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# BACTERIOPHAGE DEGRADATION OF *Klebsiella* K44 POLYSACCHARIDE: AN N.M.R. STUDY AND CHEMICAL PROOF OF THE POSITION OF THE ACETATE GROUP*

GUY G. S. DUTTON AND D. NEDRA KARUNARATNE
*Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, B.C. V6T 1Y6 (Canada)*

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## ABSTRACT

Bacteriophages ($\phi$) have been used to degrade polysaccharides into oligosaccharides containing one or more of their repeating units. The capsular polysaccharide from *Klebsiella* K44 contains an acetate group, and n.m.r. spectroscopy and chemical methods have been employed to prove its linkage to O-6 of the 4-linked glucose residue. Phage $\phi$44 was shown to be an $\alpha$-glucosidase not influenced by the acetate moiety and thus able to depolymerize the polysaccharide into pentasaccharide repeating units, some of which contained acetate on O-6 of the reducing glucose residue. The two oligosaccharides were studied by $^{1}H$- and $^{13}C$-n.m.r. spectroscopy, and their spectra were compared with those of the native and the deacetylated polysaccharide. $^{13}C$-n.m.r. was a useful tool for locating the 6-linked acetate, the position of which was confirmed by the method of temporary protection using methyl vinyl ether. The importance of using bacteriophages to obtain oligosaccharides is highlighted by the better results obtained with the oligosaccharide in comparison to the polysaccharide, both in n.m.r. spectroscopy and the temporary protection method.

## INTRODUCTION

In recent years, bacteriophages have been used in our laboratories as a means of generating simple oligosaccharide repeating units from *Klebsiella* exopolysaccharides[1–7]. The enzymic activity of bacteriophages on several *Klebsiella* host capsules has been studied[8] and phage $\phi$44 was reported to be an endoglucanase of unknown specificity. The isolation of a single repeating unit enabled us to determine the nature of the reducing glucose residue by chemical methods and by $^{1}H$- and $^{13}C$-n.m.r. spectroscopy. We now report the isolation, by the use of $\phi$44, of the pentasaccharide repeating unit from the *Klebsiella* K44 polysaccharide of known

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structure[9], and the location of the position, hitherto unknown, of the acetate group.

## RESULTS AND DISCUSSION

*Preparation of polysaccharide.* — Capsular polysaccharide from *Klebsiella* K44 was purified as previously described[10]. Due to its very low degree of polymerization ($\overline{M}_w$ 2 × $10^5$) the polysaccharide is extremely lyophilic, and on removal of the *O*-acetate with mild base shows no observable change in viscosity. The acetate group is relatively stable in mild base (0.01M sodium hydroxide, 24 h, room temperature), but is easily removed with a stronger concentration of sodium hydroxide (0.3M) under the same conditions.

*Generation and isolation of oligosaccharides.* — Bacteriophage $\phi$44 was propagated on the host strain *Klebsiella* K44 by using a nutrient broth medium[1]. Depolymerization of the polysaccharide[2] with $\phi$44 generated two pentasaccharides, **1** and **2**, corresponding to the repeating unit of the polysaccharide. These were isolated by preparative paper chromatography.

$$\beta\text{-D-Glc}p\text{A-}(1\rightarrow2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-D-Glc}\sim\text{OH}$$
$$\begin{array}{l} 6 \\ | \\ \text{OR} \end{array}$$

**1** R = H **2** R = Ac
**3** Alditol of **1** **4** Alditol of **2**

K44 Oligosaccharides

*Quantitation of the acetate groups*[11]. — Spectrophotometric determination of the hydroxamic acid formed by the acetate, using acidic ferric chloride to generate a coloured compound, indicated that the polysaccharide contained 0.64 mole of acetate per mole of repeating unit. Oligosaccharide **2** was shown to contain one mole of acetate per mole. This was in accordance with the yields of compounds **1** (34%) and **2** (66%) isolated.

