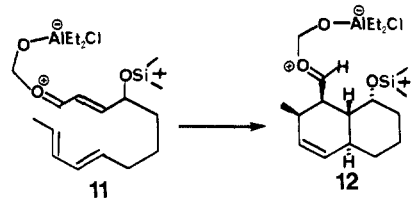
tramolecular [4 + 2] cycloaddition[17] or Lewis acid-mediated ring opening of the dioxin to produce oxonium ion **11** which cyclizes to **12** and then ejects complexed formaldehyde to afford cycloadduct **6i**.



In summation, we have shown that the anion **4** represents an improvement in β-acyl vinyl anion (**1**) methodology since the desired α,β-unsaturated aldehydes can be generated under exceptionally mild conditions. This methodology can be extended by using dioxins **6** as metalation substrates. Moreover, the carbon–carbon double bond of substituted dioxins participate in stereoselective hydrogenation, oxidation, cycloaddition reactions, etc. These investigations will be reported in due course.

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(17) For an investigation of the catalyzed intramolecular Diels–Alder cyclizations of conjugated aldehydes, see: (a) Marshall, J. A.; Audia, J. E.; Grote, J. *J. Org. Chem.* **1986**, *51*, 1157. (b) Marshall, J. A.; Audia, J. E.; Grote, J.; Shearer, B. G. *Tetrahedron* **1986**, *42*, 2893. (c) Marshall, J. A.; Audia, J. E.; Grote, J. *J. Org. Chem.* **1984**, *49*, 5277.

A General Strategy for the Chemical Sequencing of Polysaccharides

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The sequencing of polysaccharides is more difficult than for proteins or nucleic acids because one must not only establish the identities and sequence of the monomers but also their ring forms, position(s) of linkage, and anomeric configurations as well. We recently described[1-10] a new method for the simultaneous determination of ring forms and positions of linkage which we refer to as the reductive cleavage method. The salient feature of this method, reductive cleavage of the glycosidic carbon–oxygen bonds in a fully methylated polysaccharide, gives rise to partially methylated *anhydroalditols* which are subsequently analyzed as their acetyl derivatives by gas–liquid chromatography–mass spectrometry (GC/MS). Depending upon the catalyst employed, either *total* or *selective* reductive cleavage[3,5,7-9] can be accomplished. The former serves to identify the monomeric species of the polymer, whereas the latter, which gives rise to small oligomers, can potentially be used to establish the sequence of the polymer and the configurations of selected glycosidic linkages.[5,7,9]

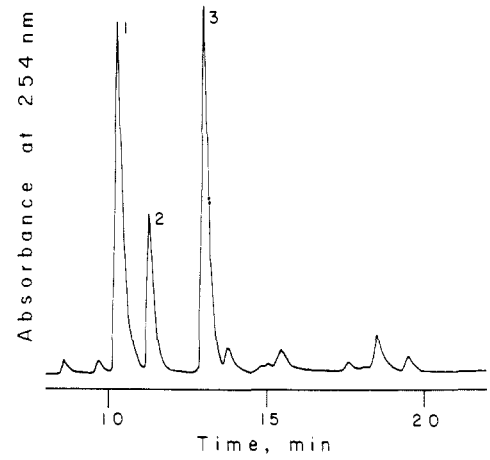

Figure 1. HPLC on a column (9.2 mm × 25 cm) of DuPont Zorbax ODS of the product obtained by $Me_3SiOSO_2CF_3$-catalyzed reductive cleavage and in situ benzoylation of the permethylated and reduced ($LiAlH_4$) K2 polysaccharide. The column was eluted with a 20-min gradient (curve 3, Waters Associates Model 660 solvent programmer) from 40% MeCN in H_2O to 80% MeCN at a flow rate of 3 mL/min.

The identification of monomeric cleavage products, however, requires comparison of their mass spectra and GC retention times to those of synthetic standards. During the course of our synthetic studies it became apparent that products of this type were readily identified from their ^1H NMR spectra, due to the fact that a different pattern of couplings between ring protons is observed for the various configurational isomers. Oligomeric cleavage products, if isolated from the reaction mixtures,[5] could also be characterized by ^1H NMR spectroscopy in order to establish the configurations of intact glycosidic linkages and, in some cases, sequence.[5,9]

The foregoing studies therefore suggested an integrated approach to the structural characterization of polysaccharides involving both total- and selective-reductive cleavage, separation of the products by high performance liquid chromatography (HPLC), and characterization of the latter by chemical ionization mass spectrometry (CIMS), ^1H NMR spectroscopy, and, where necessary, further chemical sequencing. The polysaccharide chosen to demonstrate the feasibility of this approach, the *Klebsiella* K2 capsular polysaccharide, is well characterized[11,12] and serves as a good model for the structural complexities that can be encountered.

