Carbohydrate Research, 144 (1986) 251–261
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

NOVEL OLIGOSACCHARIDES OBTAINED BY BACTERIOPHAGE DEGRADATION OF THE POLYSACCHARIDE FROM *Klebsiella* SEROTYPE K26

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(Received December 17th, 1984; accepted for publication, March 29th, 1985)

ABSTRACT

Bacteriophage ϕ26 was used to depolymerize the polysaccharide of *Klebsiella* K26, yielding three oligosaccharides. The major product was the heptasaccharide repeating unit, with one of the minor products being the fourteen-sugar oligosaccharide corresponding to two repeating units. The other minor product was unusual since it was a hexasaccharide devoid of the terminal, pyruvate-containing galactose unit present in the side chain of the normal repeating unit. Phage ϕ26 was shown to act as a β-galactosidase, and hence it may have the ability to remove the terminal β-galactose residue in the side chain.

INTRODUCTION

A bacteriophage competent for infection often exhibits the capability for enzymatic attack on the capsular exopolysaccharide surrounding its host bacterial cell. The need to gain access to the interior of the cell would seem to explain the fact that most polysaccharide-degrading enzymes from bacteriophages act as depolymerases, although instances are known of debranching activities, which remove only side chains, leaving the capsule intact[1].

Oligosaccharides containing 1-carboxyethylidene substituents (pyruvate acetals) can only be obtained by the enzymatic depolymerization of polysaccharides. As a continuation of our studies on oligosaccharide production by bacteriophage action, we now report on the nature of the products obtained by the depolymerization of the capsular polysaccharide of *Klebsiella* K26.

RESULTS AND DISCUSSION

Klebsiella K26 was depolymerized using phage ϕ26, which was isolated from sewage and propagated on P-medium[2] instead of nutrient broth[3]. Dialysis of the generated oligomers and gel filtration of the dialyzate yielded **P1a** (8.2%), **P1** (54.1%), and **P2** (11.9%), where **P1a** was a hexasaccharide, **P1** the heptasaccharide repeating unit, and **P2** the dimer of **P1**. No higher oligomers were observed.

0008-6215/85/$ 03.30

TABLE I

DETERMINATION OF THE DEGREE OF POLYMERIZATION AND THE REDUCING END OF OLIGOSACCHARIDES **P1a**, **P1**, AND **P2** FROM *Klebsiella* K26

Peracetylated derivative of	T^a	*Mole %*		
		P1a	**P1**	**P2**
Mannononitrile	1.00	32.6	30.3	31.4
Glucononitrile	1.05	44.3	31.9	35.3
Galactononitrile	1.09	—	17.4	23.2
Galactitol	1 41	23.1	20.2	10.0

[a]Retention time relative to peracetylated mannononitrile, determined on a column of DB17 programmed at 180° for 2 min, then increasing at 5°/min to 220°.

TABLE II

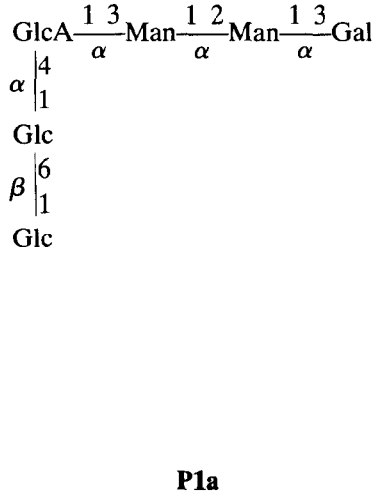
METHYLATION ANALYSES OF OLIGOSACCHARIDES **P1a**, **P1**, AND **P2**

Partially methylated alditol acetate of[a]	T^b	*Mole %*[c]					
		P1a		**P1**		**P2**	
		A[d]	*B*	*A*	*C*	*B*	*C*
1,2,4,5,6-Gal	0.76	—	—	—	8 8	—	5 3
2,3,4,6-Glc	1.00	25.8	20.4	—	—	—	—
3,4,6-Man	1.38	30.1	17.5	20.8	23.1	17.5	28.6
2,5,6-Gal	1.46	15.0	5.3	11.3	—	3.9	—
2,3,6-Glc / 2,3,4-Glc	1.51	21.8	36.3	43.2	48.8	46.1	45.7
2,4,6-Man	1.53						
2,4,6-Gal	1.58	7.3	2.9	4.0	—	10 1	10.4
2,3-Glc	2.13	—	17.3	—	—	6.6	—
2,3-Gal	2.18	—	—	20.7	19.3	9.6	19.6
3-Glc	2.73	—	—	—	—	6 2	—

