

STRUCTURES OF THE O-SPECIFIC POLYMERS FROM THE LIPOPOLYSACCHARIDES OF THE REFERENCE STRAINS FOR *Pseudomonas cepacia* SEROGROUPS O3 AND O5

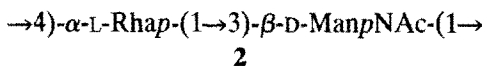
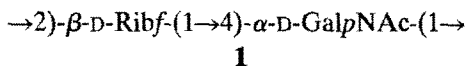
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ABSTRACT

The putative O-specific polymers of lipopolysaccharides from two reference strains of *Pseudomonas cepacia* have been isolated and characterized. Both polymers have disaccharide repeating-units. Structure **1** was established for the O3 polymer, and structure **2** for the O5 polymer. Polymers with the same repeating units have been found previously as the O antigens of other bacteria.



INTRODUCTION

The nutritional versatility of *Pseudomonas cepacia* and the importance of the organism as an opportunistic pathogen^{1–3} have prompted numerous studies of selective growth media^{4–7}, typing methods^{8–11}, possible virulence factors^{12–16}, and the origins of antibiotic resistance^{17–21}. Particular attention has recently been paid to lipopolysaccharides of the organism because of the low permeability^{17–20} of the outer membrane, of which lipopolysaccharide is a component, and because typing schemes based on the O-antigenic side-chains have been devised^{8,9}. Structures of O antigens have been reported for strain IMV 3181 (ref. 22), strain IMV 4137 (ref. 23), strain IMV 673/2 (ref. 24), and strains belonging to serotypes⁹ B and E (ref. 25).

As part of a general study of the surface chemistry of *P. cepacia*, we have characterized the fatty acids and novel polar lipids²⁶ of reference strains for the serogroups defined by Heidt *et al.*⁸. We now report the structures of the putative O antigens for two of these strains, representing serogroups O3 and O5.

RESULTS

The O3 polymer. — The lipopolysaccharide isolated in 15% yield from lipid-free cell walls was subjected to mild acid hydrolysis in the presence of sodium dodecyl sulphate²⁷. Two polymeric fractions were obtained by chromatography of the water-soluble products on Sephadex G-50. The fraction (F1a) of higher M_r (yield, 8% of the lipopolysaccharide) gave matching elution profiles for carbohydrate and phosphorus, and was assumed to contain undegraded lipopolysaccharide, although the ^1H -n.m.r. spectrum of the material was almost indistinguishable from the fraction (F1b) eluted slightly later.

Fraction 1b (yield, 33%), which was used for structural studies, gave D-ribose (28.2%) and 2-amino-2-deoxy-D-galactose (35.7%) on acid hydrolysis. A regular disaccharide repeating-unit for the polymer was evident from its n.m.r. spectra. The ^1H -n.m.r. spectrum contained anomeric signals (each 1 H) at δ 5.32 (unresolved) and 5.20 ($J_{1,2}$ 2.7 Hz) and a methyl singlet at δ 2.19, showing that the hexosamine was present as its *N*-acetyl derivative. The ^{13}C -n.m.r. spectrum contained 13 discrete signals, including anomeric signals at δ 107.04 and 95.85 and those for the *N*-acetyl group at δ 174.69 and 22.12, consistent with the compositional data. From the spectroscopic data, it could also be inferred that the polymer was constructed from β -ribofuranosyl and 2-acetamido-2-deoxy- α -galactopyranosyl residues. These inferences were confirmed by methylation analysis, which also showed that the ribose residues were 2-substituted and the hexosamine residues 4-substituted. Thus, structure **1** can be assigned to the repeating unit of the O3 polymer. The ^{13}C -n.m.r. data for the polymer (Table I) were entirely consistent with this assignment.

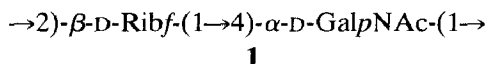


TABLE I

 ^{13}C -N.M.R. DATA FOR THE O3 POLYMER

Carbon atom	Chemical shift (p.p.m.) ^a	
	$\rightarrow 2)\text{-}\beta\text{-D-Ribf-(1}\rightarrow$	$\rightarrow 4)\text{-}\alpha\text{-D-GalpNAc-(1}\rightarrow$
C-1	107.04	95.85
C-2	79.40	50.24
C-3	70.30	67.64
C-4	82.76	76.46
C-5	62.98	71.14
C-6		61.54
-NHC(O)CH ₃		174.69
-NHC(O)CH ₃		22.12

^aThe spectrum for the sample in D₂O was recorded at 100.62 MHz and 50° with tetramethylsilane as the external reference.