Shown in Figure 1 is the HPLC profile of the product obtained when the methylated and reduced ($LiAlH_4$)[13] polysaccharide was subjected to $Me_3SiOSO_2CF_3$-catalyzed reductive cleavage and in situ benzoylation (benzoic anhydride).[14] The numbered peaks were identified as methylated/benzoylated anhydroalditols by CIMS (NH_3, positive) and were characterized by 300 MHz ^1H–^1H COSY NMR spectroscopy as solutions in deuteriochloroform.

†On leave from Kangweon National University, Korea.

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(13) GC/MS analysis of the anhydroalditol acetates derived by reductive cleavage of the permethylated polysaccharide had demonstrated that a uronic acid was present (ref 9).
(14) After workup in the usual way (ref 5), the product was applied to a column of silica gel in chloroform. Elution with chloroform to remove benzoic acid followed by 5% methanol in chloroform afforded the mixed benzoyl esters.

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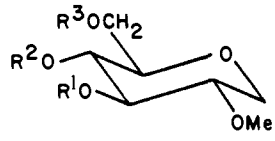
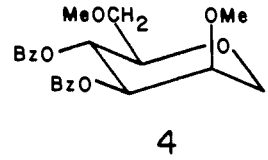
Structural assignments were made based upon a straightforward analysis of the multiplicities and coupling constants of ring proton resonances. Peak 1 was identified by ^{1}H NMR as a 1:1 mixture of 1,5-anhydro-3-*O*-benzoyl-2,4,6-tri-*O*-methyl-D-glucitol (**1**) and 1,5-anhydro-4-*O*-benzoyl-2,3,6-tri-*O*-methyl-D-glucitol (**2**), and these assignments were confirmed with pure samples of **1** and **2** isolated from the reductive cleavage products of laminarin and pullulan, respectively. Peak 2, which was absent when the unreduced polymer was analyzed, was identified as 1,5-anhydro-6-*O*-benzoyl-2,3,4-tri-*O*-methyl-D-glucitol (**3**). The remaining major component (peak 3, Figure 1) was identified as 1,5-anhydro-3,4-di-*O*-benzoyl-2,6-di-*O*-methyl-D-mannitol (**4**). Integration of Figure 1 established that compounds **1**–**4** were present in relative proportions of 1.00:0.93:0.76:0.90, respectively. This experiment therefore established the presence of a repeating unit containing 3-linked- and 4-linked-D-glucopyranosyl residues, a 3,4-linked-D-mannopyranosyl residue, and a terminal (nonreducing) D-glucopyranosyluronic group.





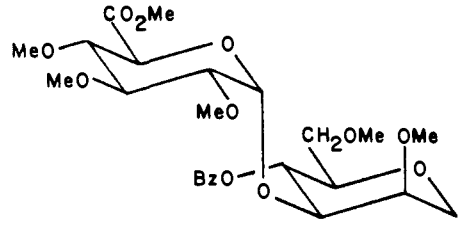
4

1, R^1 = Bz, R^2 = R^3 = Me

2, R^2 = Bz, R^1 = R^3 = Me

3, R^3 = Bz, R^1 = R^2 = Me

The sequence of the repeating unit was established by selective reductive cleavage. The HPLC profile of the benzoylated product obtained by BF_3·Et_2O-catalyzed[5] reductive cleavage was similar to that shown in Figure 1, except for the absence of peak 2 and the presence of two new components at elution times of 10.0 and 11.0 min. The component at 10 min was characterized as **5** on



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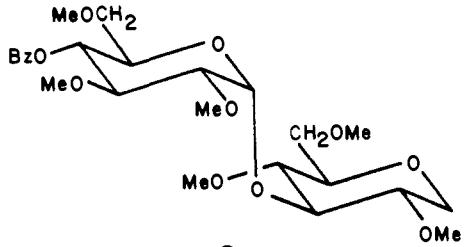