*Characterization of oligosaccharides* **1** *and* **2**. — The degree of polymerization of the oligosaccharides was determined by the reduction of each to the oligosaccharide alditol **3**, which was analyzed by the aldononitrile procedure[1,12]. The ratios of rhamnononitrile, glucononitrile, and glucitol obtained were 2:1:1, and on reduction of the uronic acid the ratio was changed to 2:2:1. Hence compounds **1** and **2** are two pentasaccharides. By reducing **2** with sodium borohydride for 30 min under mild conditions, the acetate was left intact (compound **4**). Methylation of compound **3** by the Hakomori method[13], reduction of a portion with lithium aluminum hydride, and analysis of both as the partially methylated alditol acetates revealed the compounds listed in Table I, columns I and II. The compounds obtained by

methylation of oligosaccharide **2** (see Table I, column III), when compared with those from the methylated oligosaccharide **3**, indicated the presence of a terminal glucuronic acid and a 4-linked, reducing glucose residue. The $^1$H-n.m.r. spectra of **1** and **2** showed the disappearance of an $\alpha$ signal in the polysaccharide spectrum and its replacement with a pair of lines corresponding to a reducing $\alpha$:$\beta$ ratio of 0.4:0.6. These reducing $\alpha,\beta$ signals were missing in the spectra of **3** and **4** (see Table II). This proves that bacteriophage $\phi$44 is an endo-$\alpha$-glucosidase.

*Location of the position of the acetate function.* — The native polysaccharide and oligosaccharide **2** were both treated with methyl vinyl ether[14] and the acetal-protected products were methylated by the Hakomori procedure[13] which, due to the basicity of the reaction conditions, removes the acetate function and permits methylation of the free hydroxyl position generated. On analysis as the alditol acetates, the compounds in Table III, columns I and II, were obtained. The presence of small amounts of 2-*O*-methylrhamnose and 2-*O*-methylglucose in the sample from the polysaccharide, in addition to 6-*O*-methylglucose (Fig. 1), may be attributed to decreased accessibility of the corresponding positions in the polysaccharide. These results prove that the acetyl group is attached to O-6 of one of the glucose residues.

In order to determine which of the two glucose residues is acetylated, two separate methods were employed. One method involved the removal of the protecting 1-methylethyl groups under mild acidic conditions, leaving the methyl group and the glycosidic linkages intact, and ethylating the deprotected hydroxyl groups. Since standards were not available, the native polysaccharide was ethylated[13] and converted into alditol acetates (see Table IV, column I). Thus, the acetal-protected, methylated polysaccharide was treated with 1% aqueous sulfuric acid–acetone (3:2) and the deprotected polymer was ethylated by the Hakomori procedure. The alditol acetates obtained (Table IV, column II) show that the 4-linked

TABLE I

METHYLATION ANALYSIS OF OLIGOSACCHARIDES **2** AND **3**

| *Methylated sugar*[a] *(as alditol acetate)* | *Relative retention time on OV-225*[b] | *Mole %*[c] | | |
|---|---|---|---|---|
| | | *I*[d] | *II*[d] | *III*[d] |
| 1,2,3,5,6-Glc | 0.44 | 18.7 | 12.8 | — |
| 3,4-Rha | 0.87 | 28.0 | 21.3 | 21.8 |
| 2,4-Rha | 0.94 | 30.8 | 24.2 | 23.3 |
| 2,4,6-Glc | 1.64 | 22.5 | 23.0 | 27.3 |
| 2,3,4-Glc | 1.95 | — | 18.7 | — |
| 2,3,6-Glc | 2.00 | — | — | 27.6 |

[a]1,2,3,5,6-Glc = 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methyl-*O*-glucitol, 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-glucitol on a column of 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 200° isothermal. [c]Values corrected using effective carbon response correction factors[18]. [d]I, methylated oligosaccharide **3**; II, methylated, reduced oligosaccharide **3**; III, methylated oligosaccharide **2**.

TABLE II

N M R DATA FOR *Klebsiella* K44 POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES[a]

