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LIPOPOLYSACCHARIDES FROM *Pseudomonas maltophilia*: COMPOSITION OF THE LIPOPOLYSACCHARIDE AND STRUCTURE OF THE SIDE-CHAIN POLYSACCHARIDE FROM STRAIN N.C.I.B. 9204

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ABSTRACT

Lipopolysaccharide was extracted from defatted cell-walls of *Pseudomonas maltophilia* N.C.I.B. 9204. The major fatty acid components were 9-methyldecanoic acid, 2-hydroxy-9-methyldecanoic acid, 3-hydroxy-9-methyldecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxy-11-methyldodecanoic acid. Monosaccharide components of the phosphorylated core-oligosaccharide were D-glucose, D-mannose, D-galacturonic acid, 2-amino-2-deoxyglucose, and a 3-deoxyoctulosonic acid. The putative O-specific polysaccharide was composed mainly of 2-amino-2-deoxy-D-glucose, D-arabinose, and 6-deoxy-L-talose, but also contained an *O*-acetyl group and small proportions of rhamnose and 6-deoxy-3-*O*-methyltalose. Degradative and n.m.r. (^{1}H and ^{13}C) studies showed that the polymer had a branched trisaccharide repeating-unit with the following structure; the *O*-acetyl group was tentatively assigned to C-2 of the 6-deoxytalopyranosyl residue.

α-D-Ara*f*
1
↓
6
→3)-β-D-Glc*p*NAc-(1→3)-α-L-6dTal*p*-(1→

INTRODUCTION

Pseudomonas maltophilia, as an opportunist pathogen, commands the attention of clinical microbiologists[1–3] and, as a very distinctive pseudomonad, attracts the interest of bacterial taxonomists[4–6]. Our studies[7,8] of the lipopolysaccharide from the type strain of the species (N.C.T.C. 10257) have revealed several structural features shared with lipopolysaccharides from some *Xanthomonas* species, supplementing the diverse evidence for a special relationship between these organisms[4,9]. In order to reinforce this inference, we have analysed the lipopolysaccharide from a second strain of *P. maltophilia* (N.C.I.B. 9204). This strain was selected as a result of a preliminary survey[10], which suggested the presence in the lipopolysaccharide of an unusual neutral sugar, here identified as 6-deoxy-L-talose.

TABLE I

FATTY ACID COMPOSITION OF THE LIPOPOLYSACCHARIDE[a]

Fatty acid[b]	*Acid hydrolysate*	*Alkaline hydrolysate*
10:0	1.3	1.3
i-11:0	14.9	14.1
Δ^3-i-11:1	0	0.3
Δ^2-i-11:1	2.8	1.1
Δ^3-12:1	0	1.9
Δ^3-i-13:1	0	2.4
Δ^2-12:1	3.1	2.2
Δ^2-i-13:1	4.5	2.7
i-15:0	0.4	0.3
2-OH-i-11:0	6.4	6.4
3-OH-10:0	1.0	0.5
3-OH-i-11:0	13.0	13.2
3-OH-11:0	2.8	1.1
3-OH-i-12:0	1.4	1.6
3-OH-12:0	16.1	16.1
3-OH-i-13:0	27.5	29.6
Unidentified acids	4.8	5.2

[a]Determined by g.l.c. of the methyl esters after hydrolysis at 105° for 5 h under nitrogen with 4M HCl or KOH. Results are given as percentages of the total peak area. [b]Shorthand designations: Δ^3-i-11:1 = 9-methyldec-3-enoic acid, 3-OH-i-11:0 = 3-hydroxy-9-methyldecanoic acid, *etc.*

RESULTS AND DISCUSSION

Lipopolysaccharide was obtained from isolated cell-walls in modest yield (average for five batches, 11%). Typical analyses were total carbohydrate, 33% (determined as glucose by the phenol–sulphuric acid method), and phosphorus, 2.4%. The lipopolysaccharide contained a 3-deoxyoctulosonic acid (0.4% by the thiobarbituric acid method), D-galacturonic acid (*vide infra*), and 2-amino-2-deoxy-D-glucose (D-glucosamine) (16%), but no galactosamine or aldoheptose. The fatty acid composition is given in Table I. Neutral sugar components and their approximate proportions (as indicated by relative peak areas in g.l.c. of the alditol acetates) were arabinose (100), Compound X (<76), glucose (38), mannose (36), ribose (4), Compound Y (3), and rhamnose (integrated with Compound X). Glucose and mannose were shown to be the D isomers by means of enzymic assays, and arabinose was identified as the D isomer by the $[\alpha]_D$ value of −99° (*c* 0.3, water); lit. −105°.