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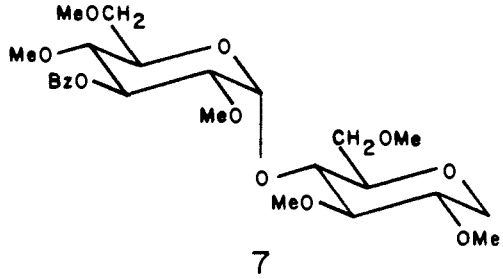

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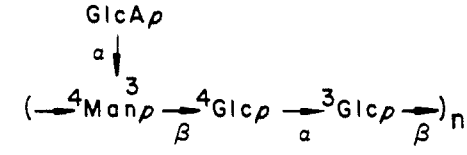
the basis of CIMS, which established its molecular weight as 528 and its ^{1}H NMR spectrum. Importantly, the magnitude of the coupling constant for the anomeric proton resonance established that the linkage was α, whereas the multiplicity of the ester methine resonance established that the benzoyl ester was present at *O*-4 of the 1,5-anhydro-D-mannitol residue. The methylated α-D-glucopyranosyluronic residue must therefore be linked to the anhydromannitol residue at *O*-3. The component at 11.0 min was identified as **6** by CIMS, ^{1}H NMR, and *chemical sequencing*. Its CI mass spectrum identified it as a mono-*O*-benzoylhexa-*O*-methylhexosylanhydrohexitol, and its ^{1}H NMR spectrum established its composition and the configuration (α) of the glycosidic linkage. The ^{1}H NMR spectrum could not distinguish between isomeric structures **6** and **7**, however, due to the fact that both would give rise to an ester methine resonance (dd or t) displaying trans-diaxial couplings. That this component was actually **6** was established by subjecting it to $Me_3SiOSO_2CF_3$-catalyzed reductive cleavage in the presence of *deuteriotriethylsilane* with subsequent in situ benzoylation. GC/MS analysis revealed the presence of **1** and **2** in a 1:1 ratio, and CIMS demonstrated that **2** was deuteriated.

From the combined data, the structure of the repeating unit is known except for the anomeric configurations of the two glycosidic linkages that were completely cleaved in the BF_3·Et_2O-catalyzed reaction. The remaining configurations were established through a comparison of the ^{1}H NMR spectra of **5**, **6**, and the fully methylated polysaccharide. The spectrum of the methylated polysaccharide displayed two axial (δ 4.33, 4.70) and two equatorial (δ 5.14, 5.32) anomeric proton resonances (br singlets), and since the latter were accounted for in the spectra of **5** (δ 5.00) and **6** (δ 5.45), the anomeric proton resonances at δ 4.33 and 4.70 can only be attributed to the remaining two β-linked residues. These experiments therefore define the complete structure of the repeating unit **8** in the K2 polysaccharide.



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The strategy for the chemical sequencing of polysaccharides that is exemplified herein is substantially different from others which involve separate determinations for composition, ring forms and positions of linkage, anomeric configurations, and sequence.[15] Although not as sensitive as "standard" methylation analysis, wherein partially methylated alditol acetates are analyzed by GC/MS, the method is applicable to most structural problems. In the present study, 50–100 μg quantities of reductive cleavage fragments were analyzed by ^{1}H NMR, but it should be possible to routinely analyze 15–25 μg of material at 500 MHz. In contrast to standard methylation analysis,[16] however, this method yields the identity of each parent sugar, making the synthesis of authentic standards or interpretation of mass spectral fragmentation patterns unnecessary.

Acknowledgment. This research was supported by NIH Grant GM34710.

Supplementary Material Available: Spectroscopic data for compounds **1**–**6** and brief comments on their interpretation (2 pages). Ordering information is given on any current masthead page.

(15) A random fragmentation method for sequencing, involving partial hydrolysis of a fully methylated polysaccharide and subsequent reduction, ethylation, separation (HPLC), and characterization (^{1}H NMR, MS) of the complex mixture of resultant oligomers, has previously been proposed (see: Valent, B. S.; Darvill, A. G.; McNeil, M.; Robertsen, B. K.; Albersheim, P. *Carbohydr. Res.* **1980**, *79*, 165–192). A similar scheme for sequencing oligosaccharides, based upon partial reductive cleavage, has also been proposed (see: Reinhold, V. N.; Coles, E.; Carr, S. A. *J. Carbohydr. Chem.* **1983**, *2*, 1–18). In the latter case, analysis of the resultant oligomers was accomplished solely by MS.

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