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STRUCTURE OF THE PUTATIVE O ANTIGEN CONTAINING 2-AMINO-2-DEOXY-L-GLUCOSE IN THE REFERENCE STRAIN FOR *Pseudomonas cepacia* SEROGROUP O1

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ABSTRACT

Polymeric material isolated from the lipopolysaccharide of the reference strain of *Pseudomonas cepacia* serogroup O1 consisted mainly of D-glucose and 2-amino-2-deoxy-L-glucose: rhamnose and *O*-acetyl groups were also present. As a result of spectroscopic and degradative studies, the disaccharide repeating-unit shown could be assigned to the major polymer present. A possible origin of the minor components is suggested.

→4)-α-D-Glc*p*-(1→3)-α-L-Glc*p*NAc-(1→

INTRODUCTION

The rise in importance of *Pseudomonas cepacia* as an opportunistic pathogen and as a cause of infection in patients with cystic fibrosis has encouraged studies of surface polysaccharides in relation to virulence[1], antibiotic resistance[2–4], serological classification[5,6], and interaction with lectins[7]. Although many strains of *P. cepacia* isolated from cystic fibrosis patients produce R-type lipopolysaccharides (lacking an O-specific side chain)[1], it is clearly desirable to establish the chemical basis for the two schemes for serotyping organisms by their heat-stable O antigens[5,6]. So far, structures have been published[8–11] for the products from five strains, and we have recently[12] characterized the O3 and O5 antigens in the scheme of Heidt *et al.*[5]. We now report the structure of the repeating unit for the O1 polymer, which surveys[5,6] have shown to be one of the most common (~20% of strains examined).

RESULTS AND DISCUSSION

Lipopolysaccharide was isolated in 21% yield from cell walls of the *P. cepacia* O1 reference strain. The major fatty acid components were tetradecanoic acid, 3-hydroxytetradecanoic acid, and 3-hydroxyhexadecanoic acid, as expected from previous studies of lipopolysaccharides from *P. cepacia*[13]. Polymeric material was isolated from two batches of lipopolysaccharide by mild acid hydrolysis, followed

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by chromatography of the water-soluble products on Sephadex G-50. From the first batch of lipopolysaccharide (hydrolysed with aq. 1% acetic acid for 2 h at 100°), the yield of polymer was 24%; from the second batch (hydrolysed in the presence of sodium dodecyl sulphate[14]), the yield was 33%. Both products gave the same ^{1}H-n.m.r. spectrum.

Monosaccharide analyses of the polymeric material showed the presence of D-glucose and an amino sugar initially identified by p.c., autoanalysis, and p.e. as 2-amino-2-deoxyglucose (each ~30%), together with some rhamnose (~8%). Because the amino sugar did not react in an enzymic assay for 2-amino-2-deoxy-D-glucose, further checks on its identity were carried out. The mass spectrum of the peracetylated aminodeoxyalditol contained the signals (*e.g.*, at *m/z* 144 and 360) diagnostic for the derivative of a 2-amino-2-deoxyhexose, and the *N*-acetyl derivative of the sugar gave a positive Morgan–Elson reaction. Further evidence for the *gluco* configuration was obtained by (*a*) g.l.c. of the peracetylated amino-deoxyalditol, (*b*) g.l.c. of the peracetate of the product from deamination–reduction, and (*c*) ^{1}H-n.m.r. spectroscopy of the peracetylated amino sugar. In all cases, the compound from *P. cepacia* behaved like a standard of 2-amino-2-deoxy-D-glucose. Confirmation of the enzymic evidence that the compound from *P. cepacia* was actually the L isomer was provided by (*a*) g.l.c. of the acetylated oct-2-yl glycosides, (*b*) g.l.c. of the acetylated but-2-yl glycosides, (*c*) an approximate value of $-60°$ for $[\alpha]_D$ of the hydrochloride (*cf.* $+72.5°$ for the D isomer), and (*d*) a c.d. curve for the peracetylated aminodeoxyalditol which mirrored that for the D isomer.

