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STRUCTURAL STUDIES OF THE O-SPECIFIC POLYSACCHARIDE FROM *Serratia marcescens* N.C.T.C. 1377

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ABSTRACT

The putative O-specific polysaccharide of *Serratia marcescens* N.C.T.C. 1377 is a partially acetylated glucorhamnan. By means of ^{1}H- and ^{13}C-n.m.r. spectroscopy, methylation analysis, and periodate oxidation, it was shown that the polymer has a disaccharide repeating-unit for which the following structure is proposed: →4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→. *O*-Acetyl groups are probably located at C-2 of the rhamnopyranosyl residues. Except for the extent of *O*-acetylation, the polysaccharide is identical with the corresponding product from *S. marcescens* Bizio (A.T.C.C. 264), for which a different structure has previously been proposed.

INTRODUCTION

Serratia marcescens is recognised to be an important agent of nosocomial infections and, in consequence, various schemes for epidemiological typing of the organism [including typing of its heat-stable O-antigens (lipopolysaccharides)] have been developed[1,2]. Although these lipopolysaccharides have been studied extensively by several groups and for various reasons, relatively little information about their structures is available. Factors which have complicated previous studies include the production by *S. marcescens* of a complex range of cellular and extracellular polysaccharides[3,4], the tenacious "binding" of protein by the lipopolysaccharides[5–8], and the tendency of the lipopolysaccharides to dissolve in, or be degraded by, phenol[6,7,9,10]. Nevertheless, the biologically active lipid A fraction has been characterised in terms of heterogeneity[11,12], fatty acid composition[3,9,13,14], and basic structure[13]. Compositional data for the core oligosaccharide[15] and the structure of the O-specific polysaccharide[16,17] have also been obtained for strains Bizio (A.T.C.C. 264) and O8. As part of a wider programme to establish serotype–chemotype relationships for *S. marcescens*, we have analysed the lipopolysaccharide from the well-studied strain N.C.T.C. 1377. We now report on the structure of the putative O-specific polysaccharide, and compare it with the corresponding product from strain Bizio.

RESULTS AND DISCUSSION

Lipopolysaccharide was obtained from the aqueous phase after the extraction of isolated cell-walls with hot, aqueous phenol only in low yield (8%), suggesting that some material may have remained in the phenolic phase. On mild hydrolysis of the lipopolysaccharide (1% acetic acid, 100°, 2 h), a black suspension was formed. Although this observation has not been reported previously for *S. marcescens*, a similar result has been obtained[18] for a lipopolysaccharide from serogroup O14, and it may indicate the release and decomposition of 4-amino-4-deoxyarabinose as suggested for other lipopolysaccharides (page 33 of ref. 19). However, the presence of the latter amino sugar in lipid A from *S. marcescens* has not yet been established[20]. After the deposition of insoluble products by centrifugation, the hydrolysate was freeze-dried and then fractionated by chromatography on Sephadex. An initial separation of products of high and low molecular weight was achieved with Sephadex G-50, and these were further resolved by using Sephadex G-100 and G-15, respectively.

The products of low molecular weight consisted of the putative core-oligosaccharide (F2) and acid-labile components of the lipopolysaccharide (in F3). By means of paper electrophoresis, F3 was shown to contain a 3-deoxy-2-octulosonic acid (KDO), another compound reactive in the thiobarbituric acid test (M_{KDO} 0.78 at pH 5.3), P_i, and an unidentified phosphate. The last component gave an elongated streak extending to about the position of glucose 6-phosphate: this behaviour was not significantly affected by including EDTA in the buffer nor by pretreatment of F3 with alkaline phosphatase. Fraction F2 did not contain phosphorus; its major components were glucose and L-*glycero*-D-*manno*-heptose (or its enantiomer). The fraction also contained D-*glycero*-D-*manno*-heptose (or its enantiomer), ribose, 2-amino-2-deoxyglucose, and a trace of rhamnose.

The major sugars in the polymeric fraction (F1) of the mild, acid hydrolysate were glucose and rhamnose; small proportions of mannose, heptoses, ribose, and 2-amino-2-deoxyglucose were also present. The carbohydrate profile for F1 on Sephadex G-50 showed two overlapping peaks. These were better resolved on Sephadex G-100, to give F1a (the first peak), F1b, and F1c (respectively the front and rear portions of the second, broad peak). All of the sub-fractions had qualitatively similar monosaccharide compositions, but F1a appeared to contain undegraded lipopolysaccharide. Thus, F1a (11% of the total lipopolysaccharide) had enhanced contents of phosphorus and 2-amino-2-deoxyglucose, showed amide absorption bands in the i.r. spectrum, and gave discoloration and a precipitate (probably lipid A or its fatty acids) during acid hydrolysis. Sub-fractions F1b and F1c (4 and 10% of the lipopolysaccharide, respectively) had closely similar i.r. spectra and probably represented bands of O-specific polysaccharides differing in chain length[21,22]. The following structural studies were based mainly on sub-fraction F1c.

