Carbohydrate Research, 167 (1987) 257-267
Elsevier Science Publishers B.V., Amsterdam - Printed in The Netherlands

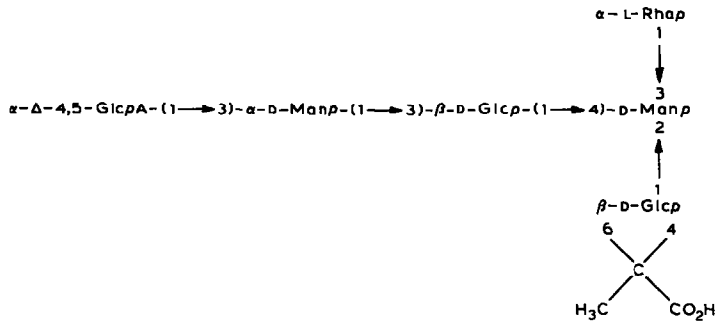
BACTERIOPHAGE-ASSOCIATED LYASE ACTIVITY AGAINST *Klebsiella* SEROTYPE K64 CAPSULAR POLYSACCHARIDE*

NEIL RAVENSCROFT, ALISTAIR M. STEPHEN, AND EDWIN H. MERRIFIELD
Departments of Organic Chemistry and Clinical Science and Immunology, University of Cape Town, Rondebosch 7700 (South Africa)

Received July 28th, 1986; accepted for publication, October 20th, 1986)

ABSTRACT

Bacteriophage ϕ64 possesses a lyase that depolymerises the capsular polysaccharide of *Klebsiella* K64 into a hexasaccharide having an unsaturated derivative of glucuronic acid at the non-reducing end (**1**). The unsaturated hex-4-enuronic acid residue generated was characterised spectroscopically (u.v. and n.m.r.) and by g.l.c.–m.s. after hydrogenation of the double bond. Partial hydrolysis, Smith degradation, methylation analysis, and n.m.r. spectroscopy have been used to establish the structures of oligosaccharides produced from the polysaccharide. Evidence from ^{1}H-n.m.r. spectroscopy indicates that the D-Man*p* residue that undergoes fission is β.



1

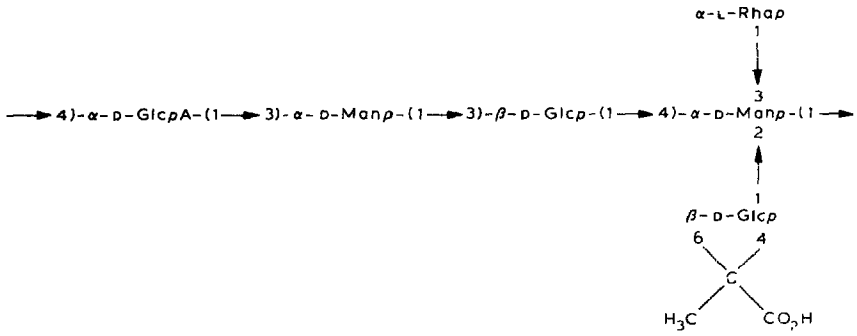
INTRODUCTION

The serotype K64 is one of two strains of *Klebsiella* whose capsular polysaccharides are composed of D-glucuronic acid, D-glucose, D-mannose, and L-rhamnose residues. Previous studies[1,2], involving partial depolymerisation by acid hydrolysis and Smith degradation, led[2] to the formulation of a repeating unit **2**; the unusual doubly-branched hexasaccharide structure includes one pyruvate ketal and

*Presented at the XIIIth International Carbohydrate Symposium, Ithaca, 10–15 August, 1986.

0008-6215/87/$03.50

some acetyl groups. Bacteriophage-borne enzymic depolymerisation of the capsular polysaccharide was conducted with a view to isolating the intact repeating-unit, which would be more amenable to further chemical and spectroscopic studies and thus permit confirmation of the repeating structure and ultimately location of the *O*-acetyl substituent. Aspects of this work have been briefly reported[3,4]. We now report on the mode of enzyme cleavage and the structural analysis of the oligosaccharides thereby obtained.



