Carbohydrate Research, 181 (1988) 153–162
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

BACTERIOPHAGE DEGRADATION OF THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* K69: LOCATION OF THE *O*-ACETYL GROUPS

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(Received November 5th, 1987; accepted for publication, January 23rd, 1988)

ABSTRACT

The *O*-acetyl group in the repeating unit of the capsular polysaccharide of *Klebsiella* K69 has been located on O-6 of the 4-linked β-mannosyl residue by a combination of ^{1}H- and ^{13}C-n.m.r. spectroscopy and f.a.b.-m.s. of the pentasaccharide obtained by degradation of the polysaccharide with a bacteriophage. Only every third repeating-unit in the polysaccharide chain is *O*-acetylated. Phage Φ69 was shown to be a β-D-mannosidase and cleaved the β-D-Man*p*-(1→4)-β-D-Glc*p* linkages in the polysaccharide.

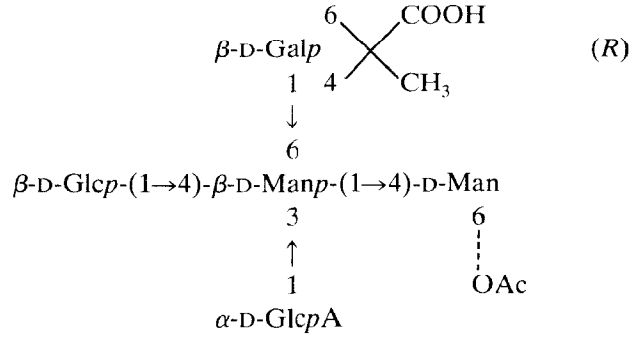
INTRODUCTION

O-Acetyl groups are frequently encountered as constituents of bacterial polysaccharides and, in many cases, have been shown to be serologically important. Their location in polysaccharides may be established by n.m.r. spectroscopy[1–4] and by chemical techniques[2,4]. The spectroscopic approach requires well-resolved spectra which are seldom obtained with polysaccharides of high molecular weight[5]. Improved spectra may be acquired by first partially depolymerising polysaccharides with acid[6]; however, labile substituents such as acetate and pyruvate may be lost during this process. The chemical techniques for locating *O*-acetyl groups involve treating the polysaccharide with methyl vinyl ether[7] under acid catalysis or with trifluoromethanesulphonate[8] in the presence of a proton scavenger. However, these methods do not always furnish unambiguous results due to incomplete derivatisation of the polysaccharide[2,4,9].

Alternative approaches which have been applied successfully to the location of *O*-acetyl groups in bacterial polysaccharides include depolymerisation with HF[5,10] or bacteriophage-borne enzymes[4], followed by ^{1}H- and ^{13}C-n.m.r. spectroscopy[4,5], f.a.b.-m.s.[5,11], or chemical analysis[4] of the *O*-acetylated oligosaccharides formed.

0008-6215/88/$ 03.50

We now report the location of the *O*-acetyl group in the repeating unit of the capsular polysaccharide of *Klebsiella* K69 (ref. 12) by ^{1}H- and ^{13}C-n.m.r. spectroscopy and f.a.b.-m.s. of the pentasaccharide (**P1**) produced by degradation of the polysaccharide with a bacteriophage.



P1

RESULTS AND DISCUSSION

Depolymerisation of Klebsiella *K69 polysaccharide with bacteriophage Φ69.* — *Klebsiella* bacteriophage Φ69, isolated from sewage water, was purified by three successive single-colony isolations and propagated on its host strain in nutrient broth. A bacteriophage solution containing 4×10^{12} plaque-forming units (PFU) was incubated with K69 polysaccharide for 72 h at 30°, after which the mixture was purified and the products were separated on Bio-Gel P4 to afford the oligosaccharides **P1** and **P2**.