| *Compound* | *$^1$H-N.m.r. data* | | | | *$^{13}$C-N.m.r. data* | |
|---|---|---|---|---|---|---|
| | *δ (p.p.m.)*[b] | *$J_{1,2}$ (Hz)* | *Integral (protons)* | *Assignment*[c] | *δ (p.p.m.)*[d] | *Assignment*[e] |
| **1** | 5.29 | s | 1 | $\underline{\ }^{2}\text{Rha}\underline{\ }_{\alpha}$ | 105.03 | $\text{GlcA}\underline{\ }_{\beta}$ |
| | 5.21 | 4 | 0.4 | $\underline{\ }^{4}\text{Glc}\sim_{\alpha}\text{OH}$ | 103.17<br>103.13 | $\underline{\ }^{3}\text{Glc}\underline{\ }_{\beta}$ |
| | 5.13 | s | 1 | $\underline{\ }^{3}\text{Rha}\underline{\ }_{\alpha}$ | 101.65 | $\underline{\ }^{2}\text{Rha}\underline{\ }_{\alpha}$ |
| | 4.67 | 8 | 1 | $\text{GlcA}\underline{\ }_{\beta}$ | 101.58 | $\underline{\ }^{3}\text{Rha}\underline{\ }_{\alpha}$ |
| | 4.65 | 8 | 0.6 | $\underline{\ }^{4}\text{Glc}\sim_{\beta}\text{OH}$ | 96.55 | $\underline{\ }^{4}\text{Glc}\sim_{\beta}\text{OH}$ |
| | 4.53 | 8 | 1 | $\underline{\ }^{3}\text{Glc}\underline{\ }_{\beta}$ | 92.61 | $\underline{\ }^{4}\text{Glc}\sim_{\alpha}\text{OH}$ |
| | 1.32 | 8 | 3 | $CH_3$ of Rha | 61.51 | C-6 of $\underline{\ }^{3}\text{Glc}\underline{\ }_{\beta}$ |
| | 1.28 | 8 | 3 | $CH_3$ of Rha | 61.0451 | C-6 of $\underline{\ }^{4}\text{Glc}\sim_{\beta}\text{OH}$ |
| | | | | | 60.92 | C-6 of $\underline{\ }^{4}\text{Glc}\sim_{\alpha}\text{OH}$ |
| | | | | | 17.40<br>17.33 | $CH_3$ of Rha |

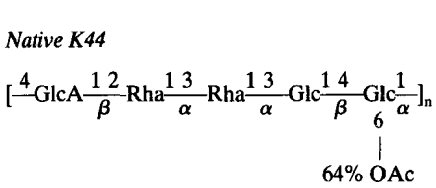
| | | | | | | |
|---|---|---|---|---|---|---|
| **2** | 5.29 | s | 1 | $\rightarrow 2)\text{-Rha}\text{-}\alpha$ | 105.04 | GlcA-$\beta$ |
| | 5.20 | 4 | 0.4 | $\rightarrow 4)\text{-Glc}\sim\text{OH}\text{-}\alpha$ | 103.39<br>103.33 | $\rightarrow 3)\text{-Glc}\text{-}\beta$ |
| | 5.12 | s | 1 | $\rightarrow 3)\text{-Rha}\text{-}\alpha$ | 101.67 | $\rightarrow 2)\text{-Rha}\text{-}\alpha$ |
| | 4.66 | 8 | 1.6 | GlcA-$\beta$, $\rightarrow 4)\text{-Glc}\sim\text{OH}\text{-}\beta$ | 101.60 | $\rightarrow 3)\text{-Rha}\text{-}\alpha$ |
| | 4.53 | 12, 2 | 1 | H-6*S* of $\rightarrow 4)\text{-Glc}\sim\sim\text{OH}\text{-}\alpha,\beta$ | | |
| | 4.47 | 8 | 1 | $\rightarrow 3)\text{-Glc}\text{-}\beta$ | 96.66 | $\rightarrow 4)\text{-Glc}\sim\text{OH}\text{-}\beta$ |
| | 4.33<br>4.29 | 12, 4<br>12, 4 | 0.5<br>0.5 | H-6*R* of $\rightarrow 4)\text{-Glc}\sim\sim\text{OH}\text{-}\alpha,\beta$ | 92.66 | $\rightarrow 4)\text{-Glc}\sim\text{OH}\text{-}\alpha$ |
| | 2.14 | 1 | 2.6 | $CH_3$ of acetate | 63.87 | C-6 of $\rightarrow 4)\text{-Glc}\sim\sim\sim\text{OH}\text{-}\alpha,\beta$ |
| | 1.32 | 8 | 3 | $CH_3$ of Rha | 61.53 | C-6 of $\rightarrow 3)\text{-Glc}\text{-}\beta$ |
| | 1.28 | 8 | 3 | $CH_3$ of Rha | 21.05 | $CH_3$ of acetate |
| | | | | | 17.41<br>17.35 | $CH_3$ of Rha |
| **3** | 5.28 | s | 1 | $\rightarrow 2)\text{-Rha}\text{-}\alpha$ | | |
| | 5.12 | s | 1 | $\rightarrow 3)\text{-Rha}\text{-}\alpha$ | | |
| | 4.68 | 8 | 1 | GlcA-$\beta$ | | |

