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Carbohydrate Research 319 (1999) 141–147

CARBOHYDRATE RESEARCH

Ozonolytic depolymerization of polysaccharides in aqueous solution

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Received 10 March 1999; accepted 29 April 1999

Abstract

The selective oxidation of β-D-glycosidic linkages of polysaccharides by ozone has great utility as a general method for depolymerization of polysaccharides. Here we describe a 'one-step' method whereby polysaccharides dissolved in water or basic solutions are depolymerized by ozonolysis. The oxidation of glycosidic linkages of unprotected carbohydrates by ozone is complicated by several side reactions. We describe here optimized conditions for carrying out ozonolysis degradation. We also characterize the major pathways for unwanted degradation by various side reactions. In the preferred oxidation pathway, the aldosidic linkage is oxidized to an aldonic ester function that hydrolyzes under the basic conditions employed to give a free aldonate, with cleavage of the polysaccharide chain. Nonselective degradation pathways include oxidative degradation by radical species that oxidize glycosyl residues to formic, acetic, and oxalic acids. The nonselective degradation caused by acids is minimized by basic buffers. The products of polysaccharide depolymerization form a size distribution around a nominal molecular weight, and the average molecular weight of the products can be controlled by the rate or amount of ozone passed through the reaction mixture. The ozonolysis method described herein provides a convenient, inexpensive, and controllable means for generating small polysaccharides or large oligosaccharide fragments. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Ozonolysis; Polysaccharide; Depolymerization; Group B *Streptococcus*

1. Introduction

Polysaccharides, abundant in nature, have broad applications ranging from specialty pharmaceuticals to commodity chemicals. The size or degree of polymerization of these molecules plays a critical role in determining their application. Native polysaccharides of large molecular size may have limited applications since they tend to be insoluble or highly viscous in solution. A decrease in molecular size, which is associated with a decrease in solution viscosity, can substantially enhance their utility. For example, we have been developing glycoconjugate vaccines against group B *Streptococcus* (GBS)—the leading cause of neonatal sepsis and meningitis [1–3]—for which depolymerized large polysaccharides are desired. These vaccines are prepared by cross-linking GBS capsular polysaccharides to protein carriers [4,5]. Some GBS polysaccharides are too large (with molecular sizes of up

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PII: S0008-6215(99)00113-5

to millions of daltons) and form insoluble gels when cross-linked to proteins. Generating smaller polymer or oligomer fragments that retain the parental repeating unit from large polysaccharides is therefore critical to the development of a myriad of vaccines.

Nine types of GBS polysaccharides have been identified thus far [6–8]. All are branched polymers that have complex repeating units composed mostly of glucose, galactose, and *N*-acetylglucosamine with β-D-linkages and sialic acid at the end of side chains with an α-D-ketosidic linkage. The sialic acid residues are crucial to the immunogenicity of the oligo- or polysaccharides and thus must be retained in fragments to be used in vaccine formulation. Sialic acids are highly susceptible to acid hydrolysis; however, most chemical methods are not suitable for depolymerizing GBS polysaccharides without removal of the sialic acids and destroying the antigenicity of the polysaccharides. Deslongchamps et al. have reported that ozone can selectively oxidize small alkyl β-D-aldosides to aldonic esters [9–12]. Since the backbones of all GBS polysaccharides contain β-D-aldosidic linkages, we explored ozonolysis as a general method of depolymerization [13].

We have previously developed a method utilizing ozone to selectively depolymerize polysaccharides that contain β-D-aldosidic linkages [13]. In this method, polysaccharides were protected by peracetylation and then treated with ozone in organic solvents. This procedure was designed to protect the substrate from unwanted side reactions that can occur in aqueous or polar protic solvents and to ensure that the products have a high degree of chemical integrity. This method is based on the selective ozonolytic oxidation of β-D-aldosides. As proposed previously [9–13], ozonolysis proceeds under strong stereoelectronic control and only if the aldoside can assume a conformation in which the acetal function at the anomeric center has two of its lone-pair electron orbitals oriented trans-antiperiplanar to the alkylidene C–H bond. This conformation is possible for β-D-aldosides in the $^4C_1$ conformation.