Characterisation of **P1** *and* **P2**. — The degree of polymerisation and identity of the reducing termini of **P1** and **P2** were determined by analysing **P1**- and **P2**-alditol, with and without reduction of the uronic acid, by the aldononitrile procedure[13,14]. The results established **P1** and **P2** to be penta- and deca-saccharides, respectively, having the composition glucuronic acid, mannose, and glucose in the ratios 1:2:1 with mannose as the reducing terminus in each case.

Methylation of **P1**- and **P2**-alditol followed by g.l.c. and g.l.c.–m.s. of the derived partially methylated alditol acetates, after reduction of the uronic acid, gave the results in Table I, columns I and II. The methylation results reported[12] for the carboxyl-reduced polysaccharide are shown for comparison in column III. The small amount of 2,3,4,6-tetra-*O*-methylgalactose formed from **P1** arises from the loss of some pyruvate during preparation of the acid form of **P1** for methylation. The results confirm mannose as the reducing end in each oligosaccharide, establish that **P1** and **P2** represent the repeating unit and double repeating unit, respectively, of the polysaccharide, and demonstrate that Φ69 is capable of distinguishing between the two mannosyl residues, and cleaves the β-D-Man*p*-(1→4)-β-D-Glc*p* linkage in the polysaccharide.

TABLE I

METHYLATION ANALYSIS OF *Klebsiella* K69 POLYSACCHARIDE, **P1**, AND **P2**

Methylated sugars[a] *(as alditol acetates)*	T[b] *(OV-225)*	*Molar ratios*[c]		
		I	*II*	*III*
1,2,3,5,6-Man	0.54	0.57	0.92	
2,3,4,6-Glc	1.00	0.82	1.30	
2,3,4,6-Gal	1.11	0.18		
2,3,6-Man	1.62		1.28	1.00
2,3,6-Glc	1.81		1.12	0.99
2,3,4-Glc	1.88	0.86	1.68	0.56
2,3-Gal	3.24	0.75	1.92	1.02
2-Man	3.90	1.00	2.00	1.00

[a]2,3,6-Glc = 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, *etc.* [b]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on DB-225 (J + W fused-silica capillary column, 0.25-μm film thickness, 30 m × 0.25 mm), isothermal at 205°, except where otherwise stated. [c]Molar ratios: I, reduced, methylated, carboxyl-reduced **P1**; II, reduced, methylated, carboxyl-reduced **P2**; III, methylated, carboxyl-reduced polysaccharide.

The numerical data for the ^{1}H- and ^{13}C-n.m.r. spectra of **P1**, deacetylated **P1**, deacetylated, depyruvalated **P1**, and **P1**-alditol are collected in Table II, and a partial ^{1}H-n.m.r. spectrum of **P1** is shown in Fig. 1. The assignment of the ^{1}H-n.m.r. signals for **P1** and derived oligosaccharides was facilitated by comparison with the assignments established for the oligosaccharides isolated from a partial hydrolysate of K69 polysaccharide[12]. The ^{13}C assignments for the various oligosaccharides were confirmed by a 2D ^{1}H–^{13}C correlation experiment (not shown) on deacetylated, depyruvalated **P1**.

The ^{1}H-n.m.r. data for **P1** demonstrated that approximately one-third of the molecules were acetylated. The proton signals of **P1** most affected by the partial acetylation were the fractional signals (due to the α/β mutarotational equilibrium) representing H-1 of the reducing mannose and the H-1 signal of the β-mannopyranose residue. These signals exhibited uncharacteristic twinning. The ratios of the twinned signals were approximately the same as the ratio of acetylated to unacetylated molecules in **P1**. The above data suggest that acetate is located on one of the mannopyranosyl residues in **P1**. The ^{1}H-n.m.r. spectrum of deacetylated **P1** contained, as expected, only fractional signals (due to the mutarotational equilibrium) for the terminal, reducing mannopyranose residues.

