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ACYLATED OLIGOSACCHARIDES FROM *Klebsiella* K63 CAPSULAR POLYSACCHARIDE: DEPOLYMERIZATION BY PARTIAL HYDROLYSIS AND BY BACTERIOPHAGE-BORNE ENZYMES

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ABSTRACT

The extracellular polysaccharide from *Klebsiella* K63 is unique in having acetic and formic ester groups attached to the D-galactopyranosyluronic residues in the trisaccharide repeating-sequence. These *O*-acyl substituents are shown to be somewhat resistant to mild hydrolysis by both acid and alkali. Bacteriophage-induced depolymerization of the polysaccharide generated a series of acylated oligosaccharides comprising one, or more, repeating unit(s). By mild hydrolysis with acid, the same series of oligomers was released from the polysaccharide, together with the corresponding non-acylated compounds and the expected acylated and non-acylated aldobiouronic acids.

A study of these oligosaccharides, as well as of a number of their related compounds, is described, with particular emphasis on the methods used to locate the formic and acetic ester groups. The location of the *O*-acyl substituents on the galactosyluronic residues was further supported by the results obtained from the high-resolution, 400-MHz, p.m.r. spectra and ^{13}C-n.m.r. spectra of a number of the oligosaccharides.

INTRODUCTION

The presence of two *O*-acyl substituents in a single polysaccharide has been reported only by Sutherland[1], who demonstrated that each tetrasaccharide repeating-unit of the capsular polysaccharide from *Klebsiella aerogenes* type 54 (strain A3) is substituted with an *O*-formyl group, and that an *O*-acetyl group is present in every second such unit. In that case, attempts to locate these esters groups by partial hydrolysis of the tetra- and octa-saccharides generated by phage-induced depolymerization of the polysaccharide, to give smaller acylated oligosaccharides, proved unsuccessful. The locating of these groups, using methods similar to those discussed herein, is reported separately[2].

The capsular polysaccharide from *Klebsiella* serotype K63 constitutes a unique chemotype among a large number of slime polysaccharides produced by this family of bacteria. The primary structure of the K63 polysaccharide has been determined by

chemical methods[3], and shown to comprise the trisaccharide repeating-sequence →3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*A-(1→3)-α-L-Fuc*p*-(1→. An estimation of the content of *O*-acetyl by p.m.r. spectroscopy of a partially hydrolyzed sample of the polysaccharide indicated that approximately one in every five repeating-units is substituted with an *O*-acetyl group[3]. Attention has also been drawn to the serological identity of this polysaccharide with that produced[4] by *Escherichia coli* serotype K42, which contains the same sequence of sugar residues, but shows a higher degree of *O*-acetyl substitution (one mol-equiv. per two repeating units).

In an earlier communication[5], we reported that formic ester groups, situated at O-4 of each D-galactosyluronic acid residue, form an integral part of the K63 polysaccharide. Subsequent studies performed on the tri-, hexa-, and nona-saccharide products obtained by bacteriophage-induced depolymerization of the K63 polysaccharide revealed that each repeating unit also contains one *O*-acetyl group. This is linked at O-2 of the D-galactosyluronic residue, forming a unique, di-*O*-acyl derivative of this acid. These *O*-acyl groups were located by using chemical methods, and the results were substantiated by n.m.r.-spectroscopic studies.

Mild hydrolysis of the K63 polysaccharide with acid released the same series of oligosaccharides that were generated by the action of the viral enzyme, together with their nonacylated products, and two disaccharides, corresponding to the di-acylated and nonacylated aldobiouronic acids. This indicates the relative stability of the formic and acetic esters to mild acid, compared with the L-fucosyl bond (which is readily hydrolyzed). The action of bacteriophage cleaves the same glycosyl linkage, thus providing the first instance (in studies of this type) in which the products of phage-induced depolymerization and chemical degradation are identical.

We now report a detailed, structural analysis of the oligosaccharides obtained both by bacteriophage depolymerization and by partial, acid hydrolysis of the *Klebsiella* K63 polysaccharide, particularly the location of the *O*-acyl groups. The effects of *O*-acylation on the galactosyluronic residue, as shown by n.m.r.-spectral studies, are also described.

RESULTS AND DISCUSSION

A. Depolymerization with bacteriophage φ63

Generation of oligosaccharides. — Bacteriophage φ63 was isolated from sewage, and propagated on the host strain *Klebsiella* K63 by using a nutrient-broth medium[6]. Capsular polysaccharide from *Klebsiella* K63, grown and purified as previously described[7], had $[\alpha]_D$ +101°, $\overline{M}_w$ ~10^7 (by gel-permeation chromatography) and moved as a single band in electrophoresis. Depolymerization of the polysaccharide with phage[8] φ63 generated oligomers that corresponded to one (trisaccharide **1**), two (hexasaccharide **2**), and three (nonasaccharide **3**) repeating-units of the polysaccharide.

The oligosaccharides **1–3** were isolated by preparative paper-chromatography, and characterized by using standard techniques. The molecular weights and optical

TABLE I

CHARACTERIZATION OF THE COMPOUNDS OBTAINED IN DEPOLYMERIZATION STUDIES ON THE CAPSULAR POLYSACCHARIDE FROM *Klebsiella* K63

Compound[a]	R_{Gal}[b]	$[\alpha]_D$ *(degrees)*	*Mol. wt.*[c]	$\overline{d.p.}$[d]	O-*Formyl* *(molar proportions)*[e]	O-*Acetyl* *(molar proportions)*[e]
1	0.77	+120	502	3.0	1.0	1.0
2	0.22	+100	986	6.0	2.0	2.0
3	0.08	+93	1480	9.0	2.7	2.6[f]
4	0.30	+122	502	3.0	—	—
5	0.11	+105	986	6.0	—	—
6	0.01	+90	1480	9.0	—	—
7	n.d.	+155	n.d.	3.0	—	—
8	n.d.	+140	n.d.	6.0	—	—
9	n.d.	+114	n.d.	9.0	—	—
10	n.d.	+150	n.d.	3.0	1.0	1.0
11	1.47	+38	340	2.0	1.0	1.0
12	0.65	+38	340	2.0	—	—
13	0.62	+90	n.d.	2.0	—	—

[a]Fucose was identified as the reducing sugar residue in all of the oligosaccharides. [b]Solvent *A*. [c]By gel-permeation chromatography on Bio-Gel P-2. [d]Determined by Morrison's method[10]. [e]By p.m.r. spectroscopy. [f]Small proportions of *O*-acyl groups were removed during the isolation.

rotations of these compounds, as well as their ^{1}H- and ^{13}C-n.m.r. spectra (see Part C), were in accord with the structures assigned. A summary of the characteristics of these oligomers and their related compounds is shown in Table I. Further studies are outlined next.

Detection of O-*acyl groups.* — Hydroxamic acid derivatives were prepared from an acid hydrolyzate of the native polysaccharide by the method of Thompson[9], and identified by paper chromatography; with ferric chloride as the developing agent, spots corresponding to both acetyl- and formyl-hydroxamic acid were detected. The presence of these esters was further confirmed by p.m.r. spectroscopy of the oligosaccharides **1–3**, which gave the proportions of acetate and formate shown in Table I.

Deacylation. — Oligosaccharides **1–3** were converted into the corresponding, nonacylated products **4–6** by deacylation with 0.03M potassium hydroxide. Attempts to remove, selectively, either the formate or the acetate group, to yield the monoesters of these oligosaccharides, by use of 0.01M and 0.02M potassium hydroxide were unsuccessful, as neither *O*-acyl group was hydrolyzed under these conditions. This behavior is in contrast to that observed for these ester groups in the oligosaccharides obtained from the K54 polysaccharide, where it was found that the acetic, but not the formic, ester could be saponified by using 0.01M potassium hydroxide. Subsequent investigations[2] have, however, shown that the *O*-acyl groups in the K54 polysaccharide are not situated on the uronic acid residues, as in the K63 polysaccharide, indicating that the susceptibility of these esters towards alkaline hydrolysis depends largely on their exact position in the repeating sequence.

