Carbohydrate Research, 147 (1986) 295–313
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE EXTRACELLULAR POLYSACCHARIDE PRODUCED BY THE BACTERIUM *Alcaligenes* (ATCC 31555) SPECIES

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(Received July 8th, 1985; accepted for publication, September 26th, 1985)

ABSTRACT

The extracellular anionic polysaccharide produced by the bacterium *Alcaligenes* (ATCC 31555) contains L-mannose, L-rhamnose, D-glucose, and D-glucuronic acid in the molar ratios 1.0:4.5:3.1:2.3. Analysis of the methylated and methylated, carboxyl-reduced polysaccharide indicated terminal non-reducing rhamnose and mannose, (1→4)-linked rhamnose, (1→3)- and (1→3,1→4)-linked glucose, and (1→4)-linked glucuronic acid to be present in the ratios 1.0:0.8:2.1:2.2:2.0:2.2. Partial acid hydrolysis and base-catalysed β-elimination gave a series of oligosaccharides that were isolated as their alkylated alditol derivatives by reverse-phase h.p.l.c. and characterised by f.a.b.-m.s., e.i.-m.s., and ^{1}H-n.m.r. spectroscopy. The repeating unit **1**, excluding *O*-acyl groups, is proposed.

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

1 R = α-L-Rha*p* and α-L-Man*p*

INTRODUCTION

The bacterium *Alcaligenes* (ATCC 31555) produces an extracellular anionic heteropolysaccharide that gives aqueous dispersions which exhibit high low-shear rate viscosities and shear-thinning behaviour at low concentrations[1,2]. The polysaccharide is thermostable when compared with Xanthan gum[1,2] and gives viscous solutions that are insensitive to pH and temperature.

Only limited data[1,2] have been published on the chemical composition of this polysaccharide. Further to preliminary results[3], we now report in detail on the

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0008-6215/86/$ 03.50

structure of the polysaccharide. Since this work was completed, Jansson *et al.*[4] have shown independently that the polysaccharide is composed of a pentasaccharide repeating-unit and proposed that the mannose is L which has been confirmed.

RESULTS

Monosaccharide composition. — The polysaccharide contained L-mannose, L-rhamnose, D-glucose, and D-glucuronic acid in the molar ratios 1.0:4.5:3.1:2.3. The presence of D-mannose was reported[3] on the basis of the g.l.c. behaviour of the acetylated glycosides of (+)-2-octanol. Jansson *et al.*[4] have proposed that the mannose is L. Therefore, mannose was isolated by preparative p.c. after partial hydrolysis (0.2M trifluoroacetic acid, 1 h at 100°) of the polysaccharide which preferentially released the bulk of the rhamnose and mannose. The isolated mannose had $[\alpha]_{589}^{20}$ −12° (water), and g.l.c. of its acetylated (+)-2-octyl glycosides gave an elution profile that was essentially the same as that obtained with authentic L-mannose. It appears that the g.l.c. column used to separate the acetylated glycosides of (+)-2-octanol from the total hydrolysis of the polysaccharide could not resolve all the derivatives satisfactorily.

The sugar ratios obtained by direct analysis reflect an underestimate of glucose due to the stability of the glucosyluronic→glucose linkage (see below). The presence of *O*-acyl groups was established from the strong i.r. absorption at 1730 cm^{-1} of the polysaccharide.

Linkage analysis. — Analysis of the methylated and carboxyl-reduced polysaccharide (Table I, column I) indicated terminal non-reducing rhamnose and mannose, (1→4)-linked rhamnose, (1→3)-, (1→2,1→3)-, and (1→3,1→4)-linked glucose, and (1→4)-linked glucuronic acid to be present in the ratios 1.0:0.8:2.3:1.6:0.5:3.5:2.3. After remethylation of the methylated and carboxyl-reduced material (Table I, column II), the 4,6-di-*O*-methylglucitol derivative could not be detected and the ratio of (1→4)-linked rhamnose to (1→3)-linked glucose became unity. This suggested that the 4,6-di-*O*-methylglucitol derivative arose from incomplete methylation of O-2 of the (1→3)-linked glucosyl residue, and was confirmed as described below. Small amounts (<3% of the identified alkylated alditol acetates) of the derivatives from (1→2,1→3)-linked 6-deoxyhexose and from (1→4)- and (1→6)-linked hexose were also detected, but the origins of these residues are unclear.

After ethylation of the methylated and carboxyl-reduced polysaccharide followed by conventional conversion into the alditol acetates, g.l.c.–m.s.(e.i.) revealed (Table I, column III) 1,3,5-tri-*O*-acetyl-2-*O*-ethyl-4,6-di-*O*-methylglucitol which was characterised from the primary fragment ions at *m/z* 132 (100%), 161 (5.1), 248 (9.6), and 291 (2.0). The amount of this derivative was estimated from the relative abundance of the ions at *m/z* 118 (2,4,6-Me_3-Glc) and 132 (2-Et-4,6-Me_2-Glc) by selective ion monitoring over the area of the peak of the 2,4,6-tri-*O*-methylglucitol derivative with which it co-eluted. These results confirmed that O-2

TABLE I

ALKYLATED ALDITOL ACETATES DERIVED FROM THE METHYLATED AND CARBOXYL-REDUCED, METHYLATED, CARBOXYL-REDUCED, AND RE-ALKYLATED AND DEGRADED *Alcaligenes* (ATCC 31555) POLYSACCHARIDE

Alkylated alditol acetate[a]	*I*[e]	*II*	*III*	*IV*	*V*	*Linkage*
2,3,4-Me_3-Rha[b]	1.0	1.0	1.0	1.0	1.0	Rha*p*
2,3-Me_2-Rha	2.3	2.1	2.1	4.2	0.3	(1→4)-Rha*p*
2,3,4,6-Me_4-Man	0.8	0.8	0.8	2.2	1.2	Man*p*
2,4,6-Me_3-Glc	1.6	2.1	1.8	2.6	0	(1→3)-Glc*p*
2,3,6-Me_3-Glc[c]	—	2.2	—	—	—	(1→4)-Glc*p*A
2,3,6-Me_3-Glc	—	—	—	1.6	—	(1→4)-Glc*p*
2,6-Me_2Glc	3.5	2.1	1.9	2.1	0.2	(1→3,1→4)-Glc*p*
4,6-Me_2Glc	0.5	0	—	0.7	—	(1→2,1→3)-Glc*p*
2,3-Me_2Glc[c]	2.3	—	—	—	—	(1→4)-Glc*p*A
2,3-Me_2-6-Et-Glc[c,d]	—	—	2.3	—	—	(1→4)-Glc*p*A
2,6-Me_2-4-Et-Glc	—	—	—	—	2.0	(1→3,1→4)-Glc*p*
4,6-Me_2-2-Et-Glc	—	—	0.5	—	—	(1→3)-Glc*p*

[a]Values calculated as molar ratios relative to 2,3,4-Me_3-Rha. [b]2,3,4-Me_3-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, *etc.* [c]6,6-Dideuterio derivative. [d]2,3-Me_2-6-Et-Glc = 1,4,5-tri-*O*-acetyl-6-*O*-ethyl-2,3-di-*O*-methylglucitol, *etc.* [e]I, methylated and carboxyl-reduced; II, methylated, carboxyl-reduced, and re-methylated; III, methylated, carboxyl-reduced, and ethylated; IV, partially acid-hydrolysed and methylated; V, methylated and β-eliminated.

of the (1→3)-linked glucose was undermethylated, and are consistent with the presence of terminal non-reducing rhamnose and mannose, (1→4)-linked rhamnose, (1→3)- and (1→3,1→4)-linked glucose, and (1→4)-linked glucuronic acid in the ratios 1.0:0.8:2.1:2.2:2.0:2.2.

Methylation analysis of the partially acid-hydrolysed polysaccharide (20mM oxalic acid, 100°, 2 h) showed (Table I, column IV) a decrease in the proportions of terminal non-reducing rhamnose and (1→3,1→4)-linked glucose, and the appearance of (1→4)-linked glucose. The terminal rhamnosyl group was therefore linked to O-3 of (1→4)-linked glucose.