The ^{13}C-n.m.r. spectrum of deacetylated **P1** contained signals at 61.38 and 61.93 p.p.m., representing C-6 of the reducing mannose and β-glucopyranosyl residues, respectively. On the other hand, the spectrum of **P1** contained a signal at 61.89 p.p.m. (C-6 of the β-D-glucopyranosyl residue) and two partial signals at 61.33 (C-6 of reducing mannose) and 63.95 p.p.m. The last signal, which was absent from the ^{13}C-n.m.r. spectrum of deacetylated **P1**, is indicative of the presence of acetate on C-6 of the reducing mannose residue. The twinning of the anomeric carbon

TABLE II

N.M.R. DATA FOR PRODUCTS OF BACTERIOPHAGE DEGRADATION

Compound[a]	*^{1}H-N.m.r. data (500 MHz)*				*^{13}C-N.m.r. data (125 MHz)*	
	δ[b] *(p.p.m.)*	*$J_{1,2}$ (Hz)*	*Integral (No. of H)*	*Assignment*[c]	*(p.p.m.)*[d]	*Assignment*[e]
P1	5.23	3.5	1	α-GlcA	103.25	β-Glc
	5.19	1.5	0.40	4-α-Man	103.06	β-Gal
	5.18	1.5	0.20	4-α-Man(6OAc)		4 6 \ / Pyr
	4.92	1.5	0.13	4-β-Man(6OAc)	102.30	α-GlcA
	4.91	1.5	0.27	4-β-Man	101.16	3,4,6-β-Man-(1→4)-α,β-Man(6OAc)
	4.79	n.o.[f]	0.67	3,4,6-β-Man-(1→4)-α,β-Man	100.94	3,4,6-β-Man-(1→4)-α,β-Man
	4.74	n.o.	0.33	3,4,6-β-Man-(1→4)-α,β-Man(6OAc)	95.73 94.57	4-α,β-Man
	4.60	8.0	1	β-Gal 4 6 \ / Pyr	94.44	4-α,β-Man(6OAc)
	4.51	7.5	1	β-Glc	63.95	C-6 of 4-α,β-Man(6OAc)
	2.15	s[g]	1	Me of acetate	61.89	C-6 of β-Glc
	1.56	s	3	Me of pyruvate	61.33	C-6 of 4-α,β-Man
					25.79	Me of pyruvate
					21.03	Me of acetate
Deacetylated **P1**	5.23	3.5	1	α-GlcA	103.32	β-Glc
	5.19	1.5	0.6	4-α-Man	103.11	β-Gal
	4.91	1.5	0.4	4-β-Man		4 6 \ / Pyr
	4.79	n.o.	0.6	3,4,6-β-Man-(1→4)-α-Man	102.34	α-GlcA
	4.78	n.o.	0.4	3,4,6-β-Man-(1→4)-β-Man	100.99	3,4,6-β-Man
	4.59	8	1	β-Gal 4 6 \ / Pyr	94.62	4-α-Man

	4.52	8	1	β-Glc	94.49	4-β-Man
	1.59	s	3	Me of pyruvate	61.93	C-6 of β-Glc
					61.38	C-6 of 4-α,β-Man
					25.78	Me of pyruvate
P1-Alditol	5.23	4	1	α-GlcA	103.54	β-Glc
	4.85	n.o.	1	3,4,6-β-Man	103.23	β-Gal
	4.57	8	1	β-Gal		4 6
				4 6		\ /
				\ /		Pyr
				Pyr	103.28	α-GlcA
	4.50	7.5	1	β-Glc	100.78	3,4,6-β-Man
	1.59	s	3	Me of pyruvate	25.79	Me of pyruvate
Deacetylated	5.18	n.o.	1	α-GlcA	103.84	β-Glc
depyruvalated	5.18	n.o.	0.6	4-α-Man	103.13	β-Gal
P1	4.90	n.o.	0.4	4-β-Man	102.41	α-GlcA
	4.77	n.o.	0.6	3,4,6-β-Man-(1→4)-α-Man	100.98	3,4,6-β-Man
	4.76	n.o.	0.4	3,4,6-β-Man-(1→4)-β-Man	94.62	4-α-Man
	4.58	8	1	β-Gal	94.49	4-β-Man
	4.47	8	1	β-Glc	61.94	C-6 of β-Glc and β-Gal
					61.32	C-6 of 4-α,β-Man