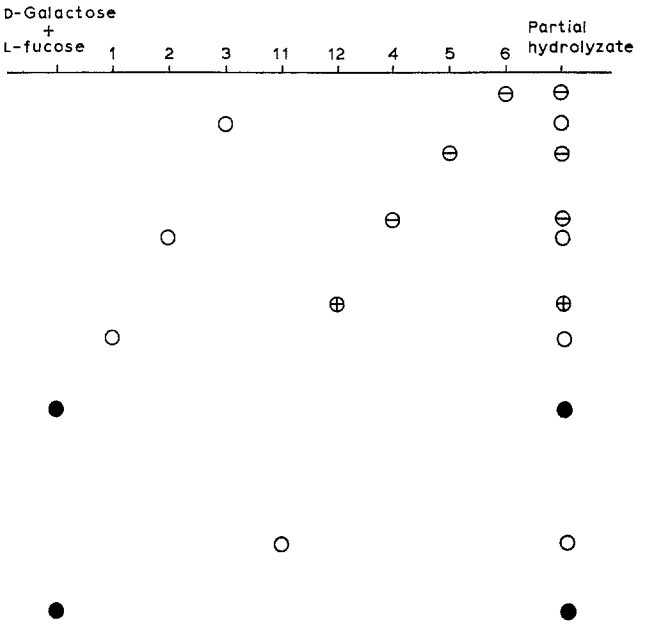


Fig. 1. Paper chromatography of the oligosaccharides from *Klebsiella* K63. (Solvent system: 18:3:1:4 ethyl acetate–acetic acid–formic acid–water. Chromatograms developed with *p*-anisidine in aqueous 1-butanol. Key: ● yellow, ○ pink, ⊕ reddish-brown, and ⊖ brownish-yellow.)

Paper chromatography. — From the R_{Gal} values given in Table I, it is evident that esterification of the 2- and 4-hydroxyl groups of D-galacturonic acid causes a marked increase in the paper-chromatographic mobility of the oligosaccharides, as illustrated in Fig. 1. Furthermore, these oligomers were detected as intensely pink spots on chromatograms developed with the *p*-anisidine reagent, due, presumably, to the presence of two additional carbonyl functions on the D-galactosyluronic ring. The corresponding, nonacylated compounds give a brownish-yellow color with this spray. (Development of the paper chromatograms with the silver nitrate reagent

TABLE II

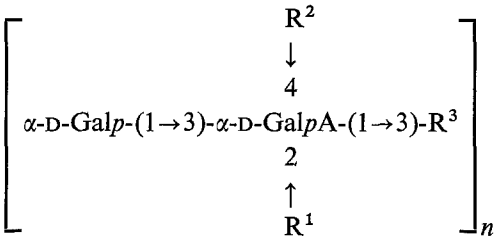
DETERMINATION OF DEGREE OF DEPOLYMERIZATION OF OLIGOSACCHARIDES FROM THE K63 POLYSACCHARIDE, AND IDENTIFICATION OF THE REDUCING SUGARS

Acetyl derivative of	*Relative retention-time on OV-225*[a]	*Mole %*[b]		
		1	**2**	**3**
Fucononitrile	0.36	—	23.9	34.2
Fucitol	0.40	48.8	23.9	17.7
Galactononitrile	**1.00**	51.2	52.2	48.1

[a]Column of 3% of OV-225 on Gas-Chrom Q (100–120 mesh), isothermally at 210°. [b]Proportions present in the hydrolyzates of the acidic oligosaccharides (*i.e.*, uronic acid not reduced).

yields little of this information, and can, indeed, lead to confusion when such mixtures of the acylated and nonacylated oligosaccharides as were obtained by partial hydrolysis of the polysaccharide are examined.)

Degree of polymerization. — The degree of polymerization of oligomers **1–3** was determined by Morrison's method[10], following their conversion into the corresponding oligosaccharide-alditols **7–9** with sodium borohydride (see Table II). Analysis, by p.m.r. spectroscopy, of the products obtained by reduction of **1–3** with borohydride for 1 h at pH ~14 revealed that, although the L-fucose residues had been completely converted into L-fucitol residues, only half of the *O*-acyl groups had been hydrolyzed under these conditions. However, treatment of **1–3** with sodium borohydride for 18 h resulted in total loss of both ester groups. In a separate experiment, the di-*O*-acylated trisaccharide **1** was reduced with a dilute solution of sodium borohydride for 35 min at pH ~9, to yield oligosaccharide **10**, which still retained the full complement of *O*-acetyl and *O*-formyl groups (as shown by p.m.r. spectroscopy). Compound **10** was used in the periodate-oxidation studies described later.



1 ($n = 1$) (acylated trisaccharide)
$R^1 = OCOCH_3$
$R^2 = OOCH$
R^3 = L-Fuc*p* ~ OH

2 ($n = 2$) (acylated hexasaccharide)

3 ($n = 3$) (acylated nonasaccharide)

4 (nonacylated trisaccharide)
$R^1 = R_2 = OH$
R^3 = L-Fuc*p* ~ OH

5 (nonacylated hexasaccharide)

6 (nonacylated nonasaccharide)

7 (reduced trisaccharide)
$R^1 = R^2 = OH$
R^3 = L-fucitol

8 (reduced hexasaccharide)

9 (reduced nonasaccharide)

10 (reduced, acylated trisaccharide)
$R^1 = OCOCH_3$
$R^2 = OOCH$
R^3 = L-fucitol

TABLE III

METHYLATION ANALYSES OF THE NATIVE K63 POLYSACCHARIDE, AND THE TRI- AND HEXA-SACCHARIDES PRODUCED BY DEGRADATION WITH BACTERIOPHAGE

Methylated sugars[a] *(as alditol acetates)*	T[b]	*Mole %*[c]						
		A[d]	*B*	*C*	*D*[e]	*E*[f]	*F*[g]	*G*
2,4-Fuc	0.89	29.9[h]	28.3	28.3	30.5	29.4	36.5[h]	32.0
2,3,4,6-Gal	**1.00**	70.1	37.4	45.3	22.0	20.5	—	—
2,4,6-Gal	1.36	—	—	26.5	15.8	20.2	63.5	37.4
2,4-Gal	2.68	—	23.8	—	23.9	25.4	—	23.6
2-Gal	3.12	—	10.5	—	7.8	4.5	—	7.0

[a]2,4-Fuc = 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol, etc. [b]Retention time of partially methylated alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol on a column of 3% of OV-225 on Gas-Chrom Q (100–120 mesh) programmed for 4 min at 180°, and then at 2° per min to 210°. [c]Values corrected by using e.c.r. molar-response factors[14]; A, methylated trisaccharide; B, methylated and reduced trisaccharide; C, re-methylated, methylated and reduced trisaccharide; D and E, methylated and reduced hexasaccharide; F, methylated polysaccharide; G, methylated and reduced polysaccharide. [d–g]Treated with methylsulfinyl anion for 1, 2, 30, and 15 min, respectively. [h]Proportions are not meaningful, owing to incomplete hydrolysis of the aldobiouronic acid linkages under the conditions used.