F1c contained mainly D-glucose and L-rhamnose (molar ratio 1.0:0.8), a small amount of L-*glycero*-D-*manno*-heptose (molar proportion ~0.16), and traces of D-*glycero*-D-*manno*-heptose, mannose, ribose, and hexosamine. The presence of an

O-acetyl substituent was indicated by an i.r. band at 1730 cm^{-1}, and by ^{13}C-n.m.r. signals at δ 173.80 and 20.54 (Fig. 1*a*) that were absent from the spectrum of the alkali-treated polysaccharide (Fig. 1*b*). The presence in the latter spectrum of twelve discrete signals, including anomeric signals at δ 100.85 and 95.79, showed that the polysaccharide had a disaccharide repeating-unit of pyranosyl residues. In the proton-

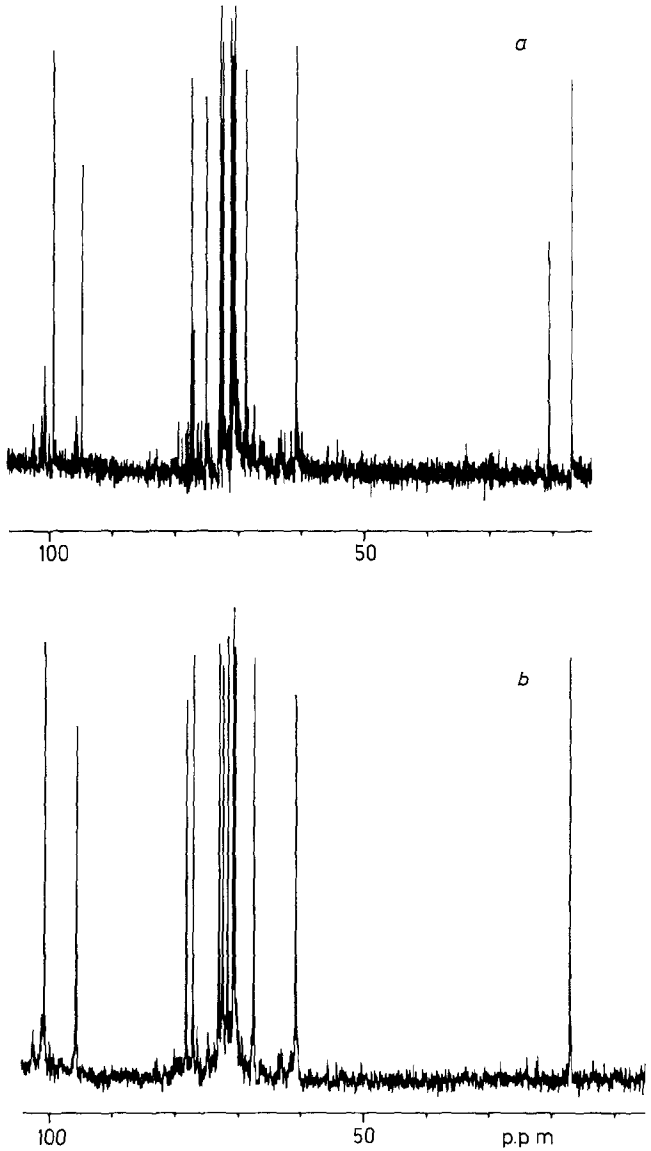

Fig. 1. ^{13}C-N.m.r. spectra for native fraction Flc (*a*) and deacetylated Flc (*b*). Spectra for the samples in D_2O were obtained at 100.62 MHz and 50° with complete proton-decoupling. In addition to the signals shown, the spectrum for native Flc contained a signal for a carbonyl carbon at δ 173.80 with reference to external Me_4Si.

coupled spectrum, the signal at δ 100.85 had a $^1J_{C,H}$ value of 162 Hz, and the signal at δ 95.79 a value of 170 Hz, indicating β and α configurations, respectively. The ^{1}H-n.m.r. spectrum of deacetylated F1c contained one-proton anomeric signals at δ 5.08 ($J_{1,2} \sim 3.5$ Hz) and 4.84 (broad singlet), and a methyl doublet ($J \sim 6$ Hz) at δ 1.30. The signals at δ 95.79 (^{13}C) and 5.08 (^{1}H) can be assigned to an α-D-glucopyranosyl residue, and the other anomeric signals to a β-L-rhamnopyranosyl residue. The $[\alpha]_D$ value of +106° (*c* 0.5, water) (actually determined for sub-fraction F1b) is consistent with this interpretation.