Compound X had high mobilities (*e.g.*, R_{Rha} 1.2, solvent 4) in p.c., similar to those of 3-*O*-methylxylose. However, it gave a yellow-brown colour with aniline hydrogenoxalate (and white fluorescence under u.v. light) and was recovered unchanged after attempted *O*-demethylation. Its alditol acetate had a g.l.c. retention time (0.63 relative to arabinitol penta-acetate on column 1) slightly greater than that of rhamnitol penta-acetate, but an identical mass spectrum (primary fragments at

m/z 303, 290, 231, 218, 159, and 146 for the product having a deuterium label at C-1, and at *m/z* 318 [M − 59]). Paper chromatography (solvent *C*) and paper electrophoresis (buffer III), using rhamnose, fucose, and quinovose as reference 6-deoxyhexoses, indicated[11] that Compound X was 6-deoxytalose. Chromatographic and electrophoretic comparisons with synthetic 6-deoxy-L-talose confirmed this diagnosis. The L configuration of the sugar from *P. maltophilia* was indicated by the direction (laevo) of optical rotation (for 6-deoxy-L-talose, $[\alpha]_D$ is $-20°$), and was confirmed by g.l.c. of the acetylated oct-2-yl glycosides[12].

Compound Y had even greater mobilities in p.c. (*e.g.*, R_{Rha} 1.4, solvent *A*) than compound X, but gave similar colour reactions with aniline hydrogenoxalate. Its alditol acetate had a g.l.c. retention time of 0.50, and a mass spectrum (primary fragments at *m/z* 203 and 190 for the product having a deuterium label at C-1) which indicated that Compound Y was a 6-deoxy-3-*O*-methylhexose. Its identification as 6-deoxy-3-*O*-methyltalose (acovenose) was based on comparisons with the reference compound by p.c. (solvents *A* and *C*), paper electrophoresis (buffer III), g.l.c. of the alditol acetate (column I), and *O*-demethylation followed by p.c. and electrophoresis of the 6-deoxytalose produced. The very small amount of Compound Y available prevented a conclusive assignment of its configuration, but the sugar seemed to be laevorotatory, indicating[11] it to be L.

Hydrolysis of the lipopolysaccharide with 1% aqueous acetic acid at 100° for 1.5 h, followed by centrifugation of the hydrolysate, gave insoluble lipid A, and water-soluble products (yield, 60–70%) which were fractionated by chromatography on Sephadex. Fraction I, the putative side-chain polysaccharide, was obtained in a yield of 30–40% (based on lipopolysaccharide) by using Sephadex G-50. Fraction II, the partly degraded core-oligosaccharide, was separated from hydrolytic fragments (Fractions III and IV) by using Sephadex G-15 (Fig. 1). Fraction III contained most of the P_i, a little ethanolamine phosphate (identified by paper electrophoresis in buffer system I and hydrolysis by alkaline phosphatase), 3-deoxyoctulosonic acid, and galacturonic acid. The two sugar acids were isolated and then separated by preparative paper electrophoresis (buffers I and II, respectively). The identity of the hexuronic acid was confirmed by p.c. (solvents *D* and *E*) and by reduction to galactose. Identical results were obtained when the galactose was determined enzymically and by the phenol–sulphuric acid method, showing that the uronic acid was D-galacturonic acid. Fraction IV contained some P_i, arabinose, and an unidentified neutral compound which reduced alkaline silver nitrate and gave a yellow colour with aniline hydrogenoxalate (R_{Glc} 0.75, solvent *A*). No products were detected when an acid hydrolysate of the compound was examined by p.c. (the parent compound was absent from the hydrolysate), nor when the compound was reduced, acetylated, and then examined by g.l.c. The same compound is apparently formed or released when the lipopolysaccharide from *P. maltophilia* N.C.T.C. 10257 is subjected to a comparable, mild hydrolysis[8] with acid. Fraction II from *P. maltophilia* N.C.I.B. 9204 contained D-glucose, D-mannose, D-galacturonic acid, glucosamine, a 3-deoxyoctulosonic acid,