[a]For sources of **P1** *etc.*, see text. [b]Chemical shift in p.p.m. relative to acetone δ 2.23 downfield from DSS. [c]4-α-GlcA refers to H-1 of a 4-linked α-glucosyluronic acid residue in the α-anomeric configuration. [d]Chemical shift in p.p.m. relative to acetone 31.07 p.p.m. downfield from DSS. [e]As for *c*, but for anomeric ^{13}C-nuclei. [f]Not observed. [g]Singlet.

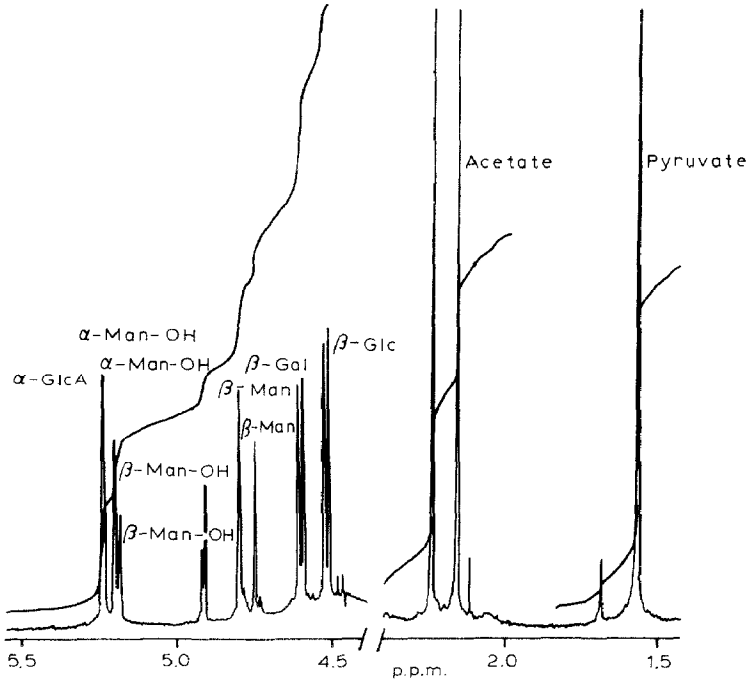


Fig. 1. Partial 500-MHz ^{1}H-n.m.r. spectrum of **P1**.

signals for the β-mannopyranosyl and reducing mannose residues in **P1**, which disappeared on deacetylation, is consistent with the ^{1}H-n.m.r. data for **P1** and deacetylated **P1**. The signal for the methyl carbon of the 1-carboxyethylidene group in **P1** occurred at 25.79 p.p.m. and the corresponding proton signal at δ 1.56, thus confirming the *R* configuration[16] for this group.

Confirmation of the amount of *O*-acetylation, the location of the *O*-acetylated residue, and the sequence of **P1** was obtained from fast-atom-bombardment (f.a.b.) studies. The negative ion f.a.b.-mass spectrum of **P1** (Fig. 2) contained molecular ion signals at *m/z* 911 and 953 corresponding to the compositions $Hex_4HexU_1Pyr_1$ and $Hex_4HexU_1Pyr_1Ac_1$, respectively. The relative intensities of these signals may not reflect relative concentrations since it is known that acylation will facilitate ionisation/desorption of the oligosaccharides. It was therefore necessary to prepare the deuterioacetyl derivative[11] of **P1**. The positive ion f.a.b.-mass spectrum of deuterioacetylated **P1** (Fig. 3) contained signals for molecular ions ($[M + NH_4]^+$) at *m/z* 1560 and 1557 corresponding to the compositions $Hex_4HexU_1Pyr_1$ and $Hex_4HexU_1Pyr_1Ac_1$, respectively. Each of these signals was accompanied by its oxonium ion resulting from loss of the C-1-deuterioacetyl function (*m/z* 1480 and 1477). The relative intensities of *m/z* 1560 and 1557 and of 1480 and 1477 indicate that **P1** contains approximately 33% of natural *O*-acetylation. Loss of the reducing hexosyl residue *via* glycosidic cleavage produces the A_1-type[15]