Here we report a simplified ozonolysis method that can be performed in aqueous solution in 'one step' and in 'one pot'. In this one-step method, unprotected polysaccharides are treated with ozone directly in aqueous solution to rapidly yield smaller fragments. The previous method of ozonolyzing derivatized polysaccharides in organic solvent is very selective in cleaving β-D-aldosidic linkages, and the depolymerizing products varied in size in such a way that repeating units of various lengths could be isolated [13]. In the present case, however, the oligomer products formed a size distribution about a specific nominal molecular weight. This relatively low selectivity can be attributed to the side reactions accompanying the ozonolysis procedure, particularly the formation of organic acids that cause nonselective degradation.

2. Results and discussion

We have applied this one-step method to the depolymerization of several polysaccharides, including type Ia [6], III [7], and V [8] GBS polysaccharides, and the type 14 capsule of *Streptococcus pneumoniae* [14] (Fig. 1); all results were similar. In a typical example, a sample of the type V GBS polysaccharide (9 mg) was dissolved in 0.1 M $NaHCO_3$ (5 mL, pH 8.6), through which ozone was bubbled at a flow rate of 3 mL/min (0.12 mmol/min) for a total of 60 min. The pH of the reaction solution decreased slightly during the reaction, and occasional adjustment of pH was attempted by titration with 0.1 M NaOH. At various intervals, aliquots (20 μL) of the reaction mixture were applied to a Superose 12 gel-filtration column to monitor the molecular size of the product. The product sizes decreased rapidly (Fig. 2), going from 150 kDa to 113, 74, 41, 32, and 25 kDa after 12, 21, 32, 45, and 60 min, respectively. The kinetics from depolymerizing type III GBS polysaccharides are similar, as shown in Fig. 2. After purification on a gel-filtration column, a fragment pool with average molecular size of 10 kDa from depolymerizing type III GBS polysaccharides was analyzed by ^{1}H NMR spectroscopy. The NMR spectra (Fig. 3) indicated that fragments retained the structure of the polymer and that the sialic acid residue was

not destroyed by ozonolysis. These results demonstrate that ozonolysis can be carried out in basic solutions to rapidly depolymerize polysaccharides that contain β-D-aldosides, without affecting acid-labile residues.

The predominant degradation mechanism is the selective oxidation of β-D-glycosides by ozone to form aldonic acid esters that are spontaneously saponified to yield smaller fragments (Scheme 1). The basis for selectivity has been suggested before [13]. The ozonolytic oxidation of aldosides proceeds under strong stereoelectronic control and prefers the aglycone conformation, in which each oxygen has one of its lone-pair orbitals antiperiplanar to the alkylidene C–H bond. Therefore, glycosidic linkages with different conformations can have different reaction rates with ozone, allowing for selectivity in cleaving β-D-linkages of polysaccharides.

Since acids are slowly formed during ozonolysis in aqueous solution, some side reactions can occur. To identify these side reactions, we examined the reaction of ozone with cellobiose [4-*O*-(β-D-glucopyranosyl)-β-D-glucopyranose] and lactose [4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranose] in water. The disaccharide solutions gradually became acidic as ozonolysis proceeded. TLC analyses indicated that both cellobiose and lactose were degraded to several smaller fragments. After ozone treatment of a sample of cellobiose (5 mg) in water (5 mL), an aliquot (20 μL) of reaction mixture was dried, and the products were converted to their trimethylsilyl derivatives. GC–MS analysis of these derivatives revealed that the products of cellobiose ozonolysis were glucopyranose, acetic acid, and oxalic acid. ^{1}H NMR analysis of the reaction mixture also indicated the formation of formic acid, as evidenced by a singlet resonance at 8.1 ppm (data not shown).