Degradation of the methylated polysaccharide by β-elimination with sodium methylsulphinylmethanide followed by ethylation and conversion into the alditol acetates gave (Table I, column V), as the major components, the acetylated derivatives of 2,3,4-tri-*O*-methylrhamnitol, 2,3,4,6-tetra-*O*-methylmannitol, and 4-*O*-ethyl-2,6-di-*O*-methylglucitol in the ratios 1.0:1.2:2.0. These data established that the glucuronosyl residue was linked to O-4 of a glucosyl residue which was also substituted through position 3 with a terminal rhamnosyl or mannosyl group. From these results, the partial structure **2** could be deduced.

→4)-D-GlcpA-(1→4)-D-Glcp-(1→
3
↑
1
R

2 R = L-Rhap and L-Manp

Sequencing of the alkylated oligosaccharide-alditols. — The sequencing was performed using e.i.-m.s. and established principles[5]. The nomenclature used is that of Kochetkov and Chizhov[6] except that the alditol fragments will be referred to[7] as *ald* to distinguish them from terminal non-reducing residues. The ion containing the alditol moiety (*e.g.*, *ald*J_2) arises[8] from loss of 60 m.u. from the *ald*J_1 fragment.

Partial acid hydrolysis. — Hydrolysis of the polysaccharide for 4 h with 0.5M H_2SO_4 followed by ion-exchange chromatography gave an acidic fraction that contained glucose and glucuronic acid. G.l.c. of the reduced and methylated acidic fraction gave one major peak which was eluted in the region for a methylated disaccharide-alditol methyl ester (*T* 1.19; *T* 1.00 for methylated cellobi-itol). G.l.c.–m.s. (ammonia c.i.) gave ions at *m/z* 486 (10.7%) and 503 (100), corresponding to $[M + 1]^+$ and $[M + 18]^+$, respectively, from a disaccharide derivative containing hexuronosyl and hexitol residues. G.l.c.–m.s.(e.i.) gave ions at *m/z* 169 (aA_3, 7.2%), 201 (aA_2, 100), 233 (aA_1, 13.4), 236 (*ald*J_2, 11.7), and 296 (*ald*J_1, 0.4), confirming the disaccharide nature of the derivative. The ratio of the intensities of the ions at *m/z* 133 and 134 of 1.0:5.4 demonstrated[4] the hexitol moiety to be substituted through position 4. These results showed that the acidic fraction contained the aldobiouronic acid D-GlcpA-(1→4)-D-Glcp.

Partial acid hydrolysis of the polysaccharide with 0.2 or 0.5M trifluoroacetic acid followed by ion-exchange chromatography gave acidic fractions that contained rhamnose, glucose, and glucuronic acid. Only traces (<5.0%) of mannose were detected in these fractions. The neutral fractions contained mainly rhamnose and mannose.

Reduction of the acidic oligosaccharides to their corresponding alditols followed by methylation and fractionation by reverse-phase h.p.l.c. (Fig. 1) gave a series of methylated oligosaccharide-alditol methyl esters. Each partial hydrolysate contained two common major components (2A and 5A; 2B and 5C, see Fig. 1) and two separate minor components (2C and 5B, see Fig. 1). The major (5A and 5C) and minor (5B and 2C) components, respectively, were characterised by f.a.b.-m.s., e.i.-m.s., and ^{1}H-n.m.r. spectroscopy.

F.a.b.-m.s. of fraction 5A gave an ion at *m/z* 690, corresponding to $[M + 1]^+$ from a methylated oligosaccharide-alditol methyl ester containing hexosyl, hexuronosyl, and hexitol residues. E.i.-m.s. gave ions at *m/z* 155 (aA_3, 10.5%), 187 (aA_2, 79.6), 219 (aA_1, 13.3), 236 (*ald*J_2, 47.4), 296 (*ald*J_1, 3.0), 373 (baA_3, 0.5), 405 (baA_2, 0.4), 437 (baA_1, 0.5), 454 (b*ald*J_2, 0.8), and 514 (b*ald*J_1, 0.5), and confirmed the

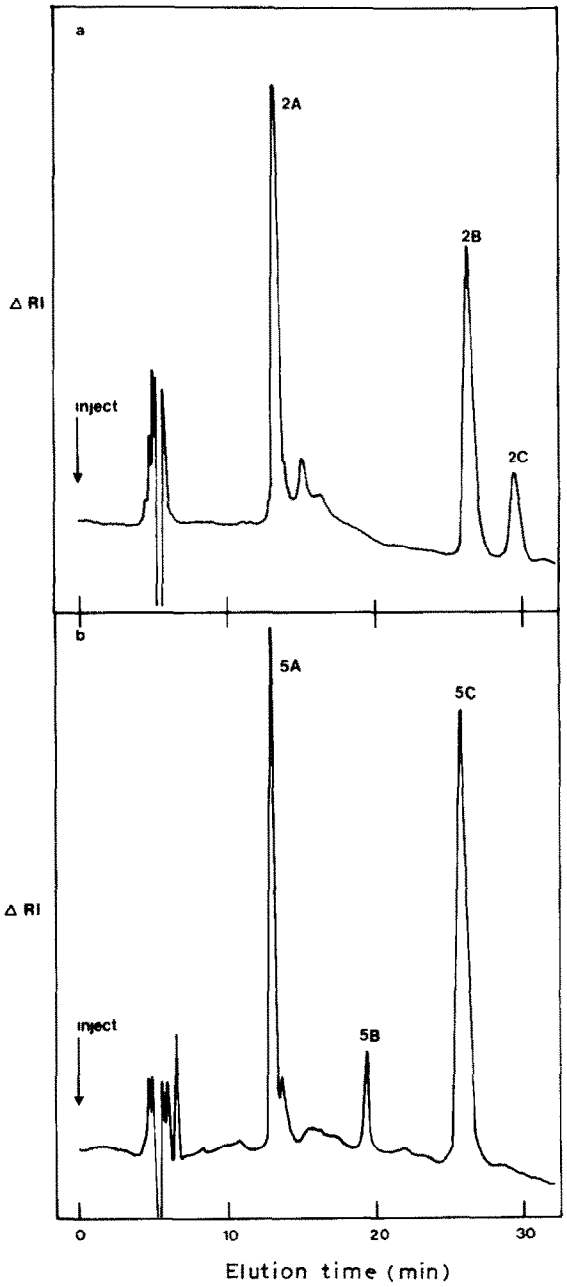


Fig. 1. Reverse-phase h.p.l.c. of the methylated oligosaccharide-alditol methyl esters obtained after partial acid hydrolysis of the *Alcaligenes* polysaccharide with (a) 0.2M and (b) 0.5M trifluoroacetic acid.

trisaccharide nature of the derivative (Fig. 2). The ratio of the intensities[4] of the ions at *m/z* 133 and 134 of 1.0:4.9 showed the hexitol moiety to be substituted through position 4. The ^{1}H-n.m.r. spectrum of fraction 5A contained signals for anomeric protons (Table II) at δ 4.27 ($J_{1,2}$ 7.6 Hz) and 4.52 ($J_{1,2}$ 7.3 Hz) which were assigned to *β-gluco* derivatives. Thus, fraction 5A originated from the aldotrio-uronic acid **3**.