TABLE II *(continued)*

N M R DATA FOR *Klebsiella* K44 POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES[a]

| *Compound* | *$^1$H-N.m.r. data* | | | | *$^{13}$C-N.m.r. data* | |
|---|---|---|---|---|---|---|
| | *δ (p.p.m.)*[b] | *$J_{1,2}$ (Hz)* | *Integral (protons)* | *Assignment*[c] | *δ (p.p.m.)*[d] | *Assignment*[e] |
| | 4.59 | 8 | 1 | $\xrightarrow{3}$Glc$_\beta$ | | |
| | 1.31 | 8 | 3 | $CH_3$ of Rha | | |
| | 1.28 | 8 | 3 | $CH_3$ of Rha | | |
| **4** | 5.33 | s | 1 | $\xrightarrow{2}$Rha$_\alpha$ | | |
| | 5.12 | s | 1 | $\xrightarrow{3}$Rha$_\beta$ | | |
| | 4.62 | 8 | 1 | GlcA$_\beta$ | | |
| | 4.55 | 8 | 1 | $\xrightarrow{3}$Glc$_\beta$ | | |
| | 4.46 | 12 | 1 | H-6*S* of $\xrightarrow{4}$Glucitol 6 \| OAc | | |
| | 4.25 | b | 1 | H-6*R* of $\xrightarrow{4}$Glucitol 6 \| OAc | | |
| | 2.15 | s | 3 | $CH_3$ of acetate | | |
| | 1.28 | b | 6 | $CH_3$ of Rha | | |

*Native K44*



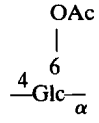
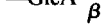
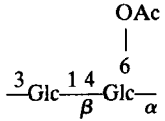
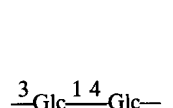
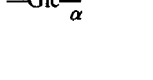
| | | | | | |
|---|---|---|---|---|---|
| 5.41 | s | 0.6 |  | 105.09 |  |
| 5.38 | s | 0.4 |  | 103.30 |  |
| 5.29 | s | 1 |  | 103.08 |  |
| 5.15 | s | 1 |  | 101.76 |  |
| 4.64 | 8 | 0.9 |  | 101.64 |  |
| 4.50 | 8 | 0.4 |  | 99.61 |  |
| 4.40 | b | 1.2 |  | 99.51 |  |
| | | |  | | |
| 2.16 | s | 1.5 | $CH_3$ of acetate | 63.44 |  |

*TABLE II (continued)*

N M R. DATA FOR *Klebsiella* K44 POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES[a]

| *Compound* | *$^1$H-N.m.r. data* | | | | *$^{13}$C-N.m.r. data* | |
|---|---|---|---|---|---|---|
| | *δ (p.p.m.)*[b] | *$J_{1,2}$ (Hz)* | *Integral (protons)* | *Assignment*[c] | *δ (p.p.m.)*[d] | *Assignment*[e] |
| | 1.32 | 8 | 3 | $CH_3$ of Rha | 61.60 | C-6 of $\xrightarrow{3}$Glc$_\beta$ |
| | 1.28 | 8 | 3 | $CH_3$ of Rha | 60.39 | C-6 of $\xrightarrow{4}$Glc$_\alpha$ |
| | | | | | 21.10 | $CH_3$ of acetate |
| | | | | | 17.44 | $CH_3$ of Rha |
| | | | | | 17.37 | $CH_3$ of Rha |
| *Deacetylated K44* | | | | | | |
| $[\xrightarrow{4}$GlcA$\beta\xrightarrow{1\,2}$Rha$\alpha\xrightarrow{1\,3}$Rha$\alpha\xrightarrow{1\,3}$Glc$\beta\xrightarrow{1\,4}$Glc$\alpha\xrightarrow{1}]_n$ | 5.37 | s | 1 | $\xrightarrow{4}$Glc$_\alpha$ | 105.08 | $\xrightarrow{4}$GlcA$_\beta$ |
| | 5.29 | s | 1 | $\xrightarrow{2}$Rha$_\alpha$ | 103.15 | $\xrightarrow{3}$Glc$_\beta$ |
| | 5.15 | s | 1 | $\xrightarrow{3}$Rha$_\alpha$ | 101.82 | $\xrightarrow{2}$Rha$_\alpha$ |
| | 4.63 | b | 1 | $\xrightarrow{4}$GlcA$_\beta$ | 101.57 | $\xrightarrow{3}$Rha$_\alpha$ |
| | 4.50 | b | 1 | $\xrightarrow{3}$Glc$_\beta$ | 99.16 | $\xrightarrow{4}$Glc$_\alpha$ |
| | 1.32 | b | 3 | $CH_3$ of Rha | 61.62 | C-6 of $\xrightarrow{3}$Glc$_\beta$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 1.28 | b | 3 | $CH_3$ of Rha | 60.60 | C-6 of $\rightarrow 4)$-Glc-$(\alpha 1\rightarrow$ |
| | | | | | 17.42 | $CH_3$ of Rha |
| | | | | | 17.32 | $CH_3$ of Rha |

