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

ANDREW D. COX AND STEPHEN G. WILKINSON
School of Chemistry, The University, Hull HU6 7RX (Great Britain)
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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.

→2)-β-D-Rib*f*-(1→4)-α-D-Gal*p*NAc-(1→
1

→4)-α-L-Rha*p*-(1→3)-β-D-Man*p*NAc-(1→
2

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 serotypes[9] 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 lipids[26] 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.

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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 sulphate[27]. 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 ^{1}H-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 ^{1}H-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.

→2)-β-D-Rib*f*-(1→4)-α-D-Gal*p*NAc-(1→

1

TABLE I

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

Carbon atom	*Chemical shift (p.p.m.)*[a]	
	→2)-β-D-Ribf-(1→	*→4)-α-D-GalpNAc-(1→*
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

[a]The spectrum for the sample in D_2O 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 hydrolysis[27] 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 ^{1}H-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 ^{13}C-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

^{13}C-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

[a]The spectrum for the sample in D_2O 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.

→4)-α-L-Rha*p*-(1→3)-β-D-Man*p*NAc-(1→
2

Further evidence for the structure above was provided by Smith degradation of the polymer. The disaccharide-alditol produced was laevorotatory, and its ^{1}H-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**.

β-D-Man*p*NAc-(1→3)-1-Deoxyerythritol
3

DISCUSSION

The putative O-specific polymers isolated from the O3 and O5 reference strains[8] 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 serotypes[9] 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]	
	β-D-ManpNAc-(1→	*→3)-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	

[a]The spectrum for the sample in D_2O 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.
[b]Not 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 described[26]. Lipopolysaccharides were extracted by using hot, aqueous phenol[32]. 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 sulphate[27], 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 buffer[36]. 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 (^{1}H and ^{13}C) of samples in D_2O were recorded with a Bruker WH-400 or JEOL JNM-GX270 spectrometer. In general, ^{1}H-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. ^{13}C-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 deionised[32]. 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 glycosides[37].

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 assay[38] 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 electrophoresis[36], and the Morgan–Elson assay[39] 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 deamination[40] 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 $NaIO_4$ (5 mL) for 5 days at 4°. The oxidised polymer was reduced ($NaBH_4$) 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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REFERENCES

1 B. HOLMES, *J. Appl. Bacteriol.*, 61 (1986) 299–314.
2 W. R. JARVIS, D. OLSON, O. TABLAN, AND W. J. MARTONE, *Eur. J. Epidemiol.*, 3 (1987) 233–236.
3 N. HØIBY, *Chest*, 94 (1988) 97s–102s.
4 O. C. TABLAN, L. A. CARSON, L. B. CUSICK, L. A. BLAND, W. J. MARTONE, AND W. R. JARVIS, *J. Clin. Microbiol.*, 25 (1987) 485–487.
5 D. F. WELCH, M. J. MUSZYNSKI, C. H. PAI, M. J. MARCON, M. M. HRIBAR, P. H. GILLIGAN, J. M. MATSEN, P. A. AHLIN, B. C. HILMAN, AND S. A. CHARTRAND, *J. Clin. Microbiol.*, 25 (1987) 1730–1734.
6 C. HAGEDORN, W. D. GOULD, T. R. BARDINELLI, AND D. R. GUSTAVSON, *Appl. Environ. Microbiol.*, 53 (1987) 2265–2268.
7 L. A. CARSON, O. C. TABLAN, L. B. CUSICK, W. R. JARVIS, M. S. FAVERO, AND L. A. BLAND, *J. Clin. Microbiol.*, 26 (1988) 2096–2100.
8 A. HEIDT, H. MONTEIL, AND C. RICHARD, *J. Clin. Microbiol.*, 18 (1983) 738–740.