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-D-Glc*p*

3

F.a.b.-m.s. of fraction 5C gave an ion at *m/z* 864, corresponding to $[M + 1]^+$ from a methylated oligosaccharide-alditol methyl ester containing two hexosyl, hexuronosyl, and 6-deoxyhexitol residues. E.i.-m.s. gave ions at *m/z* 155 (aA_3, 28.5%), 187 (aA_2, 90.6), 206 (*ald*J_2, 72.0), 219 (aA_1, 18.8), 266 (*ald*J_1, 20.6), 373 (baA_2, 2.4), 405 (baA_2, 0.8), 410 (*cald*J_2, 1.4), 437 (baA_1, 1.1), 470 (*cald*J_1, 0.3),

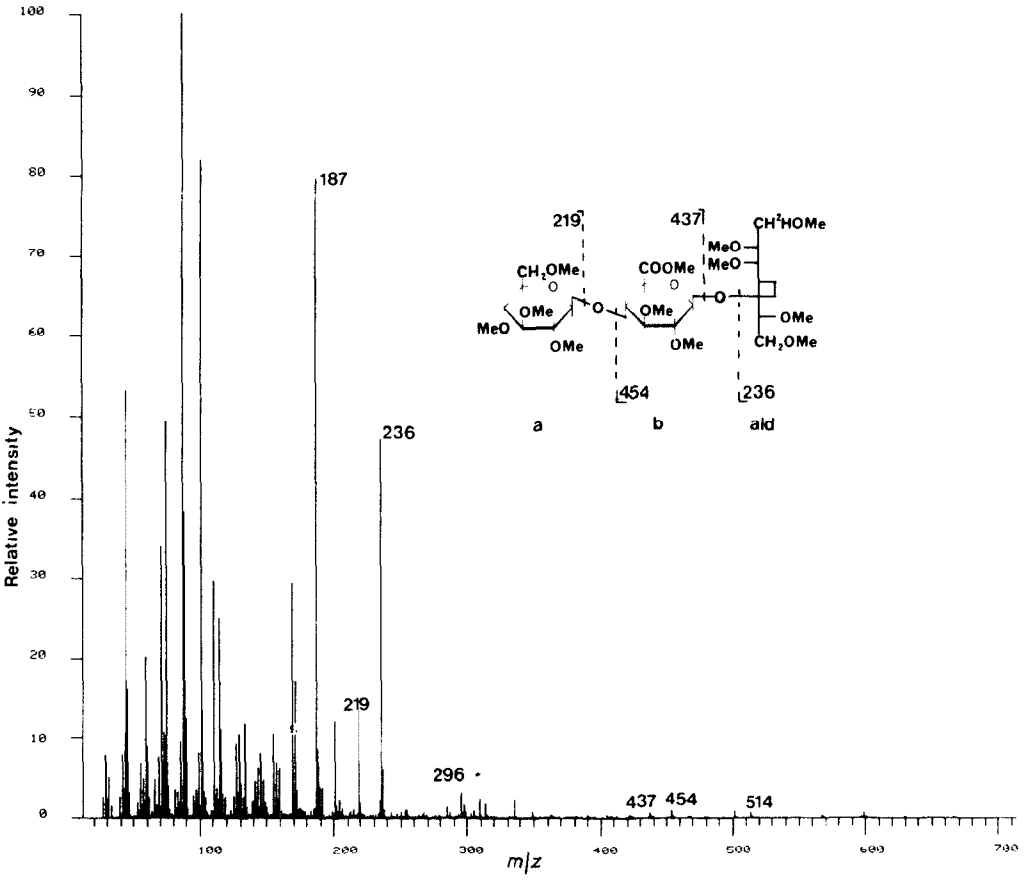


Fig. 2. Mass spectrum and fragmentation pattern of fraction 5A.

TABLE II

^{1}H-N.M R. DATA FOR THE ANOMERIC PROTONS OF THE ALKYLATED *Alcaligenes* POLYSACCHARIDE AND SELECTED ALKYLATED OLIGOSACCHARIDE-ALDITOLS

Alkylated derivative	*H-1(δ)*	$J_{1,2}$ *(Hz)*	*Integral*	*Assignment*
Methylated polysaccharide	4.33	5.8	1.8	
	4.43	6.0	1.9	
	4.70	6.7	2.4	
	5.32	~1	1.0	
	5.36	~1	1.8	
	5.42	~1	1.0	
Alkylated oligosaccharide-alditol				
3	4.27	7.6	1.0	β-Glc*p*
	4.52	7.3	1.0	β-Glc*p*A
4	4.24	7.7	1.0	β-Glc*p*
	4.29	7.9	1.0	β-Glc*p*
	4.45	7.9	1.0	β-Glc*p*A
7	4.63	7.9	1.0	β-Glc*p*
	5.40	2.1	1.0	α-Rha*p*
	5.49	1.3	1.0	α-Man*p*
9	4.30	7.9	1.0	β-Glc*p*
	5.51	1.5	1.0	α-Man*p*
10	4.30	8.1	1.0	β-Glc*p*
	5.39	1.5	1.2	α-Rha*p*

577 (cbaA_3, 0.1), 609 (cbaA_2, 0.1), 628 (bc*ald*J_2, 0.2), 641 (cbaA_1, 0.1), and 688 (bc*ald*J_1, 0.8), and confirmed the tetrasaccharide nature of the derivative (Fig. 3). The ratio of intensities of the ions at *m/z* 133 and 134 of 1.0:11.2 showed the 6-deoxyhexitol residue to be substituted through position 4. The ^{1}H-n.m.r. spectrum of fraction 5C contained signals for anomeric protons (Table II) at δ 4.24 ($J_{1,2}$ 7.7 Hz), 4.29 ($J_{1,2}$ 7.9 Hz), and 4.45 ($J_{1,2}$ 7.9 Hz) consistent with two β-linked glucosyl residues and one β-linked glucuronosyl residue. These data, in combination with the results of methylation analysis of the polysaccharide (Table I, column II), showed that the derivative in fraction 5C was derived from the aldotetraouronic acid **4**.

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Rha*p*

4

F.a.b.-m.s. of fraction 5B gave an ion at *m/z* 660, corresponding to $[M + 1]^+$ from a trisaccharide-alditol derivative containing hexuronosyl, hexosyl, and 6-deoxyhexitol residues. E.i.-m.s. gave ions at *m/z* 169 (aA_3, 7.9%), 201 (aA_2, 97.4), 206 (*ald*J_2, 46.9), 233 (aA_1, 13.1), 266 (*ald*J_1, 14.3), 373 (baA_3, 0.3), 405 (baA_2, 0.5), 410 (b*ald*J_2, 0.2), 437 (baA_1, 0.4), and 470 (b*ald*J_1, 0.1) which defined the sequence Hex*p*A→Hex*p*→6-Deoxyhex. In combination with **3** and **4**, the derivative in fraction 5B was derived from the aldotriouronic acid **5**.

