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PREPARATION OF BRANCHED HEXASACCHARIDES BY THE ACTION OF A VIRAL LYASE ON *Klebsiella* K14 POLYSACCHARIDE

HARALAMBOS PAROLIS, LESLEY A. S. PAROLIS,
School of Pharmaceutical Sciences, Rhodes University, Grahamstown, 6140 (South Africa)

AND GUY G. S. DUTTON
Department of Chemistry, University of British Columbia, Vancouver, B.C., V6T 1Y6 (Canada)

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ABSTRACT

Klebsiella K14 capsular polysaccharide was degraded by a bacteriophage-borne enzyme to afford oligosaccharides **A**–**C** which were studied by one- and two-dimensional n.m.r. spectroscopy. **A** and **B** were the repeating-unit hexasaccharide and pyruvylated hexasaccharide, respectively, while **C** was a dodecasaccharide. Each oligomer was terminated by a reducing mannose and a non-reducing 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue, indicating that the phage enzyme had cleaved the β-D-Man*p*-(1→4)-β-D-Glc*p*A linkages in the polysaccharide by a lyase, rather than the more common glycosidase, activity found with other *Klebsiella* bacteriophages. In this respect, the depolymerisation resembles those reported for the capsular polysaccharides of *Klebsiella* K5 and K64

INTRODUCTION

Bacteriophages that infect encapsulated bacteria possess enzymes in their tail spikes which depolymerise the capsular polysaccharide, thus facilitating penetration of the virus particles into the bacterial cells[1]. These enzymes are specific and the product of depolymerisation is often the repeating-unit oligosaccharide of the capsular polysaccharide. Several workers have reported bacteriophage-mediated degradations of *Klebsiella* polysaccharides[2–6]. The depolymerases are usually specific endoglycosidases and, for the bacteriophages which depolymerise the polysaccharides of *Klebsiella* K5[7] and K64[8], they are lyases. The bacteriophage that depolymerises the capsular polysaccharide of *Klebsiella* K14, which has[9] the repeating unit **1**, produces[1] oligosaccharides with Man reducing groups, but the nature of the cleavage or the structure(s) of the product(s) was not studied. We now report on the oligosaccharides produced by the degradation of K14 polysaccharide by a lyase-containing bacteriophage.

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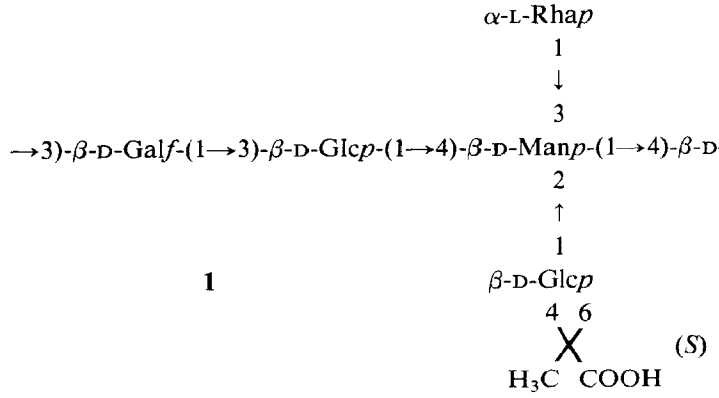
taining bacteriophage.



RESULTS AND DISCUSSION

A bacteriophage (phage) which infects *Klebsiella* K14 was isolated from sewage water and propagated on its host strain, purified, and concentrated by precipitation with ammonium sulphate[2]. Incubation of the capsular polysaccharide with the phage gave the oligosaccharides **A**–**C** which were isolated by gel-permeation chromatography.

The sugar analyses for **A**–**C** and the depyruvylated polysaccharide are presented in Table I. The ^{1}H- and ^{13}C-n.m.r. spectra of **A** and **B** (Tables II and III) indicated that each was a hexasaccharide with Man as the reducing terminus, and that **B** was pyruvylated **A**. Comparison of the ^{1}H-n.m.r. spectra of **A** and **B** with that of the depyruvylated polysaccharide indicated that something other than a simple hydrolysis had taken place during the depolymerisation. The β-D-Man*p* H-1 signal at δ 4.77 for the polysaccharide was replaced for **A** by partial signals at δ 5.29 and δ 4.97, confirming that the phage had cleaved the Man*p* linkages. Moreover, the H-1 signal for the β-Glc*p*A residue in the polymer was replaced by two doublets at δ 5.38 (^3J 4.1 Hz) and 6.18 (^3J 4.3 Hz), and similar resonances were noted for **B**. The

TABLE I

SUGAR ANALYSIS DATA FOR THE CAPSULAR POLYSACCHARIDE OF *Klebsiella* K14 AND OLIGOSACCHARIDES **A**, **B**, AND **C**.