2

RESULTS AND DISCUSSION

Polysaccharide capsular material from *Klebsiella* K64, after isolation and purification[2], had properties and constituents in agreement with those found earlier. Negligible OAc was present, however, according to ^{1}H-n.m.r. spectroscopy. Bacteriophage ϕ64 was propagated on its host strain until $\sim 10^{13}$ plaque-forming units (p.f.u.) were obtained (sufficient to degrade $\sim$1 g of polysaccharide[5]). Depolymerisation then yielded oligomers which were isolated and separated into monomeric (*P1*) and dimeric (*P2*) fractions by gel filtration combined with partition chromatography on silica gel[3].

The results of the analysis and measurement of d.p. by the method of Morrison[6] (Table I) confirmed that *P1* is a hexasaccharide, that *P2* is the dimer, and that both have a mannose residue as the terminal, reducing unit. Cleavage could have occurred at either mannoside linkage (*cf.* **2**). Reduction of *P1* with sodium borodeuteride ($NaBD_4$) followed by methylation analysis (Table II, A and B) yielded a peak, not present among the partially methylated alditol acetates derived from unreduced *P1* (A), which corresponded to the acetylated derivative of 1,5,6-tri-*O*-methylmannitol[3] deuterium-labelled at C-1. Referring to structure **2**, cleavage must have occurred between the highly substituted mannose and the glucuronic acid residues, a favoured position, with respect to the acid group, in several known cases of bacteriophage action on *Klebsiella* polysaccharides[8]. Prior to methylation analysis, the sample had been used for high temperature n.m.r. studies, so that the detection of terminal glucose would be expected from the partial loss of the acid-labile pyruvic

TABLE I

DETERMINATION OF D.P. AND CHARACTERISATION OF THE REDUCING END OF THE OLIGOSACCHARIDES *P1* AND *P2* DERIVED FROM *Klebsiella* K64

Peracetylated derivative of	T[a]	*Mol%*	
		P1	P2
Rhamnononitrile	0.30	17	16
Mannononitrile	0.67	17	25
Gluconononitrile	1.00	40	44
Mannitol	1.15	26	15

[a]Retention times, determined on column *1* at 220°, relative to 2,3,4,5,6-penta-*O*-acetylgluconononitrile.

acid ketal; this is corroborated by a decrease in the peak due to 2,3-di-*O*-methylglucose (Table II). The appearance of terminal mannose at the expense of the 2,4,6-tri-*O*-methylmannose can be explained only by removal of what had been the terminal glucuronic acid residue[4]. Uronic acid glycosides are resistant to acid hydrolysis[9] and therefore the glycosidic link must have been destabilised during bacteriophage action. It has now become apparent that the bacteriophage ϕ64 enzyme acts as a lyase[10], catalysing the β-elimination reaction of the highly substituted mannosyl

TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDES A - D

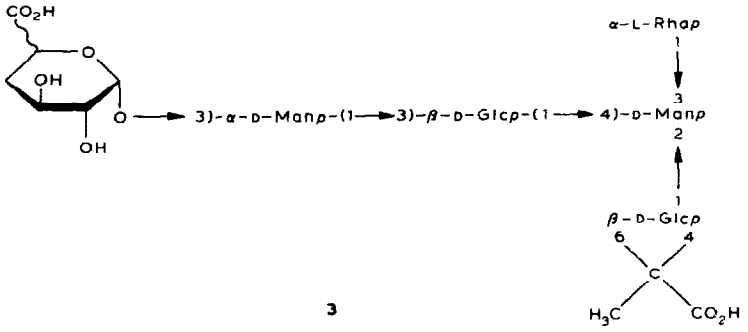
Partially methylated alditol acetates[a]	T[b]	*Mol%*[c]			
		A	*B*	*C*	*D*[d]
2,3,4-Rha	0.68	20	16	15	18
1,3,5,6-Man[e]	0.88	—	—	5	—
2,3,4,6-Man	0.99	9[f]	17[f]	43	26
2,3,4,6-Glc	1.00				
1,5,6-Man[e]	1.16	—	24	14	7
2,4,6-Glc	1.48	22	18	18	24
2,4,6-Man	1.52	20	16	5	10
6-Man	2.56	15	—	—	7
2,3-Glc	2.74	14	9	—	8[g]