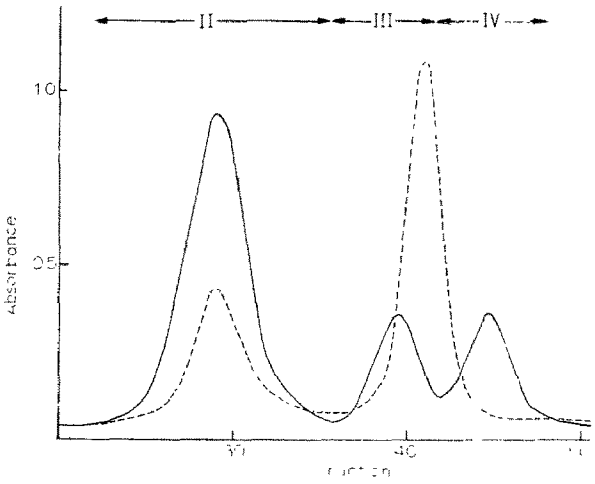


Fig. 1. Separation of the partly degraded core-oligosaccharide (Fraction II) from hydrolytic fragments (Fractions III and IV). After hydrolysis of the lipopolysaccharide with 1% acetic acid at 100° for 1.5 h, lipid A was isolated by centrifugation and the putative O-specific polysaccharide by chromatography on Sephadex G-50. The solutes of low molecular weight were eluted from a column (84 × 1.5 cm) of Sephadex G-15 with aqueous pyridine–acetic acid buffer (pH 5.4) at a flow rate of 5 mL h^{-1}. Fractions (2 mL) were analysed for total carbohydrate by the phenol–sulphuric acid method (A_{490}, — — —) and for phosphorus (A_{830}, - - -).

and phosphorus. The results of structural studies of this phosphorylated oligosaccharide will be published elsewhere.

The major components of the polymeric Fraction I, $[\alpha]_D$ +11° (*c* 1.8, water), were D-arabinose, 6-deoxy-L-talose, and D-glucosamine. Quantitative data are given in Table II. Because of an unexpected result obtained during methylation analysis of Fraction I, special care was taken over identification of the hexosamine. The compound from Fraction I was indistinguishable from glucosamine by p.c. (solvent

TABLE II

COMPOSITION OF THE POLYMERIC FRACTION I[a]

Component	*Composition (%)*
D-Arabinose	20.1
6-Deoxy-L-talose	16.3
D-Glucosamine	20.9
Rhamnose	3.2
Mannose	2.9
Glucose	2.5
Acovenose	~1
Ribose	Trace
Phosphorus	0.36

[a]Neutral sugars were estimated by g.l.c. of the alditol acetates, and glucosamine by autoanalysis.

E), paper electrophoresis (buffer I), cation-exchange chromatography[13], autoanalysis (continuous elution with citrate buffer having pH 5.28, or stepwise elution with citrate buffers having pH 3.25, 4.25, and 6.65), and g.l.c. of the alditol acetate (column II). As expected, reactivity in the Elson–Morgan assay was insignificant when the treatment with alkaline pentane-2,4-dione was carried out at 0° (ref. 14). Degradation of the hexosamine with ninhydrin gave arabinose (identified by p.c. and by g.l.c. of the alditol acetate), and deamination gave 2,5-anhydromannose (identified by p.c. of the anhydroalditol and g.l.c. of its tetra-acetate). The $[\alpha]_D$ value of the hydrochloride, isolated by cation-exchange chromatography[13], was +70.5° (*c* 1.5, water; based on the Elson–Morgan assay[14]); lit. +72.5° for D-glucosamine hydrochloride.

The analytical data for Fraction I (Table II) are indicative of a trisaccharide repeating-unit, while the small proportions of phosphorus, glucose, and mannose are consistent with the presence of a terminal core-oligosaccharide. The significance of the other minor components (consistent in all five batches of lipopolysaccharide) is more problematic. Although contamination of the side-chain polysaccharide is an obvious explanation, one of the sugars (rhamnose or acovenose?) could be present as a modified, terminal repeating-unit.