Compound	*GlcA*	*Glc*	*Gal*	*Rha*	*Man*
K14 Polysaccharide[9]	0.9	1.8	0.9	1.1	1.0
Oligosaccharide **A**		1.9	1.0	1.0	1.0
B		2.0	0.9	1.0	1.0
C	1.0	3.9	1.9	1.9	2.0

TABLE II

^{1}H-N.M.R. DATA[a] FOR K14 POLYSACCHARIDE AND PHAGE-PRODUCED OLIGOSACCHARIDES

Compound	*Chemical shift (p.p.m.)*[b] ^{3}J (*Hz*)										
	α-Man	*β-Man*	*β-GlcA*	*EnUA*	*H-4 EnUA*	*β-Galf*	*β-Glc*	*→3)-β-Glc*	*α-Rha*	*CH_3Rha*	*CH_3Pijr*
A	5.29(0.8)	4.97(0.2)		5.38(1)	6.18(1)	5.33(1)	4.50(1)[c]	4.47(1)[c]	5.05(1)[c]	1.26(3)[c]	
	n.o.[d]	n.o.		4.1	4.3	n.o.	7.9	7.8	n.o.	6.6	
A-alditol				5.39(1)	6.19(1)	5.35(1)	4.62(1)[e]	4.61(1)[e]	5.17(1)	1.27(3)	
				4.4	4.3	1.3	7.8	7.9	1.6	6.2	
B	5.28(0.8)	4.96(0.2)		5.38(1)	6.20(1)	5.33(1)	4.54(1)[c]	4.48(1)[c]	4.92(1)[c]	1.26(3)[c]	1.53(3)
	1.8	n.o.		4.1	4.6	n.o.	7.9	7.9	n.o.	6.3	
B-alditol				5.39(1)	6.25(1)	5.35(1)	4.70(1)	4.57(1)	5.09(1)	1.26(3)	1.56(3)
				4.3	4.2	n.o.	7.2	8.0	n.o.	6.2	
Polysaccharide[g] (depyruvylated)		4.77(1)	4.68(1)[f]			5.33(1)	4.68(1)[f]	4.49(1)	5.05(1)	1.26(3)	
		n.o.	n.o.			n.o.	n.o.	7.4	n.o.	5.7	

[a]Chemical shifts, integrals (in parentheses) and coupling constants are given for the anomeric protons, unless otherwise stated, of the sugar residues as indicated in the structure of the repeating-unit oligosaccharide. [b] Chemical shift in p.p.m. relative to internal acetone at δ 2.23. [c]Signals twinned by the mutarotation of the reducing Man; δ value is for the major component. [d]Not observed. [e]Values may be interchanged. [f]Overlapped signals. [g]For assignments, see ref. 9.

TABLE III

^{13}C-N.M.R. DATA[a] FOR K14 POLYSACCHARIDE AND PHAGE-PRODUCED OLIGOSACCHARIDES

Compound	*Residue*													
	α-Man	*β-Man*	*β-GlcA*	*EnUA*	*C-4 EnUA*	*β-Galf*	*β-Glc*	*→3)-β-Glc*	*α-Rha*	*CH_3Rha*	*CH_3P*[b]	*COOHP*[b]	*C-6EnUA*	*C-5EnUA*	*C-6GlcA*
A	92.83[c] (170.8)	94.75 161.2)		100.19 (172.8)	111.81 (175.5)	109.01 (176.6)	103.09 (160.7)	102.63 (162.2)	96.85[d] (170.2)	17.22			166.92	142.32	
A-alditol				100.23	112.03	109.10	104.33	103.10	101.20	17.51			166.86	141.86	
B	92.82	94.63		100.25	112.18	109.02	103.79	102.63	96.83[d]	17.22	25.30	174.62	166.76	[e]	
B-alditol				100.35	112.87	109.13	104.55	103.16	100.74	17.52	25.32	174.12	166.30	141.22	
Polysaccharide (depyruvylated)		101.24	102.59[f]			108.99	104.47	102.77[f]	96.54	17.20					174.26

[a]Chemical shifts and one-bond C–H coupling constants (in parentheses) for the anomeric carbons, unless otherwise stated, for the sugar residues as indicated in the structure of the repeating-unit oligosaccharide. [b]P = pyruvate. [c]Chemical shift in p.p.m. relative to internal acetone at δ 31.07. [d]Signals twinned due to mutarotaion of the reducing-end Man; δ value is for the major component. [e]Not observed. [f]Assignments may have to be reversed.

latter resonance is consistent with that of a vinylic 4-proton of a hex-4-enopyranosyluronic acid[10].