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* [b]Retention times, determined on column *3* at 215°, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. Assignments confirmed by g.l.c.-m.s. [c]Values were corrected by use of carbon-response factors given by Sweet *et al.* [7]. [d]Oligosaccharide A, *P1* (1); B, 1 reduced with $NaBD_4$; C, 1 autohydrolysed and $NaBD_4$-reduced; D, *P2* autohydrolysed and $NaBD_4$-reduced. [e]C-1 deuterated according to g.l.c.-m.s. [f]From some loss of acid (uronic, or pyruvic ketal). [g]C-6 dideuterated according to g.l.c.-m.s.

residue from position 4 of the glucuronic acid within the polysaccharide chain. In this way, an unsaturated derivative of glucuronic acid is generated at the non-reducing end of the oligomers produced (1). Degradation of heteroglycans by lyase action on bacterial products is generally common[11], but only one other example of bacteriophage-associated lyases has been published in the *Klebsiella* field: Van Dam *et al.*[12] showed that the lyase action of bacteriophage $\phi 5$ splits a pyruvylated mannose residue from position 4 of D-glucuronic acid, to generate an unsaturated acid-containing trisaccharide.

Characterisation of the unsaturated uronic acid residues. — Colorimetric assay for glucuronic acid[13] in *P1* gave a negligible result. Indirect evidence for a modified residue was initially obtained by the absence of detectable (p.c.) glucuronic acid from an acid hydrolysate of *P1*; likewise, methylation analysis of *P1* showed the absence of any uronic acid derivatives. These observations are consistent with the presence of the unsaturated derivative of glucuronic acid, which is known to be degraded during acid hydrolysis[9,14]. The unsaturated acid residue, as its Na^+ salt, was characterised by ultraviolet (λ_{max} 233 nm) and infrared [ν (C=C) at 1664 cm^{-1}] absorptions which accorded with those previously reported[4,11,12,15]. The ^{1}H-n.m.r. spectrum of *P1* contained downfield doublets, not present in the original polymer, typical[12,15-17] of the olefinic (H-4) and anomeric (H-1) protons of an α-linked unsaturated acid residue, while C-5 (145.6 p.p.m.) and C-4 (109.3 p.p.m.) were readily identified by ^{13}C-n.m.r. spectroscopy[4]. Protonation of the Na^+ salt of *P1* caused the expected downfield shifts of the signals for H-4 and H-1 (Table III), confirming the assignments. The lability of the unsaturated acid residue was demonstrated[4] during the course of high-temperature n.m.r. experiments in which the signals assigned to H-1 and H-4 gradually disappeared, and were replaced by signals between δ 6.6 and 8.3. Purification of the resulting oligosaccharide product by gel filtration yielded a neutral pentasaccharide which showed no abnormal low-field signals (Table IV); therefore, these signals must have arisen after release and further degradation of the unsaturated acid moiety[14].

P1 (as the Na^+ salt) was hydrogenated to yield a product (3) having no u.v. absorption at 233 nm and an absence of low-field doublets in the ^{1}H-n.m.r. spectrum, this being indicative of the saturation of the uronic moiety to 4-deoxyuronate;



3

TABLE III

^{1}H-N.M.R. DATA (90 MHz) FOR *PI* (Na^+) AND *PI* (H^+)

