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STRUCTURAL STUDIES OF A POLYSACCHARIDE (S-194) ELABORATED BY *Alcaligenes* ATCC 31961

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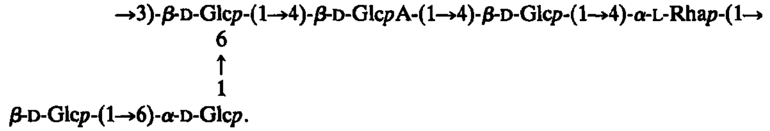
ABSTRACT

The structure of the polysaccharide (S-194) elaborated by *Alcaligenes* ATCC 31961 has been investigated, methylation analysis, specific degradations, and ^{1}H- and ^{13}C-n.m.r. spectroscopy being the main methods used. It is concluded that the polysaccharide is composed of hexasaccharide repeating-units with the structure:



$$\begin{array}{l}
\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow\\
\qquad\qquad\qquad 6\\
\qquad\qquad\qquad \uparrow\\
\qquad\qquad\qquad 1\\
\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p.
\end{array}$$

The polysaccharide further contains approximately one *O*-acetyl group per repeating unit, distributed over secondary positions.

INTRODUCTION

The search for bacterial polysaccharides of potential industrial application[1–3] has, *inter alia*, resulted in xanthan gum from *Xanthomonas campestris*, gellan gum from *Pseudomonas elodea*, and a polysaccharide with the working name S-130[4] and the tradename welan gum from *Alcaligenes* ATCC 31555. We now report structural studies of another of these polysaccharides, S-194, with the tradename rhamsan gum, from *Alcaligenes* ATCC 31961[5].

RESULTS AND DISCUSSION

The polysaccharide, on acid hydrolysis, yielded a mixture of L-rhamnose and

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D-glucose in the relative proportions 22:78. It further contained D-glucuronic acid (9–13%[5]) and *O*-acetyl groups (4–13%[5]). Glyceric acid, which is present to some extent in gellan gum[6], could not be found in S-194. This is further discussed below.

The absolute configurations of the sugars were determined by the method devised by Gerwig *et al.*[7]. Methylation analysis[8], without and with carboxyl-reduction of the methylated polysaccharide, gave the sugars listed in Table I, columns *A* and *B*, respectively. These results indicate that S-194 is composed of hexasaccharide repeating-units, containing one residue each of L-rhamnose linked through O-4, terminal D-glucose, D-glucose linked through O-4, D-glucose linked through O-6, branching D-glucose linked through O-3 and O-6, and D-glucuronic acid linked through O-4. The n.m.r. spectra, and other evidence discussed below, demonstrate that all of the sugars are pyranosidic.

S-194 gave highly viscous, aqueous solutions and, consequently, poor n.m.r. spectra. In order to obtain better spectra, the polysaccharide was deacylated by treatment with base and then degraded by treatment with acid under mild conditions. The ^{1}H-n.m.r. spectrum of this material (Fig. 1), which was still polymeric, showed six signals of equal intensities in the region for anomeric protons. Four of these, with $J_{1,2}$ values of 7–8 Hz, could be assigned to β-pyranosides with the D-*gluco* configuration. One signal, at δ 4.95 ($J_{1,2}$ 3.4 Hz), could be assigned to an α-pyranoside with the D-*gluco* configuration, and another, at δ 5.14 ($J_{1,2}$ 1.2 Hz), to an α-L-rhamnopyranoside. In addition, a signal for a *C*-methyl group was present at δ 1.31 ($J_{5,6}$ 6.1 Hz). In accordance with this conclusion, the ^{13}C-n.m.r. spectrum showed six signals in the region for anomeric carbons. Four of these, between 103.8 and 103.1 p.p.m., could be assigned to β-pyranosides with the D-*gluco* configuration, one, at δ 99.1, to an α-pyranoside with the D-*gluco* configuration, and one, at

TABLE I

METHYLATION ANALYSIS OF S-194 AND DEGRADATION PRODUCTS[a]

Sugar[b]	T[c]	*Mole %*				
		A	B	C	D	E
1,2,3,4,5-Glucitol	0.58				24	
2,3-Rha	0.94	15	17			
2,3,4,6-Glc	1.00	29	29		53	28
2,3,4,6-Glc				70[d]		
2,3,6-Glc	1.58	15	19			22
2,3,4-Glc	1.67	32	18	30	23	33
2,3-Glc	2.36		14			17
2,4-Glc	2.41	8	3			

[a]*A*, Native polysaccharide; *B*, methylated polysaccharide reduced with $LiBH_4$; *C*, uronic acid-degraded polysaccharide; *D*, $NaBD_4$-reduced trisaccharide; *E*, $NaBD_4$-reduced hexasaccharide. [b]2,3-Rha = 2,3-di-*O*-methyl-L-rhamnose, *etc.* [c]Retention time of the corresponding alditol acetate on an SE-54 column at 150° (2 min)→220° at 2°/min., relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [d]Contains 30% of CD_3-labelling at O-4.