The formation of these organic acids suggests that the reaction most likely proceeded

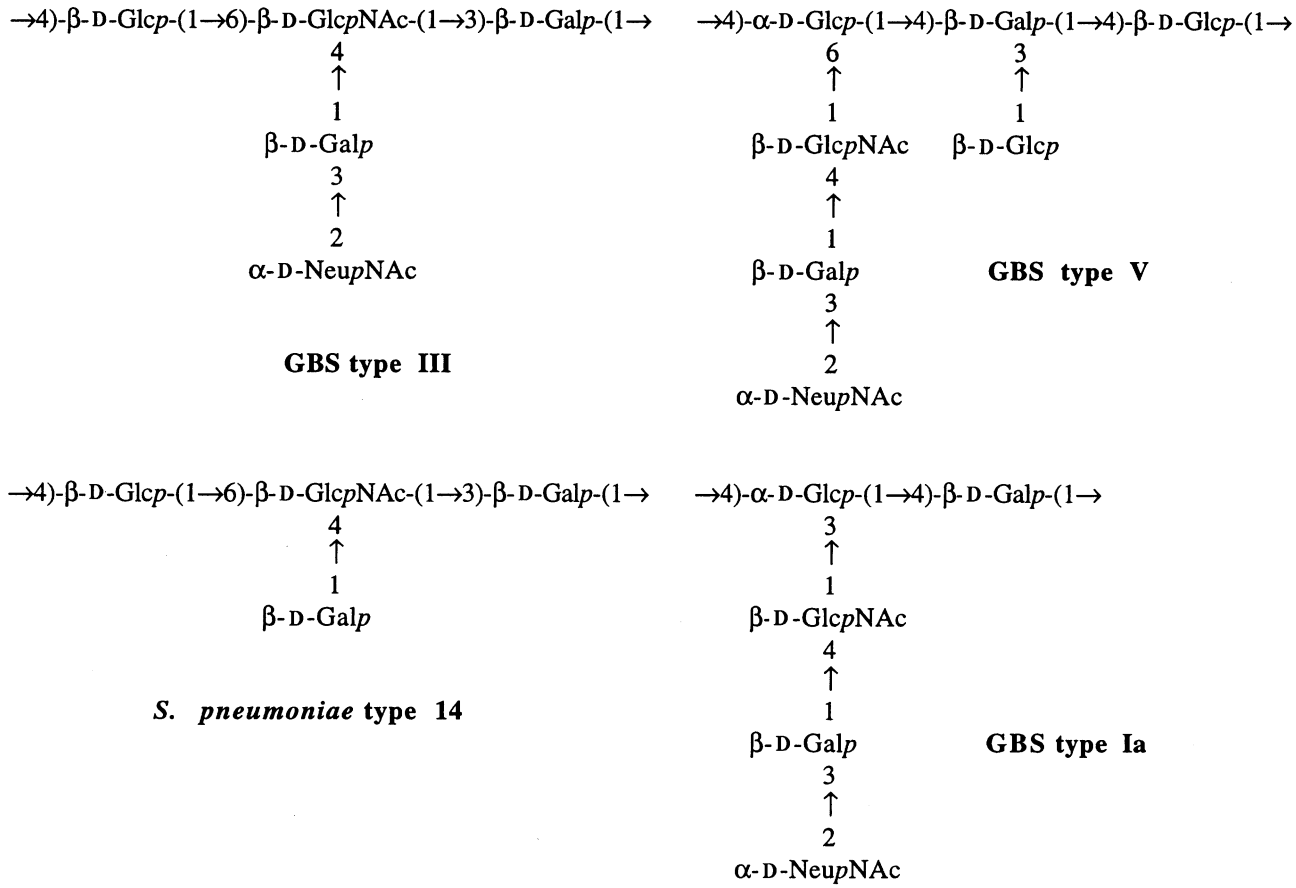


Fig. 1. The repeating unit structures of polysaccharides from GBS type Ia [6], III [7], V [8], and *S. pneumoniae* type 14 [14]. These polysaccharides were depolymerized by the present ozonolysis method.