Linkages in the disaccharide repeating-unit of F1c were initially identified by methylation analysis. Only two significant peaks (A and B, relative areas 0.83 : 1.00) were detected by g.l.c. of the methylated alditol acetates. By means of g.l.c. retention times and g.l.c.–m.s., peak A was identified as the product from a 3-substituted rhamnopyranosyl residue, and peak B as the product from a 4-substituted glucopyranosyl residue. These linkages were confirmed by periodate oxidation of F1c. After 5 days, the consumption of periodate was 4.3 μmol.mg^{-1} (expected, 3.3 μmol.mg^{-1} for pure glucorhamnan), and analysis of the oxidised product showed that the rhamnose had survived but that the glucose had been destroyed by cleavage of the C-2–C-3 bond (erythritol was a major product after borohydride reduction and acid hydrolysis). Essentially the same results (for the rate and extent of periodate consumption, and the production of erythritol from glucose) were obtained on oxidation of a sample of polysaccharide containing few *O*-acetyl substituents (see below).

As the ^{13}C-n.m.r. spectrum of F1c showed that O-6 of glucose is unsubstituted (δ 60.86), the *O*-acetyl group must be placed at position 2 or 4 of rhamnose. A choice between these alternatives should be possible from an interpretation of the spectra for native and deacetylated F1c (Fig. 1), assuming that structure **1** represents the disaccharide repeating-unit. In both spectra, there are 8 signals in the range δ 67–79, of which the two at lowest field (δ 78.26 and 77.19 for deacetylated F1c) must correspond to the *O*-glycosylated carbon atoms. Tentatively, the signal at δ 78.26 is assigned to C-3 of the β-L-rhamnopyranosyl residue (the signal for C-3 in methyl β-L-rhamnopyranoside[23] is at δ 74.1, to which an α-D-glucosylation shift[24–27] of 4–7 p.p.m. might reasonably be added; the signal for C-4 in methyl α-D-glucopyranoside[28] is at $\delta \sim 70.6$, to which a β-L-rhamnosylation shift[23,25,27,29] of 7–10 p.p.m. might reasonably be added). The other distinctive signal for deacetylated F1c, at δ 67.65, can be assigned to C-2 of the rhamnosyl residue: for methyl β-L-rhamnopyranoside[23], the corresponding signal is at δ 71.8, but an upfield shift of $\sim$4 p.p.m. due to the β-effect of 3-substitution on C-2 with its axial hydroxyl group can be expected. An effect of this magnitude is found for the 3-methyl ethers of D-mannose[30], L-rhamnose[31], and methyl α-L-rhamnopyranoside[32], and should be diagnostic for α-D-glucosylation in the present case[27,33]. Assignments for the remaining signals in the ^{13}C-n.m.r. spectrum are suggested in Table I.

→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→

1

TABLE I

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

Carbon atom	*Native Flc*[b]		*Deacetylated Flc*	
		OAc | 2		
	→4-α-Glc-1→	*→3-β-Rha-1→*	*→4-α-Glc-1→*	*→3-β-Rha-1→*
C-1	94.85	99.83	95.79	100.85
C-2	71.26	68.87	71.67	67.65
C-3	72.53	75.12	72.49	78.26[c]
C-4	77.48	70.97[d]	77.19[c]	70.86[e]
C-5	70.67[d]	72.94	70.61[e]	73.11
C-6	60.86	17.00	60.89	17.07
-OC(O)CH_3		173.80		
-OC(O)CH_3		20.54		

[a]Chemical shifts are given in p.p.m. downfield from external Me_4Si. [b]Minor signals attributable to the presence of non-acetylated residues are not listed. [c,d,e]Pairs of signals for which the assignments may be interchanged within each pair.

The presence in the ^{13}C-n.m.r. spectrum of native Flc of several minor signals which matched major signals for the deacetylated polymer showed that *O*-acetylation was not stoichiometric. A comparison of related pairs of signals suggested that acetylation of the specific hydroxyl groups was 80–90% complete. The major differences from the spectrum of deacetylated Flc were in the anomeric region (upfield shifts of 1.47 p.p.m. for the rhamnosyl signal and 0.94 p.p.m. for the glucosyl signal), in the shifts for *O*-glycosylated carbons (signals at δ 77.48 and 75.12), and in the signal for C-2 of the rhamnosyl residue (downfield shift of 1.22 p.p.m.). These differences point to location of the *O*-acetyl group at position 2 of the rhamnosyl residue. Although the substituent effects at C-1 and C-2 of the rhamnosyl residue are smaller than some acetylation shifts that have been reported, they are not unreasonable[34]. An upfield shift of 1.2 p.p.m. for C-1 of a 3-substituted α-L-rhamnopyranosyl residue on *O*-acetylation at C-2 has been described[35], while the effect at C-3 for Flc could be as large as 3.14 p.p.m., depending on the assignments made (Table I).