fragment ions at *m/z* 1183 and 1180. The fact that the signal at *m/z* 1180 is very much less intense with respect to *m/z* 1183 than either *m/z* 1477 with respect to *m/z* 1480, or *m/z* 1557 with respect to *m/z* 1560, indicates that the majority of natural *O*-acetylation is located on the reducing terminal hexose of **P1**. Data which are in potential conflict with this conclusion were observed in the low-mass region of the spectrum where the pair of signals at *m/z* 343 and 340 can be attributed to A_1-type ions for non-reducing Hex and HexAc, respectively. Alternatively, *m/z* 340 could be due to the A_1-type ion for the NH_4^+ salt of HexPyr. The latter was found to be correct when Na^+ dosing experiments were performed. Thus, when sodium acetate was added to the matrix and spectra of the deuterioacetylated samples were obtained, the signal at *m/z* 340 was almost totally absent (Fig. 4). Also, there was no signal at *m/z* 323 (HexPyr), indicating that the introduction of sodium into the sample had altered the fragmentation pattern such that pyruvylated hexose did not form the A_1-type ion. Since the signal at *m/z* 343 (Hex) is still observed in the absence of the signal at *m/z* 340, this is evidence that there was no natural *O*-acetylation on the non-reducing terminal hexose.

Additional A_1-type fragment ions were present at *m/z* 323 (HexPyr) and *m/z* 872 (OH_1Hex_3Pyr arising from the loss of non-reducing HexU from $HexUHex_3Pyr$ by β-cleavage). Again, the absence of a significant signal at *m/z* 869 is further evidence for the natural acetyl group being located on the reducing residue of **P1**.

Preliminary f.a.b.-m.s. analysis of **P2** indicates the presence of some di-acetylated molecules, suggesting that not all of the *O*-acetyl groups are distributed regularly on every third repeating-unit.

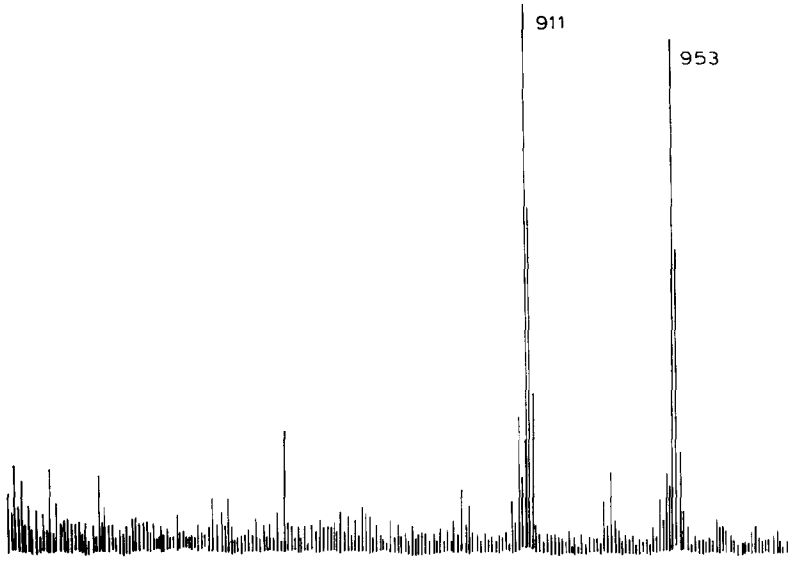


Fig. 2. Molecular ion region of the negative ion f.a.b.-mass spectrum of **P1**.