Compound	δ	$J_{1,2}$	*Assignment*	
PI (Na^+ form)	5.76	3.2 ($J_{3,4}$)	α-Hex-4-ene*p*A	H-4
	5.33	2.8	α-Hex-4-ene*p*A	H-1
	5.24	s	α-Man*p*-OH	H-1
	5.15	1.7	α-Man*p* (c)[a]	H-1
	4.89	s	α-Rha*p* (t)[b] β-Man*p*-OH	H-1
	4.51	7.5	β-Glc*p* (c)	H-1
	4.48	7.6	β-Glc*p* 4 6 \ / P	H-1
	1.44	s	$CH_3-\overset{\displaystyle|}{\underset{\displaystyle|}{C}}-$ COO^-Na^+	
	1.24	6.2 ($J_{5,6}$)	CH_3 of Rha	
PI (H^+ form)	6.20	3.2 ($J_{3,4}$	α-Hex-4-ene*p*A	H-4
	5.36	2.8	α-Hex-4-ene*p*A	H-1
	1.50	s	$CH_3-\overset{\displaystyle|}{\underset{\displaystyle|}{C}}-$ COOH	
	Other peaks unchanged			

[a]Glycosidic, in-chain (c). [b]Glycosidic, terminal group (t).

the proportion of β-L-*arabino* and α-D-*xylo* C-5 epimers formed is known to vary with experimental conditions[11]. Sugar analysis of the alditol acetates (after methanolysis and methyl ester reduction with $NaBD_4$, followed by acid hydrolysis and treatment with $NaBH_4$) gave the results shown in Table V. In addition to derivatives of the neutral sugars that were expected, three components each containing deuterium label were detected; on the basis of retention times and g.l.c–m.s. analysis[18], these were clearly identified as the 6,6-dideuterated, acetylated derivatives of a 4-deoxyhexitol, a 1,6-anhydro sugar, and a lesser amount of a 3,4-dideoxyhexitol

TABLE IV

^{1}H-N.M.R. DATA (90 MHz) FOR THE PENTASACCHARIDE OBTAINED BY PARTIAL HYDROLYSIS OF *P1*

Compound	δ	$J_{1,2}$	*Assignment*	
Pentasaccharide	5.26	2.2	α-Man*p*-OH	H-1
	5.19	1.7	α-Man*p* (t)	H-1
	5.00	1.7	αRha*p* (t)	H-1
	4.90	0.8	β-Man*p*-OH	H-1
	4.49	7.6	β-Glc*p* (t)	H-1
	4.47	7.6	β-Glc*p* (c)	H-1
	1.24	6.3 ($J_{5,6}$)	CH_3 of Rha	
Reduced pentasaccharide	5.22	1.5	α-Man*p* (t)	H-1
	5.10	1.6	α-Rha*p* (t)	H-1
	4.61	7.4	β-Glc*p* (t)	H-1
			β-Glc*p* (c)	H-1
	1.26	6.3 ($J_{5,6}$)	CH_3 of Rha	

(consequent upon β-elimination). 4-Deoxy-*arabino*-hexoses form 1,6-anhydrides readily[19] and were found together with the 4-deoxy and 3,4-dideoxy sugar derivatives upon acid hydrolysis of a (1→2)-linked 4-deoxy-β-D-*arabino*-hexopyranosyl homopolymer[18]. The products obtained from the present series of reactions and the location of the deuterium labels are consistent only with hydrogenation of a hex-4-enuronic acid group in *P1*, leading to a 4-deoxy derivative, having taken place.

Linkage and sequence of sugar residues. — Autohydrolysis of *P1* and *P2*, prior to borodeuteride reduction and methylation, ensured quantitative removal of the labile groups mentioned earlier (namely, pyruvate and hexenuronate); the results of methylation analysis are shown in Table II (C and D). The appearance of derivatives of 6-*O*-methylmannose and deuterated 2,3-di-*O*-methylglucose on analysis of *P2* (D), but not *P1* (C), shows that the reducing mannopyranose residue of one repeating unit is joined to O-4 of the glucuronic acid of the next unit[4]. Thus, all of the proposed linkage assignments are confirmed; anomeric configurations for **1** and its autohydrolysed product follow from the ^{1}H-n.m.r. spectra (Tables III and IV). Proton integrals for the respective H-1 signals and for Rha and pyruvate CH_3 were in conformity with these assignments. The loss of the labile hexenuronic acid group and the appearance of greatly enhanced terminal mannose on methylation analysis

