Carbohydrate Research, 126 (1984) 261–269
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PREPARATION OF A BRANCHED HEPTASACCHARIDE BY BACTERIOPHAGE DEPOLYMERIZATION OF *Klebsiella* K60 CAPSULAR POLYSACCHARIDE

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(Received August 6th, 1983; accepted for publication, August 26th, 1983)

ABSTRACT

Bacteriophage ϕ60 possesses an endoglucosidase that depolymerizes the capsular polysaccharide of *Klebsiella* K60 into a heptasaccharide having two single β-D-glucopyranosyl side-chains. This bulky oligosaccharide may be used as a probe to examine the combining sites of immunoglobulins. The ^{1}H- and ^{13}C-n.m.r.-spectral data of the oligosaccharide are reported.

INTRODUCTION

Oligosaccharides of known structure and of different structural patterns are required for many types of investigation ranging from studies of conformations in solution[1] to the binding properties of immunoglobulins[2]. The realization that bacterial polysaccharides have regular structures based on the concept of a repeating unit[3], and that endoglycanases, capable of producing oligosaccharides in high yield, are present in bacteriophages[4] has greatly improved the selection of oligosaccharides available.

Despite the time that has elapsed since the pioneering work of Kabat[5] on the binding properties of immunoglobulins, there is still a paucity of data on the behavior of bulky oligosaccharides[6,7]. It is in the context of such investigations that the present paper reports on the preparation and n.m.r. spectroscopy of the heptasaccharide obtained from the capsular polysaccharide[8] of *Klebsiella* serotype K60 by a phage-borne endoglucosidase[4]. Many of the *Klebsiella* capsular polysaccharides have a single side-chain[4] per repeating unit, and give linear oligosaccharides on depolymerization[9]. The structure of the polysaccharide from *Klebsiella* K60 is unique in the series in having three side-chains per repeating unit and yielding a heptasaccharide with two branches. This oligosaccharide thus represents a bulky probe with which to study reactions of immunoglobulins.

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0008-6215/84/$ 03.00

RESULTS

Klebsiella ϕ60 was isolated from sewage, purified, and propagated on its host strain in nutrient broth until 10^{13} plaque-forming units (P.F.U.) were obtained, sufficient to depolymerize 1 g of polysaccharide[10,11]. Depolymerization was conducted at 37° for 2 d, when dialysis gave a mixture of oligosaccharides from which **P1** and **P2** were obtained by gel filtration. Analysis[12] showed **P1** to be a heptasaccharide corresponding to the repeating unit, and **P2** to be the dimer thereof. Some higher oligomers were obtained in a fraction, designated **P3**, that was not further examined.

The results of the analysis and measurement of d.p. by the method of Morrison[12] are presented in Table I, and confirm that **P1** is a heptasaccharide, **P2** is the dimer, and both have a glucose residue as the terminal, reducing unit. Comparison of the methylation data for **P1** with those for the original polysaccharide shows that 2-*O*-methylglucose (from glucuronic acid) is replaced in **P1** by 2,3-di-*O*-methylglu-

TABLE I

DETERMINATION OF THE DEGREE OF POLYMERIZATION AND THE REDUCING END OF OLIGOSACCHARIDES **P1** AND **P2** FROM *Klebsiella* K60

Peracetylated derivative of	R_T[a]	*Mole%*	
		P1	**P2**
Mannononitrile	**1.00**	15	15
Glucononitrile	1.27	56	64
Galactononitrile	1.38	14	14
Glucitol	1.60	15	7

[a]Determined on a column of OV-225 at 210°.