The ^{1}H-n.m.r. spectrum of the polymeric material contained two prominent anomeric signals (each 1 H) at δ 5.06 ($J_{1,2}$ 3.6 Hz) and 4.95 ($J_{1,2}$ 3.5 Hz), indicating that both major sugars were present as α-pyranosyl residues, together with a methyl singlet at δ 2.02. The spectrum also contained a range of minor signals indicating

TABLE I

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

Carbon atom	*Chemical shift (p.p.m.)*[a]	
	→4)-α-D-Glcp-(1→	*→3)-α-L-GlcpNAc-(1→*
C-1	100.31	98.53
C-2	72.48	54.28
C-3	72.48	77.09
C-4	79.26	68.98
C-5	71.65	72.77
C-6	60.32	60.76
-NHC(O)CH_3		~175
-NHC(O)CH_3		22.69

[a]The spectrum for the sample in D_2O was recorded at 100.62 MHz and 27° with proton decoupling and 1,4-dioxane as the internal reference. The assignments listed are provisional.

contamination, heterogeneity, or the presence of a significant proportion of core oligosaccharide. These minor signals included a multiplet at δ 1.3 (attributable to H-6 of rhamnose), and others between δ 2.09 and 2.15 and in the anomeric region (notably an unresolved signal at δ 5.30) indicating *O*-acetyl substitution at various locations. Consistent with this interpretation, treatment of the polymeric material with alkali removed most of the minor signals (including that at δ 5.30), and also simplified the signal at δ ~1.3 without affecting the chemical shifts of the major anomeric doublets. The possible origin and significance of the minor components of the polymeric material are considered later.

The ^{13}C-n.m.r. spectrum of the *O*-deacetylated polymeric material contained 13 major signals (Table I), including one of double intensity. These signals included anomeric signals at δ 100.31 and 98.53 (consistent with a disaccharide repeating-unit for the major polymer), signals for a 2-acetamido group at δ ~175, 54.28, and 22.69, and two for unsubstituted hydroxymethyl carbons at δ 60.76 and 60.32. Methylation analysis showed that the repeating unit was constructed from 4-substituted glucopyranosyl and 3-substituted 2-acetamido-2-deoxyglucopyranosyl residues as in structure **1**.

→4)-α-D-Glc*p*-(1→3)-α-L-Glc*p*NAc-(1→

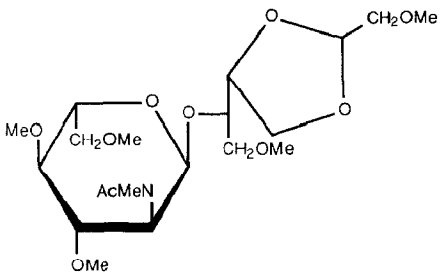
1

Further evidence for structure **1** was obtained by Smith degradation of the *O*-deacetylated material. Although gel permeation h.p.l.c. gave virtually a single peak, with an elution volume corresponding to that of a disaccharide-alditol, further studies showed that the product did not consist simply of the expected glycosylerythritol (**2**). The anomeric region of the ^{1}H-n.m.r. spectrum contained three major signals at δ 5.16 (d, 0.7 H, 3.8 Hz), 5.05 (d, 1 H, 3.8 Hz), and 4.98 (t, 0.7 H, 3.5 Hz), as well as several minor signals (each <0.2 H), *e.g.*, at δ 5.01 (d, 1.6 Hz), 5.00 (s), and 4.94 (d, 1.7 Hz). Other significant ^{1}H signals were a methyl singlet (equivalent to 5.1 H) at δ 2.07 and a methyl doublet (equivalent to 1 H, 6.3 Hz) at δ 1.29. These data suggested the presence of two major glycosides of 2-acetamido-2-deoxyglucose (molar ratio 1.0:0.7) and of minor component(s) containing rhamnose (see later). The ^{13}C-n.m.r. spectrum of the Smith-degradation products supported the above inferences. Again, there were three signals in the anomeric region (at δ 103.52, 98.58, and 98.35), and the signals due to the 2-acetamido group (at δ 54.73, 54.61, 22.74, and 22.64; carbonyl resonances were not recorded) were split. Also, five signals in the range δ 61.32 to 63.17, corresponding to unsubstituted hydroxymethyl carbons, were detected.