TABLE V

SUGAR ANALYSIS OF HYDROGENATED *P1* (3)

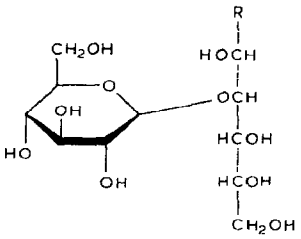
Peracetylated derivative of	T[a]	*Proportion*[b] *(mol %)*	*M.s.* (m/z)
1,6-Anhydro-4-deoxyhexitol-*6,6-d*$_2$	0.15	7	83, 84, 85, 102, 130, 172
Rhamnitol	0.29	16	[c]
3,4-Dideoxyhexitol-*6,6-d*$_2$	0.38	3	83, 85, 101, 103, 143, and 145
4-Deoxyhexitol-*6,6-d*$_2$	0.71	7	71, 81, 96, 103, 131, 145, 156, 201, 203, 233, and 303
Mannitol	0.83	29	[c]
Glucitol	1.00	38	[c]

[a]Retention time determined on column *3* at 215°. [b]Uncorrected peak areas. [c]Identical with standards.

of C and D (relative to A and B) confirms the α-Δ-4,5-Glc*p*A-(1→3)-α-D-Man*p* linkage. However, the positions of attachment of the other three sugars to the mannose at the reducing end was known at this stage only by inference from the polysaccharide structure (**2**), and these were confirmed as follows.

Loss of some rhamnose during the autohydrolysis of *P1* resulted in the appearance, on methylation analysis, of acetylated 1,3,5,6-tetra-*O*-methylmannitol deuterated at C-1 (Table II, C), at the expense of the 1,5,6-tri-*O*-methylmannitol derivative, confirming that the rhamnosyl group is linked at O-3. Smith degradation of autohydrolysed *P1* yielded β-D-Glc-(1→2)-pentitol (**4**) and β-D-Glc-(1→3)-hexitol (**5**) as the major and minor products, respectively. The pentitol moiety of **4** must have arisen by fission of the C-5–C-6 bond of the ring-opened, reducing-end mannose. The loss of C-6 from the hexose derivative **5** shifts the position of attachment of the glucosyl group from C-3 in **5** to C-2 in pentose derivative **4**, which corresponds to glycosylation at C-4 of mannose in the original structure **2**. The 3-linked (and therefore periodate-resistant) glucosyl residue in the main chain is therefore linked at O-4, and the pyruvylated glucosyl group at O-2, of the mannose residue at the reducing end of *P1*.

Although n.m.r. evidence obtained after Smith degradation of the polysaccharide[2] suggested both mannose residues to be α-linked, comparison with the spectra of a doubly branched oligosaccharide of similar structure[20] indicates that the signal at δ 4.74 in the spectrum of *P2*, which persisted after $NaBH_4$ reduction, is



4 (R = H)

5 (R = CH_2OH)

attributable to a β-mannosyl residue. Inspection of the ^{1}H-n.m.r. spectra obtained earlier[2] allows a similar interpretation to be made of an unresolved signal at δ 4.76, on the shoulder of the HOD peak. Formula **2** accordingly requires modification in this respect. This is the anomeric configuration of the substituted mannose in K5 that undergoes elimination by the lyase action of ϕ5.

EXPERIMENTAL

General methods. — Solvent systems used in paper and thin-layer chromatography were *A*, 20:20:7 chloroform–methanol–water; *B*, 97:3 chloroform–methanol; and *C*, 8:2:1 ethyl acetate–pyridine–water. Sugars in t.l.c. were detected by spraying with *p*-anisaldehyde–sulphuric acid–ethanol (1:1:18) or *p*-anisidine hydrochloride (2%) in sulphuric acid–ethanol (1:20) followed by heating at 110° for 5–10 min. Sugars on paper were detected in the usual manner[2]. G.l.c. and g.l.c–m.s. analyses were performed with columns: *1*, a packed column (2 m × 3 mm i.d.), containing 3% of OV-17 on Chromosorb W-HP (80–100 mesh); *2*, a packed column as above containing 3% of OV-225; or *3*, a quartz capillary (30 m × 0.32 mm i.d.) with OV-225 (0.25-μm film thickness) as the bonded phase (Durabond DB-225; J. and W. Scientific).