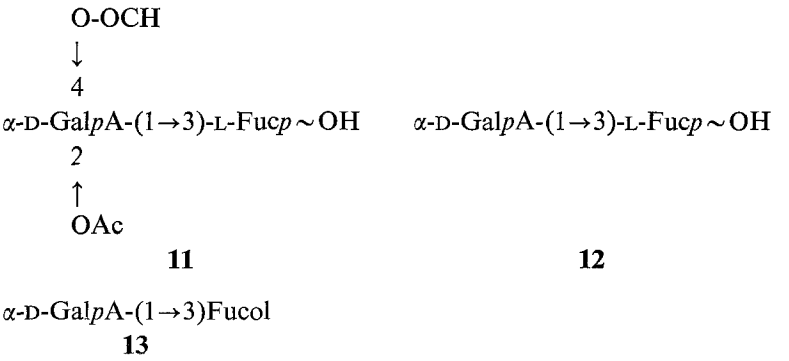
Methylation analysis; location of the O*-formyl groups.* — Trisaccharide **1** was methylated by the Hakomori[11] procedure, but was exposed to the base for only 1 min before addition of the methylating agent. P.m.r. spectroscopy of the methylated derivative indicated that a substantial proportion of the formic ester groups was still present; no acetyl groups were detected. Examination of this methylated product by gas–liquid chromatography (g.l.c.) in a column of OV-17 showed the presence of four components, corresponding to the anomers of the methylated, and methylated-formylated, trisaccharide. Hydrolysis of a sample of the mixture, followed by g.l.c. analysis of the neutral sugars present in the hydrolyzate (as the derived alditol acetates) gave the proportions of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-L-fucose shown in Table III (column A). No evidence was obtained, either from the infrared spectrum of the methylated products, or from g.l.c., to suggest undermethylation. Reduction of a further sample with lithium aluminum hydride in oxolane, followed by g.l.c. on OV-17, again gave four components, but having retention characteristics different from those of the unreduced derivatives. This was to be expected, as treatment of the mixture containing both methylated and methylated-formylated trisaccharides with lithium aluminum hydride gives rise to oligosaccharides having free hydroxyl groups at C-6, and at both C-4 and C-6, of D-galacturonic acid, respectively. Hydrolysis of a portion of the reduced mixture, and g.l.c. analysis of the sugars present in the hydrolyzate (as the derived alditol acetates) gave the results shown in Table III (column B). Remethylation of a second portion with the Purdie reagents[12], followed by g.l.c. analysis on OV-17, gave only two

components, corresponding to the anomers of the fully methylated trisaccharide. Analysis of the sugars present in a hydrolyzate of this material gave the results shown in Table III (column C).

Similar methylation studies were performed on the hexasaccharide **2**, which was treated with the methylsulfinyl anion for periods of 3 and 30 min before the addition of methyl iodide, and also on the original capsular polysaccharide (15 min in the base). Methylation analyses of the reduced, methylated products obtained in each reaction were made as for the trisaccharide **1**; the results are shown in Table III (columns D, E, and G).

The presence of both 2,4-di- and 2-*O*-methyl-D-galactose in the hydrolyzates of the methylated products following carboxyl reduction indicates that both of these sugars must have been derived from the D-galactosyluronic residues. No evidence was obtained to suggest that these residues were present as a result of undermethylation. The foregoing methylation studies indicate that a substantial proportion of the formic ester groups survives treatment with the strong base, and that they are subsequently removed by reduction, resulting in a free hydroxyl group at C-4 of D-galacturonic acid. Carboxyl reduction of these D-galactosyluronic residues gives rise to the proportions of 2-*O*-methyl-D-galactose found in the hydrolyzates of the reduced, methylated products.

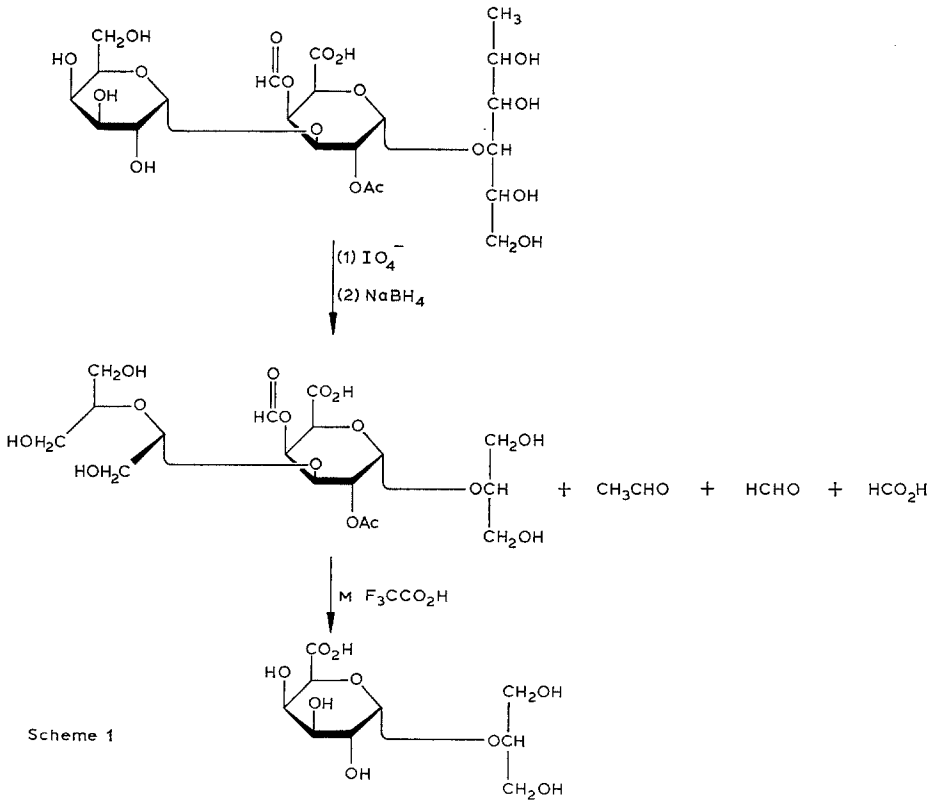
Hydrolysis with enzymes. — Hydrolysis of the diacylated trisaccharide **1** with α-D-galactosidase, followed by paper chromatography of the hydrolyzate, showed the presence of D-galactose, and of a faster-moving component (**11**); the latter was separated from the monosaccharide and the unhydrolyzed material by preparative paper-chromatography. Oligosaccharide **11** was identified as the aldobiouronic acid 3-*O*-(α-D-galactosyluronic acid)-L-fucose, and was shown by p.m.r. spectroscopy to contain equimolar proportions of formate and acetate. Treatment of **11** with 0.03M potassium hydroxide gave the corresponding, nonacylated aldobiouronic acid **12**, which was found identical with authentic material isolated from a partial hydrolyzate of the K63 polysaccharide. Reduction of **11** with sodium borohydride for 18 h yielded the reduction product **13**.



Samples of the hexasaccharide **2** and the nonasaccharide **3** were subsequently treated with α-D-galactosidase, and the products were examined by paper chromatography. Both hydrolyzates showed the presence of D-galactose and a component having a chromatographic mobility higher than those of the original, unhydrolyzed oligosaccharides **2** and **3**. These components were not isolated, but there is little doubt that they were the penta- and octa-saccharides resulting from the loss of the terminal D-galactosyl groups in **2** and **3**, respectively.

These results not only confirm that the D-galactose occurs in the terminal, nonreducing position in oligosaccharides **1–3**, but also eliminate the possibility that these residues are a site for the attachment of the *O*-acetyl substituents.

Periodate oxidation; location of the O-*acetyl groups.* — The foregoing studies indicate that both the formate and acetate groups are situated on the aldobiouronic acid portion of the trisaccharide repeating-sequence. Methylation studies had already shown that the formyl groups are attached to O-4 of D-galacturonic acid, thus leaving O-2 of these residues and O-2 and O-4 of L-fucose as possible sites for *O*-acetyl substitution; both sugars are linked at O-3 in the polysaccharide chain. As mentioned earlier, the intense coloration of the oligosaccharides **1–3**, as well as of the aldobiouronic acid **11**, on paper chromatograms developed with *p*-anisidine was attributed



Scheme 1. Periodate oxidation and Smith degradation of the diacylated, reduced trisaccharide **10**.

to the presence of both *O*-acyl groups on the uronic acid residue; this would indicate that the *O*-acetyl groups are situated at O-2 of the D-galactosyluronic residue.

In order to test these hypotheses, and to eliminate the 2- and 4-hydroxyl groups of L-fucose as possible sites of *O*-acetyl substitution, the trisaccharide alditol **10** was subjected to the sequence of reactions involving periodate oxidation, reduction with sodium borohydride, and Smith hydrolysis, shown in Scheme 1. The resulting mixture of products was analyzed by paper chromatography, which showed the presence of glycolaldehyde, glycerol, and a slower-moving component, **14**, which was isolated by preparative paper-chromatography.