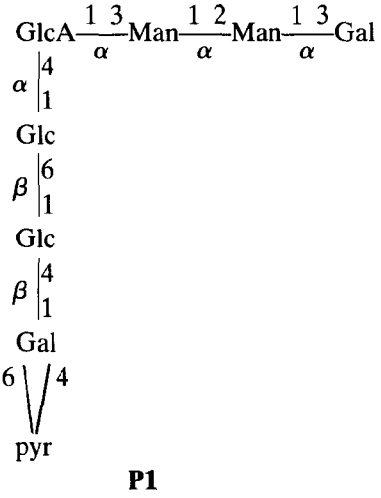
[a]1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, etc. [b]Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, determined on a column of DB17 programmed at 180° for 1 min, then increasing at 2°/min to 250°. [c]Values corrected using e.c.r. factors given by Albersheim *et al.*[13]. [d]A, Methylated oligosaccharides; B, methylated, $LiAlH_4$-reduced oligosaccharides, C, $NaBH_4$-reduced and methylated oligosaccharides.

Analysis of **P1a** and **P1** revealed that each had a galactose residue at the reducing end, the difference between the two being the absence of the terminal, pyruvate-containing galactose in **P1a** (see Table I). Oligosaccharide **P2** also contained a reducing galactose unit, thus confirming the presence of a β-galactosidase activity in ϕ26. Methylation analysis of **P1a** confirmed the absence of the terminal galactose unit, and showed the replacement of the 4-linked glucose by a terminal (tetra-*O*-methyl) glucose residue. Product **P1** contained the terminal galactose

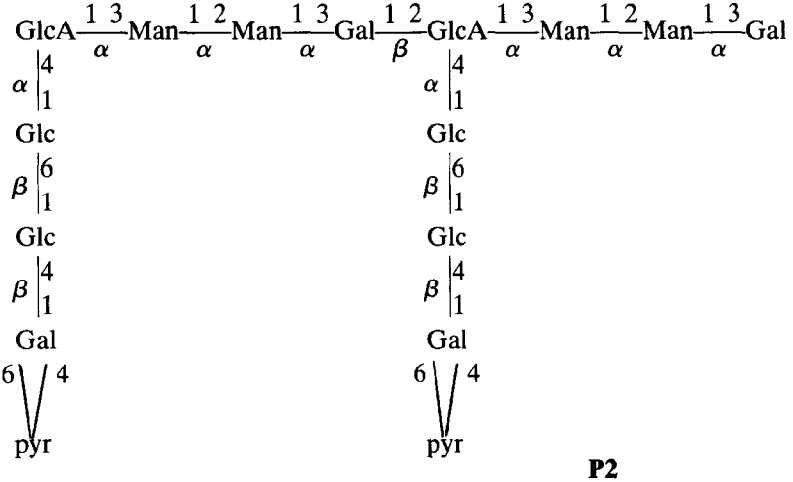
bearing the pyruvic acetal substituent at positions 4 and 6 (see Table II). Comparison of the methylation data for **P1a** with those for the original polysaccharide[4] showed the replacement of the 3-*O*-methylglucose (from glucuronic acid) by 2,3-di-*O*-methylglucose. Interestingly, the predominance of 2,5,6-tri-*O*-methylgalactose was noted, and attributed to the existence of 60% galactofuranose at the reducing end. In a similar comparison of the methylation data for **P2**, the reducing galactose residue of one repeat unit was shown to be joined to O-2 of the glucuronic acid of the second unit. Using the results from the original structure elucidation of the K26 polysaccharide[4] together with the present data, the structures of **P1a, P1**, and **P2** can be given as follows:



P1a



P1



P2

TABLE III

400 MHz ^{1}H-N M R DATA FOR OLIGOSACCHARIDES **P1a, P1**, AND **P2** FROM THE BACTERIOPHAGE DEGRADATION OF *Klebsiella* K26 POLYSACCHARIDE