α-L-Glc*p*NAc-(1→2)-Erythritol

2

The n.m.r. evidence for heterogeneity of the Smith-degradation product was confirmed by g.l.c. Two major, two minor, and one trace component were detected in the methylated oligosaccharide-alditols. G.l.c.–m.s. studies showed that the most abundant products (S3 and S4) and the trace product (S5) each contained a 2-acetamido-2-deoxyglucopyranosyl group. Diagnostic ions on e.i.-m.s. were members of the aA series[15] at *m/z* 260 and 228. The alditol residue in S3 was identified as erythritol from the ions at *m/z* 147 and 115 (the bA series) and at *m/z* 207 (J_1). Conformation was provided by c.i.-m.s. with ammonia as the reagent gas: although the base peak was at *m/z* 260 (aA_1), diagnostic ions included the quasimolecular ion at *m/z* 424 $[M + H]^+$ and an ion at *m/z* 165 $[147 + NH_4]^+$. Thus, the parent disaccharide-alditol of S3 could be assigned structure **2** (the classical Smith-degradation product). Similar studies of the other major product S4 indicated that it was derived from a substituted 1,3-dioxolane (or possibly a 1,3-dioxane). Thus, ions derived from the "alditol" residue were shifted by 28 mass units compared with the corresponding ions for S3, to *m/z* 175, 143, and 235 (e.i.-m.s.) or *m/z* 193 and 452 (c.i.-m.s.). Thus, the parent compound for S4 probably has structure **3**. Such by-products occur through acid-catalysed transacetalation during the hydrolysis step of the Smith degradation[16–18], and formation of the 5-membered ring seems to be favoured with α-glycans[19]. The production of compound **3** would explain the pseudoanomeric signals at δ 4.98 (^1H) and δ 103.52 (^{13}C) observed for the total Smith-degradation products. No structural information was obtained for the trace product S5. However, the formation of an ion with *m/z* 406 on e.i.-m.s. for both S4 (corresponding to M − 45) and S5 indicated that the compounds were isomeric.



3

During the characterization of the repeating unit of the major polymer as structure **1**, some evidence for the presence and structural features of a minor polymer was obtained. As already noted, the polymeric material contained rhamnose and *O*-acetyl groups. From the ^1H-n.m.r. data, the ratio of these components was ~1.0:0.9, while the effect of *O*-deacetylation on the signal for H-6 of rhamnose suggested a common polymeric location. Methylation analysis also showed that the rhamnose occurred as 3-substituted pyranosyl residues. Finally,

g.l.c.-m.s. studies of the methylated Smith-degradation products showed that S1 and S2 were the rhamnosyl counterparts of S3 and S4. Thus, ions of the aA series at *m/z* 189, 157, and 125 were detected for both compounds by e.i.- and c.i.-m.s., and the appropriate "alditol"-derived ions described for S3 and S4 were also detected. Tentatively, it is suggested that the minor polymer may be an acetylated glucorhamnan of partial structure **4**. Polymers with such repeating units have been found as the side chains of lipopolysaccharides from *Serratia marcescens,* for example[20].

→4)-Glc*p*-(1→3)-Rha*p*-(1→

4

As far as we aware, the major polymer with repeating unit **1** is unique among bacterial polysaccharides in containing 2-amino-2-deoxy-L-glucose, although this sugar (as its *N*-methyl derivative) is well known as a component of streptomycin. Assuming that lipid A in the O1 reference strain of *P. cepacia* has a conventional structure, the presence in the lipopolysaccharide of both enantiomers of 2-amino-2-deoxyglucose may be inferred. It seems that the metabolic versatility of *P. cepacia* extends to the biosynthesis of unusual sugars, as D-rhamnose and D-fucose have also been found as components of O-specific polymers[8,11].

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharide. — Cultures of the reference strain[5] for *P. cepacia* O1 were grown for 24 h at 37° and used to prepare cell walls[21]. The yields of wet cells and freeze-dried cell walls, respectively, were as follows: batch 1, 92 g and 2.22 g; batch 2, 90 g and 1.73 g. Lipopolysaccharide was obtained by hot, aqueous phenol extraction of the defatted cell walls[12] and was hydrolysed with aq. 1% acetic acid at 100° or by the method of Caroff *et al.*[14]. Phosphorus-free, polymeric material was isolated by chromatography of the water-soluble products on Sephadex G-50.

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 in pyridine–acetic acid buffer. Equipment used for g.l.c., n.m.r. spectroscopy, and the measurement of optical rotation was that previously described[12]. C.d. spectra were recorded at the S.E.R.C. national service (Birkbeck College, London). E.i.-m.s. was done with a Finnigan 1020B instrument (by direct insertion or by coupling with g.l.c.). Further studies by g.l.c.–m.s. with e.i. or c.i. (ammonia) were carried out by the S.E.R.C. service (University College of Swansea). ^{1}H-N.m.r. spectra were recorded at 70° (polysaccharides), 60° (oligosaccharide-alditols), or 21° (peracetylated amino sugars) with D_2O or $CDCl_3$ as the solvent (as appropriate), and external sodium

trimethylsilylpropanoate-d_4 or internal tetramethylsilane as the reference. ^{13}C-N.m.r. spectra for samples in D_2O were recorded at 21 or 27° with 1,4-dioxane as the internal reference.