δ 101.5, to an L-rhamnopyranoside. A signal at δ 17.7 could be assigned to C-6 of the rhamnosyl residue. The native polysaccharide was also depolymerised with anhydrous hydrofluoric acid, which should leave *O*-acyl groups intact, in order to detect the possible presence of esters of glyceric acid. ^{13}C-N.m.r. spectroscopy of this material showed no signals in the region δ 63–66, demonstrating the absence of both primary *O*-acetyl groups and the structural element $-CHOH-CH_2OH$, as in esters of glyceric acid. In the ^{1}H-n.m.r. spectra, several signals for *O*-acetyl groups were observed in the region δ 2.1–2.2, indicating that the *O*-acetyl groups were distributed over several positions.

The methylated S-194 was treated with sodium methylsulfinylmethanide in dimethyl sulfoxide and the product remethylated with trideuteriomethyl iodide. The methylated sugars in a hydrolysate of this material were analysed, as the alditol acetates, by g.l.c.–m.s. (Table I, column *C*). The 2,3,6-tri-*O*-methyl-D-glucose obtained in the analysis of unmodified S-194 had been replaced by 2,3,4,6-tetra-*O*-methyl-D-glucose, with a trideuteriomethyl group at O-4, and the 2,4-di-*O*-methyl-D-glucose had disappeared. The course of the degradation proved to be analogous to those reported for gellan gum[9] and S-130[10]. By arguments analogous to those used in these investigations, this demonstrates the presence of the structural

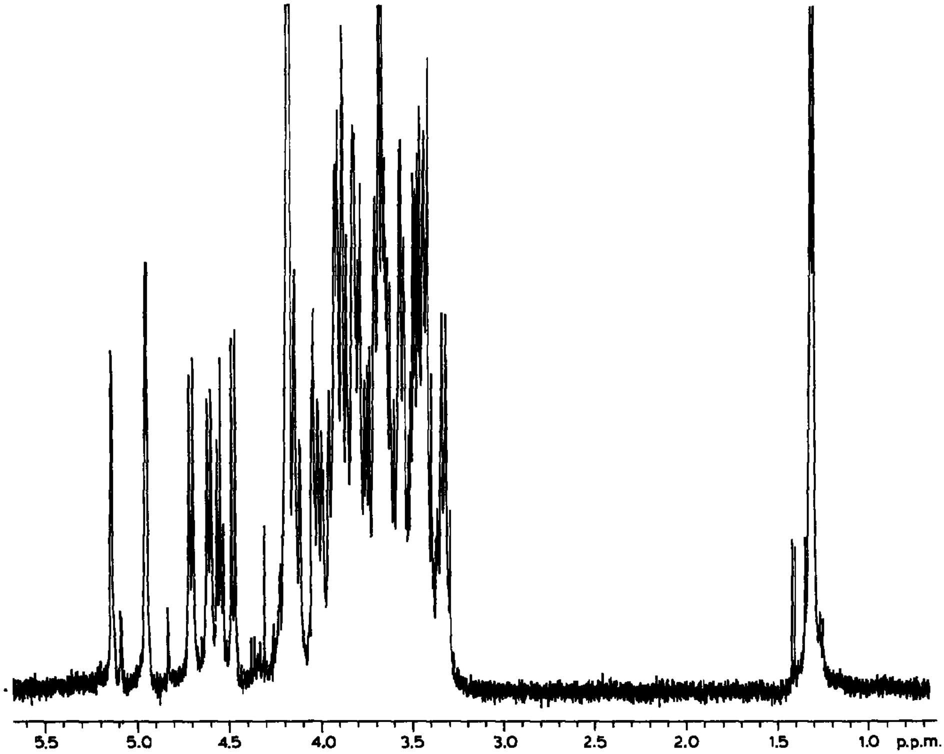
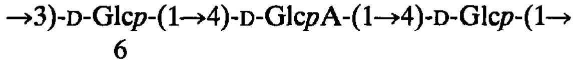