The 2D ^1H-^1H spin-correlated spectra (COSY) of **A** (data not shown) and **A**-alditol (Table IV) established that the signals at δ 5.38 and 6.18 for **A** and δ 5.39 and 6.19 for **A**-alditol were part of the same spin system which contained only four protons. The u.v. absorption at 227 nm of **A** and **B** was in keeping with 4-deoxyhex-4-enopyranosyluronic acids[11,12]. Furthermore, **A** gave a pink chromophore with λ_{max} 459 nm in the thiobarbituric acid assay[13]. These data confirm that the signals at δ 5.38 and 6.18 for **A** (and the corresponding signals for **A**-alditol) may be assigned to H-1 and H-4, respectively, of an unsaturated uronic acid. The COSY spectra of **A** and **A**-alditol revealed long-range coupling (~1 Hz) of H-1 of the unsaturated uronic acid to H-3 and similar coupling (~1 Hz) of H-4 to H-2. These long-range couplings are consistent with those observed for a 4-deoxy-α-L-*threo*-hexopyranosyluronic acid residue[10]. Such a residue could only have arisen by elimination of the β-D-Man*p* residue from O-4 of the β-D-Glc*p*A in the polysaccharide.

The ^{13}C-n.m.r. spectra of **A**-alditol and the polysaccharide also exhibited differences. Apart from the expected disappearance of the signal for an anomeric carbon atom at 101.24 p.p.m. for the β-D-Man*p* residue, the signal for the carboxyl group of the uronic acid had shifted upfield to 166.92 p.p.m., whereas the signal for

TABLE IV

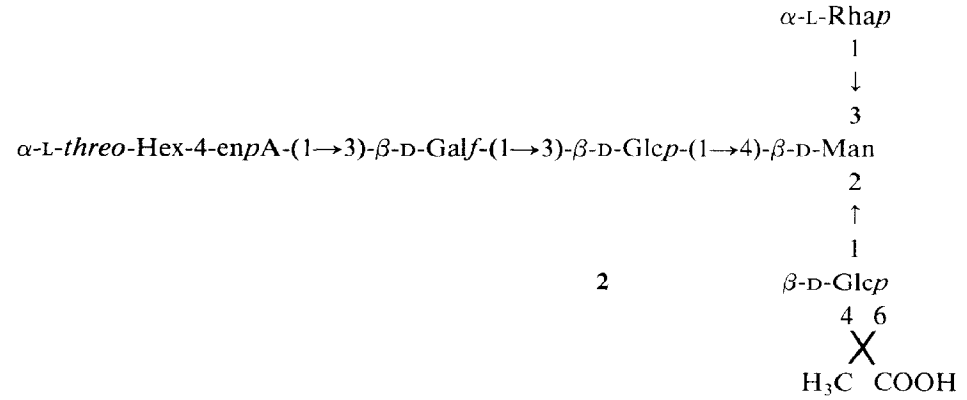
N.M.R. DATA[a] FOR **A**-ALDITOL

Atom	*Residue EnUA*	*α-Rha*	*β-Glc*[b]	→3)-β-Glc[b]	→3)-β-Galf	*Mannitol*
H-1	5.391(4.4)	5.168(1.6)	4.614(7.8)	4.606(7.9)	5.354(1.3)	4.076,3.705(12.4)
C-1	100.229	101.200	104.332	103.098	109.104	62.969
H-2	3.961(3.9)	4.024(3.4)	3.366(9.4)	3.440(9.4)	4.370(2.3)	4.253(8.2,2.1)
C-2	69.847	70.948	74.348	74.492	80.165	85.556
H-3	4.173(3.9)	3.786(9.8)	3.504(9.4)	3.665(9.4)	4.297(5.4)	4.525(2.5)
C-3	66.115	70.900	76.437	82.448	84.986	72.921
H-4	6.193(4.3)	3.450	3.414(8.9)	3.491(8.9)	4.210(3.9)	4.035(8.2)
C-4	112.027	72.695	70.351	68.442	83.277	79.767
H-5		3.902	3.442(5.5)	3.475	3.931(7.2,4.3)	3.850
C-5	141.856	70.057	76.691	76.082	71.350	71.522
H-6		1.275(6.2)	3.758(12.4)	3.804	3.613(12.0)	3.782
C-6	166.863	17.512	61.665	61.198	63.603	62.756
H-6′			3.890	3.885	3.659	3.903

[a]Chemical shifts and 3-bond coupling constants (in parentheses) for **A**-alditol with acetone as internal standard, δ 2.230 and δ 31.070 for ^1H and ^{13}C, respectively. [b]Values for H-5, H-6, and H-6′, and C-5 and C-6 may have to be interchanged between these residues.

the anomeric carbon atom of the acid had shifted to 100.23 p.p.m. In addition, two nuclei were substantially deshielded, and the resonances appearing at 112.03 and 141.86 p.p.m. were assigned to C-4 and C-5, respectively, of the hex-4-enuronic acid residue[14].