Compound	*Chemical shift*[a] *(p.p.m. δ)*	$J_{1,2}$ *(Hz)*	*No. of protons*	*Assignment*[b]
P1a	5.43	s	1	$\xrightarrow{6}$Glc$_{\alpha}$
	5.33	s	1	$\xrightarrow{4}$GlcA$_{\alpha}$
	5.29	1	0.3	$\xrightarrow{3}$Gal*p*$_{\alpha}$OH, $\xrightarrow{3}$Gal*f*$_{\beta}$OH
	5.25	s	1	$\xrightarrow{3}$Man$_{\alpha}$
	5.16	6	0.25	$\xrightarrow{3}$Gal*f*$_{\alpha}$OH
	5.06	s	1	$\xrightarrow{2}$Man$_{\alpha}$
	4.62	8	0.4	$\xrightarrow{3}$Gal*p*$_{\beta}$OH
	4.48	8	1	Glc$_{\beta}$
	4.41	8	1	H-5 of $\xrightarrow{4}$GlcA$_{\alpha}$
P1	5.43	s	1	$\xrightarrow{6}$Glc$_{\alpha}$
	5.33	s	1	$\xrightarrow{4}$GlcA$_{\alpha}$
	5.30	bs	0.3	$\xrightarrow{3}$Gal*p*$_{\alpha}$OH, $\xrightarrow{3}$Gal*f*$_{\beta}$OH
	5.25	s	1	$\xrightarrow{3}$Man$_{\alpha}$
	5.17	6	0 25	$\xrightarrow{3}$Gal*f*$_{\alpha}$OH
	5.07	s	1	$\xrightarrow{2}$Man$_{\alpha}$
	4.63	8	0.4	$\xrightarrow{3}$Gal$_{\beta}$OH
	4.52	8	2	$\xrightarrow{4}$Glc$_{\beta}$, pyr⟨Gal$_{\beta}$
	4.42	8	1	H-5 of $\xrightarrow{4}$GlcA$_{\alpha}$
	1.57	s	3	CH_3 of pyr

TABLE III *(continued)*

Compound	*Chemical shift[a] (p.p.m. δ)*	$J_{1,2}$ *(Hz)*	*No. of protons*	*Assignment[b]*
P2	5.50	3	1	$\xrightarrow{2,4}$GlcA
	5.46	4	1	$\xrightarrow[\alpha]{6}$Glc
	5.43	4	1	$\xrightarrow[\alpha]{6}$Glc
	5.33	4	1	$\xrightarrow[\alpha]{4}$GlcA
	5.29	4	0.3	$\xrightarrow[\alpha]{3}$Gal*p*OH, $\xrightarrow[\beta]{3}$Gal*f*OH
	5.25	s	2	$\xrightarrow[\alpha]{3}$Man
	5.17	8	0.2	$\xrightarrow[\alpha]{3}$Gal*f*OH
	5.07	s	2	$\xrightarrow[\alpha]{2}$Man
	4.66	8	1	$\xrightarrow[\beta]{3}$Gal
	4.63	8	0.5	$\xrightarrow[\beta]{3}$Gal*p*OH
	4.52	8	4	$\xrightarrow[\beta]{4}$Glc, pyr$\langle\rangle$Gal$_{\beta}$
	4.45	8	1	H-5 of $\xrightarrow[\alpha]{2,4}$GlcA
	4.43	8	1	H-5 of $\xrightarrow[\alpha]{4}$GlcA
	1.58	s	6	CH_3 of pyr
alditol of **P1a**	5.49	4	1	$\xrightarrow[\alpha]{6}$Glc
	5.29	b	2	$\xrightarrow[\alpha]{4}$GlcA, $\xrightarrow[\alpha]{3}$Man
	5.10	s	1	$\xrightarrow[\alpha]{2}$Man
	4.53	8	1	Glc$_{\beta}$