[a]For the sources of **1, 2, 3,** and **4,** see text. [b]Chemical shift relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), measured from internal acetone, $\delta$ 2.23. [c]The numerical prefix indicates the position at which the sugar is substituted; $\alpha$ or $\beta$, the configuration of the glycosidic bond, or the anomer in the case of a reducing terminal sugar residue. Thus $\rightarrow 2)$-Rha-$(\alpha$ refers to the anomeric proton of a 2-linked glycosyl residue in the $\alpha$-anomeric configuration. The absence of a numerical prefix indicates a nonreducing terminal group. [d]Chemical shift relative to DSS, measured from internal acetone, $\delta$ 31.07 p.p.m. [e]As in *c* but for $^{13}C$ nuclei.

TABLE III

SUGAR ANALYSIS OF THE ACETAL-PROTECTED, METHYLATED POLYSACCHARIDE AND OLIGOSACCHARIDE **2**

| *Alditol acetate*[a] | *Relative retention time*[b] | *Mole %* | | |
|---|---|---|---|---|
| | | *I*[c] | *II*[c] | *III*[c] |
| 2-Rha | 0.22 | 2.2 | — | — |
| Rha | 0.28 | 45.8 | 42.3 | 44.4 |
| 6-Glc(1-*d*)[d] | 0.54 | — | — | 27.8 |
| 6-Glc | 0.58 | 17.7 | 20.3 | — |
| 2-Glc | 0.70 | 9.3 | — | — |
| Glc | 1.00 | 25.0 | 37.3 | 27.8 |

[a]2-Rha = 2-*O*-methyl-1,3,4,5-tetra-*O*-acetyl-L-rhamnitol, etc. [b]Retention time relative to that of glucitol hexaacetate on a column of 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 220° isothermal. [c]I, polysaccharide; II, oligosaccharide **2**; III, deuterium-labelled oligosaccharide **2**. [d]6-*O*-Methyl-1,2,3,4,5-penta-*O*-acetyl[1-$^2$H]-D-glucitol

glucose contained a 6-*O*-methyl group but the 3-linked glucose was fully ethylated (proved by g.c.-m.s.[15]). The acetal-protected, methylated oligosaccharide was also deprotected in the same manner and divided into two portions. One was reduced with sodium borohydride to its oligosaccharide alditol and then ethylated as before. The alditol acetates obtained (Table IV, column III) showed the 3-linked glucose to be present as its ethylated derivative, but the 4-linked glucose, at the reducing end, had a methyl group at position 6. The second method involved reducing the remaining portion of the deprotected oligosaccharide with sodium borodeuteride in order to label the reducing glucose residue. Hydrolysis with 2M trifluoroacetic acid and g.c.-m.s. of the alditol acetates (see Table III, column III) proved that only the 6-*O*-methylglucitol contained deuterium, while glucitol was unlabelled. All these experiments confirm that the methyl group is on O-6 of the 4-linked glu-

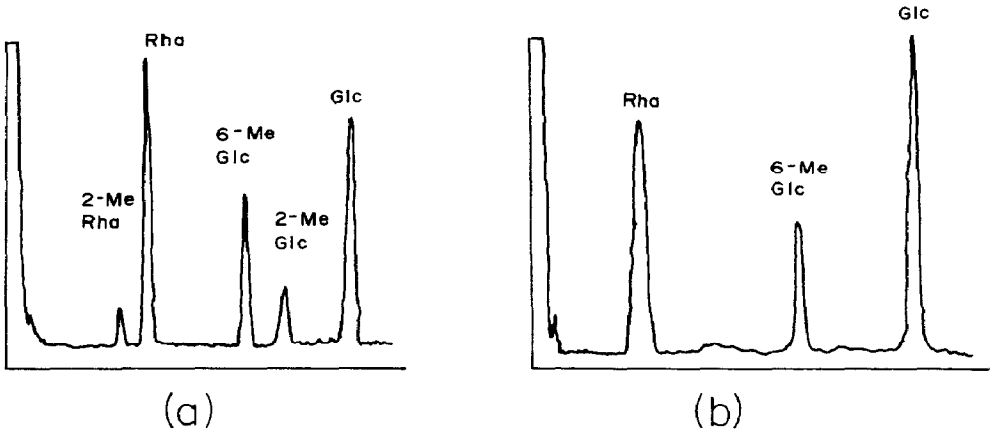



Fig. 1. Gas-liquid chromatogram of methylated alditol acetates derived from (a) K44 polysaccharide, (b) pentasaccharide obtained by action of the bacteriophage.

