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Structural analysis of tamarind seed xyloglucan oligosaccharides using β-galactosidase digestion and spectroscopic methods *

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ABSTRACT

The borohydride-reduced forms (oligoglycosyl alditols) of two isomeric octasaccharides (Glc_4Xyl_3Gal) that are released from xyloglucans of various plant species upon treatment with a fungal *endo*-(1 → 4)-β-glucanase were isolated and structurally characterized. A mixture of oligosaccharides that is released from tamarind seed xyloglucan by the *endo*-(1 → 4)-β-glucanase was digested with a commercially available β-galactosidase (*Aspergillus niger*). The β-galactosidase selectively hydrolyzed the galactosyl residue of one of the two isomeric octasaccharides present in the mixture. A homogeneous preparation of the β-galactosidase-resistant octasaccharide was prepared by high-resolution gel-permeation chromatography of the enzyme-digestion products. Spectroscopic characterization of the oligoglycosyl alditol prepared by reduction of this octasaccharide confirmed the previously proposed structure that had been based on analysis of the mixture of isomeric octasaccharides. The availability of large amounts of the pure, reduced octasaccharide and of a pure, reduced pentasaccharide (Glc_3Xyl_2) made it possible to completely assign their ^1H and ^{13}C NMR spectra. In addition, the borohydride-reduced form of the β-D-galactosidase-susceptible octasaccharide isomer was purified by high pH anion-exchange chromatography of the *endo*-(1 → 4)-β-glucanase-released octasaccharides from rapeseed xyloglucan (no β-galactosidase treatment), and its ^1H and ^{13}C NMR spectra were assigned. Additional correlations between specific structural features of xyloglucan oligoglycosyl alditols and the positions of specific resonances in their NMR spectra were deduced and added to the extensive list that we have compiled. The effects of recording the NMR spectra of the xyloglucan oligoglycosyl alditols in the presence of borate salts, which could lead to incorrect structural assignments, are also described.

INTRODUCTION

Xyloglucans (XGs) are a class of hemicellulosic polysaccharides that are found in noncovalent association with microcrystalline cellulose fibers in the cell walls of higher plants[1–4]. The xyloglucan backbone consists of β-(1 → 4)-linked D-Glc*p*

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residues that are substituted at C-6 with α-D-Xyl*p*, β-D-Gal*p*-(1 → 2)-α-D-Xyl*p*, and α-L-Fuc*p*-(1 → 2)-β-D-Gal*p*-(1 → 2)-α-D-Xyl*p* side chains[5]. In addition, α-L-Ara*f* and β-D-Xyl*p* substituents have been found at C-2 of some of the β-D-Glc*p* residues in the backbone of the xyloglucan produced by suspension-cultured *Acer pseudoplatanus* cells[6,7], and α-L-Ara*f* residues are found at C-2 of the α-D-Xyl residues in XGs produced by members of the Solanaceae[8,9]. XGs are proposed to be major load-bearing structures in primary (growing) plant cell walls by virtue of their potential to crosslink cellulose fibers, and as such, are thought to play an important role in regulating plant cell-wall extension[10]. Furthermore, very low concentrations of xyloglucan oligosaccharides produced by the action of *endo*-(1 → 4)-β-glucanases can affect the growth of plant tissues[11,12].