TABLE III *(continued)*

Compound	*Chemical shift*[a] *(p.p.m. δ)*	$J_{1,2}$ *(Hz)*	*No. of protons*	*Assignment*[b]
alditol of **P1**	5.43	4	1	$\xrightarrow{6}$Glc$\xrightarrow[\alpha]{}$
	5.34	4	1	$\xrightarrow{4}$GlcA$\xrightarrow[\alpha]{}$
	5.24	s	1	$\xrightarrow{3}$Man$\xrightarrow[\alpha]{}$
	5.09	s	1	$\xrightarrow{2}$Man$\xrightarrow[\alpha]{}$
	4.53	8	1	$\xrightarrow{4}$Glc$\xrightarrow[\beta]{}$
	4.52	8	1	pyr⟨Gal$\xrightarrow[\beta]{}$
	4.45	8	1	H-5 of $\xrightarrow{4}$GlcA
alditol of **P2**	5.47	b	2	$\xrightarrow{6}$Glc$\xrightarrow[\alpha]{}$, $\xrightarrow{2,4}$GlcA$\xrightarrow[\alpha]{}$
	5.44	b	1	$\xrightarrow{6}$Glc$\xrightarrow[\alpha]{}$
	5.25	s	3	$\xrightarrow{4}$GlcA$\xrightarrow[\alpha]{}$, $\xrightarrow{3}$Man$\xrightarrow[\alpha]{}$
	5.07	s	1	$\xrightarrow{2}$Man$\xrightarrow[\alpha]{1\ 3}Gal\xrightarrow[\beta]{}$
	5.05	s	1	$\xrightarrow{2}$Man$\xrightarrow[\alpha]{1\ 3}$galactitol
	4.66	8	1	$\xrightarrow{3}$Gal$\xrightarrow[\beta]{}$
	4.53	8	2	$\xrightarrow{4}$Glc$\xrightarrow[\beta]{}$
	4.50	8	2	pyr⟨Gal$\xrightarrow[\beta]{}$

[a]Chemical shift downfield from Me_4Si: measured from internal acetone, $\delta = 2.23$ p.p.m. Spectra were recorded at 95 ±5°. [b]The numerical prefix indicates the position at 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 $\xrightarrow{6}$Glc$\xrightarrow[\alpha]{}$ refers to the anomeric proton of a 6-linked glucosyl residue in the α-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. The 1-carboxyethylidene group (from pyruvic acid) is indicated by pyr.

TABLE IV

100 MHz ^{13}C-N M R DATA FOR **P1a, P1**, AND **P2** ISOLATED FROM *Klebsiella* K26

Compound	*Chemical shift*[a] *(p.p.m. δ)*	*Assignment*[b]
P1a	173.78	C-6 of GlcA
	103.42	Glc-β
	102.95	$\rightarrow$3)-Man-α
	101.15	$\rightarrow$6)-Glc-α
	99.90	$\rightarrow$4)-GlcA-α
	97.18	$\rightarrow$3)-Gal-β-OH
	95.49	$\rightarrow$2)-Man-α-1,3)-Gal-β-OH
	95.22	$\rightarrow$2)-Man-α-1,3)-Gal-α-OH
	93.08	$\rightarrow$3)-Gal-α-OH
P1	175.05	C-1 of pyr
	174.12	C-6 of GlcA
	103.64	pyr$\langle\rangle$Gal-β
	103.26	$\rightarrow$4)-Glc-β
	102.99	$\rightarrow$3)-Man-α
	101.18	$\rightarrow$6)-Gal-α
	99.82	$\rightarrow$4)-GlcA-α
	97.20	$\rightarrow$3)-Gal-β-OH
	95.50	$\rightarrow$2)-Man-α-1,3)-Gal-β-OH
	95.22	$\rightarrow$2)-Man-α-1,3)-Gal-α-OH

TABLE IV *(continued)*

Compound	*Chemical shift*[a] *(p.p.m. δ)*	*Assignment*[b]
	93 10	$\xrightarrow[\alpha]{3}$Gal—OH
	25.85	CH_3 of pyr
P2	174.44	C-1 of pyr
	173.72	C-6 of GlcA
	105.14	$\xrightarrow[\beta]{3}$Gal—
	103.64	pyr⟨⟩Gal$\xrightarrow[\beta]{}$ (× 2)
	103.24	$\xrightarrow[\alpha]{3}$Man—, $\xrightarrow[\beta]{4}$Glc— (× 2)
	103.07	$\xrightarrow[\alpha]{3}$Man—
	102.97	$\xrightarrow[\alpha]{6}$Glc—
	101.19	$\xrightarrow[\alpha]{6}$Glc—
	100.69	$\xrightarrow[\alpha]{2,4}$GlcA—
	99.95	$\xrightarrow[\alpha]{4}$GlcA—
	97.18	$\xrightarrow[\beta]{3}$Gal—OH
	95.49	$\xrightarrow[\alpha]{2}$Man$\xrightarrow[\alpha]{1\ 3}Gal\xrightarrow[\beta]{1}$
	95.44	$\xrightarrow[\alpha]{2}$Man$\xrightarrow[\alpha]{1\ 3}Gal\xrightarrow[\beta]{}$OH
	95.16	$\xrightarrow[\alpha]{2}$Man$\xrightarrow[\alpha]{1\ 3}Gal\xrightarrow[\alpha]{}$OH
	93.08	$\xrightarrow[\alpha]{3}$Gal—OH
	25.77	CH_3 of pyr