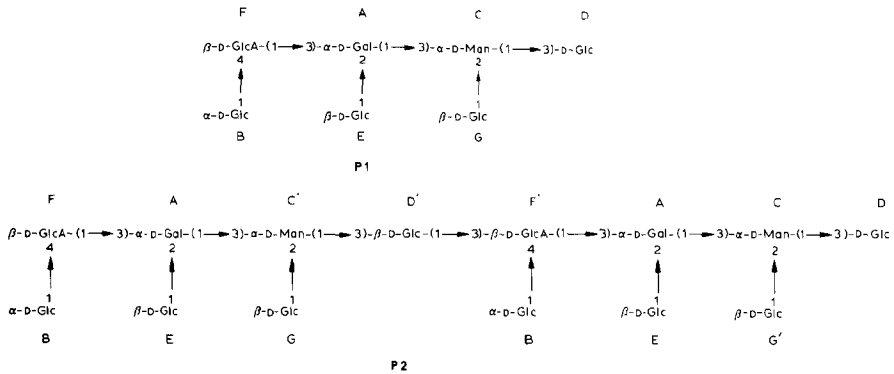
TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDES **P1** AND **P2** FROM BACTERIOPHAGE DEGRADATION OF *Klebsiella* K60

Partially methylated alditol acetates[a]	R_T[b]	*Mole %*[c]	
		P1	**P2**
2,3,4,6-Glc	**1.00**	46.7	44.4
2,4,6-Glc	1.83	10.0	13.3
4,6-Man	2.96	12.9	14.0
4,6-Gal	3.26	15.2	15.1
2,3-Glc	4.63	15.3	7.4
2-Glc	7.61	—	5.7

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, etc. [b]Determined on a column of OV-225 at 170°. [c]Values were corrected by use of carbon-response factors given by Albersheim *et al.*[13].

cose. The same result was obtained by comparing the methylation data for **P1** and **P2** (see Table II), and indicated that the reducing glucopyranose residue of one repeating unit is joined to O-3 of the glucuronic acid of the second unit. These results, together with the original investigation of the K60 polysaccharide, enable the structures of **P1** and **P2** to be written as follows.



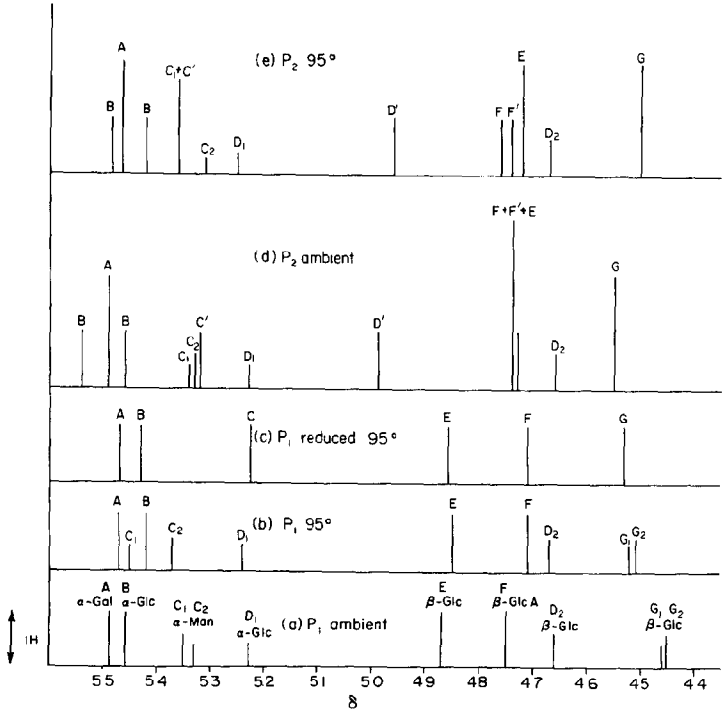


Fig. 1. N.m.r. spectra (400 MHz) of **P1** and **P2**. [(a) **P1** (H^+ form) at ambient temperature, (b) **P1** at 95°, (c) **P1**-alditol at 95°, (d) **P2** (H^+ form) at ambient temperature, and (e) **P2** at 95°.]