The presence in the polysaccharide of equal amounts of *O*-acetyl and *N*-acetyl groups was evident from the i.r. spectrum (bands at 1740, 1660, and 1550 cm^{-1}), the ^{1}H-n.m.r. spectrum (singlets at δ 2.15 and 2.06, each 3 H), and the ^{13}C-n.m.r. spectrum (signals at δ 174.78, 173.06, 22.85, and 20.85). In the n.m.r. spectra for the alkali-treated polysaccharide, the corresponding signals at δ 2.05 (^{1}H), 174.70, and 22.67 (^{13}C) could be attributed to the *N*-acetyl group. The ratio of total acetyl (by g.l.c.) to *O*-acetyl (by the ferric hydroxamate assay) was 2.16:1, essentially in agreement with the spectroscopic data.

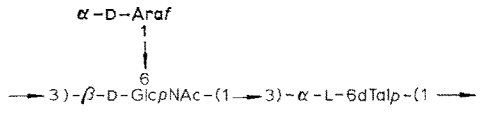
Other signals in the ^{1}H-n.m.r. spectrum of native Fraction I included a 3-proton doublet (*J* 6.4 Hz) at δ 1.25, corresponding to the methyl group of 6-deoxytalose, and four 1-proton signals in the anomeric region at δ 4.74 ($J_{1,2}$ ~8 Hz), 4.96, 5.00, and 5.05 (all low *J* values). *O*-Deacetylated Fraction I gave three anomeric signals at δ 4.74 ($J_{1,2}$ ~8 Hz), 4.97, and 5.06, indicating that the signal lost could be attributed to the proton attached to the acetoxylated carbon. As other studies (*vide infra*) showed that the arabinose is present as a furanosyl group, the signal at δ 4.74 must correspond to the anomeric proton of a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. A trisaccharide repeating-unit for Fraction I was also confirmed by the ^{13}C-n.m.r. spectra; signals for anomeric carbons were found at δ 107.83, 100.86, and 99.19, of which that at lowest field can be attributed to C-1 of an α-D-arabinofuranosyl residue[15–17]. For *O*-deacetylated Fraction I, the anomeric signals occurred at δ 107.75 ($^1J_{CH}$ 173.9 Hz), 101.71 ($^1J_{CH}$ 169.2 Hz), and 99.87 ($^1J_{CH}$ 165.5 Hz). Selective, heteronuclear $^{13}C\{^1H\}$ irradiation at δ 4.74 confirmed that the ^{13}C-signal at δ 99.87 was due to C-1 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue, and indicated therefore that the signal at δ 101.71 was due to C-1 of a 6-deoxy-α-L-talopyranosyl residue (assuming the $^1C_4$ conformation). During oxidation of the peracetylated polysaccharide with chromium trioxide, destruction of all three major

monosaccharide components occurred rapidly and was essentially complete after 3 h at 50°. This result was expected for the arabinofuranosyl and β-glucosaminyl residues[18], and could reflect low conformational stability in the case of the 6-deoxytalopyranosyl residues.

Linkages in the polysaccharide were identified by methylation analysis. G.l.c. of the methylated alditol acetates from the neutral sugars gave three significant peaks (A, B, and C; relative areas, 1.00:0.94:0.29) and several minor ones. Peak A corresponded to 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol in its retention times on columns I, III, and IV, and in g.l.c.–m.s. (primary ions at *m/z* 161, 118, and 45 for the product having a deuterium label at C-1). Peak B had the following retention times relative to those of peak A: column I, 1.73; column III, 1.61; column IV, 1.58. The mass spectrum (primary ions at *m/z* 234, 131, and 118 for the product having a deuterium label at C-1) indicated that peak B corresponded to 1,3,5-tri-*O*-acetyl-6-deoxy-2,4-di-*O*-methyltalitol. Peak C had a retention time (column I) close to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, which[19], combined with the results of g.l.c.–m.s. (primary ions at *m/z* 234, 203, 131, and 118), suggested that it was derived from both 3-substituted and 4-substituted rhamnopyranosyl residues. The significance of these residues was not determined.