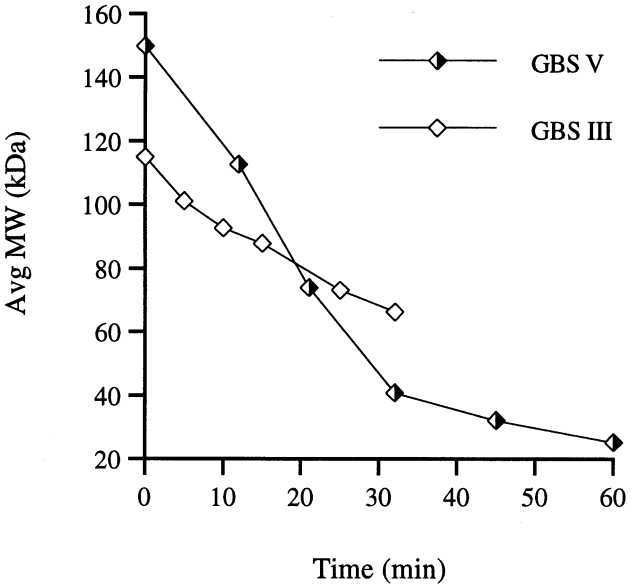


Fig. 2. Average molecular sizes of the products vs. ozonolysis time from depolymerizing type III and V GBS polysaccharides.

by a radical mechanism in which ozone attacked water and formed oxy- and/or hydroxy-radicals that led in turn to degradation (Schemes 2–4). The free-radical mechanism is similar to that observed in the radiation of carbohydrates in water [15]. Oxalic acid is formed by hydrogen abstraction at the 2-position, as outlined in Scheme 3, and the formation of acetic acid is expected at a high pH by the route outlined in Scheme 4.

Radicals and acids can both degrade polysaccharides, and such degradations are not specific for β-D-glycosides. To test whether ozonolysis can also degrade polysaccharides containing no β-D-aldosides, we examined dextran, a polymer composed predominantly of 1,6-linked α-D-glucoses. In one experiment, a sample of dextran (4.9 mg) was dissolved in 0.03 M $NaHCO_3$ (2.4 mL) and subjected to ozonolysis for 2.5 h. The decrease in pH of the solution was slow during the first hour but accelerated as the reaction proceeded and reached 1 after 2.5 h. The average molecular mass of the products decreased only slowly during the first hour of the reaction but decreased rapidly to 300 Da by the reaction's end. These results demonstrate that polysaccharides containing α-D-linkages can be depolymerized by ozonolysis in aqueous solution.

In conclusion, ozonolysis provides a simple and inexpensive method for depolymerizing polysaccharides. Carbohydrates are degraded by ozone in aqueous solution by several mechanisms: ozonolytic oxidation of β-D-aldosidic linkages, oxidative degradation by radical species, and acid hydrolysis. Ozonolytic oxidation of β-D-aldosides leads to selective depolymerization of polysaccharides, whereas radical reactions and pH-induced ionic reactions lead to nonselective degradation pathways. Overall, the present method, a simple one-step procedure, can be used to degrade any polysaccharide. It is particularly useful for generating small polymer or large oligomer fragments from large polysaccharides for applications in which some minimal degree of structural difference can be tolerated.

3. Experimental

Materials.—Type Ia, III, and V GBS polysaccharides were isolated and purified as described previously [2–4]. Desialylated type III polysaccharides were obtained by mild acid hydrolysis (6% AcOH at 80 °C for 30 min). Dextran was purchased from Pharmacia; cellobiose and lactose from Difco. Ozone was generated by passing compressed air through an electronic ozone generator (More-Zone, Taiwan).