The ^{1}H and ^{13}C resonances for all six spin systems in **A**-alditol (Table IV) were assigned using a combination of COSY, RELAYED COSY, and ^{1}H–^{13}C shift correlation spectroscopy. ^{1}H–^{1}H coupling constants were obtained from the 1D spectrum and the 2D *J*-resolved spectrum. The assignment of the resonances for the β-D-Gal*f* residue was facilitated by the long-range H-1,3 and H-3,5 couplings. Long-range coupling between H-1 and H-3 of the terminal α-L-Rha*p* group was also observed. The above data, when considered together with the known structure of K14 capsular polysaccharide, permit structure **2** to be written for oligosaccharide **B**.



Oligosaccharide **C**, which was produced in least quantity during the depolymerisation, was shown by sugar analysis (Table I) and ^{1}H-n.m.r. spectroscopy to be a dodecasaccharide with an intact 4-linked β-D-Glc*p*A residue (H-1, δ 4.54, ^{3}J 7.9 Hz), a reducing Man terminus, and a 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue (H-1, δ 5.36; H-4, δ 6.09).

The depolymerisation of *Klebsiella* K14 polysaccharide is the third example of a bacteriophage-associated lyase activity towards a *Klebsiella* capsular polysaccharide. The two other examples involve K5[7] and K64[8]. Each of these three capsular polysaccharides possesses a trisubstituted β-D-Man*p* residue linked to O-4 of the Glc*p*A residue. It is not surprising that β-elimination reactions, rather than the more frequently encountered glycosidic hydrolyses, are effected by the phages in depolymerising such sterically hindered substrates. In spite of the similarity of the linkages cleaved in each of the three polysaccharides, each phage was specific for its host polysaccharide. The lack of cross-reaction between phage 64 and *Klebsiella* 14, and phage 14 and *Klebsiella* 64 is surprising since the β-D-Man*p* residue eliminated is substituted identically in both polysaccharides.

EXPERIMENTAL

The general methods used have been previously described[15,16]. COSY, RELAYED COSY, 2D *J*-resolved spectroscopy, and heteronuclear shift-correlation spectroscopy were performed on a Bruker WM-500 spectrometer, using standard Bruker software.

Bacteriophage degradation. — The isolation and purification of the K14 capsular polysaccharide were performed as described previously[9]. A bacteriophage, active on *Klebsiella* K14, was isolated from Grahamstown sewage water by standard procedures and propagated on the bacteria in nutrient broth (Difco) until a solution having a total of 10^{13} plaque forming units (PFU) was obtained. The phage particles were precipitated by the addition of ammonium sulphate, and a solution in water was dialysed against phosphate buffer. Polysaccharide (500 mg) was dissolved in the phage solution (175 mL, 9.75 x 10^{12} PFU) and the solution was stirred gently at 32° in the presence of a small amount of chloroform. After 96 h, the digest was lyophilised and then dialysed against distilled water, and the diffusates were combined and freeze-dried (550 mg). The product was passed down an ice-jacketed column of Amberlite IR-120(H^+) resin and then subjected to gel-permeation chromatography on a column (2.6 x 70 cm) of Bio-Gel P-4 with 0.1M pyridinium acetate (20 mL/h), to afford oligosaccharides **A** (64 mg), $[\alpha]_D$ −77° (*c* 0.8, water); **B** (107 mg), $[\alpha]_D$ −71° (*c* 0.8, water); and **C** (10 mg).

Analysis of oligosaccharides. — Oligosaccharides **A** and **B** were each reduced with sodium borohydride, and **A**, **A**-alditol, **B**, **B**-alditol, and **C** were examined by ^1H- and ^{13}C-n.m.r. spectroscopy. 2D-N.m.r. experiments were performed on **A**-alditol (COSY, RELAYED COSY, *J*-resolved, ^1H–^{13}C shift-correlated spectroscopy) and **B** (COSY, ^1H–^{13}C shift-correlated spectroscopy).

Depyruvylated K14 polysaccharide.— A solution of the polysaccharide (60 mg) in water (15 mL) was decationised, then heated for 30 min at 100°, and dialysed, the retentate was lyophilised, and the residue was analysed by ^1H- and ^{13}C-n.m.r. spectroscopy.

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