[a]Chemical shift downfield from Me_4Si; measured from internal acetone, $\delta = 31.07$ p.p m. [b]As in Table III, but for ^{13}C nuclei.

N.m.r. data. — The anomeric signals in the ^{1}H- and ^{13}C-n.m.r. spectra of **P1a** indicated the presence of six sugar residues, while those of **P1** indicated seven (Tables III and IV). Assignment of these signals was made possible by comparison of the spectral data for **P1a, P1**, and **P2** with data for the original K26 polysaccharide. The presence of reducing galactofuranose was seen in the high temperature ^{1}H-n.m.r. spectra of each of **P1a, P1**, and **P2**, but furanose signals were not detectable in either the ^{13}C or the ^{1}H spectra recorded at ambient temperature. The signals for H-1 and H-5 of the 4-linked glucuronic acid at δ 5.33 and 4.42 shifted upfield to δ 5.25 and 4.17, respectively, on conversion of this residue to its sodium salt. The signal at δ 4.17 was observed only in the spectra run at ambient temperature, since it was overlapped by the HOD peak at high temperature. All signals showed slight variation in chemical shift dependent on the temperature at which the spectra were recorded[5]. Assignment of the signals for α-galactofuranose and β-galactofuranose was based on their $J_{1,2}$ values, the β-anomer usually having $J_{1,2}$ ~1 Hz as compared to 4-5 Hz for the α-anomer[6,7].

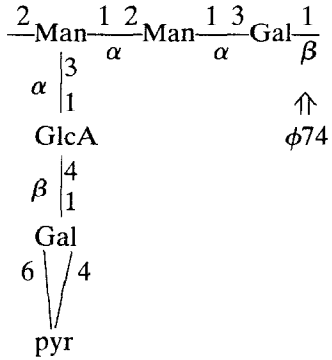
The ^{13}C-n.m.r. spectra (Table IV) were also interpreted by comparison and were easily assigned. The anomeric signal of the 2-linked mannose showed a twinning[8] caused by α-β anomerism in the adjacent, reducing galactose residue[4]. The substitution of **P1a** by pyruvate-containing galactose caused a small upfield shift of the ^{13}C signal of the terminal β-glucose, and a shift to lower field of the corresponding ^{1}H signal. In the ^{1}H- and ^{13}C-n.m.r. spectra of **P2** the signals for the 2,4-linked glucuronic acid unit were downfield of those for the monosubstituted uronic acid. The sugars adjacent to glucuronic acid, namely 3Man$\underset{\alpha}{\text{——}}$ and 6Glc$\underset{\alpha}{\text{——}}$,

showed two ^{13}C signals each because of the dual environments resulting from the substitution of one of the uronic acid units at position 2. In contrast, the ^{1}H-n.m.r. spectrum showed only one signal for the two mannose residues, but gave a signal for each of the two 6-linked α-glucose units. This observation is an indication of the versatility of ^{13}C-n.m.r. in distinguishing small changes in environment. The signal at 105.14 p.p.m. in the ^{13}C-n.m.r. spectrum of **P2** indicates that the in-chain 3-linked galactose is β, in accordance with the signal in the ^{1}H spectrum at δ 4.66 ($J_{1,2}$ = 8 Hz). The presence of this signal in the spectrum of **P2** only permits its assignment to the in-chain 3-linked β-galactose unit.