By means of g.l.c. using columns II and V, peaks corresponding to two methylation products from glucosamine were detected (relative areas, ~8:1). The major peak had retention times relative to those of glucosaminitol hexa-acetate of ~0.9 (column II) and ~1.1 (column V). The latter result notwithstanding, g.l.c.–m.s. showed that the product was derived from a 3,6-disubstituted *N*-acetylhexosamine residue, and that part of the product had been *N*-methylated. The minor peak, with relative retention times of ~0.4 (column II) and ~0.6 (column V), corresponded to the analogous products from a 3-substituted *N*-acetylhexosamine residue. Thus, the results of methylation analysis indicated that Fraction I was constructed from a branched-trisaccharide repeating-unit and that the main chain was substituted at *N*-acetylglucosamine by arabinofuranosyl groups, some of which had probably been lost during the mild, acid hydrolysis used to prepare Fraction I.

In accord with the above results, only arabinose of the major components was destroyed during periodate oxidation of native Fraction I, and the oxidation was complete within 24 h. The production of formaldehyde was negligible; of the periodate consumed (2.17 μmol.mg^{-1}), 70% could be accounted for by the oxidation of the arabinofuranosyl groups. As expected, arabinose was the only monosaccharide released when Fraction I was hydrolysed under mild conditions (0.05M HCl, 100°, 30 min; or 0.1M trifluoroacetic acid, 85°, 1.5 h). After hydrolysis under the latter conditions, polymeric and oligomeric products (both depleted in arabinose) were isolated by chromatography on Sephadex. The oligomeric products contained comparable amounts of 6-deoxytalose, glucose, and mannose, indicating an enhanced content of core oligosaccharide. Monosaccharide and methylation analyses of the polymeric products showed that ~80% of the arabinofuranosyl residues had been released, and that the ratio of 3-substituted to 3,6-disubstituted *N*-acetylglucosaminyl



1

residues was ~4:1. Structure **1** can therefore be assigned to the repeating unit of *O*-deacetylated Fraction I.

The results of periodate oxidation of Fraction I, together with a signal at δ 61.44 in the ^{13}C-n.m.r. spectrum (which must correspond to unsubstituted C-5 of arabinose), showed that the arabinofuranosyl groups were not *O*-acetylated. Also, if C-4 of the *N*-acetylglucosaminyl residue (the only position available) had been acetoxylated, the signal at δ 5.00 in the ^{1}H-n.m.r. spectrum would have shown measurable splitting because of the *trans*-diaxial relationship of H-4 to H-3 and H-5. Thus, possible sites for the *O*-acetyl group were limited to the axial hydroxyl groups at C-2 and C-4 of the 6-deoxytalopyranosyl residue.

In order to distinguish between these possibilities, more-detailed analysis of the ^{13}C-n.m.r. spectra was attempted. The assignment of signals to the carbon atoms of the unsubstituted arabinofuranosyl group (Table III) was facilitated by their prominence (*e.g.*, Fig. 2 for *O*-deacetylated Fraction I), their insensitivity to *O*-deacetylation, and the use of literature data[15-17]. Readily identified signals for the β-*N*-acetylglucosaminyl residue in the *O*-deacetylated polymer were those at δ 55.60 (C-2), 66.04 (C-6; a triplet in the proton-coupled spectrum), and 81.35 (*O*-glycosyl-

TABLE III

ASSIGNMENT OF SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF FRACTION I AND *O*-DEACETYLATED FRACTION I[a]

Carbon atom	↓ 6 →3-β-GlcpNAc-1→		(OAc) | 2? →3-α-6dTalp-1→		α-Araf-1→	
C-1	99.87	(100.91)[b]	101.71	(99.19)[b]	107.75	(107.82)
C-2	55.60	(55.38)	69.86[c]	(69.00)[d]	81.13	(81.05)
C-3	81.35	(81.96)	74.50[e]	(74.44)[f]	76.72	(76.78)
C-4	70.21[c]	(69.89)[d]	68.28[g]	(67.95)[h]	84.02	(83.96)
C-5	74.33[e]	(74.18)[f]	67.79[g]	(67.26)[h]	61.43	(61.44)
C-6	66.04	(65.77)	15.54	(15.31)		
-NHCOCH$_3$	174.70	(174.78)				
-NHCOCH$_3$	22.67	(22.85)				
-OCOCH$_3$				(173.06)		
-OCOCH$_3$				(20.85)		

[a]Chemical shifts are given in p.p.m. downfield from external tetramethylsilane. Values in parentheses are those for the native Fraction I. [b–h]Pairs of signals for which the assignments may be interchanged within each pair.