α-D-Gal*p*A-(1→2)-Glycerol
14

Hydrolysis of **14** gave D-galacturonic acid (detected by paper chromatography) and glycerol (identified by g.l.c. as the triacetate). The ^{13}C-n.m.r. spectrum of **14** was in accord with the structure assigned. This compound could only have been generated by the series of reactions employed if both C-2 and C-4 of L-fucitol, and, hence, of L-fucose in the original trisaccharide **1**, carry free hydroxyl groups. The removal of the *O*-acyl groups occurs during the prolonged, mild treatment with acid, as p.m.r. spectroscopy showed their presence prior to hydrolysis.

B. Mild depolymerization with acid

Hydrolysis of the K63 polysaccharide with 0.5M trifluoroacetic acid, and paper chromatography of the hydrolyzate, showed the presence of the aldobiouronic acids **11** and **12**, together with the oligosaccharides **1–3** and their corresponding, non-acylated derivatives **4–6**. These oligomers were identified by their paper-chromatographic mobility, and by the color developed with the *p*-anisidine reagent. In some cases, the pure compounds were isolated by preparative paper-chromatography, and were shown to be identical in all aspects with those obtained earlier. This is illustrated in Table IV, which compares the characteristics of the aldobio- and aldotrio-uronic acids obtained by both depolymerization methods.

TABLE IV

ANALYTICAL DATA FOR COMPOUNDS **1** AND **11** OBTAINED BY THE METHODS OF ENZYMIC AND ACID HYDROLYSIS OF THE K63 POLYSACCHARIDE

Compound[a]	*Method of hydrolysis*	R_{Gal}[b]	$[\alpha]_D$ *(degrees)*	*Mol. wt.*[c]	$\overline{d.p.}$[d]	O-*Formyl* *(molar proportions)*[e]	O-*Acetyl* *(molar proportions)*[e]
1	enzymic	0.77	+120	502	3.0	1.0	1.0
	acid	0.77	+118	502	3.0	1.0	1.0
11[f]	enzymic	1.47	+38	340	2.0	1.0	1.0
	acid	1.47	+42	340	2.0	1.0	1.0

[a–e]As for Table I. [f]Isolated following treatment of diacylated trisaccharide **1** with α-D-galactosidase.

The hydrolyzate also contained an appreciable quantity of D-galactose, together with traces of D-galacturonic acid and L-fucose, and a component having a molecular weight of 5000 which was separated from the mono- and oligo-meric material by dialysis. Proton magnetic resonance spectroscopy of this polymer indicated that ~50% of the acetic and formic esters were still present.

These results indicate that the *O*-acyl substituents in the K63 polysaccharide are relatively more stable towards mild acid than those encountered in other bacterial polysaccharides. This stability is attributed to the fact that both of these groups are linked to D-galacturonic acid, allowing cleavage of the acid-labile, L-fucosyl bonds in the polysaccharide without removal of the ester groups. The oligosaccharides that are released therefore correspond to the intact, repeating-unit (**1**), and to multiples thereof (**2** + **3**). Removal of the *O*-acyl substituents, before or after hydrolysis of the L-fucosyl bonds, generates the nonacylated oligomers (**4**–**6**).

The resistance of these substituents to mild acid was further demonstrated by heating an aqueous solution of trisaccharide **1** with an acidic resin. Paper chromatography of the hydrolyzate showed that D-galactose and the diacylated aldobiouronic acid **11** were the major products released. This also accounts for the substantial proportion of D-galactose found in the partial hydrolyzate of the K63 polysaccharides. The aldobiouronic acid linkage is extremely resistant to acid hydrolysis[3], resulting in only traces of D-galacturonic acid and L-fucose being found in the hydrolyzate.

C. ^{1}H- and ^{13}C-n.m.r.-spectral studies

Studies on the aforementioned, *O*-acylated oligosaccharides and their related compounds by using ^{13}C-n.m.r. and high-resolution, 400-MHz, p.m.r. spectroscopy revealed that a number of conclusions may be drawn as to the effects that the *O*-acyl substituents have on the chemical shifts of the D-galactosyluronic ring protons and carbon atoms. It should be emphasized that these conclusions are based on the knowledge gained from the earlier chemical studies on these oligosaccharides, which indicated substitution at O-4 with formyl and at O-2 with acetyl groups. No direct evidence may be obtained from n.m.r. spectroscopy to differentiate between these positions of substitution, although it may be reasonable to assume that formate will have a larger electron-withdrawing effect than acetate. Furthermore, it should be noted that, although many n.m.r. studies have been performed on fully acetylated mono- and oligo-saccharides, little is known concerning the effect of a *single* *O*-acetyl group on the position of substitution and on the other ring atoms. Many of the observations made for the *O*-acetyl substituent in the K63 oligosaccharides have been substantiated by the results obtained[2] for the *O*-acetylated oligosaccharides from *Klebsiella* K54. For convenience, the following study is discussed under appropriate headings.

^{1}H-N.M.R. SPECTROSCOPY

The 400-MHz, high-resolution, p.m.r. spectra of the oligosaccharides and related compounds from *Klebsiella* K63 were recorded at room temperature, with

TABLE V

PROTON ASSIGNMENTS[a] (400 MHz) FOR THE OLIGOSACCHARIDES AND RELATED COMPOUNDS ISOLATED FROM *Klebsiella* K63 CAPSULAR POLYSACCHARIDE

Compound	α-D-*Galactose* *H-1*	α-L-*Fucose* *H-1*	β-L-*Fucose* *H-1*	α-D-*Galacturonic acid*					O || *O-CH*	*O-Ac*
				H-1	*H-2*	*H-3*	*H-4*	*H-5*		
12	—	5.22	4.62	5.31	3.87	4.04	4.38	4.72	—	—
11	—	5.22	4.61	5.33	3.87	4.22	4.70	4.68	5.67	2.13
13	—	—	—	5.18	3.87	4.05	4.36	4.65	—	—
1	5.19	5.22	4.61	5.41	4.03	4.35	4.91	4.68	5.94	2.13
4	5.20	5.20	4.60	5.36	3.96	4.16	4.62	4.70	—	—
10	5.20	—	—	5.27	3.93	4.28	4.86	4.65	5.93	2.12
7	5.20	—	—	5.25	3.89	4.12	4.65	4.77	—	—
2	5.20	5.22	4.61	5.42	4.02	4.36	4.86	4.67	5.96	2.12
5	5.20	5.22	4.61	5.37	3.98	4.19	4.63	4.74	—	—
8	5.20	5.22	—	5.35 5.25	3.96	4.19	4.63	4.68	—	—
3	5.20	5.22	4.61	5.42 5.37	4.02	4.36 4.20	4.88 4.63	4.67	5.96	2.12
6	5.20	5.22	4.61	5.37	3.98	4.19	4.63	4.67	—	—
9	5.20	5.22	—	5.37 5.25	3.95	4.17	4.63	4.67	—	—

[a]All chemical shifts are relative to internal acetone, taken as δ 2.23.

simultaneous irradiation of the HOD signal[14]. In certain spectra, signals due to H-4 and H-5 of D-galacturonic acid were masked by the residual HOD peak, and, in these cases, the chemical shifts were assigned by reference to the corresponding, low-resolution spectrum recorded at ~90°. Proton resonances attributed to D-galactosyluronic acid residues in the compounds studied were determined by selective-decoupling experiments, and are shown in Table V.