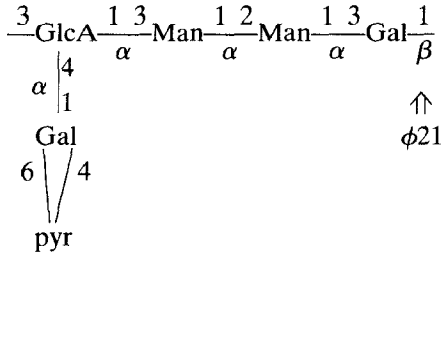
CONCLUSION

In contrast to the previous cases where the use of bacteriophages straightforwardly gave oligosaccharides corresponding to single or multiple repeat units, phage ϕ26 gave a hexasaccharide in addition to the heptasaccharide repeating unit of its substrate. One possible reason for this result is that ϕ26 is weakly active against the pyr$\overset{6}{\underset{4}{\diagup\!\diagdown}}Gal\underset{\beta}{\overset{1\ 4}{\text{——}}}$Glc linkage due to its close resemblance to the phage-

labile $\overset{3}{\text{——}}\text{Gal}\overset{1\ 2}{\underset{\beta}{\text{——}}}\text{GlcA}$ linkage. Secondly, there is a possibility that ϕ26 as isolated contained a second enzymatic activity, perhaps from a contaminating bacteriophage, that is specific for the former linkage. *Klebsiella* K26 polysaccharide is unusual, among those studied, in having a second sugar and linkage similar to the phage-labile structure. The polysaccharide of *Klebsiella* K74 (ref. 9) is the closest analogue to K26, having two β-D-galactopyranosyl residues but only a single cleavage site. *Klebsiella* K21 polysaccharide (ref. 10) also possesses two galactose units but these have opposite anomeric configurations and simultaneous hydrolysis would not be expected.



K74



K21

The enhanced spectral dispersion now obtainable in n.m.r. studies of oligosaccharides enabled more specific signal assignments to be made than had been possible in the original investigation of the K26 polysaccharide[4] (Tables III and IV). The presence of the furanose form of galactose is seen in aqueous solution at elevated temperatures and it predominates over the pyranose form in dimethyl sulfoxide as seen from the methylation data (Table II).

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded with a Bruker WH-400 instrument at 90 ±5°, or at ambient temperature. Acetone was used as the internal standard (δ 2.23). ^{13}C-N.m.r. spectra were recorded with the same instrument at ambient temperature. I.r. spectra were recorded with a Perkin-Elmer model 457 spectrophotometer. Analytical g.l.c. separations were carried out on a Hewlett Packard 5890A gas chromatograph, fitted with a Durabond DB17 capillary column. A Nermag R 10-10 mass spectrometer fitted with capillary columns coated with DB-17 or DB-225 was used for g.l.c.–m.s. analysis.

Propagation of bacteriophage and depolymerization. — Bacteriophage ϕ26 was isolated from sewage, and purified by a series of propagations of single plaques on the host *Klebsiella* K26 (ref. 3). The purified ϕ26 was propagated on its host in a dialyzable P-medium[2] to yield 7×10^{12} p.f.u. After dialysis of the medium and concentration to 200 mL, the concentrate was added to the polysaccharide (1 g/100 mL water) and the mixture was incubated for 48 h at 37°. This solution was then concentrated to 80 mL and dialyzed against distilled water (3×1 L). The dialyzates were combined and freeze dried (yield, 780 mg). The residue was applied to a column of Bio-Gel P4 (2.5×70 cm) to yield the three oligosaccharides **P1a** (64 mg), **P1** 422 mg), and **P2** (93 mg) on elution with 250:2.5:1 (v/v) water–pyridine–acetic acid.

Analysis of the oligosaccharides. — Aqueous solutions of the oligosaccharides were reduced with sodium borohydride, and the products after workup were hydrolyzed with 2M trifluoroacetic acid, then converted into the peracetylated aldononitriles[11] (see Table I).

Methylations of the oligosaccharides, and of the sodium borohydride-reduced oligosaccharides, were performed according to the method of Hakomori[12]. Methylated oligosaccharides **P1a** and **P2** were treated with lithium aluminum hydride in anhydrous oxolane to reduce the uronic esters. These compounds were all hydrolyzed with 2M trifluoroacetic acid and converted into the permethylated alditol acetates. The results obtained are presented in Table II.

ACKNOWLEDGMENTS

We thank Dr. I. Ørskov for a culture of *Klebsiella* K26. We are grateful to N.S.E.R.C. (Ottawa) for continued financial support and for the award of a MacMillan Bloedel Graduate Scholarship and a University Graduate Fellowship to J. L. Di F.

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