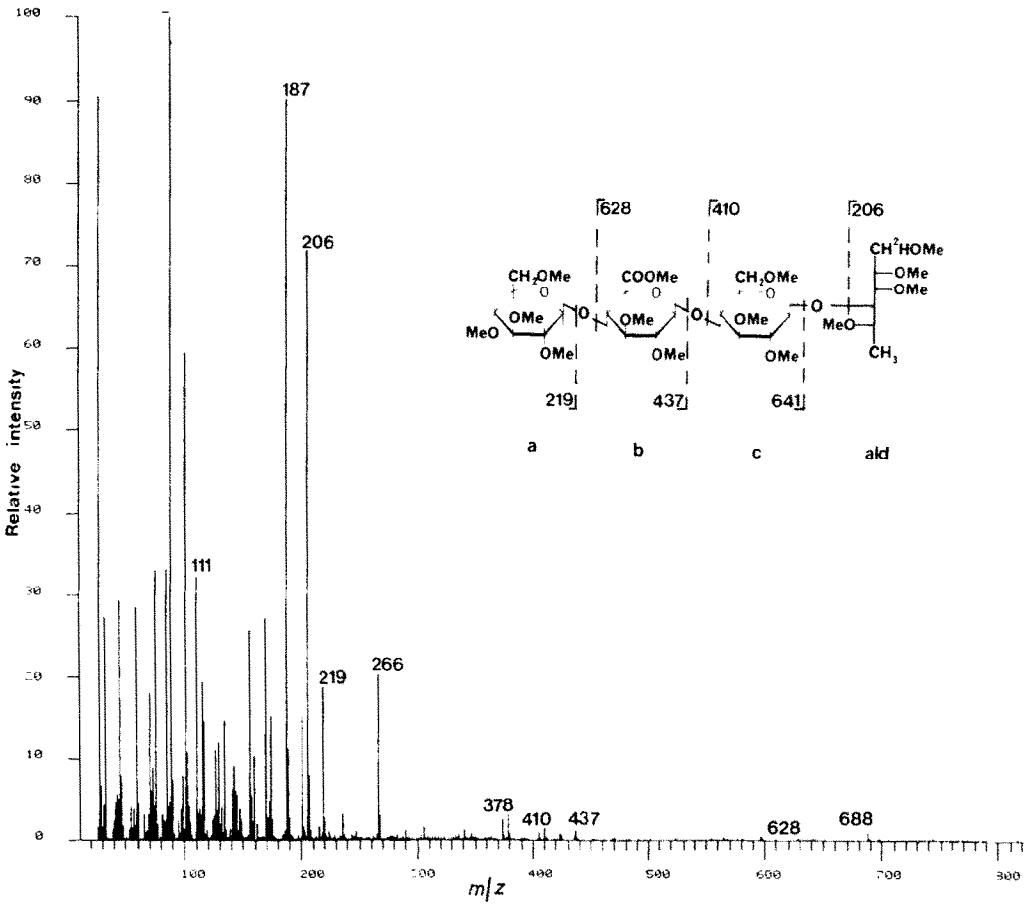


Fig. 3 Mass spectrum and fragmentation pattern of fraction 2C.

β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Rha*p*

5

F.a.b.-m.s. of fraction 2C gave an ion at *m/z* 1068, corresponding to $[M + 1]^+$ from a pentasaccharide-alditol derivative containing hexuronosyl, 3 hexosyl, and 6-deoxyhexitol residues. E.i.-m.s. gave ions at *m/z* 206 (*ald*J_2, 12.0%) and 219 (aA_1, 31.7) which demonstrated the presence of 6-deoxyhexitol and terminal non-reducing hexosyl residues, respectively. Due to the low intensity of the ions in the mass spectrum, further evidence for the sequence of glycosyl residues in the pentasaccharide-alditol methyl ester could not be obtained.

From the results of methylation analysis, partial acid hydrolysis, and base-catalyzed degradation, a series of overlapping sequences (**2–5**) were characterised, and the partial structure **6** for the polysaccharide was defined.

→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Rha*p*-(1→
3
↑
1
R

6 R= L-Rha*p* and L-Man*p*

β-Elimination with diazabicyclo[5.4.0]undec-7-ene[9]. — In order to confirm the partial structure **6**, establish the anomeric configurations of the (1→4)-linked rhamnosyl residue and the terminal non-reducing rhamnose and mannose substituents, and also establish the presence of the sequence →3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→, the methylated polysaccharide was subjected to base-catalysed β-elimination with diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene–acetic anhydride[9]. The products were de-acetylated, reduced, and ethylated, and the resulting alkylated oligosaccharide-alditols were subjected to reverse-phase h.p.l.c. (Fig. 4). The compounds in the four peaks (1–4) were isolated and characterised by m.s. and ^{1}H-n.m.r. spectroscopy. The components eluted between 10–30 min were probably excess reagents, which are known to be difficult to remove[10] and were not examined.

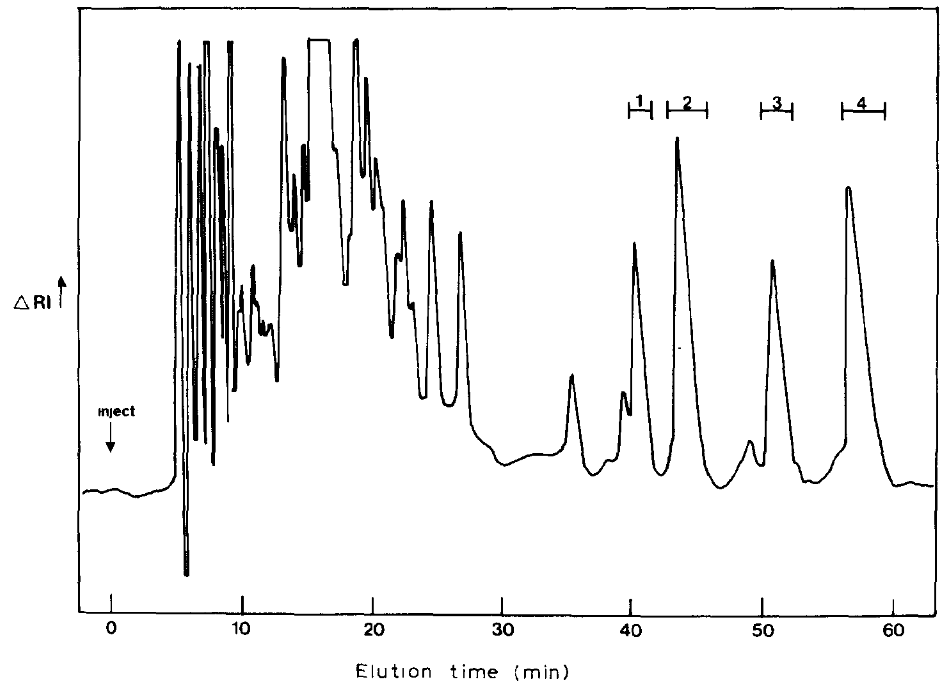


Fig. 4. Reverse-phase h.p.l.c. of the alkylated oligosaccharide-alditols obtained from β-elimination of the methylated *Alcaligenes* polysaccharide with DBU.

F.a.b.-m.s. of fraction 1 gave an ion at *m/z* 892, corresponding to $[M + 1]^+$ from an alkylated tetrasaccharide-alditol composed of 2 hexosyl, 6-deoxyhexosyl, and hexitol residues, and containing three *O*-ethyl groups. E.i.-m.s. gave ions at *m/z* 219 (aA_1, 69.8%), 264 ($aldJ_2$, 38.5), 437 (baA_1, 4.0), 438 ($caldJ_2$, 3.6), 611 ($cbaA_1$, 0.2), and 656 ($bcaldJ_2$, 0.7) which defined the sequence Hex*p*→Hex*p*→6-Deoxyhex*p*→Hexitol (Fig. 5). The mass of the baA_1 ion (*m/z* 437) showed that it contained a single *O*-ethyl group, and this was confirmed from the ions at *m/z* 391 (baA_1 − 46, 1.2%) and 359 (baA_1 − 78, 2.0). The mass of the $aldJ_2$ fragment (*m/z* 264) and the ions at *m/z* 103 (30.4%) and 147 (6.8) demonstrated the presence of a 1,5-di-*O*-ethylhexitol residue. These ions, in combination with the ion at *m/z* 324 ($aldJ_1$, 7.3%), showed the hexitol moiety b to be substituted through position 3. The absence of an ion at *m/z* 498 ($caldJ_1$) and the presence of an ion at *m/z* 484 ($caldJ_0$, 1.1%) were consistent with the terminal hexosyl group being attached[11] to