TABLE IV

ETHYLATION ANALYSIS OF NATIVE K44 POLYSACCHARIDE AND THE 6-*O*-METHYL POLYSACCHARIDE AND OLIGOSACCHARIDE

| *Ethylated sugar*[a] *(as alditol acetate)* | *Relative retention time on OV-225*[b] | *Mole %*[c] | | |
|---|---|---|---|---|
| | | *I*[d] | *II*[d] | *III*[d] |
| 3,4-Et-Rha | 0.85 | 56.9 | 43.9 | 22.2 |
| 2,4-Et-Rha | 0.89 | | | 31.0 |
| 2,4,6-Et-Glc | 1.58 | 12.3 | 25.4 | 32.3 |
| 2,3,6-Et-Glc | 1.88 | 30.8 | — | — |
| 2,3-Et-6-Me-Glc | 1.81 | — | 30.7 | — |
| 1,2,3,5-Et-6-Me-Glc | 0.46 | — | — | 14.5 |

[a]3,4-Et-Rha = 3,4-di-*O*-ethyl-1,2,5-tri-*O*-acetyl-6-rhamnitol. [b]Retention time of partially alkylated alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a column of 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 200° isothermal. [c]Values corrected using effective carbon response correction factors[18]. [d]I, native K44 polysaccharide; II, acetal-protected, methylated polysaccharide; III, acetal-protected, methylated, deprotected, $NaBH_4$-reduced oligosaccharide **2**.

cose, which is also the reducing sugar, and prove the structure of oligosaccharide **2**.

*$^1$H-N.m.r.* — The $^1$H-n.m.r. spectra of the native polysaccharide (sodium salt) and the deacetylated polysaccharide were not as well resolved as those of oligosaccharides **1** and **2** (see Table II). The twinning of the signals for H-1 of α- and β-glucose in the spectrum of the native polysaccharide is assumed to be due to the partial nature of the acetyl substitution, since multiple signals are not evident in the spectrum of the deacetylated polysaccharide. There is no twinning in the spectra of **1, 2, 3** or **4** because these compounds are either totally devoid of acetate or have their acetate groups 100% intact. Two doublets of doublets in the range δ 4.27–4.35 (see Table II), observed in the spectrum of oligosaccharide **2**, were assigned to H-6*R* of the reducing glucose, which is acetylated at the 6-position[16]. The doublet of doublets at δ 4.53 was assigned to H-6*S* of the reducing glucose. These signals are not observed in **1, 3,** or the deacetylated polysaccharide, but a broad doublet at δ 4.46 and a broader signal at δ 4.25 in the spectrum of **4** arise from H-6*S* and H-6*R* of the glucitol residue acetylated at O-6. A similar pattern is also seen in the spectrum of the native polysaccharide. The twinning of the acetate signal, observed only for compound **2**, is probably due to the fact that this group is located on the reducing residue, and is thus influenced by the α/β mutarotational equilibrium.

*$^{13}$C-N.m.r.* — Since the C-6 signals of hexoses appear upfield from the ring carbon region, the shift towards lower field caused by the 6-acetate is clearly observed (Fig. 2). Again, the partial nature of the acetyl substitution in the polysaccharide is obvious from the two signals obtained for C-6 of the α-glucosyl residue at 60.39 and 63.44 p.p.m. (Fig. 2a), which collapse to one signal at 60.60 p.p.m. on deacetylation (Fig. 2b). The anomeric signals of β-glucose and α-glucose both show twinning, which disappears on deacetylation and hence may be attributed to the presence of acetate[17].