Chemical shifts of O-*acetyl and* O-*formyl groups.* — The *O*-formyl and *O*-acetyl groups present in the *O*-acylated oligosaccharides obtained from the *Klebsiella* K63 polysaccharide are readily identified by p.m.r. spectroscopy; their chemical-shift values are shown in Table V. The signal due to the methyl group of *O*-acetyl appears as a sharp singlet in all of the p.m.r. spectra at a chemical shift characteristic of these esters[15]. Conversely, the signal in these spectra that is due to the proton of the formic ester shows neither the form (sharp singlet) nor the chemical shifts (δ ~8) observed for the corresponding proton in such esters as methyl and ethyl formate. This is evident from the values given in Table V, and is illustrated in the high-resolution, p.m.r. spectrum of the di-*O*-acylated oligosaccharide **1**, shown in Fig. 2(a), from which it is evident that the formate signal is split into a doublet having a small coupling-constant (~3 Hz). Furthermore, it is apparent that removal of the D-galactosyl group from O-3 of D-galacturonic acid in oligosaccharide **1**, to yield the di-*O*-acylated aldobiouronic acid **11**, results in a marked shielding of the formate proton. This is

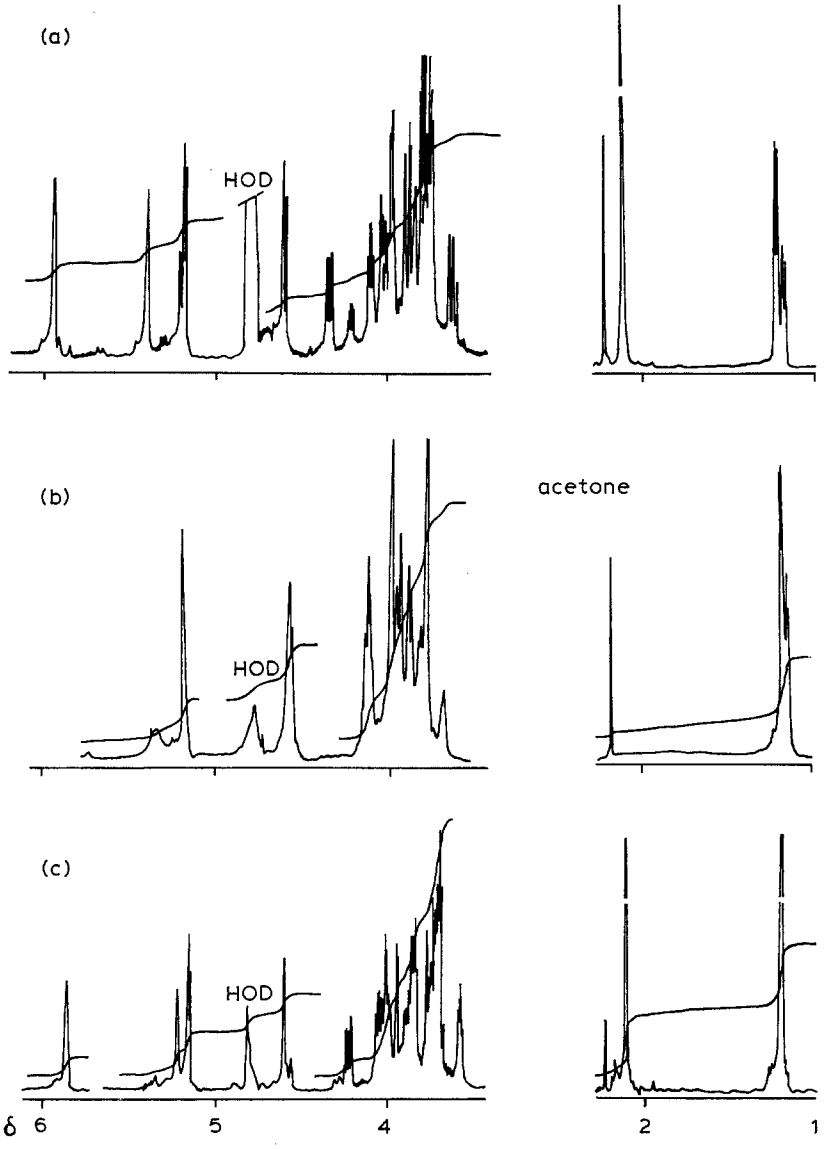


Fig. 2. Proton n.m.r. spectra (400 MHz) of (a) compound **1**, (b) compound **4**, and (c) compound **10** (see text).

evident from Table V, and suggests that this ester is somewhat sensitive to such changes, possibly because of its axial position on the ring and the presence of the nearby carboxyl group. The acetic ester situated in an equatorial position on C-2 of D-galacturonic acid remains insensitive to the removal of the D-galactosyl group.

The strong shielding experienced by the proton of the formic ester in the oligosaccharides from K63 could possibly be due to some form of hydrogen bonding, or to the fact that these ester groups are attached to a carbohydrate ring-system, and the

effect is even more pronounced[2] in the spectra of the oligosaccharides from *Klebsiella* K54, where the corresponding proton resonates at δ 4.7.

^{1}H-Chemical shifts for α-D-galacturonic acid; effects of O-acylation. — The signals due the ring protons of the D-galactosyluronic residues in the compounds studied were, in most cases, well resolved in the high-resolution spectra, and could be unambiguously assigned. From the chemical shifts listed in Table V, it is evident that formylation at O-4 of D-galacturonic acid has a marked deshielding effect on H-4, the signal of which is shifted downfield by 80–100 Hz in the spectra of most of the oligosaccharides studied. This effect is even more apparent in the data obtained for the aldobiouronic acid **11** and the corresponding, nonacylated compound **12**, where a shift to lower field of 128 Hz was observed. Furthermore, it may be seen that the formation of a glycosidic linkage at C-3 of the aldobiouronic acid **12**, to yield the corresponding, nonacylated trisaccharide **4** [Fig. 2(b)], also results in a shift of the H-4 signal to much lower field. This proton is found at a similar chemical-shift in the spectra of the nonacylated, higher oligosaccharides **5** and **6**, and is shifted by ~100 Hz downfield in the spectra of the corresponding di-*O*-acylated oligosaccharides (**2** and **3**). The 400-MHz spectrum of the nonasaccharide **3** gave signals from both acylated and nonacylated D-galactosyluronic acid residues, owing to the fact that ~20% of the formic and acetic esters had been saponified during isolation.

Acetylation of the 2-hydroxyl group of D-galacturonic acid apparently results in a deshielding of H-3 (on the adjacent carbon atom) by ~80 Hz; the signal due to H-2 is, however, only slightly deshielded. This is somewhat surprising, and suggests that the *O*-acetyl groups in these oligosaccharides may be so oriented that the carbonyl group is in close proximity to the proton on C-3. Examination of a scale model of the di-*O*-acylated trisaccharide **1** shows that acetylation at O-2 greatly increases the steric hindrance in the ring, and that these effects may be lessened by placing the carbonyl, rather than the methyl, group of the ester near H-3.

Comparison of the high-resolution spectrum of oligosaccharide **1** with that of its reduced derivative **10** [Fig. 2(c)] clearly indicates that complete reduction of the L-fucose residues can be achieved without saponification of the formic and acetic esters.

^{13}C-N.M.R. SPECTROSCOPY

In addition to the foregoing p.m.r. studies, a number of the oligosaccharides and their related compounds were also examined by ^{13}C-n.m.r. spectroscopy. The range of oligomers examined allowed the unambiguous assignment of all of the resonances due to anomeric carbon atoms, as well as many of those attributed to the ring, and other, carbon atoms. This investigation was, in some ways, simplified by the fact that all three sugar residues in the repeating unit of the K63 polysaccharide not only have similar relative configurations, but are also linked in the same mode (α-linkage) and through the same position (C-3). The data compiled in the present

study can be used to re-assign certain of the resonances in the ^{13}C-n.m.r. spectrum of the K63 polysaccharide[3].