Fig. 1. ^{1}H-N.m.r. spectrum of deacetylated, partially degraded S-194.

element **1** in S-194. As all of the 2,3-di-*O*-methyl-L-rhamnose had also disappeared, the L-rhamnopyranosyl residue is probably linked to O-3 of the branching D-glucopyranosyl residue. In a duplicate analysis, part of the 2,3-di-*O*-methyl-L-rhamnose, but no 2,4-di-*O*-methyl-D-glucose, survived.



$$\rightarrow 3)\text{-}\underset{6}{\text{D-Glc}p}\text{-}(1\rightarrow 4)\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-D-Glc}p\text{-}(1\rightarrow$$

1

Hydrolysis of deacylated S-194 by treatment with 0.2M trifluoroacetic acid at 100° for 2 h yielded a mixture of mono- and oligo-saccharides, which was reduced with sodium borodeuteride and fractionated on a Biogel P-2 column. Two pure oligosaccharides were obtained. Methylation analysis of the smaller of these (Table I, column *C*) showed that it was a trisaccharide containing terminal D-glucose, D-glucose linked through O-6, and D-glucitol linked through O-6. The ^{1}H-n.m.r. spectrum showed signals for anomeric protons at δ 4.94 ($J_{1,2}$ 3.4 Hz) and δ 4.48 ($J_{1,2}$ 7.3 Hz), demonstrating that one of the D-glucopyranosyl residues is β-linked and the other α-linked. In agreement with this conclusion, the ^{13}C-n.m.r. spectrum showed two signals for the anomeric carbons at δ 103.5 and 99.2. It is concluded, from these results, that the trisaccharide-alditol has the structure **2**, or an alternative structure, in which the anomeric configurations of the two D-glucopyranosyl residues are reversed.

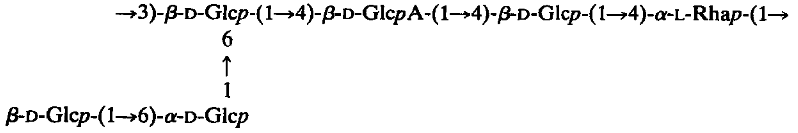
β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-glucitol-1-*d*

2

On substitution at O-6 of a D-glucopyranosyl residue with a β- or an α-D-glucopyranosyl group, the signal given by C-6 of the former residue is shifted to δ ~69 and to δ ~67, respectively[11]. The appearance of a signal at δ 69.9 and the absence of peaks between δ 66 and 69 show that structure **2** is correct.

As the only α-linked sugar with the D-*gluco* configuration has been located, the anomeric natures of all the sugar residues in the repeating unit are consequently defined.

The fact that the D-glucitol is linked through O-6, in conjunction with the results of the uronic acid degradation, demonstrates that it derives from the branching D-glucopyranosyl residue in S-194. From the results presented above, structure **3** for the hexasaccharide repeating-unit of S-194 has thus been established.



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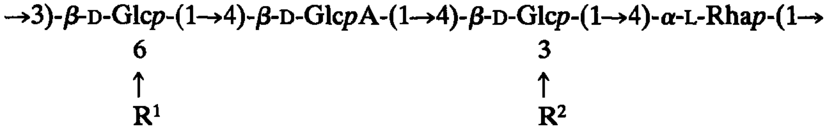
3

This structure was corroborated by analysis of the larger oligosaccharide-alditol obtained by partial hydrolysis and reduction. Methylation analysis of this substance, with carboxyl-reduction of the methylated product, gave the sugars listed in Table I, column *E*. In this analysis, the L-rhamnitol derivative was lost due to its high volatility. N.m.r. evidence (see below), however, demonstrates its presence. The analysis demonstrates that it is a linear hexasaccharide-alditol and that the α-L-rhamnopyranosyl residue in S-194 is linked to the 3-position of the branching β-D-glucopyranosyl residue. The ^{1}H-n.m.r. spectrum showed five signals in the region for anomeric protons, at δ 4.97 ($J_{1,2}$ 3.7 Hz), 4.62 ($J_{1,2}$ 7.1 Hz), 4.61 ($J_{1,2}$ 7.8 Hz), 4.55 ($J_{1,2}$ 7.8 Hz), and 4.49 ($J_{1,2}$ 7.8 Hz) and a signal for methyl protons at δ 1.3 ($J_{5,6}$ 6.1 Hz). The ^{13}C-n.m.r. spectrum showed five signals in the region for anomeric protons at δ 104.1, 103.7, 103.2 (2 C), and 99.1.