9 Y. NAKAMURA, S. HYODO, E. CHONAN, S. SHIGETA, AND E. YABUUCHI, *J. Clin. Microbiol.*, 24 (1986) 152–154.
10 J. R. W. GOVAN AND G. HARRIS, *J. Clin. Microbiol.*, 22 (1985) 490–494.
11 J. J. LIPUMA, J. E. MORTENSEN, S. E. DASEN, T. D. EDLIND, D. V. SCHIDLOW, J. L. BURNS, AND T. L. STULL, *J. Pediatr.*, 113 (1988) 859–862.
12 A. I. MCKEVITT AND D. E. WOODS, *J. Clin. Microbiol.*, 19 (1984) 291–293.
13 T. NAKAZAWA, Y. YAMADA, AND M. ISHIBASHI, *J. Clin. Microbiol.*, 25 (1987) 195–198.
14 M. K. LONON, D. E. WOODS, AND D. C. STRAUS, *J. Clin. Microbiol.*, 26 (1988) 979–984.
15 C. L. POH AND G. K. LOH, *J. Clin. Microbiol.*, 26 (1988) 607–608.
16 P. A. SOKOL AND D. E. WOODS, *Microb. Pathogen.*, 5 (1988) 197–205.
17 R. A. MOORE AND R. E. W. HANCOCK, *Antimicrob. Agents Chemother.*, 30 (1986) 923–926.
18 T. R. PARR, R. A. MOORE, L. V. MOORE, AND R. E. W. HANCOCK, *Antimicrob. Agents Chemother.*, 31 (1987) 121–123.
19 S. C. ARONOFF, *Antimicrob. Agents Chemother.*, 32 (1988) 1636–1639.
20 J. L. BURNS, L. A. HEDIN, AND D. M. LIEN, *Antimicrob. Agents Chemother.*, 33 (1989) 136–141.
21 A. PRINCE, M. S. WOOD, G. S. CACALANO, AND N. X. CHIN, *Antimicrob. Agents Chemother.*, 32 (1988) 838–843.
22 Y. A. KNIREL, A. S. SHASHKOV, B. A. DMITRIEV, N. K. KOCHETKOV, N. V. KASYANCHUK, AND I. Y. ZAKHAROVA, *Bioorg. Khim.*, 6 (1980) 1851–1859.
23 Y. A. KNIREL, B. A. DMITRIEV, N. K. KOCHETKOV, N. V. TANATAR, AND I. Y. ZAKHAROVA, *Bioorg. Khim.*, 11 (1985) 536–538.
24 Y. A. KNIREL, N. V. TANATAR, M. A. SOLDATKINA, A. S. SHASHKOV, AND I. Y. ZAKHAROVA, *Bioorg. Khim.*, 14 (1988) 77–81.
25 Y. A. KNIREL, A. S. SHASHKOV, M. A. SOLDATKINA, N. A. PARAMONOV, AND I. Y. ZAKHAROVA, *Bioorg. Khim.*, 14 (1988) 1208–1213.
26 A. D. COX AND S. G. WILKINSON, *Biochim. Biophys. Acta*, 1001 (1989) 60–67.
27 M. CAROFF, A. TACKEN, AND L. SZABO, *Carbohydr. Res.*, 175 (1988) 273–282.
28 K.-G. ROSELL AND H. J. JENNINGS, *Can. J. Biochem. Cell Biol.*, 61 (1983) 1102–1107.
29 C. JONES AND F. CURRIE, *Carbohydr. Res.*, 184 (1988) 279–284.
30 C. J. BRIGDEN, S. FURN, AND S. G. WILKINSON, *Carbohydr. Res.*, 139 (1985) 298–301.
31 D. OXLEY AND S. G. WILKINSON, *Carbohydr. Res.*, 172 (1988) 275–286.
32 C. J. BRIGDEN AND S. G. WILKINSON, *Carbohydr. Res.*, 115 (1983) 183–190.
33 D. OXLEY, *Ph.D. Thesis, University of Hull*, 1988.
34 Y. A. KNIREL, A. S. SHASHKOV, B. A. DMITRIEV, N. K. KOCHETKOV, E. S. STANISLAVSKY, AND G. M. MASHILOVA, *Bioorg. Khim.*, 11 (1985) 1265–1269.
35 Y. A. KNIREL, N. A. KOCHAROVA, A. S. SHASHKOV, L. D. VARBANETS, N. K. KOCHETKOV, E. S. STANISLAVSKY, AND G. M. MASHILOVA, *Bioorg. Khim.*, 12 (1986) 1268–1273.
36 M. J. CRUMPTON, *Biochem. J.*, 72 (1959) 479–486.
37 K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62 (1978) 359–362.
38 J. M. SEMPERE, C. GANCEDO, AND C. ASENSIO, *Anal. Biochem.*, 12 (1965) 509–515.
39 J. L. STROMINGER, J. T. PARK, AND R. E. THOMPSON, *J. Biol. Chem.*, 234 (1959) 3263–3268.
40 B. LINDBERG AND J. LÖNNGREN, *Methods Enzymol.*, 50 (1978) 3–33.