The O5 polymer. — Extraction of cell walls from *P. cepacia* O5 gave the lipopolysaccharide in 21% yield. Mild acid hydrolysis²⁷ followed by gel-permeation chromatography of the water-soluble products gave a single, phosphorus-free polymeric fraction (yield, 39%). Total acid hydrolysis of the polymer gave L-rhamnose (23.6%) and an amino sugar (~21%, determined by autoanalysis and expressed as 2-amino-2-deoxygalactose). Although the recoveries were low, the n.m.r. data below revealed no more components for the polymer. The amino sugar was identified as a 2-amino-2-deoxyhexose by the positive Morgan–Elson reaction of its *N*-acetyl derivative, and by e.i.-m.s. of the acetylated aminodeoxyhexitol. An equatorial H-2 was indicated by the low values (less than 2 Hz) for $J_{1,2}$ in both pyranose anomers of the free amino sugar. Further studies of the amino sugar identified it as 2-amino-2-deoxy-D-mannose by the following criteria: (a) p.c. of the compound and of its *N*-acetyl derivative, (b) paper electrophoresis of the compound (at pH 5.3) and of its *N*-acetyl derivative (in borate buffer at pH 10), (c) g.l.c. of the acetylated aminodeoxyhexitol and of the acetylated products of deamination–reduction, (d) enzymic assay for D-glucose after deamination of the compound.

The ¹H-n.m.r. spectrum of the polymer contained three signals (each 1 H) in the anomeric region at δ 4.97 (unresolved), 4.93 (unresolved), and 4.66 (d, J 3.8 Hz), as well as methyl signals at δ 2.03 (s) and 1.29 ($J_{5,6}$ 6.2 Hz). Treatment of the polymer with alkali produced no change in the spectrum, demonstrating the absence of an *O*-acyl substituent. Selective irradiation showed that the signal in the anomeric region at δ 4.66 was coupled to that at δ 4.97, and could in fact be assigned^{28,29} to H-2 of a 2-acetamido-2-deoxy- β -D-mannopyranosyl residue. The ¹³C-n.m.r. spectrum of the polymer supported a disaccharide repeating-unit. The 14 discrete signals included anomeric signals at δ 99.62 (J_{CH} 166 Hz) and 96.63 (J_{CH} 171 Hz), the latter showing that the rhamnosyl residue had the α configuration.

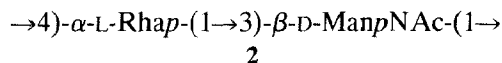
TABLE II

¹³C-N.M.R. DATA FOR THE O5 POLYMER

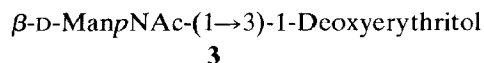
Carbon atom	Chemical shift (p.p.m.) ^a	
	→4)- α -L-Rhap-(1→	→3)- β -D-ManpNAc-(1→
C-1	96.63	99.62
C-2	70.52	49.67
C-3	70.14	75.97
C-4	79.59	65.00
C-5	67.36	76.09
C-6	16.85	60.32
-NHC(O)CH ₃		174.98
-NHC(O)CH ₃		21.83

^aThe spectrum for the sample in D₂O was recorded at 100.62 MHz and 27° with tetramethylsilane as the external reference.

The results of methylation analysis showed that the polymer was built from 4-substituted rhamnopyranosyl and 3-substituted 2-acetamido-2-deoxymannopyranosyl residues, leading to structure **2** for the repeating unit. The ^{13}C -n.m.r. data (Table II) are in accord with this structure.



Further evidence for the structure above was provided by Smith degradation of the polymer. The disaccharide-alditol produced was laevorotatory, and its ^1H -n.m.r. spectrum contained one anomeric signal at δ 4.92 (unresolved), the H-2 signal for the 2-acetamido-2-deoxy- β -D-mannopyranosyl group at δ 4.52, and methyl signals at δ 2.08 (s) and 1.16 (d). The ^{13}C -n.m.r. spectral data (Table III) were also consistent with the expected product of structure **3**.



DISCUSSION

The putative O-specific polymers isolated from the O3 and O5 reference strains⁸ of *P. cepacia* both have simple, disaccharide repeating-units of common sugars. Disaccharide repeating-units also occur in the polymers from strain IMV 4137 (ref. 23) and strains of serotypes⁹ B and E (ref. 25), although the last two polymers contain the rare sugar D-fucose. Another unusual sugar, D-rhamnose, is present in the polymers from strains IMV 3181 (ref. 22) and IMV 673/2 (ref. 24), both of which have linear, trisaccharide repeating-units.

TABLE III

^{13}C -N.M.R. DATA FOR THE SMITH-DEGRADATION PRODUCT FROM THE O5 POLYMER

Carbon atom	Chemical shift (p.p.m.) ^a	
	$\beta\text{-D-ManpNAc}\text{-(1}\rightarrow 3)\text{-1-Deoxyerythritol}$	
C-1	99.14	17.86
C-2	54.01	67.41
C-3	72.53	83.62
C-4	67.31	61.01
C-5	76.74	
C-6	60.93	
-NHC(O)CH ₃	n.d. ^b	
-NHC(O)CH ₃	22.46	

^aThe spectrum for the sample in D₂O was recorded at 100.62 MHz and 27° with tetramethylsilane as the external reference. Assignments of signals with closely similar chemical shifts may be interchanged.