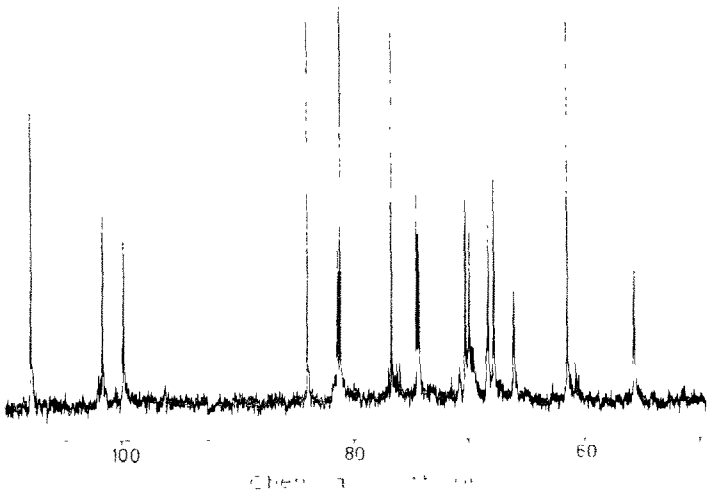


Fig. 2. ^{13}C-N.m.r. spectrum for *O*-deacetylated Fraction I. The sample was dissolved in D_2O, and the spectrum was obtained at 100.62 MHz and 40° with complete proton-decoupling. In addition to the signals shown, the spectrum contained signals at δ 174.70, 22.67, and 15.54 with reference to external tetramethylsilane.

ated C-3). Tentative assignments for the remaining signals (Table III) were derived by using data for methyl 2-acetamido-2-deoxy-β-D-glucopyranoside[20] and α-D-talopyranose[21], and the effect of a 6-deoxy group on chemical shifts of C-4 and C-5 for other aldohexoses[22]. It was also assumed that *O*-β-D-glycosylation at C-3 in the 6-deoxy-L-talopyranosyl residue would have no significant β-effect at C-2 and C-4, which carry axial hydroxyl groups[23,24].

A comparison of the ^{13}C-n.m.r. spectra for native Fraction I and *O*-deacetylated Fraction I did not reveal any striking differences that could be attributed to acetylation shifts, although the possibility that signals were falsely correlated was not excluded. Assuming no major, undetected acetylation shifts, the largest changes caused by the presence of an *O*-acetyl group were in the shifts of the four signals between δ 67.79 and 70.21 (corresponding to C-2,4,5 of the 6-deoxytalosyl residue and C-4 of the glucosaminyl residue), a downfield shift of 0.61 p.p.m. of the signal previously at δ 81.35 (C-3 of the glucosaminyl residue), and the shifts of the two anomeric signals near δ 100. It appeared that the *O*-acetyl group caused an upfield shift of both anomeric signals, or inverted their relative positions. Because of overlapping signals, the issue was not unambiguously resolved by a proton-coupled spectrum, but this did indicate that the high-field signal (δ 99.19) had $^1J_{C,H} \sim 170$ Hz, as expected for C-1 of an α-talopyranosyl residue. If valid, this observation places the *O*-acetyl group at C-2. It should be noted that the chemical shift of C-3 was insensitive to *O*-acetylation, whether it was at position 2 or 4.