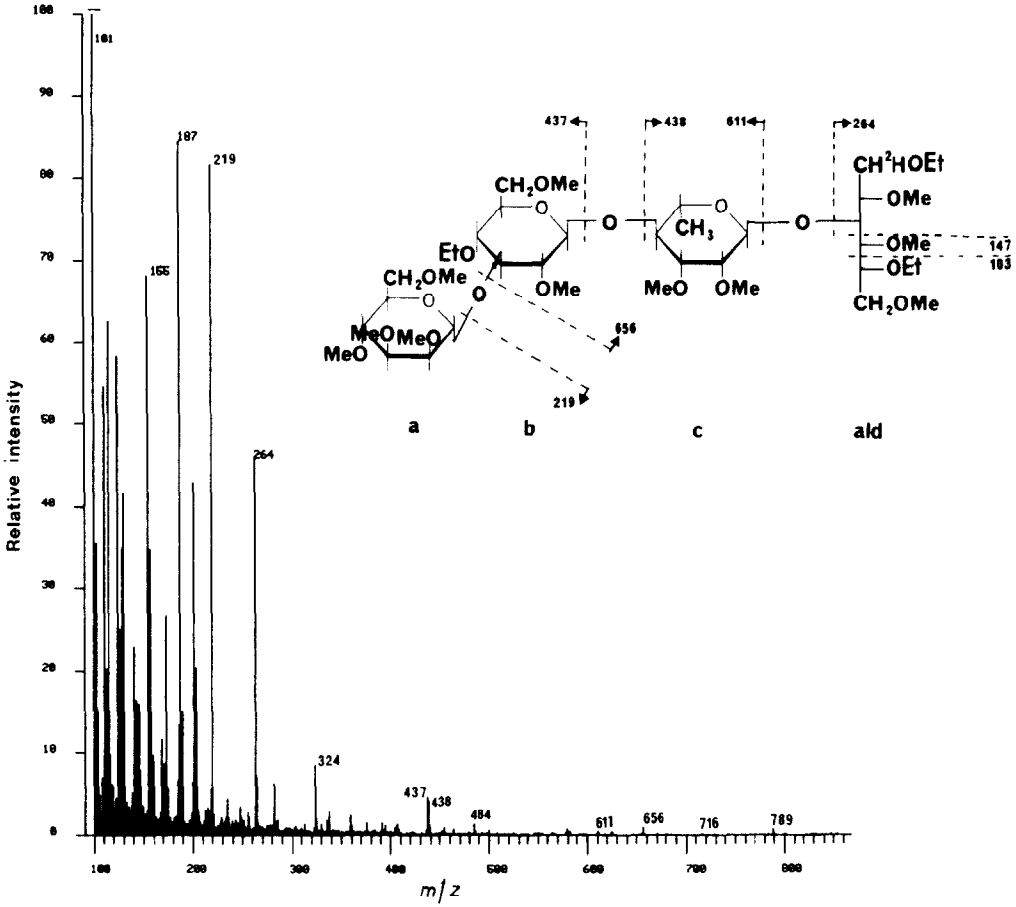
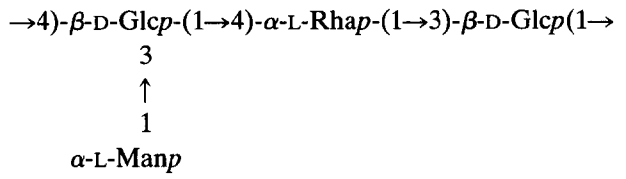


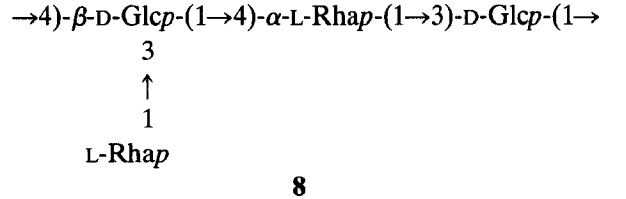
Fig. 5. Mass spectrum and fragmentation pattern of fraction DBU-1.

position 3 of the internal hexosyl residue. The ^{1}H-n.m.r. spectrum of fraction 1 contained three signals for anomeric protons (Table II) at δ 4.63 ($J_{1,2}$ 7.9 Hz), 5.40 ($J_{1,2}$ 2.1 Hz), and 5.49 ($J_{1,2}$ 1.3 Hz), which were assigned to β-glucosyl, α-rhamnosyl, and α-mannosyl residues, respectively. The data established that fraction 1 was obtained from the sequence **7** in the polysaccharide.



7

F.a.b.-m.s. of fraction 2 gave an ion at *m/z* 862, corresponding to $[M + 1]^+$ from an alkylated tetrasaccharide-alditol composed of hexosyl, two 6-deoxyhexosyl, and hexitol residues, and containing three *O*-ethyl groups. E.i.-m.s. gave ions at *m/z* 189 (aA_1, 64.5%), 264 ($aldJ_2$, 28.7), 324 ($aldJ_1$, 5.5), 407 (baA_1, 2.9), 438 ($caldJ_2$, 2.1), 484 ($caldJ_0$, 0.8), 656 ($bcaldJ_2$, 0.5), and 716 ($bcaldJ_1$, 0.1) which defined the sequence 6-Deoxyhex*p*→Hex*p*→6-Deoxyhex*p*→Hexitol (Fig. 6). The mass of the baA_1 fragment (*m/z* 407) demonstrated a single *O*-ethyl group to be present on residue b (Fig. 6). The mass of the $aldJ_2$ fragment (*m/z* 264) and ions at *m/z* 103 (18.1%) and 147 (2.6) showed that the alditol moiety contained 1,5-di-*O*-ethyl substituents and was linked through position 3. The data, in combination with the results of methylation analysis and partial acid hydrolysis, established that fraction 2 was obtained from the sequence **8** in the polysaccharide.



8

F.a.b.-m.s. of fraction 3 gave an ion at *m/z* 688, corresponding to $[M + 1]^+$ from an alkylated derivative composed of two hexosyl and 6-deoxyhexitol residues and containing three *O*-ethyl groups. E.i.-m.s. gave ions at *m/z* 219 (aA_1, 100%), 234 ($aldJ_2$, 71.6), 280 ($aldJ_0$, 5.2), 437 (baA_1, 19.3), 452 ($baldJ_2$, 5.0), and 512 ($baldJ_1$, 1.6) which demonstrated the sequence Hex*p*→Hex*p*→6-Deoxyhexitol (Fig. 7). The mass of the baA_1 fragment (*m/z* 437) showed the presence of a single *O*-ethyl substituent on residue b. The presence of ions at *m/z* 104 (29.7%) and 148 (6.3) demonstrated the 6-deoxyhexitol to be substituted at position 4. The ^{1}H-n.m.r. spectrum of fraction 3 contained signals for anomeric protons (Table II) at δ 4.30 ($J_{1,2}$ 7.9 Hz) and 5.51 ($J_{1,2}$ 1.5 Hz). These were assigned to β-glucose and

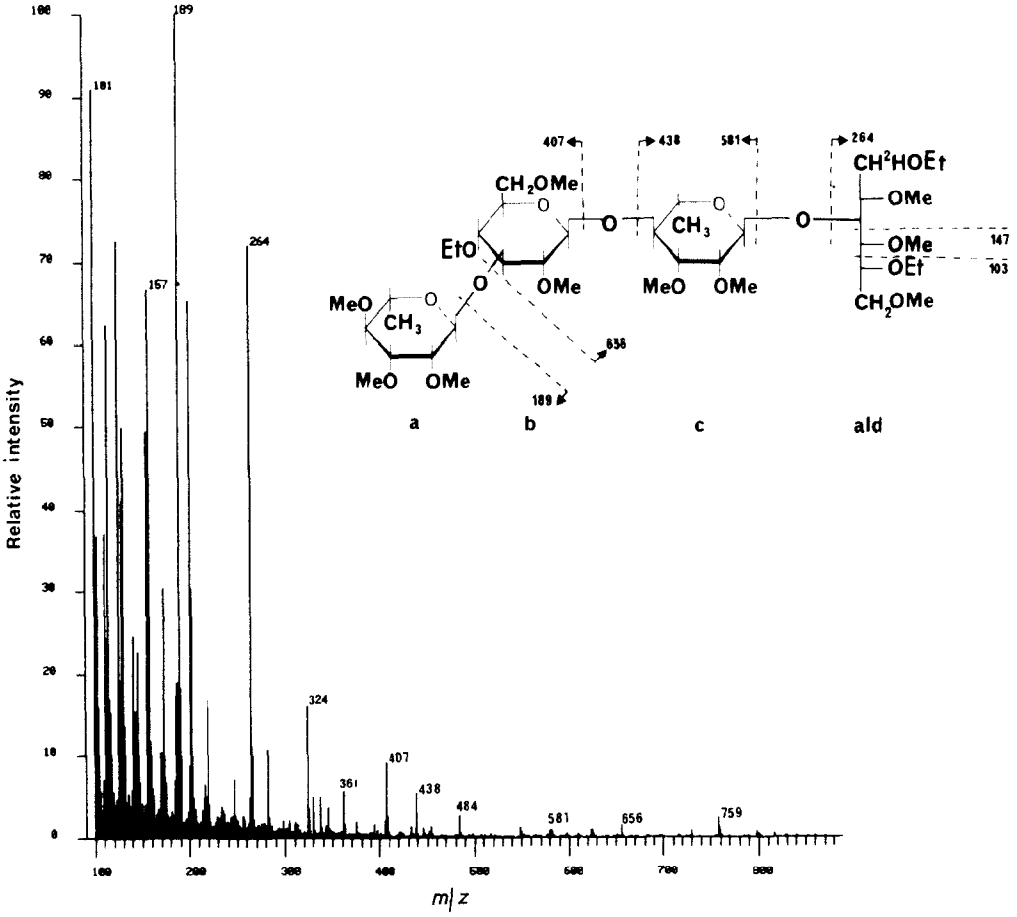


Fig. 6. Mass spectrum and fragmentation pattern of fraction DBU-2.