TABLE III

^{1}H-N.M.R. DATA (400 MHz) FOR **P1** AND **P2** ISOLATED FROM *Klebsiella* K60

Compound[a]	*Δ*[b]		$J_{1,2}$ *(Hz)*	*Integral*		*Assignment*[c]	
	Ambient	*95°*		*Ambient*	*95°*		
P1	5.49	5.47	—[d]	1	1	2,3-Gal$_{\alpha}$—3-Man	A
	5.46	5.42	3	1	1	Glc$_{\alpha}$—4-GlcA	B
	5.35	5.37	—	0.6	0.55	2,3-Man$_{\alpha}$—Glc$_{\beta}$—OH	C_1
	5.33	5.45	—	0.4	0.45	2,3-Man$_{\alpha}$—Glc$_{\alpha}$—OH	C_2
	5.23	5.24	3	0.4	0.45	3-Glc$_{\alpha}$—OH	D_1
	4.87	4.85	8	1	1	Glc$_{\beta}$—2-Gal	E
	4.75	4.72	8	1	1	4-GlcA$_{\beta}$—3-Gal	F
	4.66	4.67	8	0.6	0.55	3-Glc$_{\beta}$—OH	D_2
	4.46	4.52	8	0.4	0.45	Glc$_{\beta}$—2-Man	G_1
	4.45	4.51	8	0.6	0.55		G_2
P1 reduced		5.47	—		1	2,3-Gal$_{\alpha}$—3-Man	A
		5.43	3		1	Glc$_{\alpha}$—4-GlcA	B
		5.22	—		1	2,3-Man$_{\alpha}$—3-glucitol	C
		4.86	8		1	Glc$_{\beta}$—2-Gal	E
		4.71	8		1	4-GlcA$_{\beta}$—3-Gal	F
		4.53	8		1	Glc$_{\beta}$—2-Man	G
P2	5.54	5.48	3	1	1	Glc$_{\alpha}$—4-GlcA	B[e]
	5.49	5.46	—	2	2	2,3-Gal$_{\alpha}$—3-Man	A
	5.46	5.42	4	1	1	Glc$_{\alpha}$—3,4-GlcA	B[e]
	5.32		—	1		2,3-Man$_{\alpha}$—3-Glc$_{\beta}$—	C′
		5.36			1.7		
	5.33		—	0.6		2,3-Man$_{\alpha}$—3-Glc$_{\beta}$—OH	C_1
	5.34	5.31	—	0.4	0.3	2,3-Man$_{\alpha}$—3-Glc$_{\alpha}$—OH	C_2
	5.23	5.25	3	0.4	0.4	3-Glc$_{\alpha}$—OH	D_1
	4.99	4.96	8	1	1	3-Glc$_{\beta}$—3-GlcA$_{\beta}$—	D′
	4.74	4.76	8	3	1	4-GlcA$_{\beta}$—3-Gal	F
	4.73	4.74	8	1	1	3,4-GlcA$_{\beta}$—3-Gal	F′
		4.72	8		2	Glc$_{\beta}$—2-Gal × 2	E
	4.66	4.67	8	0.6	0.6	3-Glc$_{\beta}$—OH	D_2
	4.55	4.50	8	2	2	Glc$_{\beta}$—2-Man	G

N.m.r. spectra. — The numerical data for the ^{1}H- and ^{13}C-n.m.r. spectra of **P1** and **P2** are collected in Tables III and IV, and the proton data are displayed diagrammatically in Figs. 1a–e. The proton data are the more informative, and are discussed next.

Fig. 1a presents the ^{1}H-n.m.r. spectrum of **P1** recorded at ambient temperature. Peaks D_1 and D_2 correspond to the α/β equilibrium of the reducing glucopyranose residue. Their ratio and chemical shifts are little influenced by raising the temperature (see Fig. 1b), but both signals disappear on reduction (see Fig. 1c). The peaks C_1 and C_2 shown in Fig. 1a are attributed to an α-D-mannopyranosyl residue which, as the peaks are twinned and in the same ratio as D_1 and D_2, is attached to the reducing-end residue[14,15]. The magnitude of the downfield shift of these signals (C_1 and C_2) when the spectrum is recorded at 95° is noteworthy. On reduction, only a single signal is obtained; see Fig. 1c. The twinning of C_1 and C_2 and their negligible coupling constants now permit the unambiguous assignment of these signals to the 3-substituted mannosyl residue. The other signal in Fig. 1a that exhibits twinning is that labelled G (G_1 and G_2). From the original[8], structural proof of the K60 polysaccharide, it is known that the single mannose residue carries a β-D-glucopyranosyl unit. Because this is attached at O-2, it is reasonable that the anomeric signal should be influenced indirectly by the α/β equilibrium of the reducing terminus. In support of this hypothesis, G becomes a single signal when **P1** is reduced (see Fig. 1c). The magnitude of the shifts of C_1 and C_2 on reduction, compared with those of G_1 and G_2, is compelling evidence that the mannosyl unit is more affected by the mutarotational equilibrium than the glucosyl units, which is consistent with placing the mannosyl unit adjacent to the reducing end.