Detection of O-*acyl groups.* — The *O*-acetyl groups in the di-*O*-acylated oligosaccharides were identified by the carbon resonance of the methyl group, at ~21 p.p.m., in their ^{13}C-n.m.r. spectra. Although no direct evidence for the presence of formate can be derived from ^{13}C-n.m.r. spectroscopy, the spectra showed a strong absorption in the carbonyl region, at ~173 p.p.m., that was absent from the spectra of the corresponding, nonacylated compounds. As this absorption is normally very weak, and is difficult to detect in the spectra of most carbonyl compounds, owing to the absence of ^{13}C–H coupling, the enhancement of this signal in the ^{13}C-n.m.r. spectra of the diacylated oligomers could be due to the presence of formate, in which the carbonyl carbon atom is ^{1}H-coupled. However, it was not possible to investigate this theory in more detail, as attempts to remove acetate selectively, to yield the solely *O*-formylated oligosaccharides were unsuccessful. Similar studies[2] on the polysaccharide from *Klebsiella* K54, and on the oligosaccharides obtained by bacteriophage degradation thereof, substantiated these findings, because, in this case, the acetyl groups that are attached to O-2 of alternate L-fucose residues can be selectively removed, to yield the corresponding *O*-formylated poly- and oligo-saccharides. The ^{13}C-n.m.r. spectra of these *O*-formylated compounds all show a strong carbonyl absorption at 173 p.p.m.

^{13}*C-Chemical shifts of* α-D-*galacturonic acid*; *effects of* O-*acylation.* — When comparing results obtained for D-galacturonic acid in the diacylated oligosaccharides (**1**–**3**, and **11**), it should be noted that these are "net" effects due to formylation of O-4 and acetylation of O-2. The chemical shift due to *O*-acylation at one or other of these positions could not be ascertained, for reasons already discussed.

From the values shown in Table VI, it is evident (**1** *vs.* **4**, and **11** *vs.* **12**) that formoxylation at C-4 results in a deshielding of this carbon atom by ~2.6 p.p.m. This shift is readily detected in the ^{13}C-n.m.r. spectrum of the aldobiouronic acid **11** when it is compared with that of the nonacylated compound **12**; the signal due to C-4 (at 73.7 p.p.m.) in the diacylated acid is well separated from the remaining ^{13}C resonances. In the trisaccharide **1**, however, the formation of a glycosidic linkage at C-3 of D-galacturonic acid causes the signal for C-4 to be shifted to much higher field (~70 p.p.m.), whereas, for the nonacylated trisaccharide **4**, the corresponding signal occurs at 67.5 p.p.m. This strong, upfield shift for C-4 was reported for methyl 3-*O*-methyl-β-D-galactopyranoside[16], and also for inositols having similar configurations[17], and has been proposed as occurring as a general rule[16]. Methylation of hexopyranose hydroxyl groups causes upfield shifts of ~4.5 p.p.m. for β-carbon atoms bearing axial hydroxyl groups.

The resonance due to C-3 of D-galacturonic acid in the spectra of the diacylated oligosaccharides (**1**–**3**) is somewhat difficult to assign, as acyloxylation at the two adjacent β-carbon atoms causes this signal to be shifted upfield into the envelope of ring-carbon atoms. Comparison of the relative intensities of the signals in the ^{13}C-n.m.r. spectra of the diacylated (**1**) and nonacylated (**4**) trisaccharides, however,

suggests the location of the C-3 signal at ~72 p.p.m., but this assignment should be regarded as tentative. In the spectrum of trisaccharide **4**, the C-3 resonance is assigned at 74.6 p.p.m., a shift of only 4.7 p.p.m. downfield from the position of the corresponding signal in the spectrum of the aldobiouronic acid **12**. This is somewhat surprising, as glycoside formation usually results in a downfield shift, for the carbon atoms involved, by 7–10 p.p.m. In the ^{13}C-n.m.r. spectrum of the K63 polysaccharide[3], the signal at 75.2 p.p.m. was attributed to C-3 of the L-fucosyl residues. However, as this spectrum was recorded for a partially hydrolyzed sample of the polysaccharide (in which ~80% of the *O*-acyl groups had been removed during hydrolysis), the present studies indicate that this resonance should be assigned to C-3 of D-galacturonic acid, and the corresponding signal, at 79.3 p.p.m., to C-3 of L-fucose.

The effect of acetoxylation at C-2 appears to have a lesser impact on the D-galactosyluronic ring, causing this carbon atom to be shielded by only 0.2 p.p.m. This is evident from the values shown in Table VI (**1** *vs.* **4**, and **11** *vs.* **12**), and is in agreement with the corresponding, small shift observed for H-2 in the p.m.r. spectra discussed earlier. However, as acetylation at O-2 has been shown to cause a strong deshielding of H-3 on the D-galactosyluronic ring, it seems reasonable to assume that C-3 will also be markedly affected, resulting in the observed shift of its signal to higher field, already referred to.

Resonances due to the anomeric carbon atom of D-galacturonic acid in these oligosaccharides are seemingly unaffected both by acylation and glycosylation at O-3, and show little variation in chemical shift. Interestingly, however, C-1 gives rise to two signals in the ^{13}C-n.m.r. spectrum of compound **14**, presumably due to re-

TABLE VI

^{13}C-CHEMICAL SHIFTS FOR α-D-GALACTURONIC ACID AND O-ACETYL IN THE OLIGOSACCHARIDES AND RELATED COMPOUNDS FROM *Klebsiella* K63

Compound	*Chemical shift*[a]					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*CH$_3$ of O-acetyl*
11	101.5	68.9	69.4	73.7	73.7[b]	21.1
12	101.5	69.1	69.9	71.0	71.0	—
13	101.2	68.9	69.7	70.9	70.9	—
14	98.7 98.5	68.6	69.7	70.8	70.8	—
1	101.4	68.6	72.5	70.0	71.6[b]	20.2
4	101.5	68.9	74.6	67.5	71.4	—
7	101.5	68.9	74.6	67.5	71.4	—
10	100.7	68.6	72.3[b]	70.0	71.6[b]	21.0
2	101.3	68.5	72.5[b]	70.0	71.2[b]	20.9
3	101.3	68.4	72.5[b]	70.0	71.7[b]	20.9

[a]All chemical shifts are relative to internal acetone, taken as 31.0 p.p.m. [b]Assignments are tentative.

TABLE VII

^{13}C-CHEMICAL SHIFTS FOR TERMINAL, AND IN-CHAIN, α-D-GALACTOSE IN THE OLIGOSACCHARIDES AND RELATED COMPOUNDS FROM *Klebsiella* K63

Compound	*Chemical shift*[a]											
	Terminal α-D-galactose						*In-chain α-D-galactose*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
1	95.2	68.0	70.0	70.0	71.6	61.9	—	—	—	—	—	—
4	96.0	68.9	70.0	70.0	71.6	61.9	—	—	—	—	—	—
7	96.0	68.9	70.0	70.0	71.7	61.8	—	—	—	—	—	—
10	95.0	68.8	70.0	70.0	71.6	62.0	—	—	—	—	—	—
2	94.9	68.5	70.0	70.0	71.6	61.9	94.9	68.5	78.0	67.9	71.6	61.9
3	94.9	68.4	70.0	70.0	71.7	61.9	94.9	68.4	78.0	67.9	71.7	61.9

[a]As for Table VI.

stricted rotation of the glycerol portion of the molecule; the latter also exhibits a difference in chemical shift for the two primary carbon atoms (see Table VI).

^{13}C-Chemical shifts of α-D-galactose and α-L-fucose. — The ^{13}C-chemical shifts of α-D-galactose in the oligosaccharides examined are shown in Table VII. Of prime interest is the fact that C-1 of this sugar is strongly shielded in these compounds, and resonates at ~95 p.p.m. in the ^{13}C-n.m.r. spectra of the diacylated oligosaccharides **1–3**. Saponification of the formic and acetic esters in trisaccharide **1** results in a shift of the C-1 signal to lower field, where it is found at ~96 p.p.m. in the ^{13}C-n.m.r. spectrum of the nonacylated trisaccharide **4**; this value is still far removed from the ^{13}C shifts observed for C-1 in α-D-galactopyranosides[18,19], and must somehow be related to its attachment to D-galacturonic acid in this case. In the ^{13}C-n.m.r. spectrum of the K63 polysaccharide[3], the signal at 96 p.p.m. was previously assigned to C-1 of L-fucose. Comparison of the ^{13}C-n.m.r. resonances of the aldobiouronic acids **11** and **12** with those of the corresponding trisaccharides (**1** and **4**), shown in Tables VI–VIII, leaves little doubt that the signal is, in fact, due to C-1 of D-galactose, and not of L-fucose.