In its fatty acid composition, the lipopolysaccharide from *P. maltophilia* N.C.I.B. 9204 closely resembles that from strain N.C.T.C. 10257 (ref. 8), and probably those of other strains of the organism[5,25–28]. Some of the distinctive features

of the core oligosaccharide[8] from strain N.C.T.C. 10257, *e.g.*, the presence of acid-labile D-galacturonic acid residues and of phosphorylated D-mannose, and the absence of an aldoheptose, also seem to apply to the core oligosaccharide from strain N.C.I.B. 9204. One point of difference between the oligosaccharides is the presence of glucosamine in the latter but galactosamine in the former. As yet there are insufficient data about the O-specific polysaccharides from *P. maltophilia* for any general features to be identified, although rhamnose is a major component for some strains[7,8,29] and for many lipopolysaccharides from *Xanthomonas* species[30,31]. The polysaccharide from *P. maltophilia* N.C.I.B. 9204 is exceptional in that two of its monosaccharide components, D-arabinose and 6-deoxy-L-talose, are rarely found in bacterial polysaccharides, although both have been identified as components of mycobacterial products[32–34]. Other sources of 6-deoxytalose are the cell wall of *Actinomyces bovis*[35], the cell wall of *Streptococcus bovis*[36], a lipopolysaccharide from *Escherichia coli*[37] (the L isomer in each case), a lipopolysaccharide from *Rhodopseudomonas palustris*[38], the capsular polysaccharide from an unidentified Gram-negative organism[39] (the D isomer in each case), and polysaccharides from *Actinomyces viscosus*[40] and *Gardnerella vaginalis*[41] (unidentified isomer). In several of these polysaccharides, the 6-deoxyhexose is accompanied by its 3-methyl ether (acovenose), as in the case of the lipopolysaccharide from *P. maltophilia* N.C.I.B. 9204.

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharide. — Batch cultures of *P. maltophilia* N.C.I.B. 9204 were grown in Nutrient Broth No. 2 (Oxoid, 20 L) for 16 h or 24 h at 37° with aeration at 20 L.min^{-1}. Lipopolysaccharide was extracted from defatted cell-walls by the aqueous phenol method[7]. The water-soluble products obtained by hydrolysis of the lipopolysaccharide with 1% aqueous acetic acid at 100° for 1.5 h were fractionated by chromatography[7,8] on columns of Sephadex G-50 and G-15.

Chromatographic and electrophoretic methods. — The following solvent systems were used for p.c.: *A*, the upper phase of ethyl acetate–pyridine–water (5:2:5); *B*, the upper phase of ethyl acetate–acetic acid–water (3:1:3); *C*, toluene–butan-1-ol (1:2) saturated with water; *D*, acetone–ethanol–propan-2-ol–0.05M sodium tetraborate (pH 10) (3:1:1:2); and *E*, ethyl acetate–pyridine–water–acetic acid (5:5:3:1). The following buffer systems were used for paper electrophoresis: I, pyridine–acetic acid–water (5:2:43, pH 5.3); II, pyridine–acetic acid–water (1:10:89) adjusted to pH 2.7 with formic acid; and III, 0.05M sodium tetraborate adjusted to pH 10.4. Detection was effected with alkaline silver nitrate, aniline hydrogenoxalate, the periodate–Schiff reagents, the Warren reagents, ninhydrin, the Hanes–Isherwood reagent (all used in previous studies[7,8]), vanillin–perchloric acid[42], *p*-aminohippuric acid–phthalic acid[11], and *p*-anisidine–hydrochloric acid[43]. T.l.c. on Silica gel G (Merck), with dichloromethane as the solvent and iodine as the detection reagent, was used for the separation of hydroxy from non-hydroxy fatty acid methyl esters.

For g.l.c., glass columns of the sizes indicated were packed with the following stationary phases: I, 3% of ECNSS-M on Gas Chrom Q (1.6 m × 2 mm); II, 3% of OV-225 on Gas Chrom Q (0.8 m × 2 mm); III, 10% of OS-138 and 1% of Adpet 80 on Chromosorb W (1.5 m × 3 mm); IV, 3% of Silar 10c on Gas Chrom Q (1.6 m × 2 mm); V, 2% of OV-17 on Gas Chrom Q (1.8 m × 2 mm). VI, 10% of di(ethylene glycol) succinate on Chromosorb W/AB (3.8 m × 2 mm). Column VII was a W.C.O.T. glass column (30 m × 0.3 mm) of SP-1000.