The spectra of the oligosaccharides **1** and **2** show the shift of the C-6 signals of the reducing glucose residue from 60.92 ($\alpha$) and 61.04 ($\beta$) to 63.87 p.p.m. on acetylation, whereas the C-6 signal of the interior $\beta$-glucose unit remains virtually unchanged (61.51 and 61.53 p.p.m.). The anomeric signal of the $\beta$-glucose exhibits a twinning that does not disappear on deacetylation and hence was thought to be caused by close proximity to the reducing end. Comparison of the anomeric signals

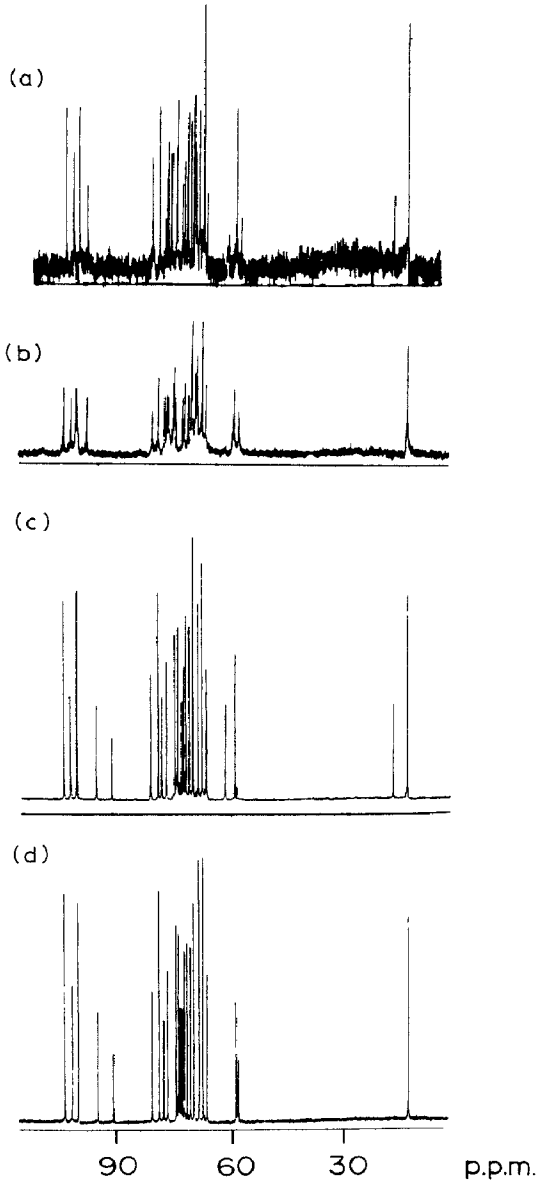



Fig. 2. The $^{13}$C-n.m.r. spectra of (a) native K44 polysaccharide, (b) deacetylated polysaccharide, (c) oligosaccharide **2**, (d) oligosaccharide **1**.

of **1** and **2** showed the downfield shifts of 0.3 p.p.m. for β-glucose (interior), 0.11 p.p.m. for β-glucose~OH, and 0.05 p.p.m. for α-glucose~OH, caused by the presence of acetate, to be similar to those of the twinned anomeric signals in the native polysaccharide. Twinning due to the presence of acetate is also observed to a small degree in the ring carbon region of the spectrum of the native polysaccharide.

*Conclusions.* — The generation of simple oligosaccharides by using bacteriophage was extremely useful in two ways. Firstly sharper and clearer n.m.r. spectra were obtained; secondly, and more importantly, two oligosaccharides were produced, one unsubstituted and one acetylated at position 6 of the reducing glucose. From the yield of the oligosaccharides obtained, it is reasonable to conclude that φ44 is an α-glucosidase insensitive to the presence of acetate at O-6.

## EXPERIMENTAL

*General methods.* — All the instrumentation used has been described previously[19]. Descending paper chromatography was performed on Whatman No. 1 paper, with the following solvent systems (ratios are v/v): (1) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water, (2) 2:1:1 1-butanol–acetic acid–water, (3) 8:2:1 ethyl acetate–pyridine–water, and (4) 4:1:5 1-butanol–ethanol–water (upper phase). Chromatograms were visualized with silver nitrate, or by spraying with *p*-anisidine hydrochloride in aqueous 1-butanol and heating the papers for 5–10 min at 110°. Preparative paper chromatography was performed by the descending method, using Whatman No. 1 paper and solvent 2. 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. Columns used were: (A) 3% of SP-2340 on Supelcoport (100–120 mesh) and (B) 3% of OV-225 on Gas Chrom Q (100–120 mesh). A Pye Unicam PU8800 u.v./vis. spectrophotometer was used for absorbance measurements. G.c.-m.s. was carried out on a Nermag R 10-10 mass spectrometer using capillary columns coated with (a) DB-225 or (b) SE-30.