The ^{13}C-n.m.r. signal of C-2 in these compounds is also shifted to higher field, but to a much lesser extent than that of C-1. Examination of the ^{13}C shifts for C-3 and C-4 (see Table VII) in the series of diacylated oligosaccharides (**1–3**) indicates that glycoside formation at C-3 results in a downfield shift from 70 to 78 p.p.m. for this carbon atom, together with the corresponding upfield shift for the C-4 signal by from 70 to 67.9 p.p.m., in accordance with the results obtained for α-D-galacturonic acid. Resonances due to C-5 and C-6 show little variation in chemical shift.

The ^{13}C shifts of the (reducing) L-fucose residue in the oligosaccharides studied showed only small differences from those obtained for the free sugar[20]. Glycoside formation at C-1 (oligosaccharides **2** and **3**), and substitution at O-3 with D-galatosyl-

TABLE VIII

^{13}C-CHEMICAL SHIFTS FOR REDUCING AND NONREDUCING α-L-FUCOSE IN THE OLIGOSACCHARIDES FROM *Klebsiella* K63

Compound	*Chemical shift*[a]																	
	α-L-*Fucose*						β-L-*Fucose*						α-L-*Fucosyl*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
11	92.9	68.0	78.8	72.5	67.1	16.2	96.8	72.0	82.0	71.7	71.5	16.2	—	—	—	—	—	—
12	92.9	68.1	79.0	72.6	67.0	16.2	96.8	72.0	82.2	71.7	71.5	16.2	—	—	—	—	—	—
1	92.9	68.1	79.0	72.5	67.0	16.2	96.8	72.0	82.2	71.6	71.4	16.2	—	—	—	—	—	—
4	92.9	68.0	78.9	72.5	67.0	16.2	96.7	72.0	82.1	71.6	71.4	16.2	—	—	—	—	—	—
2	92.9	67.9	79.1	72.5	67.0	16.2	96.7	72.0	82.2	71.6	71.2	16.2	101.3	67.9	78.5	72.5	67.0	16.0
3	92.9	67.9	79.1	72.5	67.0	16.2	96.8	72.0	82.3	71.7	71.1	16.2	101.3	67.9	78.5	72.5	67.5	16.0

[a]As for Tables VI and VII.

uronic acid, resulted in deshielding of these carbon atoms by ~8 and 10 p.p.m., respectively. Table VIII lists the values assigned to the L-fucose and L-fucosyl residues in the oligosaccharides studied. The signal, at 16.2 p.p.m., from C-6 in the (reducing) L-fucose residue was particularly well resolved from that of *O*-acetyl (~21 p.p.m.) in the spectra of oligosaccharides **1**–**3**, and was shifted to slightly higher field (16.0) for the L-fucosyl residues of the higher oligosaccharides **2** and **3** (see Table VIII). The rest of the ring-carbon atoms (C-2, C-4, and C-5) were less affected by the changes at C-1 and C-3.

EXPERIMENTAL

General methods. — Descending paper-chromatography was performed on Whatman No. 1 paper, with the following solvent-systems (all v/v): (*A*) 2:1:1 1-butanol–acetic acid–water, (*B*) 4:1:5 (upper phase) 1-butanol–ethanol–water, (*C*) 8:2:1 ethyl acetate–pyridine–water, and (*D*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water. Chromatograms were developed by heating the papers for 5–10 min at 110° after spraying with *p*-anisidine hydrochloride in aqueous 1-butanol[21], or by developing the chromatograms with silver nitrate reagent[22]. Preparative paper-chromatography was performed with Whatman No. 3 MM paper and freshly prepared solvent *A*.

Optical rotations were measured at 23 ±2° with a Perkin–Elmer model 141 polarimeter (10-cm cell). I.r. spectra were recorded with a Perkin–Elmer 457 spectrophotometer. Unless otherwise stated, hydrolyses were conducted with 2M trifluoroacetic acid (TFA) for 18 h on a steam bath. For methylation analysis by g.l.c., sugars were converted into the corresponding alditol acetates by reduction with sodium borohydride followed by acetylation with 1:1 acetic anhydride–pyridine.

Analytical-g.l.c. separations were performed in stainless-steel columns (1.8 m × 3 mm) with a carrier-gas flow-rate of 20 mL.min^{-1}. The columns used were (*1*) 3% of OV-225 on Gas-Chrom Q (100–120 mesh) programmed for 4 min at 180° and then at 2° per min to 220°, and (*2*) 3% of OV-17 on Gas-Chrom Q (100–120 mesh), isothermal at 280°. G.l.c.–m.s. was performed with a Micromass 12 instrument fitted with a Watson–Biemann separator. Spectra were recorded at 70 eV, with an ionization current of 100 μA and an ion-source temperature of 200°.

High-resolution, proton magnetic resonance spectra were recorded at ambient temperature with a Bruker WH-400 spectrometer. The intensity of the HOD signal was lessened by performing a spin–lattice relaxation-time (T_1) experiment[14]. Proton signals obscured by the residual, HOD peak were assigned from the corresponding, low-resolution spectrum recorded at ~90° with a Bruker WP-80 spectrometer. Samples were prepared for p.m.r.-spectral study by de-ionization with Amberlite IR-120 (H^+) resin, and then "exchanged", and freeze-dried, four or five times in 99.7% D_2O. Spectra were recorded for solutions in 99.7% D_2O, with acetone [δ 2.23, measured against aqueous sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS)] as the internal standard. The ^{13}C-n.m.r. spectra were recorded with a Bruker WP-80

instrument, and were obtained at ambient temperature for solutions in 99.7% D_2O, with acetone (31.07 p.p.m. downfield from DSS) as the internal standard.

A. Depolymerization with bacteriophage φ63

Preparation of oligosaccharides. — Capsular polysaccharide from *Klebsiella* K63 was degraded with phage φ63 according to the methods previously described[8], to yield oligosaccharides **1–3**, which were isolated by preparative paper-chromatography. Proton magnetic resonance spectroscopy of the oligomers showed signals due to formate (broad singlet at δ 5.95) and acetate (sharp singlet at δ 2.13), and also indicated that L-fucose was the reducing residue in each oligosaccharide. Integration of the spectra gave equimolar proportions of formate and acetate per three-sugar repeating-unit, as shown in Table I. The homogeneity of each oligomer was confirmed by paper chromatography (solvents *A* and *D*) and by gel-permeation chromatography on Bio-Gel P-2. The molecular weights and paper-chromatographic mobilities are listed in Table I.

Hydroxamic acid test. — Hydroxamic acid derivatives were prepared by treatment of an acid hydrolyzate (0.5M TFA, 45 min) of the K63 polysaccharide with 5% hydroxylamine hydrochloride in ethanol, and compared with authentic compounds by paper chromatography in solvent *B*. Components were revealed by spraying with 10% ferric chloride solution and then drying at room temperature. *O*-Acetyl- and *O*-formyl-hydroxamic acid were detected as purple and red spots, respectively.

Deacylation. — Samples (20 mg) of oligosaccharides **1–3** were each stirred with 0.03M potassium hydroxide (2 mL) for 18 h at room temperature, acidified with Amberlite IR-120 (H^+) resin, and then freeze-dried. P.m.r. spectroscopy of the products (**4–6**) in each case indicated that the formic and acetic esters had been saponified. Similar treatment of **1–3** with 0.01M and 0.02M solutions of potassium hydroxide failed to remove either of the *O*-acyl groups.

Reduction with borohydride. — Oligosaccharides **1–3** (10 mg each) in water were reduced with sodium borohydride at pH ~14 for 1 h, and the resulting compounds were examined by p.m.r. spectroscopy, which indicated complete reduction of L-fucose, but only ~50% removal of the formate and acetate. Treatment of **1–3** with sodium borohydride at pH ~14 for 18 h yielded the reduced, nonacylated oligomers **7–9**, which were hydrolyzed, and the products analyzed by g.l.c. in column *1* according to the method of Morrison[10]. The results are shown in Table II. Reduction of the aldobiouronic acid **11** for 18 h yielded the reduced disaccharide **13**.