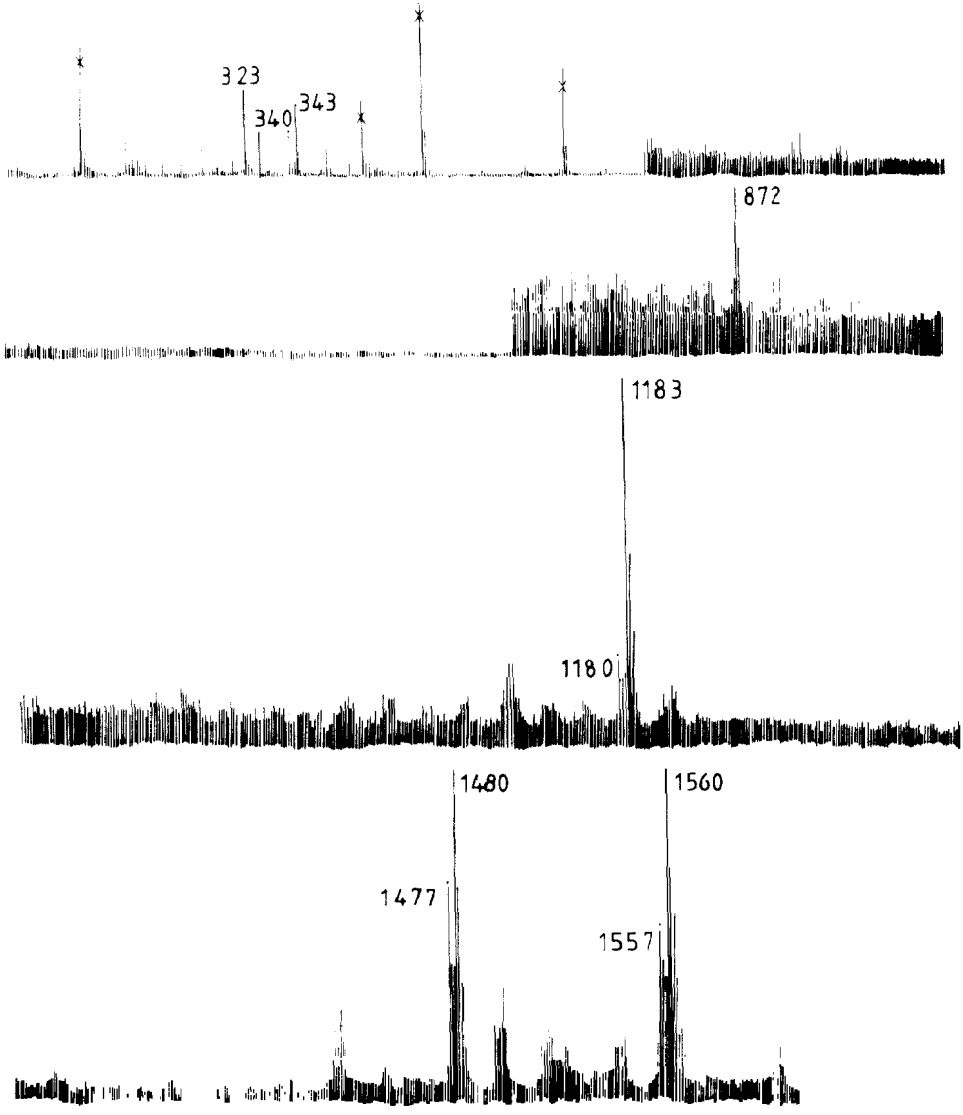


Fig. 3. Positive ion f.a.b.-mass spectrum of deuterioacetylated **P1**; the signals marked with a cross are derived from the matrix.