It should be noted that ^{1}H-n.m.r. spectra of reduced oligosaccharides are often easier to interpret that those of reducing oligomers, partly on account of the obvious reason that perturbations caused by mutarotational equilibria are eliminated, but also because the noise level of such reduced samples often appears to be much lower. It is also true that the chemical shifts of anomeric protons in reduced oligosaccharides exhibit less temperature-dependence. This is important if it is wished to utilize chemical shifts as a diagnostic tool to indicate the position of substitution of a sugar unit.

The most striking feature of the spectrum of **P2**, recorded at ambient temperature (see Fig. 1d), in comparison with that of **P1**, is the signal at δ 4.99. On the basis of chemical shift alone, definitive assignment as α or β would be inadvisable,

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[a]For source of **P1** and **P2**, see text. [b]Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [c]The numerical prefix indicates the position in which the sugar is substituted; the α or β, the configuration of the glycosidic bond, or the anomer in the case of a (terminal) reducing sugar residue. Thus, 3-Gal$\underset{\alpha}{\text{——}}$ refers to the anomeric proton of a 3-linked galactosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. [d]Coupling constant too small to be measured. [e]These two assignments may have to be interchanged.

TABLE IV

^{13}C-N.M.R. DATA FOR **P1** AND **P2** ISOLATED FROM *Klebsiella* K60.

Compound[a]	*Chemical shift*[b] *(p.p.m.)*	*Assignment*
P1	104.20	4-GlcA-β-3-Gal Glc-β-
	102.31	Glc-β-
	100.54	Glc-α-
	99.67	2,3-Man-α-
	99.23	2,3-Gal-α-
	99.74	3-Glc-β-
	93.03	3-Glc-α-
P2	104.34	4-GlcA-β- 3,4-GlcA-β- Glc-β- × 2
	103.04	3-Glc-β-
	102.38	Glc-β- × 2
	100.75	Glc-α-3,4-GlcA
	100.59	Glc-α-4-GlcA
	99.68	2,3-Man-α- × 2
	99.25	2,3-Gal-α- × 2
	96.70	3-Glc-β-OH
	93.02	3-Glc-α-OH

[a]For source of **P1** and **P2**, see text. [b]Chemical shift, in p.p.m., downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [c]As in *c*, Table III, but for ^{13}C nuclei.

but the $J_{1,2}$ value (8 Hz) clearly shows that, in **P2** (and, therefore, in the polymer), the in-chain glucopyranosyl unit is β-linked.

In **P2**, the two α-D-glucopyranosyl units are nonequivalent; one is terminal, and the other, lateral. The signals at δ 5.54 and 5.46 may, thus, be assigned to these residues, and that at δ 5.49 to the two (equivalent) α-D-galactopyranosyl units.

Of the two α-D-mannopyranosyl residues, one is adjacent to the reducing end of **P2**, whereas the other is far removed. Accordingly, these units are represented (Fig. 1d) by one signal of unit intensity (at δ 5.32) and a pair of twinned signals, at δ 5.33 (0.6 Hz) and 5.34 (0.4 Hz). These signals show a significant, downfield shift when the spectrum is recorded at 95° (see Fig. 1e).

No evidence was observed for the twinning of signal G in spectra of **P2** obtained either at ambient or elevated temperatures. This suggests a further advantage of deducing as much structural information as possible from the oligomer representing the single repeating-unit, rather than from the polysaccharide.

