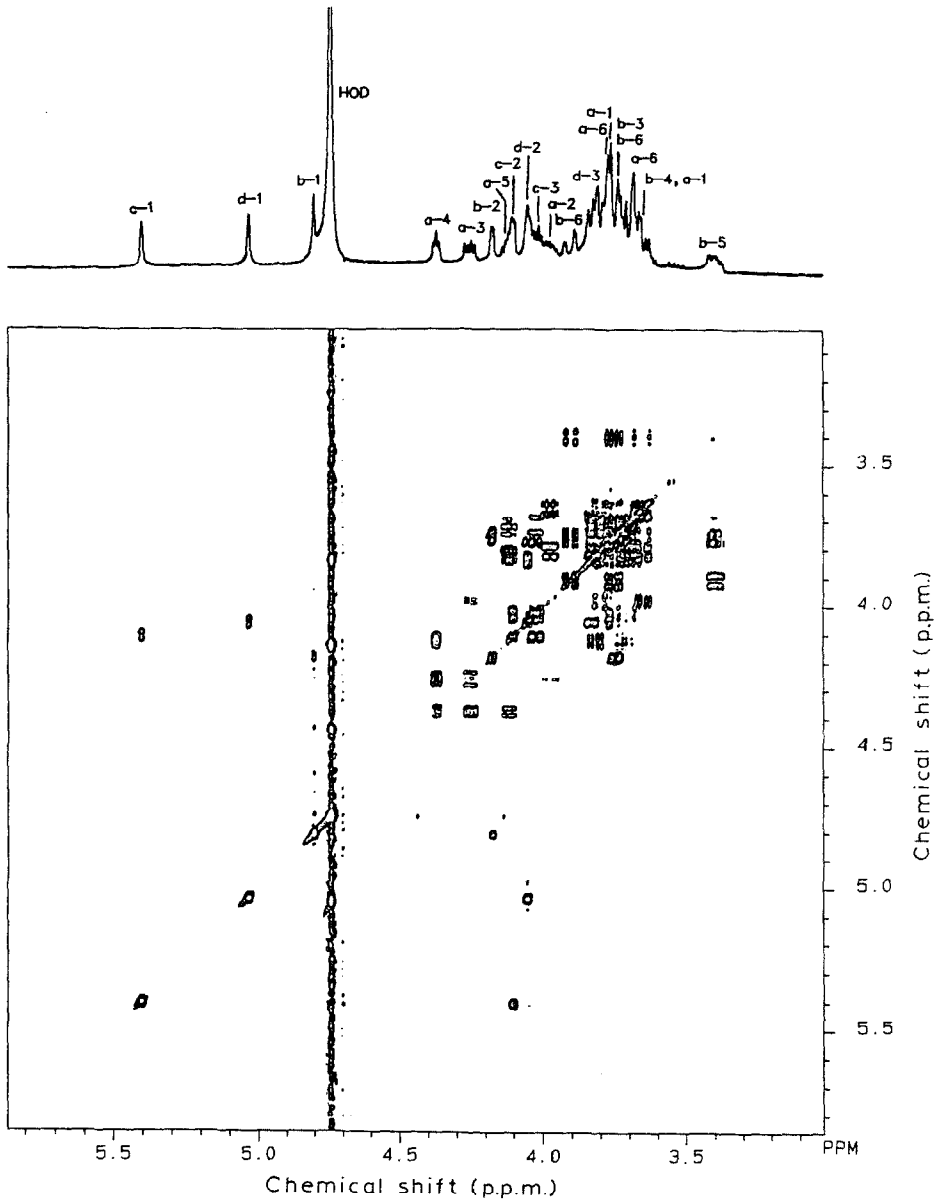


Fig. 2. The 360-MHz COSY spectrum of oligosaccharide D, with (top) the conventional proton spectrum: *a–d* refer to the units in the tetrasaccharide from the "reducing" terminus.

## EXPERIMENTAL

*Citrobacter* strain 87/57 was kindly provided by Dr. J. Sourek (Institute of Hygiene and Epidemiology, Prague). LPS was isolated from acetone-dried bacteria by extraction with phenol–water[11] and purified by filtration[12] through Sepharose 2B. LPS was hydrolysed with aqueous 1% AcOH at 100° for 2 h, lipid A was removed, and the supernatant solution was fractionated on a column (2 × 100 cm) of Sephadex G-50 equilibrated with pyridine–acetic acid buffer (pH 5.75).

The polysaccharide was *O*-deacetylated by adjusting the pH of a solution (28 mg/15 mL of $H_2O$) to 11 with aqueous ammonia. After storage for 4 h at room temperature, the solution was concentrated and freeze-dried. The *O*-deacetylated polysaccharide was eluted from a column of Sephadex G-50 in the same volume as was the native polysaccharide and no degradation was detected.