^bNot determined.

It is interesting to note that the O3 polymer (repeating unit **1**)* and the O5 polymer (repeating unit **2**) have both been described previously as the side chains of lipopolysaccharides from other organisms. Structure **1** constitutes the repeating unit in strains of *Serratia marcescens* belonging to serogroups O12 (ref. 30), O13 (ref. 31), and O14 (refs. 32, 33), and also in the O12 antigen (Lányi classification) of *Pseudomonas aeruginosa*³⁴. By coincidence, structure **2** is also the repeating unit in an O antigen of *P. aeruginosa* (type X in the Meitert classification)³⁵.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of the lipopolysaccharides.

— Cultures of the O3 and O5 reference strains of *P. cepacia* were grown and used for the preparation of cell walls as described²⁶. Lipopolysaccharides were extracted by using hot, aqueous phenol³². From 20-L batch cultures, the yields of wet cells, freeze-dried cell walls, and lipopolysaccharides were as follows: O3, 117 g, 2.69 g, and 416 mg, respectively; O5, 80 g, 2.65 g, and 562 mg, respectively. Mild acid hydrolysis of the lipopolysaccharides was done in the presence of 1% of sodium dodecyl sulphate²⁷, and the water-soluble products were fractionated by chromatography on Sephadex G-50 (ref. 32).

General methods. — The following solvent systems were used for p.c.: A, ethyl acetate–pyridine–water (13:5:4); B, ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Paper electrophoresis was carried out at pH 5.3 (ref. 32) or at pH 10 in a borate buffer³⁶. G.l.c. separations were performed with fused-silica capillary columns (25 m) of BP1 or BP10 in a Carlo Erba Mega 5160 chromatograph. A Finnigan model 1020B instrument was used for g.l.c.–m.s. Optical rotations were measured with a Bendix polarimeter model 143A. N.m.r. spectra (¹H and ¹³C) of samples in D₂O were recorded with a Bruker WH-400 or JEOL JNM-GX270 spectrometer. In general, ¹H-n.m.r. spectra were recorded at 60° (O3 polymer), 80° (O5 polymer), or 70° (O5 Smith-degradation product) with sodium trimethylsilylpropanoate-*d*₄ as the external reference. ¹³C-N.m.r. spectra were recorded at 27° or 50° with tetramethylsilane as the external reference.

Identification and determination of neutral sugars. — Samples and standards were treated with 2M HCl for 2 h at 105°, then neutralised, and deionised³². Analyses were carried out by p.c. (solvent A) and by g.l.c. of the alditol acetates. Sugars were assigned to stereochemical series by g.l.c. of the acetylated oct-2-yl glycosides³⁷.

Identification and determination of amino sugars. — Samples were hydrolysed with 6.1M HCl for 4 h at 105°. When necessary, amino sugars in the dried hydroly-

*Since this paper was submitted, we have learnt that structure **1** has been determined independently for the O3 antigen of *P. cepacia* [Y. A. Knirel, N. V. Tanatar, N. A. Soldatkina, and I. Y. Zakharova, *Bioorg. Khim.*, 14 (1988) 1684–1689].

sates were adsorbed onto Dowex 50 (H^+) resin, then recovered from the water-washed resin by elution with M HCl. Free amino sugars were analysed by p.c. (solvent *B*), paper electrophoresis (pH 5.3), and autoanalysis (Locarte), which provided quantitative data. An enzymic assay³⁸ was also used to identify 2-amino-2-deoxy-D-galactose.

The amino compound from the O5 polymer was also examined by p.c. (solvent *B*), paper electrophoresis³⁶, and the Morgan–Elson assay³⁹ after *N*-acetylation. Further samples of the amino sugar were examined (*a*) by g.l.c. and m.s. of the acetylated aminodeoxyalditol, (*b*) by deamination⁴⁰ and assay for D-glucose using hexokinase (EC 2.7.1.1) in conjunction with D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and (*c*) by g.l.c. of the reduced and acetylated deamination products.

Degradative methods. — Methylation analyses were carried out by standard methods^{31,32} and were monitored by g.l.c. and m.s. A sample (20 mg) of the O5 polymer was treated with 50mM NaIO₄ (5 mL) for 5 days at 4°. The oxidised polymer was reduced (NaBH₄) and then treated with M trifluoroacetic acid (1 mL) at room temperature overnight. The Smith-degradation product was purified by h.p.l.c. on a TSKgel G-Oligo-PW column (Anachem) eluted with water (1 mL.min⁻¹) at room temperature. Polymers were checked for the presence of *O*-acyl substituents (using n.m.r. monitoring) by treatment with 0.1M NaOH at room temperature overnight.

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