Considering that gellan gum[9], S-130[10], and S-194 have been picked from a large number of extracellular bacterial polysaccharides because of their properties in aqueous solution, there is a remarkable similarity between their structures. Gellan gum is linear polysaccharide (**4** $R^1 = R^2 = H$), and the other two contain the same backbone with branching at the 3-linked β-D-glucopyranosyl residue [R^1 = β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→, R^2 = H] for S-194 and at the 4-linked β-D-glucopyranosyl residue [R^1 = H, R^2 = α-L-Rha*p*-(1→ or α-L-Man*p*-(1→] for S-130.



$$\begin{array}{llll}
\rightarrow 3)\text{-}\beta\text{-D-Glc}p & \text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-} & \beta\text{-D-Glc}p & \text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \\
\quad\quad 6 & & \quad 3 & \\
\quad\quad \uparrow & & \quad \uparrow & \\
\quad\quad R^1 & & \quad R^2 &
\end{array}$$

4

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure or by flushing with air at <40° (bath). For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates and partially methylated alditol acetates were made on an SE-54 column, using a temperature programme, 150° (2 min)→220° at 2°/min. G.l.c.–m.s. was performed on a Hewlett–Packard 5970 MSD instrument. All identifications of mass spectra were unambiguous and are not discussed. Absolute configurations of the sugars were determined according to the procedure of Gerwig *et al.*[7]. N.m.r. spectra of solutions in deuterium oxide were recorded at 70° (^{13}C) or 85° (^{1}H) with a JEOL FX-100 or GX-400 instrument. Chemical shifts are reported in p.p.m., using internal 1,4-dioxane (δ 67.40) for ^{13}C and internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δ 0.00) for ^{1}H.

Preparation of a sample for n.m.r. studies. — A slightly turbid solution of S-194 (0.4 g) in water (600 mL) was *O*-deacylated by adding sodium hydroxide to a concentration of 0.1M and keeping at room temperature for 15 h. An aqueous solution of the neutralised and desalted material was heated to 100°, trifluoroacetic acid was added to a concentration of 0.1M, and the solution was kept at 100° for 20 min. The product was recovered by freeze-drying.

Another sample was prepared from native material by treatment with anhydrous hydrofluoric acid at −70° for 15 min. The reaction was quenched with $CaCO_3$ in dichloromethane as described earlier[12].

Methylation analysis was performed essentially as previously described. Methylated material was recovered by using a Sep-Pak C_{18} cartridge according to the procedure of Waeghe *et al.*[13].

Carboxyl-reduction of methylated S-194. — The methylated polysaccharide was dissolved in freshly distilled tetrahydrofuran (1 mL), lithium borohydride (10 mg) was added, and the sealed tube was heated at 70° for 2 h. Excess of lithium borohydride was decomposed with glacial acetic acid, chloroform was added, and the solution was washed several times with water.

Uronic acid degradation. — A trace of toluene-*p*-sulfonic acid and 2,2-dimethoxypropane (0.1 mL) was added to a solution of methylated S-194 (1 mg) in dimethyl sulfoxide (1 mL). The vial was sealed and flushed with nitrogen. After 30 min, sodium methylsulfinylmethanide in dimethyl sulfoxide (2M, 0.9 mL) was added and the solution was kept at room temperature for 15 h. The solution was cooled with ice–water, trideuteriomethyl iodide (0.5 mL) was added, and the mixture was kept for 1 h. The methylated product was recovered as described above.

Partial acid hydrolysis. — S-194 (100 mg), prepared as described above, was dissolved in water (100 mL) at 100°. Trifluoroacetic acid was added to a concentration of 0.2M, and the solution was kept at 100° for 2 h, cooled, and concentrated. After distillation of water (2 × 10 mL) from the residue, the material was reduced by sodium borodeuteride (100 mg) in M aqueous ammonium hydroxide (10 mL). After work-up as described above, the material was applied to a column (2.5 × 90 cm) of Biogel P-2 and eluted with mM aqueous formic acid containing 2.7mM 1,1,1-trichloro-2-methyl-2-propanol. The fractionation was monitored by using a differential refractometer (Optilab Multiref 902).

ACKNOWLEDGMENT

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