Analytical determinations of molecular-weight distributions were made using columns of Sepharose 4B (60 × 0.9 cm) or Bio-Gel P-2 (55 × 2.5 cm)[1]. Preparative steric exclusion chromatography (s.e.c.) was performed on column *A*, Bio-Gel P-2 (92 × 2 cm) eluted with water at 5 mL/h; or column *B*, Trisacryl GF05 (20 × 1.2 cm) eluted with water at 30 mL/h. Fractions were monitored by the phenol–H_2SO_4 assay, optical rotation, or t.l.c. (solvent *A*).

^{1}H-N.m.r. spectra were recorded for deuterium-exchanged (× 3) samples with a Bruker WH-90 instrument, either at ambient temperature or 80–90°, with sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard. ^{13}C-N.m.r. spectra were recorded with a Bruker WM-500 instrument at ambient temperature; chemical shifts were measured with respect to TSP (sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate), but are expressed relative to Me_4Si = 0.

Preparation and properties of K64 capsular polysaccharide. — A culture of

Klebsiella K64 was grown as previously described[2], and the polysaccharide was purified by precipitation once with cetyltrimethylammonium bromide. The purified polysaccharide (~3 g) from four batches of four trays (each 38 × 27 × 1.5 cm) typically had $[\alpha]_D$ +28° (*c* 0.1); N, 0.4%; and contained Rha 17 (19), Man 32 (27), Glc 35 (38), and GlcA 16. Neutral sugars were analysed as alditol acetates by g.l.c., and the uronic acid by colorimetric assay[13]. Corresponding values for those of an earlier preparation[2] are given, also as mol %, in brackets. Analysis by gel chromatography (Sepharose 4B) showed it to be homogeneous with an average molecular weight of ~1.6 × 10^6 (calibration with dextran standards).

Propagation of bacteriophage ϕ64. — Bacteriophage ϕ64 was propagated on host strain *Klebsiella* K64 in nutrient broth until 1 L of lysate containing ~10^{13} p.f.u. had been obtained. The phage solution was concentrated and dialysed against running tap-water (2 days), the dialysate finally being concentrated (to 100 mL). Two such preparations were made.

Depolymerisation of the polysaccharide and isolation and purification of the oligosaccharides. — (*i*) Phage solution (100 mL containing 3 × 10^{13} p.f.u.) was added to the polysaccharide (570 mg) in water (150 mL), and the mixture was stirred for 3 days at 37°. (*ii*) Polysaccharide (0.8 g) was added directly to the phage solution (5 × 10^{13} p.f.u. in 100 mL) and stirred for 5 days at 37° in the presence of chloroform (3 mL). The oligomers produced were isolated by dialysis and purified by successive passages through a column of Amberlite IR-120 (H^+) resin. Treatment with resin was repeated until a colourless solution was obtained which, on freeze-drying after neutralisation with $NaHCO_3$, afforded oligosaccharides (Na^+ form) in yields of >50% (*i*, 300 mg; *ii*, 620 mg). The oligosaccharide products typically had $[\alpha]_D$ +17° (c 0.5) (*cf.* +22° calc. on the basis of Hudson's rules), and contained 60% of the monomer (according to s.e.c. on a Bio-Gel P-2 analytical column). Paper chromatography (solvent *C*) showed traces of uronic acid in a hydrolysate, while g.l.c. analysis (column *2* at 220°) of the derived alditol acetates confirmed the presence of rhamnose (1 mol), mannose (2), and glucose (2). A portion (100 mg) of the oligomers was fractionated on column *A*, resulting in incomplete separation of *P1* and *P2*, as shown by t.l.c analysis (solvent *A*). Passage of the *P1*-rich fraction through column *B* yielded *P1* (42 mg). A further portion (300 mg) was fractionated on a column (30 × 1.5 cm) of silica gel (elution with solvent *A*) followed by passage through column *B* to yield purified *P1* (135 mg) and *P2* (96 mg).