General procedure for ozonolysis.—A polysaccharide sample (10 mg/mL) was dissolved in water, phosphate-buffered saline (PBS, 0.05 M phosphate and 0.15 M NaCl, pH 7.2), or 0.1 M $NaHCO_3$ (pH 8.6). A few glass beads (2 mm diameter) were added to help disperse ozone gas. The solution was then bubbled with ozone at a flow rate of 3 mL/min (0.12 mmol/min). The pH of the solution was monitored. At various time intervals, e.g., every 5 min, an aliquot (20 μL) was removed and screened on gel-filtration chromatography to check the product molecular size. The reaction was stopped after the desired sizes, as determined from gel-filtration chromatography, were obtained. The total reaction time varied from several minutes to 2 days. When ozonolysis was carried out in water or PBS, the solution gradually turned acidic, and 0.1

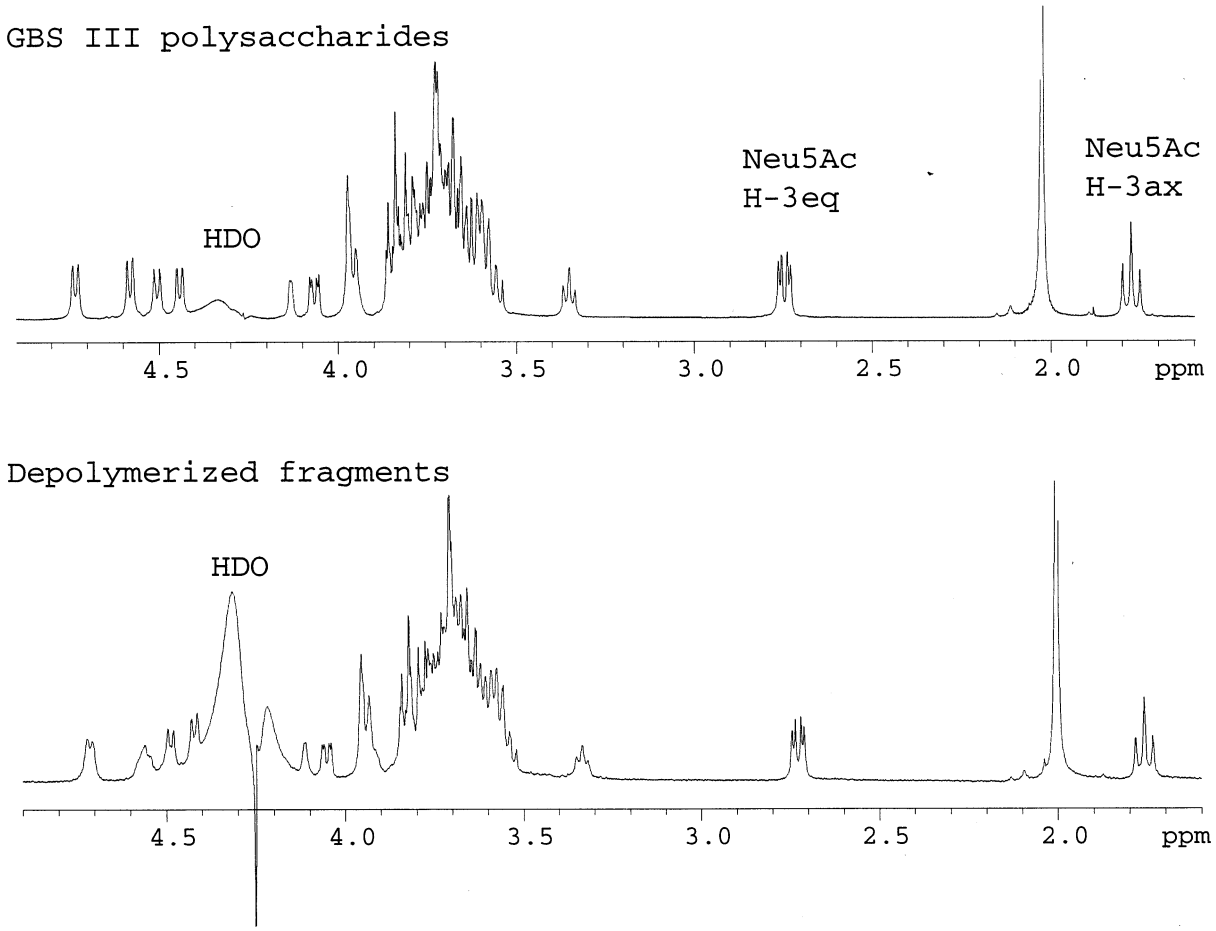
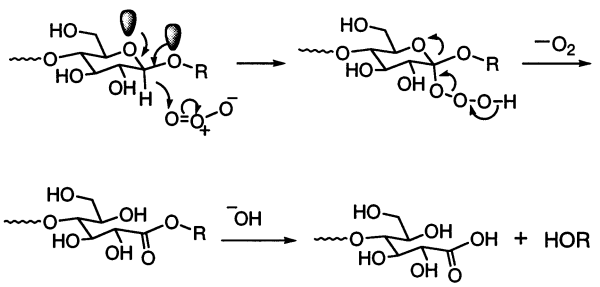