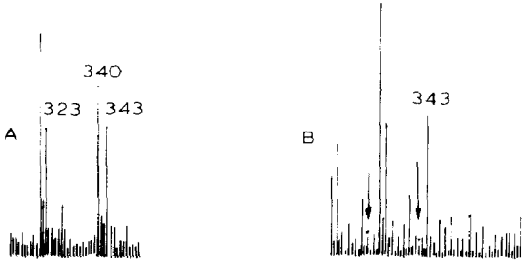


Fig. 4. Low-mass end of the f.a.b.-mass spectrum of deuterioacetylated **P1** before (A) and after (B) the addition of sodium acetate. Arrows in B indicate where the signals at *m/z* 323 and 340 would be.

The repeating unit of the capsular polysaccharide of *Klebsiella* K69 is very similar to the structure of *Klebsiella* K30 polysaccharide[17] which also has only one-third of the repeating units acetylated. The two polysaccharides differ only in the location of the 1-carboxyethylidene group on the galactose residue.

EXPERIMENTAL

The analytical methods used have been described previously[12]. F.a.b.-m.s. spectra were recorded with a VG Analytical High Field ZAB-1F mass spectrometer as described previously[18].

Isolation and purification of bacteriophage Φ69. — Bacteriophage Φ69 was isolated from sewage, and purified by successive replating of single plaques on *Klebsiella* K69 bacteria. The phage count was increased by successive test-tube and small-flask lyses as described previously[19]. The phage suspension (600 mL, 10^{10} PFU per mL) was dialysed, and concentrated in a rotary evaporator at 35° to afford 150 mL of a suspension which contained $\sim 4 \times 10^{12}$ PFU.

Depolymerisation of polysaccharide K69. — A solution of the polysaccharide (500 mg) in water (100 mL) was added to Φ69 suspension (150 mL) and the mixture was stirred at 30°, in the presence of chloroform (5 mL) to discourage bacterial growth. After 72 h, the mixture was freeze-dried, and a solution of the residue in water (50 mL) was dialysed (6-8000 M_w cut-off) against distilled water (8 × 100 mL). The diffusates were freeze-dried (260 mg), a solution of the residue in water (10 mL) was treated with Amberlite IR-120 (H^+) resin at 4°, and the mixture was separated on a column (2.6 × 50 cm) of Bio-Gel P-4 with pyridine–acetic acid–water (5:2:500). The retentate was treated similarly and like material was combined to afford **P1** (55 mg) and **P2** (103 mg).

Analysis of **P1** *and* **P2**. — **P1** had $[\alpha]_D$ +11° (water) and **P2** had $[\alpha]_D$ −8° (water). **P1** (7 mg) and **P2** (8 mg) were reduced with sodium borohydride, and each product was treated with boiling methanolic 3% hydrogen chloride (16 h). One portion of the product was hydrolysed with 2M trifluoroacetic acid, and the products were converted into the acetylated aldononitriles[14] and analysed by g.l.c. The other portion was first reduced with sodium borohydride, and then treated as above.

P1 and **P2** (6 mg of each) were reduced (NaB^2H_4 in D_2O), methylated[20], methanolysed, reduced, hydrolysed with 2M trifluoroacetic acid, reduced, acetylated, and examined by g.l.c. (Table II).

Deacetylated **P1**. — **P1** (15 mg) was treated with 10mM KOH under nitrogen at room temperature for 5 h, as described previously[21], to afford deacetylated **P1** (10 mg).

ACKNOWLEDGMENTS

Financial support from the M.R.C. (to A.D.), the FRD (to H.P. and P.L.H.), and the S.E.R.C. (to P.R.T.) is gratefully acknowledged. We thank Mr.

I. Antonovitz of the National Chemical Research Laboratory (Pretoria) for recording the n.m.r. spectra.

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