Analysis of the oligosaccharides. — A sample (5 mg) of each oligosaccharide was dissolved in water (2 mL) and treated with sodium borohydride (10 mg) overnight. The reduced oligosaccharides recovered were hydrolysed (2M CF_3CO_2H, 18 h, 100°) and the products were converted into peracetylated aldononitrile derivatives for analysis (Table I). All methylations were conducted on 2–20 mg of sample by the method of Hakomori[21] as modified by Phillips and Fraser[22]. Samples containing carboxyl groups were deionised with Amerlite IR-120 (H^+) resin and freeze-dried prior to methylation. Reduction of methyl carboxylate groups was effected by the method of Åman *et al.*[23]. Hydrolysis of the methylated products with 2M

CF_3CO_2H (8–18 h at 100°), borohydride reduction, and acetylation gave the partially methylated alditol acetates, which were analysed by g.l.c. and g.l.c.–m.s. (see Table II).

The process of autohydrolysis of *P1* and *P2*, carried out by heating the deionised sample (10 - 20 mg in 1 mL of D_2O) at 100° for 2 h, was followed by t.l.c. analysis (solvent *A*) and ^{1}H-n.m.r. spectroscopy.

Hydrogenation experiments were conducted under pressure (30 bar), the samples (20 mg in 3 mL of water) being stirred with palladium-on-charcoal (10 mg) for 2 h. The solutions were filtered through Celite, centrifuged, and freeze-dried, yielding hydrogenated products (~15 mg). Low-pressure hydrogenations (1 bar), using Pd/C or Pt as catalyst, were unsuccessful. Hydrogenated *P1* was treated with $NaBH_4$ (5 mg), and the product (15 mg) was methanolysed as follows[24]. A solution of the sample (5 mg) in dry methanol (1 mL, 1M with respect to HCl) was heated for 18 h at 100°, then neutralised with Ag_2CO_3, and treated overnight with $NaBD_4$ (10 mg) in ethanol. Acidification with acetic acid followed by passage through Amberlite IR-120 (H^+) resin yielded a mixture of methyl glycosides, which were hydrolysed (2M CF_3CO_2H, 120°, 1 h) to aldoses, and converted into alditol acetates in the usual way. The results of g.l.c.–m.s. analysis are shown in Table V.

Smith degradation. — A portion (120 mg) of oligosaccharide product (*ii*) was acidified using Amberlite IR-120 (H^+) resin, and then autohydrolysed for 2 h at 100°. The product was purified by passage through column *B*. The collected product (90 mg) was treated with 0.1M sodium periodate (12.5 mL) in the dark at room temperature. The consumption[9] of periodate became constant after 3 days at 1.2 mol per sugar residue (value expected, 1.35, based on a molar ratio of *P1*/*P2* = 4). Excess of periodate was decomposed and the oxidised material reduced by $NaBH_4$ (1.35 g) overnight. Following decomposition of the hydride with acetic acid, the solution was freeze-dried, methanol was added to the residue and removed by evaporation (three times), and the product was fractionated on column *B*. The major component of low molecular weight (~350) was kept in M CF_3CO_2H (4 mL) at room temperature. Hydrolysis was monitored by t.l.c., which indicated a single component (R_{RHA} 0.73, solvent *A*) after 4 days. The ^{1}H-n.m.r. spectrum of the recovered material (30 mg) showed a single β-anomeric signal (δ 4.58, $J_{1,2}$ 7.4 Hz). The permethylated product gave a single spot in t.l.c. (solvent *B*), but g.l.c. analysis (column *3* at 215°) showed the presence of two components **4** and **5** at retention times 0.66 (80%) and 1.03 (20%) relative to permethylated cellobi-itol. G.l.c.–m.s. showed **4** to be a Hex*p*-(1→2)-pentitol with characteristic fragments (*m*/*z* 45, 88, 89, 101, 127, 155, 159, 187, 191, 219, and 251), and **5** to be a Hex*p*-(1→3)-hexitol (*m*/*z* 45, 88, 89, 101, 155, 171, 187, 219, 235, and 295). The β configuration of the terminal linkage was confirmed by the ^{1}H-n.m.r. spectrum of the permethylated product in $CDCl_3$ (δ 4.36, $J_{1,2}$ 7.2 Hz). Hydrolysis (2M CF_3CO_2H, 6 h, 100°) followed by g.l.c–m.s. (column *3* at 215°) of the derived alditol acetates showed 3 peaks at 0.47 (24%), 0.61 (9%), and 1.00 (67%), corresponding to the acetylated derivatives of 1,3,4,5-tetra-*O*-methylpentitol, 1,2,4,5,6-penta-*O*-methylhexitol, and 2,3,4,6-tetra-*O*-methylglucitol, respectively.