*Isolation of the polysaccharide.* — A culture of *Klebsiella* K44 was grown and harvested by the usual procedure[10]. The acidic polysaccharide was isolated by one precipitation with Cetavlon. The molecular weight was found to be $2 \times 10^5$ by gel permeation chromatography on a column of Sephadex 4B (courtesy of Dr. S. C. Churms, University of Cape Town, South Africa).

*Propagation of bacteriophage and depolymerization.* — Bacteriophage φ44 was propagated on the host *Klebsiella* serotype K44 in the usual way[1,20]. The K44 capsular polysaccharide (1.0 g) was degraded with the bacteriophage φ44 according to the method previously described[2]. Oligosaccharides **1** and **2** were isolated by preparative paper chromatography in solvent 2 for 57 h. The yields of **1** and **2** obtained were 83.2 mg and 176.8 mg, respectively.

*Determination of acetate content*[11]. — To the acetate containing solution in sodium acetate buffer 2M hydroxylamine hydrochloride (1.0 mL) and 3.5M NaOH (1.0 mL) were added, and the mixture was swirled on a vortex mixer and left stand-

ing at room temperature for 2 min. Then one part of conc. HCl in two parts of water (1.0 mL) was added, the mixture swirled, 0.37M $FeCl_3 \cdot 6H_2O$ in 0.1M HCl (1.0 mL) was added, and the mixture swirled again. The purple-brown colour was measured immediately against a reagent blank at 540 nm.

*Deacetylation of the native polysaccharide.* — The K44 polysaccharide (54 mg) was treated with 0.3M NaOH (20 mL), the solution was stirred at room temperature for 2 d, then dialyzed and freeze dried. Removal of the acetate group was confirmed by both $^{13}C$- and $^{1}H$-n.m.r.

*Treatment with methyl vinyl ether*[14]. — A dried sample of polysaccharide or oligosaccharide was dissolved in dimethyl sulfoxide, together with *p*-toluenesulfonic acid, the vessel was sealed and flushed with $N_2$, and the solution was left stirring overnight. Methyl vinyl ether was introduced in two aliquots (3 mL each) at −60°, with stirring for 4 h each time. The product was purified on a column of Sephadex LH-20 by elution with acetone.

*Methylation of the acetal-protected derivatives.* — The protected sample was dissolved in dimethyl sulfoxide and treated with methylsulfinylmethyl carbanion for 2 h. Iodomethane was added to the cooled solution, which was then stirred for 1 h and extracted with $CHCl_3$. The product was purified on Sephadex LH-20 with methanol as the eluent.

*Removal of the acetal protection.* — The methylated, protected product was dissolved in 2:3 acetone–1% aqueous $H_2SO_4$ and left stirring for 24 h at room temperature. The acid was neutralized with 2M sodium hydroxide and the solution was evaporated to dryness. The residue was dissolved in dimethyl sulfoxide, and the solution was filtered and evaporated to yield the product.

*Ethylation of the methylated, deprotected polysaccharide.* — The ethylation of the native polysaccharide and the methylated deprotected polysaccharide was performed as follows. The sample was dissolved in dimethyl sulfoxide under nitrogen, methylsulfinylmethyl carbanion was added, and the solution was stirred for 3.5 h. Iodoethane was added to the cooled solution, which was stirred a further 1 h. The reaction mixture was dialyzed overnight, then the product was extracted into chloroform, hydrolyzed with 2M trifluoroacetic acid, and converted into alditol acetates in the usual way.

*Treatment of the methylated, deprotected oligosaccharide.* — (*a*) One portion was reduced with sodium borodeuteride, the solution was deionized with IR 120-$H^+$ and evaporated to dryness, and methanol was evaporated from the residue. The deuterium-labelled oligosaccharide alditol was hydrolyzed with 2M trifloroacetic acid and transformed into a mixture of peracetylated alditols. G.l.c. was carried out on OV-225 at 220° isothermally, and g.c.-m.s. on a DB-225 capillary column programmed at 150° for 1 min, then increasing 10°/min to 220°.

(*b*) The remainder was reduced with $NaBH_4$, ethylated as described above, and extracted into chloroform prior to dialysis. The product was hydrolyzed with 2M trifluoroacetic acid and converted into the perethylated alditol acetates, which were analyzed by g.l.c. on OV-225 at 200° isothermally and by g.c.-m.s. on column b, programmed at 80° for 1 min, then increasing 5°/min to 250°.

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