Methods of quantitative analysis. — Colorimetric methods used to determine total phosphorus, total carbohydrate, aldopentose, aldoheptose, and 3-deoxyoctulosonic acid, as well as enzymic methods for the determination of D-glucose, D-mannose, and D-galactose, were those listed previously[7,8]. Neutral monosaccharides, released by hydrolysis of samples with M HCl at 105° for 4 h, were also determined by g.l.c. of the alditol acetates (columns I or IV). Standard mixtures of sugars subjected to the same acid treatment were used for all calibrations (response factors determined for rhamnose were used for the determination of 6-deoxytalose). Amino sugars, released by hydrolysis of samples with 6.1M HCl at 105° for 4 h under nitrogen, were determined by autoanalysis (Locarte bench analyser). Glucosamine was also determined by variations[14,44] of the Elson–Morgan assay. Fatty acid composition was determined by g.l.c. (column VI) of the methyl esters, prepared using diazomethane, after hydrolysis of the lipopolysaccharide at 105° for 5 h under nitrogen with 4M HCl or 4M KOH. Total acetyl groups were determined by g.l.c.[45], and *O*-acetyl groups by the ferric hydroxamate method[46] with α-D-glucopyranose penta-acetate as the standard.

Methylation analysis. — A standard procedure[19] was followed for the permethylation of Fraction I. Three treatments for the release of partially methylated sugars were tried: *a*, 90% formic acid at 100° for 2 h, followed by 0.25M H_2SO_4 at 100° for 15 h; *b*, 0.5M trifluoroacetic acid at 100° for 16 h; *c*, 90% formic acid at 100° for 6 h, dilution with water (4 vol.), and further heating at 100° for 2 h. Method *c* (ref. 47) gave the best yields of neutral sugar derivatives. After reduction with $NaBH_4$ or $NaBD_4$, the products were converted into methylated alditol acetates by heating with acetic anhydride at 100° for 1 h, using either pyridine or sodium acetate as the catalyst.

Periodate oxidation. — Fraction I was oxidised with 50mM sodium periodate at 4° for up to 70 h. The consumption of periodate was measured by the method of Avigad[48], and the formation of formaldehyde by the chromotropic acid method.

Graded acid hydrolysis. — The release of monosaccharides from Fraction I, monitored by p.c. (solvent 4), was determined for various conditions of acid hydrolysis: *a*, 0.05M or 0.1M HCl at 100° for periods ranging from 10 min to 2 h; *b*, 0.05M H_2SO_4 at 80° for 3 h; *c*, 0.1M trifluoroacetic acid at 85° for 1.5 h. Method *c* appeared to give the most selective release of arabinose, and was used for the preparation of arabinose-depleted polymeric and oligomeric products, which were fractionated on Sephadex G-50 and subjected to monosaccharide and methylation analyses.

Miscellaneous methods. — Peracetylation of Fraction I and oxidation of the

product with chromium trioxide were carried out by standard methods[18]. *O*-Deacetylation of Fraction I was carried out with 0.1M NaOH at room temperature for 16 h, followed by passage of the hydrolysate down a column of Dowex 50 (H^+) resin. Previously described methods were used for the *O*-demethylation of acetylated sugar ethers[10], enzymic dephosphorylation of *O*-phosphoethanolamine[8], the reduction of galacturonic acid[8], and the isolation[13], deamination[8], and ninhydrin-degradation[49] of glucosamine. The preparation and g.l.c. (column VII) of acetylated oct-2-yl glycosides, using either (+)- or (−)-octan-2-ol (Fluka AG), was used to assign the configuration[12] to a sample of 6-deoxytalose: the authentic L isomer was used as a reference.

Physico-chemical methods. — I.r. spectra were recorded with a Unicam SP200 spectrophotometer; samples were dispersed in potassium chloride. Optical rotations were determined with a Bendix polarimeter (model 143A). N.m.r. spectra were recorded for solutions in D_2O with Bruker WH-360 and WH-400 spectrometers. ^1H-Spectra were recorded at 85° with sodium 4,4-dimethyl-4-silapentane-1-sulphonate as the external standard, and ^{13}C spectra (with complete proton-decoupling or with gated decoupling) were recorded at 40° with tetramethylsilane as the external standard. G.l.c.–m.s. analyses were carried out by Dr. S. Binns (Reckitt and Sons Ltd., Hull), staff of the Department of Organic Chemistry, University of Stockholm, and staff of the Physico-Chemical Measurements Unit, Harwell.

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