CONCLUSIONS

The heptasaccharide **P1** is required for other studies, and its preparation by bacteriophage degradation of the capsular polysaccharide from *Klebsiella* K60 was the motivation for the results described here. In the course of this work, the ^{1}H-n.m.r. spectra of **P1** and **P2** were re-examined, and certain fine details, not previously observed in the spectra of the polysaccharide[8], were noticed. Such details, although less striking than when 1-carboxyethylidene groups are present[15], are, nevertheless, useful in sequencing oligosaccharides.

An experimental difficulty, encountered when working with oligo- or polysaccharides, is the presence of a residual HOD peak even after several exchanges. This peak may be eliminated by a water-null experiment, but such a technique is likely to perturb anomeric signals in the same region of the spectrum, and thus give misleading information. In the present study, the possibility of using relatively concentrated solutions and displacing the HOD signal upfield by operating at 95° gave spectra of a quality higher than in the original structural investigation[8]. This permitted certain signals to be reassigned (with greater assurance than earlier[8]); these new assignments are given in Table III. It must be emphasized that these minor changes in no way alter the anomeric configurations originally attributed to the individual monosaccharide units in the polymer but, in fact, confirm the assignments made previously[8]. No changes to the ^{13}C-n.m.r. data were needed.

EXPERIMENTAL

General method. — The instrumentation used has been described[15]. Bacteriophage φ60 was isolated from sewage, purified by repicking single plaques, and propagated on its host strain, *Klebsiella* K60, in nutrient broth[10,11]. A solution of bacteriophage containing 10^{13} PFU in 400 mL of broth was concentrated to one third its volume, dialyzed for 2 d against running tap-water, and concentrated to 150 mL. This solution was added to the polysaccharide (1 g in 150 mL of water), and depolymerization was conducted for 30 h at 37° in the presence of chloroform (3 mL).

Isolation and purification of oligosaccharides. — After 30 h, the foregoing

solution was concentrated to 50 mL, and dialyzed against distilled water (3 × 1 L). The dialyzates were combined, evaporated to dryness, and dissolved in water (50 mL). Amberlite IR-120 (H^+) resin was added, and the solution was stirred for 20 min, and then passed through a column of the same resin. The treatment was repeated until a colorless solution was obtained which, on freeze-drying, yielded 760 mg of oligosaccharides. A portion (500 mg) was separated on a column (80 × 2.5 cm) of Bio-Gel P-4, using as the eluant 500:5:2 water–pyridine–acetic acid. Three fractions were collected: **P3** (5–10 mL), **P2** (11–16 mL; 145 mg), and **P1** (25–32 mL; 300 mg).

Analysis of the oligosaccharides. — **P1** had $[\alpha]_D^{23}$ +61° (*c* 0.7, water; calc.[16] +62°) and **P2** had $[\alpha]_D^{23}$ 45° (*c* 0.6 water; calc.[16] +56°). A sample (10 mg) of each oligosaccharide was dissolved in water (5 mL), and reduced with sodium borohydride (15 mg) for 2 h. The reduced oligosaccharides recovered were refluxed overnight with methanolic hydrogen chloride (3%) and, after neutralization (Ag_2CO_3), the uronic esters were reduced with sodium borohydride (15 mg) in anhydrous methanol (5 mL). The neutral oligosaccharides were then hydrolyzed (TFA), and the products converted into peracetylated aldononitriles (PAAN)[15,17]. The results are presented in Table I.

Samples (10 mg each) of **P1** and **P2** were separately methylated by the method of Hakomori[18], and the uronic esters were reduced with lithium aluminum hydride prior to hydrolysis. The analytical results are shown in Table II.

ACKNOWLEDGMENTS

We thank Dr. I. Orskøv for a culture of *Klebsiella* K60, and Professor S. Stirm for the bacteriophage ϕ60. We are grateful to N.S.E.R.C. (Ottawa) for continued financial support, to CSIR (Pretoria) for an overseas allowance (to H.P.), and for the award of a MacMillan Bloedel Graduate Scholarship and a University Graduate Fellowship (to J.L.D.F.).

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