α-mannose residues, respectively. The above data established that the derivative in fraction 3 was obtained from the sequence **9** in the parent polysaccharide.

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

9

F.a.b.-m.s. of fraction 4 gave an ion at m/z 658, corresponding to $[M + 1]^+$ from an alkylated trisaccharide-alditol composed of 6-deoxyhexosyl, hexosyl, and 6-deoxyhexitol residues and containing three *O*-ethyl groups. E.i.-m.s. gave ions at m/z 104 (22.2%), 148 (5.2), 189 (aA_1, 86.7), 234 ($aldJ_2$, 58.1), 280 ($aldJ_0$, 5.5), 407

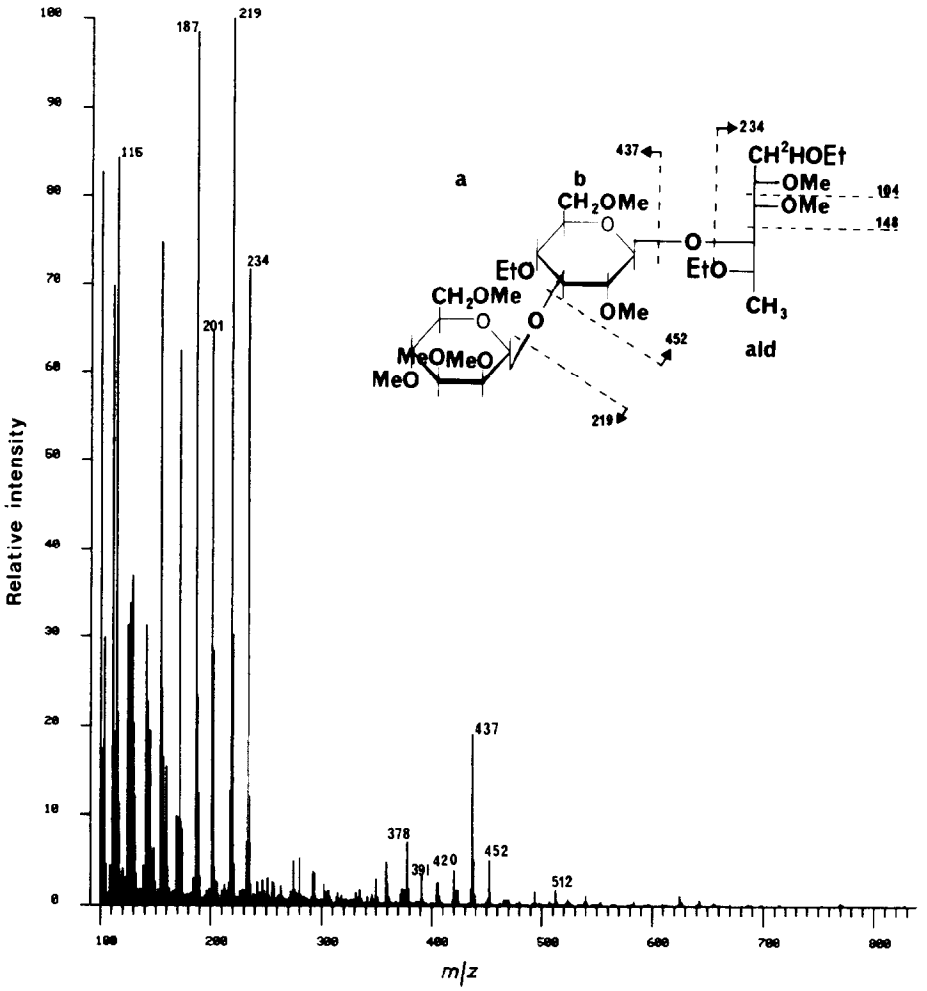


Fig. 7. Mass spectrum and fragmentation pattern of fraction DBU-3.

(baA$_1$, 12.6), 452 (b*ald*J$_2$, 6.1), and 512 (cb*ald*J$_1$, 0.5), demonstrating the sequence 6-Deoxyhex*p*→Hex*p*→6-Deoxyhexitol (Fig. 8). The ^{1}H-n.m.r. spectrum of the derivative in fraction 4 contained signals for anomeric protons (Table II) at δ 4.30 ($J_{1,2}$ 8.1 Hz) and 5.39 ($J_{1,2}$ 1.5 Hz). These were assigned to β-glucose and α-rhamnose residues, respectively. These data showed that fraction 4 was obtained from the sequence **10** in the polysaccharide.