Fig. 3. ^{1}H NMR spectra of the native type III GBS polysaccharides (top) and fragments with average molecular mass of 10 kDa generated from ozonolytic depolymerization (bottom). The spectra were recorded in D_2O at 70 °C and chemical shifts were referenced relative to the HDO signal at 4.25 ppm. Note that the spectra were identical and also that the sialic acid (Neu5Ac) residue was retained in the depolymerization product.

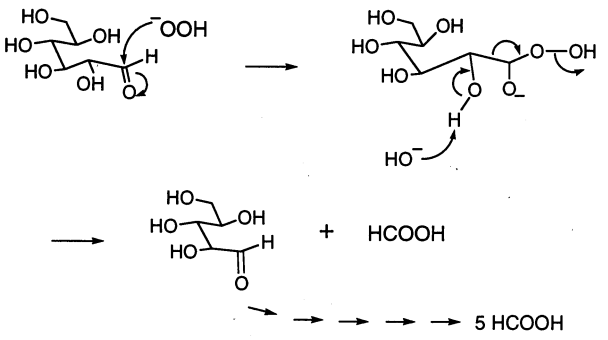
M NaOH was added as needed to neutralize the solution. When ozonolysis was carried out in 0.1 M $NaHCO_3$ solution, the pH of the solution changed much more slowly. Thus, 0.1 M $NaHCO_3$ or other basic buffers are preferred to limit acid-associated side reactions.

Ozonolysis of cellobiose and lactose.—A sample of cellobiose or lactose (38.8 mg) was dissolved in water (6 mL), and the solution was bubbled with ozone at a flow rate of 7.2 mmol/h for a total of 56 h. The reaction was monitored by TLC on Silica Gel 60 plates (EM Separations Technology, Germany). A mixture of 6:4:1 2-propanol–NH_4OH–water was used as the eluent, and the components were detected by orcinol spray (2% orcinol in 20% H_2SO_4 solution in EtOH). The products were analyzed by ^{1}H NMR spectroscopy. A portion (40 μL) of the products was dried under a stream of nitrogen and treated with $CF_3C(=NSiMe_3)OSiMe_3$ (0.1 mL) and pyridine (0.1 mL) at room temperature for 40 min. The Me_3Si derivatives were then subjected to GC–MS analysis.