ACKNOWLEDGMENTS

We thank the C.S.I.R. (Foundation for Research and Development) and the University of Cape Town for financial grants, and the Head of the Department of Microbiology, Ms. Charlene Steyn, Dr. Shirley C. Churms, Mr. Wilfred T. Mabusela, and Ms. Bridget Williamson (Mass Spectrometry Unit) for advice and assistance.

REFERENCES

1 S. C. Churms and A. M. Stephen, *Carbohydr. Res.*, 35 (1974) 73–86.
2 E. H. Merrifield and A. M. Stephen, *Carbohydr. Res.*, 74 (1979) 241–257.
3 N. Ravenscroft, E. H. Merrifield, and A. M. Stephen, *S. Afr. J. Sci.*, 81 (1985) 380.
4 N. Ravenscroft, A. M. Stephen, and E. H. Merrifield, *S. Afr. J. Sci.*, 81 (1985) 381–382.
5 H. Thurow, N. Niemann, and S. Stirm, *Carbohydr. Res.*, 41 (1975) 257–271.
6 I. A. Morrison, *J. Chromatogr.*, 108 (1975) 361–364.
7 D. P. Sweet, R. H. Shapiro, and P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217–225.
8 D. Reiger-Hug and S. Stirm, *Virology*, 113 (1981) 363–378.
9 G. O. Aspinall, in G. O. Aspinall (Ed.), *Polysaccharides*, Vol. 1., Academic Press, New York, 1982, pp. 35–131.
10 Report of the Commission on Enzymes, *I. U. B. Symp. Ser.*, 20 (1961); R. J. Linhart, P. M. Galliher, and C. L. Cooney, *Appl. Biochem. Biotechnol.*, 12 (1986) 135–176.
11 J. Kiss, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 229–303.
12 J. E. G. Van Dam, H. Van Halbeek, J. P. Kamerling, and J. F. G. Vliegenthart, *Carbohydr. Res.*, 142 (1985) 338–343.
13 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
14 B. Lindberg, J. Lönngren, and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 31 (1976) 185–240.
15 J. W. Llewellyn and J. M. Williams, *Carbohydr. Res.*, 22 (1972) 221–224.
16 A. S. Perlin, D. M. Mackie, and C. P. Dietrich, *Carbohydr. Res.*, 18 (1971) 185–194.
17 G. K. Hamer and A. S. Perlin, *Carbohydr. Res.*, 49 (1976) 37–58.
18 E. Romanowska, A. Romanowska, C. Lugowski, and E. Katzenellenbogen, *Eur. J. Biochem.*, 121 (1981) 119–123.
19 S. Hanessian, *Adv. Carbohydr. Chem.*, 21 (1966) 143–207.
20 H. Parolis and L. A. S. Parolis, personal communication.
21 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
22 L. R. Phillips and B. A. Fraser, *Carbohydr. Res.*, 90 (1981) 149–152.
23 P. Åman, L.-E. Franzén, J. E. Darvill, M. McNeil, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 103 (1982) 77–100.
24 W. F. Dudman, L.-E. Franzén, J. E. Darvill, M. McNeil, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 117 (1983) 141–156.