→4)-β-D-Glc*p*-(1→4)-L-Rha*p*-(1→
3
↑
1
α-L-Rha*p*

10

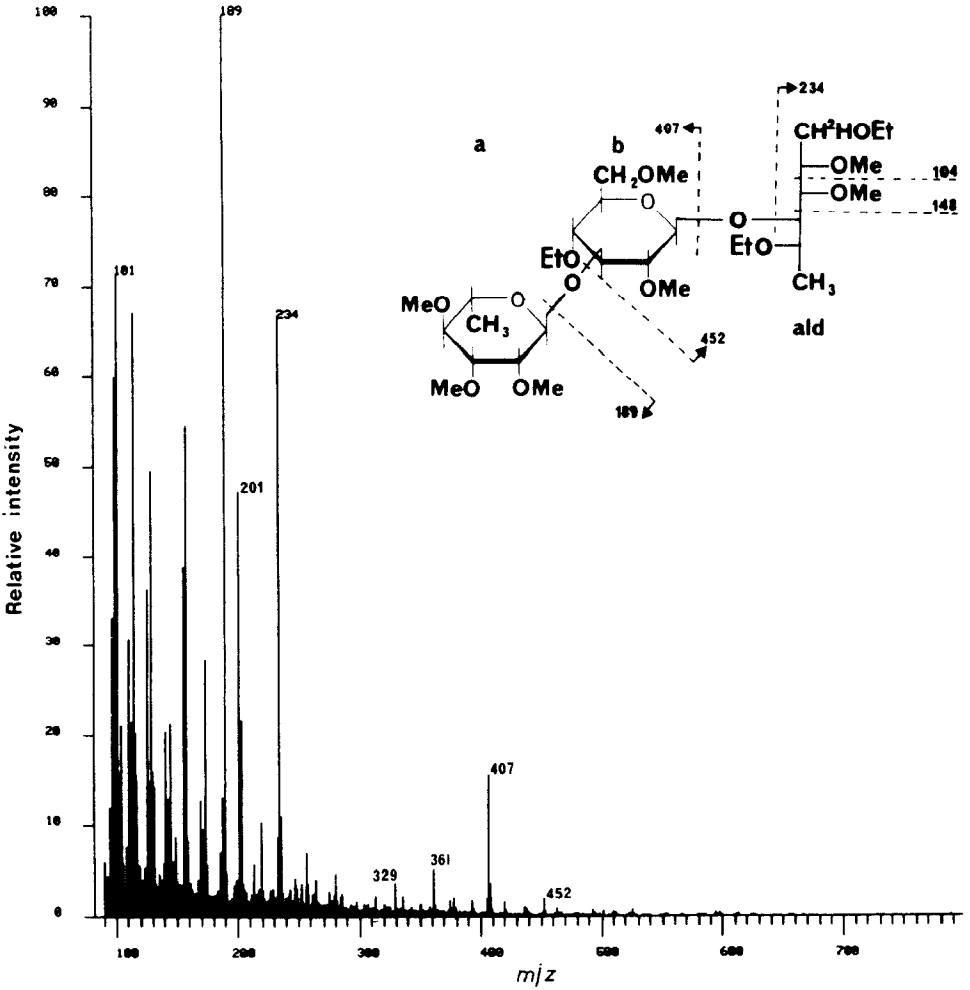
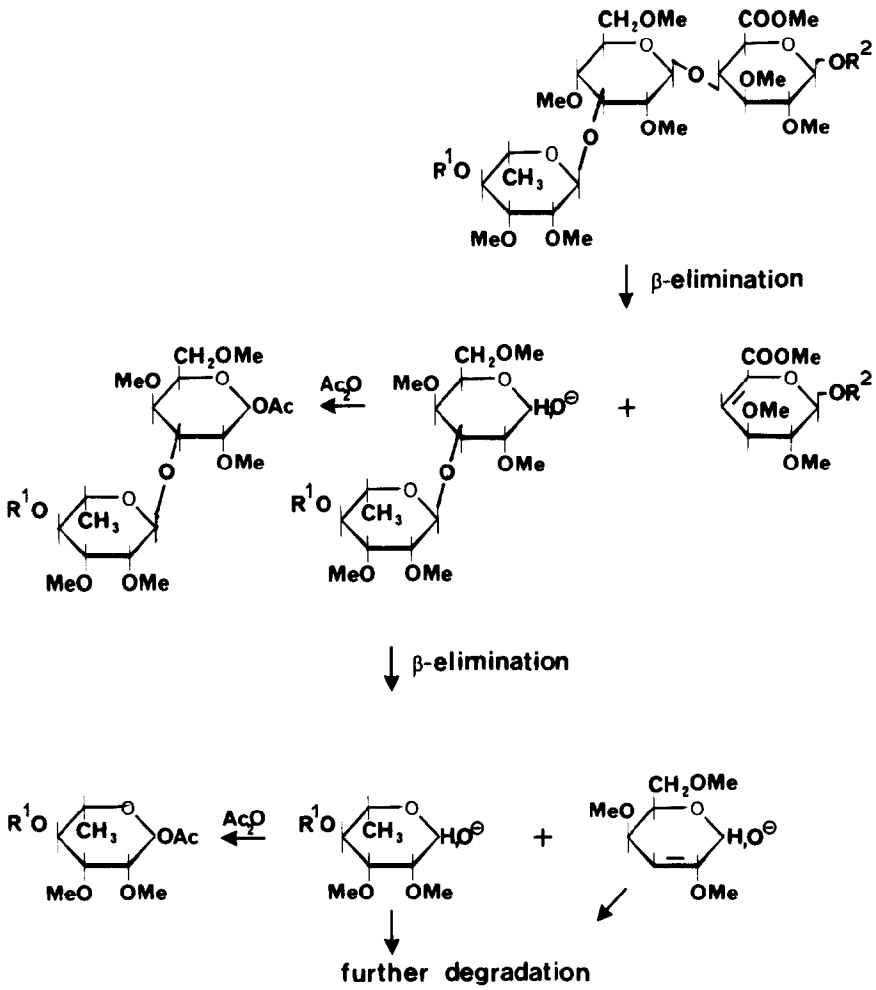


Fig. 8. Mass spectrum and fragmentation pattern of fraction DBU-4.

Base-catalysed degradation of the partial structure **6** with DBU in benzene–acetic anhydride was expected to give two tetrasaccharide derivatives (fractions 1 and 2, Fig. 4). However, two trisaccharide derivatives (fractions 3 and 4, Fig. 4) were also obtained. It is conceivable that the backbone of the polysaccharide is composed of alternating tetra- (**4**) and tri-saccharide (**5**) repeating-units in which both (1→4)-linked β-D-glucosyl residues are substituted through position 3 with terminal α-rhamnosyl and α-mannosyl groups. Methylation analysis of such a structure should give a ratio of 1:1 for the terminal non-reducing rhamnose and (1→3)-linked glucose. However the experimentally obtained ratio of 1.0:2.1 was consistent with the tetrasaccharide repeating-unit **4**. The alkylated trisaccharide-alditols were probably further degradation products of the corresponding tetrasaccharides formed by the reaction with DBU. It is possible that a proportion of the reducing groups, produced on elimination of the glycosyl residue from position 4 of the



Scheme 1

hexuronate, may have undergone a second β-elimination prior to their acetylation resulting in the loss[12] of the substituent on position 3 (Scheme 1). β-Eliminative degradation with DBU has found limited application[10] in the structural analysis of polysaccharides due to the difficulties in removing the excess of reagents. The proposal that other degradative processes occur (Scheme 1) may further complicate the use of this procedure, although these degradations may confirm the structural features deduced by alternative methods.

DISCUSSION

On the basis of the results of methylation analysis and selective chemical degradations in conjunction with mass spectral and n.m.r. analyses, the chemical repeating-unit **1** is proposed for the polysaccharide produced by the bacterium *Alcaligenes* (ATCC 31555). The results of β-elimination with DBU confirmed the structure **1**, but indicated that further degradations may occur with this procedure.

In the present study, the distribution of the terminal mannosyl and rhamnosyl groups along the polymer backbone was not determined. It is possible that the polysaccharide is a mixture of two polymers, one containing terminal rhamnosyl groups and one containing terminal mannosyl groups. If terminal rhamnosyl and mannosyl groups occur within a single polymer, then their distribution could be regular, in blocks, or random. In previous reports[2,4] and this study, it was found that the proportion of terminal non-reducing mannose was consistently lower than that of terminal non-reducing rhamnose. Even if the bulk of the polymer contained alternating terminal non-reducing rhamnose and mannose side-chains a small proportion (~10%) of the polymer would contain contiguous terminal non-reducing rhamnose residues.

The evidence presented here suggests that the polysaccharide produced by *Alcaligenes* (ATCC 31555) has the same tetrasaccharide backbone as gellan gum, a linear anionic gelling-polysaccharide[13,14]. However, the former polysaccharide contains terminal α-rhamnosyl and α-mannosyl groups linked to position 3 of the (1→4)-linked β-D-glucosyl residues. Jansson *et al.*[4] established that ~50% of the pentasaccharide repeating-units are mono-*O*-acetylated, although the location of the substituent is not known. Gellan gum and the *Alcaligenes* polysaccharide have considerably different rheological properties[1], and preliminary X-ray fibre diffraction data obtained in our laboratory and elsewhere[15] suggest that they have different secondary structures. Thus, it is concluded that the addition of terminal glycosyl groups to the gellan gum backbone alters the secondary structure of the polymer and this in turn is reflected in the rheology of aqueous dispersions.

EXPERIMENTAL

Materials. — A sample of the dried, alcohol-precipitated culture broth (Batch No. EX 4881) was a gift from Dr. R. Moorhouse (Kelco, San Diego, California, U.S.A.). The crude polysaccharide, commercially known as S130, was purified by dissolution in methyl sulphoxide followed by centrifugation to remove cell debris[16]. The polymer was recovered by precipitation with ethanol, dialysed, and freeze-dried. The extent of clarification was monitored by phase-contrast microscopy of aqueous dispersions[16].