Gel-filtration chromatography.—The molecular size of polysaccharides and oligosaccharides were determined from gel-filtration



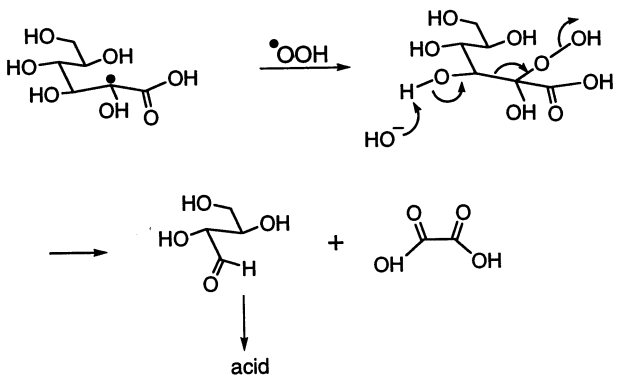
Scheme 1.



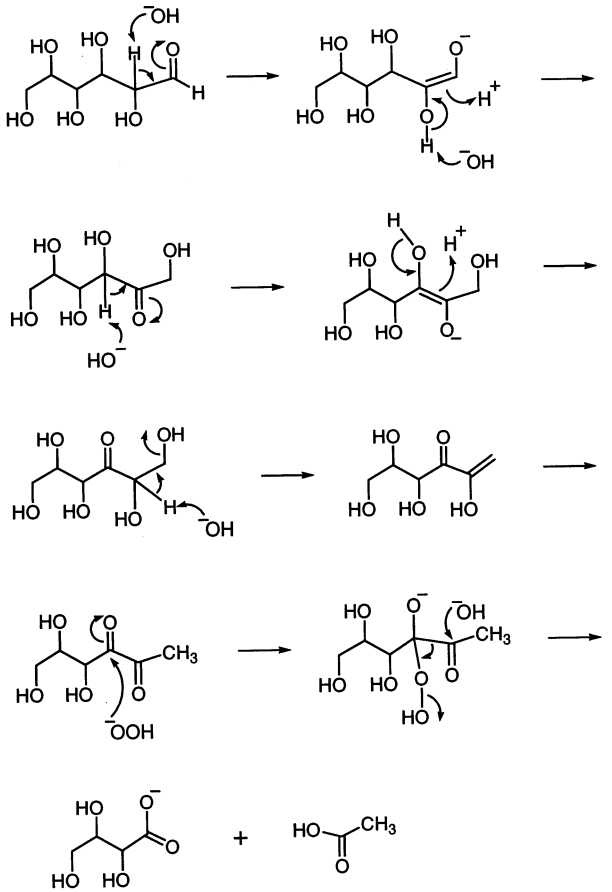
Scheme 2.

chromatography with an FPLC system (Pharmacia Biotech, Piscatway, NJ). Fractions were monitored by a refractive index detector. A Superose 12 column was used to fractionate large saccharides with molecular weights between 10 and 300 kDa; 0.1 M PBS with 0.025% azide was used as the eluent at a flow rate of 0.5 mL/min. Superdex 75 column was used to fractionate saccharides between 0.5 and 10 kDa; 0.03 M PBS with 0.025% azide was used at an eluting rate of 1 mL/min. Both columns were calibrated with oligosaccharides and dextrans of known molecular weight. The average relative molecular weights of products were estimated from column calibration.

Instrumental analysis.—^{1}H NMR spectra were recorded on a Varian Unity 500 instrument (Palo Alto, CA) with a proton frequency of 500 MHz. GC–MS analysis was performed on an HP 6890/5973 GC–MS instrument (Hewlett–Packard, Wilmington, DE) on a DB17 column (Alltech, Deerfield, IL). A GC temperature series was as follows: an initial temperature of 100 °C and maintained for 10 min, increased to 260 °C at a rate of 10 °C/



Scheme 3.



Scheme 4.

min, and then held at the final temperature at 260 °C for 20 min.

Acknowledgements

This work was supported in part by grants AI75326 and AI39576 and merit award AI23339 to D.L.K. from the National Institute of Allergy and Infectious Diseases. We thank Barbara Reinap for technical assistance, and April Blodgett, Ken Johnson, Julieanne Pinel, Lawrence Paoletti, and Robin Ross for providing the GBS polysaccharides.

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