Perhaps the least secure feature of the structural proof for the disaccharide repeating-unit of Flc is the evidence for the anomeric configuration of the L-rhamnopyranosyl residue. It is generally accepted that ^{13}C-chemical shifts for C-1 in rhamnosyl residues have little diagnostic value, although those for C-3 and C-5 can be useful[23,25,36]. Similar comments apply to ^{1}H-n.m.r. spectra for both the chemical shifts and the coupling constants of the anomeric protons[37], although differentiations based on δ values have been made for simple glycosides[25] and for bacterial polysaccharides (*e.g.* ref. 38). Shifts identical with, or very close to, that (δ 4.84) for the

anomeric proton of deacetylated F1c have been reported for 3-substituted L-rhamnopyranosyl residues having either the α or the β configuration (*e.g.*, refs. 29, 37–41). Thus, the assignment of the β configuration for F1c rests mainly on the value of 162 Hz for $^1J_{C,H}$ of the anomeric carbon. Although this value is typical for β-rhamnosides, values down to 164 Hz have been obtained for some α-rhamnosides[25,42].

The presence in F1c of sugars typical of F2 suggests that the polymer is terminated by a core oligosaccharide at the reducing end, but the number of disaccharide repeating-units and the identity of the non-reducing terminal group have not been ascertained. Conceivably, the product from a terminal glucopyranosyl group from methylation analysis might not have been resolved, by g.l.c., from that from the 3-substituted rhamnopyranosyl residues. However, even if such a group had been detected, its significance would have remained ambiguous, as methylation analysis of F2 showed the presence of a terminal glucopyranosyl group.

Polysaccharide F1c is not the first glucorhamnan to be isolated from *S. marcescens*. Glucorhamnans containing 3-substituted L-rhamnopyranosyl residues and 4-substituted D-glucopyranosyl residues (molar ratio 1:2), with glucose at the non-reducing terminus, have been obtained from the capsule and the culture filtrate of *S. marcescens* N.R.C. S-29 (ref. 3). Evidence for the presence of a glucorhamnan among the cellular polysaccharides of this strain has also been presented[4]. A more thoroughly studied glucorhamnan is the O-specific polysaccharide from *S. marcescens* Bizio (A.T.C.C. 264), for which a disaccharide repeating-unit of structure **2** has been proposed[16]. A sample of this polysaccharide (*not* that used in the previous studies[16]) was therefore compared with F1c. Unexpectedly, essentially the same results were obtained for both polysaccharides by methylation analysis and by periodate oxidation. The polysaccharides differed in the extent of *O*-acetylation; the i.r. and ^{13}C-n.m.r. spectra of the Bizio polysaccharide indicated that specific *O*-acetylation was only 20–30% complete. After deacetylation, the polysaccharides gave identical ^{13}C-n.m.r. spectra. The Bizio polysaccharide used by us must therefore have a repeating unit of structure **1**, not **2**. Although the present results cannot be fully reconciled with those of the previous study[16], several aspects of that study call for comment. (*a*) the g.l.c. column used (ECNSS-M) does not permit a conclusive differentiation of the methylated alditol acetates from 4-substituted and 6-substituted glucopyranosyl residues[43]. (*b*) the ^{1}H-n.m.r. spectrum shown for the Bizio polysaccharide does not contain the expected methyl signal for rhamnose; (*c*) there are inconsistencies in the properties or structures given for two disaccharides obtained by partial, acid hydrolysis of the polysaccharide. Thus, disaccharide D1, with a p.c. mobility less than that of glucose, was identified as **3**, whereas authentic **3** [as well as the isomeric β-glucosylrhamnoses linked (1→3) or (1→4)] has a mobility greater than that of glucose[44,45]. Disaccharide D2, which had an even lower mobility in p.c. and was cleaved by treatment with hespiridinase, was assigned structure **4** [described as rutinose; it is now accepted that rutinose has an α-(1→6) linkage[36,37] and that commercial hesperidinase contains

both an α-L-rhamnosidase and a β-D-glucosidase[46,47]]. The disaccharides D1 and D2 clearly merit closer study.