A sample of the diacylated trisaccharide **1** was reduced with sodium borohydride at pH ~9 for 35 min. The solution was cooled in ice, acidified with Amberlite IR-120 (H^+) resin, and the borate removed by co-distillation with methanol. The p.m.r. spectrum of the product (**10**) showed complete conversion of L-fucose into L-fucitol, without any saponification of the formic and acetic esters.

Methylation. — Where it is stated that methylation was conducted for a specified time, the time cited refers to the time period during which the substrate was exposed to the dimsyl anion. Methylation of poly- and oligo-saccharides was performed by

the method of Hakomori[11], according to the following procedure. Samples were dissolved in anhydrous dimethyl sulfoxide, stirred with 2M methylsulfinyl anion at room temperature, and then frozen by immersion of the reaction vessel in a bath containing an acetone–Dry Ice mixture. Following the addition of methyl iodide, the mixture was allowed to warm to room temperature, and was then stirred until a clear solution was obtained (~1 h). Methylated oligosaccharides were isolated by partitioning between water and chloroform; dimethyl sulfoxide was removed *in vacuo* by heating with an i.r. lamp. Methylated K63 polysaccharide was first dialyzed overnight against running tap-water, and then extracted into chloroform. Complete methylation in each reaction was indicated by the absence of hydroxyl absorption in the i.r. spectrum of the methylated product, and by methylation analysis using g.l.c.

Trisaccharide **1** was treated with dimsyl ion for 1 min, and methylated, and the products were analyzed by g.l.c. using column *2*. The gas chromatograms showed four components (absolute retention times, 7.4, 9.5, 10.0, and 11.3 min), indicating the presence both of methylated and of methylated, formylated trisaccharides; formate, but not acetate, was detected in the p.m.r. spectrum of the mixture in chloroform-*d*. Hydrolysis, followed by paper chromatography (solvent *B*) of the hydrolyzate, showed the presence of 2,3,4,6-tetra-*O*-methylgalactose and 2,4-di-*O*-methylfucose; these were reduced, the alditols acetylated, and the acetates analyzed by g.l.c. in column **1**. A low proportion of 2,4-di-*O*-methylfucose was indicated, proving incomplete hydrolysis of the aldobiouronic acid linkages in the methylated products. This behavior had also been noted in earlier studies[2].

Carboxyl-reduction of the methylated derivatives with lithium aluminum hydride in anhydrous oxolane, and g.l.c. analysis (column *2*) of the products, showed four components, having retention times of 5.4, 7.4, 8.8, and 9.4 min. Hydrolysis, and analysis of the sugars present in the hydrolyzate, by paper chromatography (solvent *B*), and by g.l.c.–m.s. of the partially methylated alditol acetates, showed 2,3,4,6-tetra-*O*-methylgalactose, 2,4-di-*O*-methylfucose, 2,4-di-*O*-methylgalactose, and 2-*O*-methylgalactose (see Table III, column B).

A sample of the carboxyl-reduced material was remethylated by using the Purdie reagents, to give two components, having g.l.c. retention-times of 6.1 and 6.5 min in column *2*. Hydrolysis, followed by paper chromatography of the hydrolyzate (solvent *B*), showed the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2,4-di-*O*-methylfucose, and 2,4,6-tri-*O*-methylgalactose. Reduction, acetylation, and g.l.c. analysis gave the proportions of sugars shown in Table III, column C.

Hexasaccharide **2** was methylated for 3 min, and the product carboxyl-reduced with lithium aluminum hydride in anhydrous oxolane. Methylation analysis of the reduced, methylated hexasaccharide gave the proportions of sugars shown in Table III, column D. A second sample was methylated for 30 min, the product carboxyl-reduced, and the alditol analyzed analogously (see Table III, column E).

Capsular polysaccharide in the acid form was methylated for 15 min, and a portion of the product was hydrolyzed. Paper chromatography of the hydrolyzate (solvent *B*) showed the presence of 2,4-di-*O*-methylfucose and 2,4,6-tri-*O*-methyl-

galactose. G.l.c. analysis of the derived alditol acetates again showed a low proportion of 2,4-di-*O*-methylfucose, indicating incomplete hydrolysis of the methylated polymer. A further portion was carboxyl-reduced, and the alditol hydrolyzed, and the sugars present in the hydrolyzate were analyzed by paper chromatography (solvent *B*) and by g.l.c. of the derived alditol acetates, to give the results shown in Table III, column G.

Enzymic hydrolysis. — Trisaccharide **1** in water was hydrolyzed with α-D-galactosidase (Sigma) for 48 h at room temperature. Paper chromatography of the hydrolyzate (solvent *C* and *D*) showed the presence of D-galactose, starting material, and a fast-moving component which was developed as a bright-pink spot on chromatograms sprayed with *p*-anisidine. This compound (14 mg) was isolated by preparative paper-chromatography, and was identified as the di-acylated aldobiouronic acid **11** by p.m.r. spectroscopy. Treatment of **11** with 0.03M potassium hydroxide for 18 h at room temperature resulted in complete saponification of the esters, to yield the aldobiouronic acid **12**.

Hydrolysis of oligosaccharides **2** and **3** with α-D-galactosidase at room temperature gave D-galactose and the corresponding penta- and octa-saccharides, as shown by paper chromatography in solvent *D* (R_{Gal} 0.28 and 0.12, respectively).

Periodate oxidation. — The diacylated trisaccharide alditol **10** (25 mg) was oxidized with 0.01M $NaIO_4$ (4 mL) in the dark for 2 h at 4°. Ethylene glycol was added, and the solution was stirred for 1 h at room temperature, reduced with sodium borohydride at pH ~9, and then acidified with Amberlite IR-120 (H^+) resin. Borate was removed by co-distillation with methanol, and the residue was hydrolyzed with M trifluoroacetic acid for 18 h at room temperature. Paper chromatography of the hydrolyzate (solvent *A*) showed the presence of glycolaldehyde, glycerol, and a slow-moving component (**14**), which was isolated by preparative paper-chromatography. Hydrolysis of a sample, and paper chromatography of the hydrolyzate (solvents *A* and *D*) showed galacturonic acid and glycerol. A portion of the hydrolyzate was acetylated with 1:1 acetic anhydride–pyridine, and the product analyzed by g.l.c. using column *1* isothermally at 165°. Glycerol triacetate was the only component detected. The ^{13}C-n.m.r. spectrum of **14** in D_2O gave signals that were in accord with the structure assigned.

B. Depolymerization with acid

Capsular polysaccharide (300 mg) was hydrolyzed with 0.5M trifluoroacetic acid on a steam bath for 45 min, the acid was removed by co-distillation with methanol, and the residue was dialyzed against distilled water (1 L) for 24 h. The dialyzate was concentrated, and then analyzed by paper chromatography (solvents *A* and *D*), which showed the presence of galacturonic acid, galactose, and fucose, together with the acylated and nonacylated oligosaccharides discussed in Part A. Gel-permeation chromatography of the mixture on Bio-Gel P-2 indicated components having mol. wts. of 1480, 986, 502, and 340. Preparative paper-chromatography permitted the isolation of the lower oligomers **1**, **4**, **11**, and **12** in pure state. The physical characteristics of these compounds were identical with those obtained earlier. The higher oligo-

mers were not isolated, but were readily identified by their mobility and color on paper chromatograms developed with *p*-anisidine.

The nondialyzable material was shown to be homogeneous by gel-permeation chromatography on Bio-Gel P-300, which gave a single peak at mol. wt. 5000. The p.m.r. spectrum of this component in D_2O indicated that $\sim 50\%$ of the formate and acetate groups had not been hydrolyzed under the conditions used to depolymerize the polysaccharide.

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