Monosaccharide analysis. — Neutral sugars, released by hydrolysis with M H_2SO_4 (100°, 2.5 h), were determined[17] by g.l.c. of their alditol acetates. The absolute configuration of the neutral monosaccharides was determined[18] by g.l.c.

of their acetylated (+)-2-octyl glycosides on a column (3 m × 2.2 mm) containing 3% of OV-225 at 210°. The absolute configuration of mannose was also assigned on the basis of its optical rotation determined with an AA-100 polarimeter (Optical Activity Ltd.) and a 2.5-cm cell. Mannose was isolated from a partial acid hydrolysate (0.2M trifluoroacetic acid, 1 h at 100°) of the polysaccharide by preparative p.c. (ethyl acetate–pyridine–water, 8:2:1). Uronic acid was determined colorimetrically[19] using D-glucuronic acid as the standard.

Methylation analysis. — A solution of the purified polysaccharide (25 mg) in methyl sulphoxide (5 mL) was methylated[20]. A portion (~5 mg) of the methylated polysaccharide was analysed[21] as the alditol acetates by g.l.c.–e.i.-m.s. A further portion (~10 mg) was carboxyl-reduced[22] with $LiAl^2H_4$ (50 mg) in refluxing dichloromethane–ether (1:4). Portions (2 mg) of the carboxyl-reduced polysaccharide were analysed as the alditol acetates by g.l.c.–e.i.-m.s. or re-alkylated with MeI and EtI, respectively, and analysed[21] as the alditol acetates by g.l.c.–e.i.-m.s. A solution of the remaining methylated polysaccharide (~10 mg) in methyl sulphoxide (2 mL) containing[23] toluene-*p*-sulphonic acid (trace) and 2,2-dimethoxypropane (75 μL) was treated[24] at room temperature for 12 h with sodium methylsulphinylmethanide (1 mL) and alkylated with ethyl iodide. The degraded, alkylated products were extracted into chloroform and analysed[21] as the alditol acetates by g.l.c.–e.i.-m.s. Methylation analysis was also carried out after partial acid hydrolysis of the polysaccharide (20mM oxalic acid, 100°, 2 h).

Partial acid hydrolysis. — (*a*) A solution of the polysaccharide (70 mg) in 0.5M H_2SO_4 (50 mL) was heated for 4 h at 100°. The hydrolysate was adjusted to pH 7.5 with $Ba(OH)_2$, filtered, concentrated to ~2 mL, and placed on a column (8 × 1.5 cm) of Dowex AG1-X2 (AcO^-) resin. The neutral material was eluted with water (20 mL) and the acidic material was eluted with 2M acetic acid (30 mL). Each fraction was concentrated and then freeze-dried to yield neutral (46.2 mg) and acidic (15.4 mg) materials. The acidic fraction was reduced with NaB^2H_4, methylated, and analysed[25] by g.l.c.–e.i.-m.s.

(*b*) A suspension of the polysaccharide (120 mg) in 0.2M trifluoroacetic acid (75 mL) was heated for 1.5 h at 100°. The hydrolysate was filtered and concentrated to dryness, and the residual acid removed by co-distillation with water (4 × 20 mL). A solution of the residue in water (1 mL) was adjusted to pH 7.5 and applied to a column (15 × 1.5 cm) containing QAE-Sephadex ($HCOO^-$ form). The neutral material was eluted with water (100 mL) and the acidic material was eluted with aqueous 10% formic acid (150 mL). Each fraction was concentrated and freeze-dried to yield neutral (58 mg) and acidic (41.3 mg) materials. The acidic fraction (~10 mg) was reduced with NaB^2H_4 and methylated, and the alkylated oligosaccharide-alditol methyl esters were isolated by using Sep-Pak C_{18} cartridges and fractionated by reverse-phase h.p.l.c. (see below).

(*c*) A suspension of the polysaccharide (115 mg) in 0.5M trifluoroacetic acid (75 mL) was heated at 100° for 1.5 h. Neutral (55.0 mg) and acidic (46.4 mg) fractions were obtained after ion-exchange chromatography on QAE-Sephadex.

The acidic fraction (~10 mg) was reduced with NaB^2H_4 and methylated, and the alkylated oligosaccharide-alditol methyl esters were isolated by using Sep-Pak C_{18} cartridges and fractionated by reverse-phase h.p.l.c. (see below).

β-Elimination with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[9]. — A solution of the methylated polysaccharide in benzene–acetic anhydride (4:1, 2.5 mL) was heated for 24 h at 100° with DBU (0.5 mL; Fluka). The cooled solution was washed with aqueous 1% HCl (3 × 1 mL) and water (3 × 1 mL), dried (Na_2SO_4), filtered, and concentrated. The resulting syrup was dispersed in aqueous 10% acetic acid (1.5 mL) and heated for 1 h at 100°. The cooled hydrolysate was concentrated to dryness and the residual acid was removed by co-distillation with water (5 × 2 mL). A solution of the residue in ethanol (1.5 mL) containing M NH_3 and 10 mg/mL of NaB^2H_4 was kept[26] for 2 h at 20° and then for 30 min at 50°. The excess of borodeuteride was decomposed with glacial acetic acid, and the borate was removed by co-distillation with methanolic 10% acetic acid (4 × 5 mL). A solution of the residue in aqueous 50% ethanol (2 mL) was desalted on a column (5 × 1 cm) of Dowex AG1-X2 (H^+) resin by elution with aqueous 50% ethanol (25 mL). The eluant was concentrated to dryness and the residue was dried over P_2O_5 at 45° under diminished pressure. A solution of the dry residue in methyl sulphoxide (1 mL) was alkylated with ethyl iodide, and the alkylated oligosaccharide-alditols were isolated by using Sep-Pak C_{18} cartridges and fractionated by reverse-phase h.p.l.c. (see below).

Isolation of the alkylated oligosaccharide-alditols using Sep-Pak C_{18} cartridges. — Sep-Pak C_{18} cartridges (Waters Assoc.) were attached to a 10-mL glass syringe, and conditioned by elution with methanol (20 mL), acetonitrile (10 mL), and water (10 mL).

The alkylation-reaction mixture containing the alkylated oligosaccharide-alditols was diluted with water to produce an aqueous 20% methyl sulphoxide solution, and the excess of alkylating reagent was distilled off with argon. The aqueous mixture (~5 mL) was eluted[27] through the conditioned Sep-Pak C_{18} cartridge followed by elution with water (10 mL) and aqueous 20% acetonitrile (8 mL). The methylated oligosaccharide-alditols were eluted with aqueous 60% acetonitrile (10 mL) and the eluant was concentrated to dryness. A solution of the residue in aqueous 60% acetonitrile (100–200 μL) was filtered through a 0.45-μm fluoropore membrane (Millipore Corp.), using a centrifugal microfilter (Alltech Assocs.), prior to reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. — A Perkin–Elmer Series 2 chromatograph fitted with a Zorbax ODS column (25 cm × 4.6 mm) and a Brownlee C_{18} guard column was used. The alkylated oligosaccharide-alditols were introduced through a Rheodyne 7125 injector, using a 100-μL loop, and eluted[8] isocratically with aqueous 60% acetonitrile (H.P.L.C. grade filtered through a 0.45-μm fluoropore membrane) at 0.5 mL/min (~3.5 MPa). The effluent was monitored by using a Waters 401 differential refractometer and the components were collected manually. The combined fractions were concentrated to dryness and a solution of the residue in acetone (50 μL) was used for f.a.b.-m.s.[28] or e.i.-m.s.

^{1}H-N.m.r. spectroscopy. — A Bruker CXP-300 n.m.r. spectrometer was used. Spectra were obtained from the alkylated oligosaccharide-alditols ($CDCl_3$ containing 0.1% of Me_4Si) at 300 MHz under non-saturating conditions. Data were acquired in 32k data points with zero-filling to 64k data points prior to Fourier-transformation. Chemical shifts (δ) are reported in p.p.m. downfield from internal Me_4Si.

ACKNOWLEDGMENTS

We thank Dr. S. Tanner (Norwich) for the n.m.r. spectroscopy, Professor E. D. T. Atkins (University of Bristol), Dr. M. J. Miles, and Dr. S. G. Ring (Norwich) for advice, and Mrs. L. Williams for typing the manuscript.

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