*Analytical methods.* — P.c. was performed on Whatman No. 1 paper, using solvent *A*, 1-butanol–pyridine–water (6:4:3), and detection with alkaline silver nitrate or ninhydrin.

T.l.c. was carried out on Kieselgel 60 $F_{254}$ (Merck), using solvent *B*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3), and detection with ninhydrin or by charring with sulfuric acid.

Carbohydrate material was determined by the phenol–sulfuric acid method[13]. Glucose and galactose were assayed, after hydrolysis of polysaccharide with 0.5M $H_2SO_4$ for 4 h at 100°, using D-glucose oxidase[14] and D-galactose oxidase[15], respectively. Hexosamines were determined after hydrolysis of the polysaccharide with 4M HCl for 18 h at 100° by a modified[16] Ludowieg–Benmaman method[17]. Free amino groups were determined by the method of Habeeb[18] and *O*-acetyl groups according to Hestrin[19].

The configuration of the mannose was determined as D, after hydrolysis of the polysaccharide with 0.5M HCl for 18 h at 100°, since it was phosphorylated by hexokinase using the method of Sturgeon[20] [0.2M ammonium acetate buffer (pH 8.0) was used instead of triethanolamine buffer]. Mannose 6-phosphate was detected by t.l.c. (solvent *B*). The configuration of the 2-amino-2-deoxygalactose was determined as D after hydrolysis of the polysaccharide with 4M HCl for 18 h at 100° followed by oxidation[15] with D-galactose oxidase.

The $[\alpha]_D$ value of the polysaccharide was determined with a Jasco ORD/UV-5 spectrophotometer.

Sugars were identified by g.l.c. of their alditol acetates[21]. G.l.c. was performed on a Varian 2100 instrument equipped with a flame-ionisation detector and glass columns (0.25 × 200 cm) containing 3% of OV-225 and operated at 180° (neutral sugars) or 205° (amino sugars).

Neutral and amino sugars were determined simultaneously after hydrolysis of the polysaccharide with 0.5M HCl for 18 h at 100° followed by deamination[22].

The poly- and oligo-saccharides were methylated by the Hakomori method[23]. Partially methylated alditol acetates were analysed by g.l.c.–m.s., using an LKB-2091 system (Central Laboratory for Macromolecular Studies, Lodz) with a 3% OV-225 Chrom Q column (0.3 × 290 cm) and temperature programmes of 150→200° at 5°/min or 165→200°, 1°/min.

*Periodate oxidation.* — The polysaccharide (10 mg) was treated with 0.05M sodium periodate (2 mL) for 72 h at 4°. The excess of periodate was reduced with ethylene glycol (0.1 mL), and the oxidised material was reduced with sodium borohydride (50 mg) overnight at room temperature. The mixture was neutralised with aqueous 50% AcOH and then dialysed against tap and distilled water. The periodate consumption was measured spectrophotometrically at 225 nm.

N-*Deacetylation and deamination.* — The polysaccharide was treated[24] with anhydrous hydrazine and hydrazine sulfate for 168 h at 105°. Hydrazine was then removed under vacuum in a desiccator over sulfuric acid. The *N*-deacetylated polysaccharide was freed from hydrazine sulfate by chromatography on a column (3 × 90 cm) of Sephadex G-25. The degree of *N*-deacetylation was ascertained by determining the free amino groups[18].

A solution of the *N*-deacetylated polysaccharide (2 mg) in water (1 mL) was applied to a small column of Dowex 50-X8 ($H^+$) resin (1 mL). Elution with water (2 mL) gave the neutral fraction and elution with 0.5M HCl (2 mL) gave the basic fraction.

The *N*-deacetylated polysaccharide was deaminated[22] using 5% $NaNO_2$ and aqueous 33% AcOH for 1 h at room temperature. After reduction with sodium borohydride followed by neutralisation with Dowex 50-X8 ($H^+$) resin, the products were fractionated on a column (2 × 90 cm) of Bio-Gel P-2 equilibrated with pyridine–AcOH buffer (pH 5.4).

*N.m.r. data.* — N.m.r. spectra were recorded for solutions in $D_2O$ at 27° with a Bruker AM-360 spectrometer ($^1H$, 360 MHz; $^{13}C$, 90 MHz). Acetone (2.22 p.p.m. relative to sodium 3-(trimethylsilyl)propanesulfonate was used as the internal standard for $^1H$ spectra.

ACKNOWLEDGMENT

This work was supported, in part, by Grant CPBP-06.01 from the Polish Academy of Sciences.

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