→6)-β-D-Glc*p*-(1→2)-β-L-Rha*p*-(1→
2

β-D-Glc*p*-(1→2)-L-Rha
3

β-L-Rha*p*-(1→6)-D-Glc
4

EXPERIMENTAL

Growth of bacteria, and isolation and fractionation of lipopolysaccharide. — Cells of *S. marcescens* N.C.T.C. 1377 were grown at 30° for 16 h in Nutrient Broth No. 2 (Oxoid) with aeration at 20 L.min^{-1}. Lipopolysaccharide was isolated from defatted cell-walls by standard methods[45]. The water-soluble products obtained by hydrolysis of the lipopolysaccharide (221 mg) with 1% aqueous acetic acid at 100° for 2 h were fractionated on a column (39 × 2.5 cm) of Sephadex G-50 by elution with pyridine–acetic acid–water (10:4:986; pH 5.4). Fractions (4 mL) were collected at a flow rate of 20 mL.h^{-1}, and were analysed for carbohydrate[48] and phosphorus[49]. Polymeric products (F1) were further resolved by chromatography on a column (81 × 1.5 cm) of Sephadex G-100, and products of low molecular weight by chromatography on a column (79 × 1.5 cm) of Sephadex G-15; in both cases, the flow rate was 5 mL.h^{-1}.

A sample of the O-specific polysaccharide from *S. marcescens* Bizio A.T.C.C. 264 was generously provided by Dr. P. Alaupovic and Dr. C.-S. Wang.

Chromatographic and electrophoretic methods. — Solvent systems used for p.c. were ethyl acetate–pyridine–water (13:5:4) and the upper phase of butan-1-ol–ethanol–water–0.88 ammonia (40:10:49:1). Detection reagents used were alkaline silver nitrate, aniline hydrogenoxalate, and the periodate–Schiff reagents. The buffer system used for paper electrophoresis was pyridine–acetic acid–water (5:2:43, pH 5.3), with or without EDTA (12mM), and the electrophoretograms were treated with ninhydrin, the Hanes–Isherwood reagent[50], and the Warren reagents[51], in addition to the reagents indicated above. For g.l.c., glass columns packed with the following stationary phases were used: *a*, 3% of Silar 10c on Gas Chrom Q (1.6 m × 2 mm); *b*, 10% of OS-138 and 1% of Adpet 80 on Chromosorb W (1.5 m × 3 mm). Column *a* was used at 190° for alditol acetates and at 170° for methylated alditol acetates; the latter compounds were also examined with column *b* at 170°. T.l.c. on silica gel 60 F_{254} (Merck) with diethyl ether–light petroleum (b.p. 40–60°) (1:1) was used for the isolation of rhamnitol penta-acetate from a mixture with glucitol hexa-acetate.

Determination of monosaccharide composition. — Samples were hydrolysed with 2M hydrochloric acid at 105° for 2 h (for the release of neutral sugars), or with 6.1M hydrochloric acid at 105° for 4 h (for the release of amino sugars). Neutral sugars were identified by p.c. and by g.l.c. of their alditol acetates. D-Glucose was

identified by using a reagent combination (Boehringer) based on D-glucose oxidase (EC 1.1.3.4). L-Rhamnose was identified by c.d. of the alditol acetate[52]. Amino sugars were identified by paper electrophoresis and by autoanalysis (Locarte bench analyser).

Degradative methods. – Deacetylation, periodate oxidation, and methylation analysis of polysaccharides were performed essentially as described previously[53]. Differences were (*a*) the use of dimsyl potassium[54] in the methylation analysis; and (*b*) the analysis of oxidised polysaccharides for sugars and alditols by p.c. and g.l.c., after borohydride reduction, desalting by gel filtration (Sephadex G-50), and acid hydrolysis. The treatment of phosphates of low molecular weight (released from lipopolysaccharide by mild, acid hydrolysis) with alkaline phosphatase (EC 3.1.3.1, Sigma) was carried out with 0.05M ammonium carbonate (pH 9.6) at 37° for 16 h under toluene.

General methods. – The optical rotation of fraction F1b was determined with a Bendix polarimeter (Model 143A). I.r. spectra were recorded with a Unicam SP200 spectrophotometer and samples dispersed in potassium chloride. N.m.r. spectra (^{13}C and ^{1}H) were recorded for solutions in D_2O with a Bruker WH-400 spectrometer. ^{1}H 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 ~50° with tetramethylsilane as the external standard. Analyses of methylated alditol acetates by g.l.c.-m.s. were carried out at the Physico-Chemical Measurements Unit, Harwell.

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