Identification and determination of neutral sugars. — Samples were hydrolysed with 2M HCl for 2 h at 105°. After neutralisation and deionisation, hydrolysates were examined by p.c. (solvent *A*) and by g.l.c. of the alditol acetates. D-Glucose was identified and determined by using D-glucose oxidase (EC 1.1.3.4) and by using the combination of hexokinase (EC 2.7.1.1) with D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49).

Identification and determination of amino sugars. — Samples were hydrolysed with 6.1M HCl for 4 h at 105° and the hydrolysates were dried repeatedly *in vacuo* over KOH and P_2O_5. When necessary, amino sugars were isolated via adsorption on Dowex 50 (H^+) resin[12]. Analyses were carried out by p.c. (solvent *B*), p.e., autoanalysis (Locarte), g.l.c. and m.s. of the acetylated aminodeoxyalditol, g.l.c. of the 2,5-anhydroalditol acetate derived via deamination–reduction[22], and ^{1}H-n.m.r. spectroscopy of the amino sugar acetates. The last derivatives were produced by treatment of the O1 amino sugar with pyridine–acetic anhydride (1:1) for 1 h at 100° and were obtained with a 2.7:1.0 ratio of α,β-pyranose anomers. Comparisons were made with the reference penta-acetates (Koch–Light) and the products obtained from 2-amino-2-deoxy-D-glucose under the same reaction conditions. *N*-Acetylation of the amino sugar, followed by the Morgan–Elson reaction, was carried out by the method of Strominger *et al.*[23].

For the assignment of configuration, the 2-amino-2-deoxyglucose from *P. cepacia* was examined by polarimetry, enzymic assay[24], c.d. of the acetylated aminodeoxyalditol dissolved in CH_3CN (ref. 25), and g.l.c. of the acetylated but-2-yl[26] and oct-2-yl[27] glycosides.

Identification of fatty acids. — Lipopolysaccharide was hydrolysed with 6.1M HCl for 4 h at 105° and the fatty acids were extracted with diethyl ether. Methyl esters were prepared by treatment with 0.7M HCl in methanol for 30 min at 100° and were identified by g.l.c. after extraction into hexane.

Smith degradation. — This was carried out on a sample (20 mg) of *O*-deacetylated polymeric material[12]: the degradation products were isolated by h.p.l.c. on a TSKgel G-Oligo-PW column (Anachem). G.l.c. of the permethylated products revealed five components (% total peak area): S1 (12), S2 (7), S3 (44), S4 (37), S5 (trace). The components were characterized by g.l.c.–m.s. (e.i. and c.i.). Significant fragment ions (relative intensities in brackets and some assignments[15] in square brackets) for the first four products on e.i.-m.s. are listed below. S1, *m/z* 88(100), 101(22), 115(21) [bA_2], 125(3) [aA_3], 129(5), 147(3) [bA_1], 157(2) [aA_2], 159(2), 189(4) [aA_1], and 207(14) [abJ_1]; S2, *m/z* 88(100), 101(34), 157(4) [aA_2], 175(14) [bA_1], 189(13) [aA_1], 235(9) [abJ_1], and 335(3) [M − 45]; S3, *m/z* 45(100), 71(60), 87(63), 115(43) [bA_2], 129(72), 142(37), 147(15) [bA_1], 207(29) [abJ_1], 228(9) [aA_2], 249(8), and 260(1) [aA_1]; S4, *m/z* 45(100), 71(38), 87(59), 129(68), 142(39), 175(15) [bA_1], 228(10) [aA_2], 235(19) [abJ_1], 260(2) [aA_1], and 406(10) [M − 45].

Other methods. — Methylation of the polymeric material and of the oligosaccharide-alditols was carried out by using potassium methylsulphinylmethanide[28], and the products were purified by using Sep-Pak cartridges[29]. Partially methylated alditol acetates were prepared and identified by standard methods[12]. *O*-Deacetylation was achieved by using 0.1M NaOH at room temperature overnight.

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