The combination of nuclear magnetic resonance spectroscopy (NMR) and fast-atom bombardment mass spectrometry (FABMS) provide a powerful method for determining the structures of oligosaccharides. We have rigorously characterized the reduced forms (oligoglycosyl alditols) of many of the commonly occurring oligosaccharide subunits of xyloglucans[5–7]. These analyses allowed us to deduce a set of diagnostic correlations between the chemical shifts in the NMR spectrum and specific substructures in the oligoglycosyl alditols. The oligosaccharide subunits, obtained by high-resolution gel-permeation chromatography (Bio-Gel P-2) of the products formed upon treatment of xyloglucan polysaccharide with *endo*-(1 → 4)-β-glucanase (*Trichoderma reesei*), are reduced with sodium borohydride and characterized spectroscopically. Various types of high-performance liquid chromatography (HPLC) are used to separate oligosaccharides that are not resolved, due to their similar size, by gel-permeation chromatography. We have previously proposed[5] structures for two isomeric octasaccharides (Glc_4Xyl_3Gal) that are difficult to separate by HPLC, and which were therefore characterized as a mixture. We now report that treatment of the oligosaccharide subunits from tamarind seed amyloid with a D-galactosidase from *Aspergillus niger* results in the selective hydrolysis of the β-D-Gal*p* residues attached to the central xylosyl residue in one of these oligosaccharides. The selectivity of this enzyme made it possible to prepare large quantities of one of the isomeric octasaccharides, which we have chemically reduced and characterized. The β-galactosidase-susceptible octasaccharide was also isolated, albeit in smaller quantities, by high pH anion-exchange chromatography (HPAE) of *endo*-(1 → 4)-β-glucanase-treated rapeseed xyloglucan. The ^1H and ^{13}C NMR spectra of the reduced octacaccharide isomers and a reduced pentasaccharide (Glc_3Xyl_2) were assigned. Additional correlations between the chemical shifts of certain resonances and the identity and distribution of side chains in xyloglucan oligoglycosyl alditols were deduced on this basis.

The correlations that we have deduced between xyloglucan oligoglycosyl alditol structures and features in their NMR spectra make it possible to quickly and accurately determine considerable information about the structures of xyloglucans by inspection of their one-dimensional ^1H NMR spectra[5,7]. In applying these correlations we have observed phenomena that, if not accounted for, could lead to

incorrect conclusions about the structures of oligoglycosyl alditols being analyzed. These phenomena include the temperature dependence of the chemical shifts of certain resonances and the strong tendency of galactose-containing xyloglucan oligosaccharides to form borate complexes. Specific examples and general approaches to control these phenomena are presented.

RESULTS AND DISCUSSION

Enzyme treatment of tamarind xyloglucan.—Tamarind xyloglucan was digested with an endo-(1 → 4)-β-glucanase from *Trichoderma reesei* and separated into molecular size classes by Bio-Gel P-2 chromatography (Fig. 1A). Oligoglycosyl alditols were prepared by borohydride reduction of the nonasaccharide, octasaccharide, and heptasaccharide fractions and analyzed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the oligoglycosyl alditols prepared by reduction of the octasaccharide-containing fractions 90–93 (Fig. 2A) clearly shows the presence of more than one component. Structures **1**–**4** were proposed[5] to be the four most abundant *endo*-(1 → 4)-β-glucanase digestion products of tamarind xyloglucan. However, analysis of structures **2** and **3** had been based on the spectra of mixtures

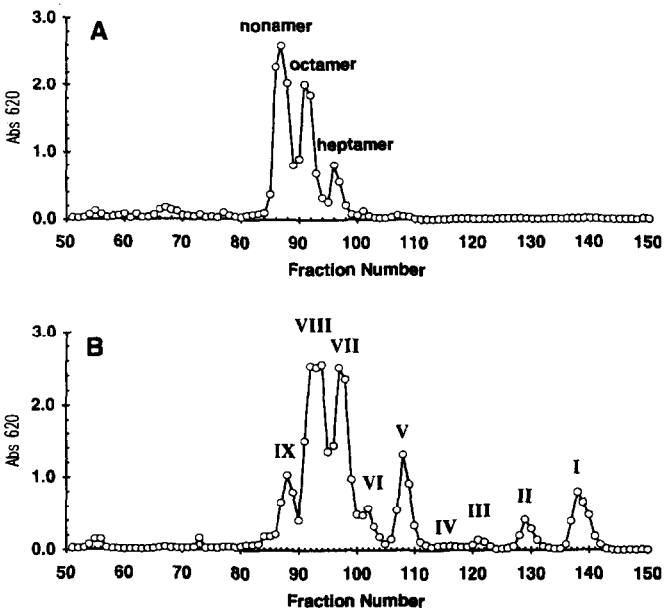
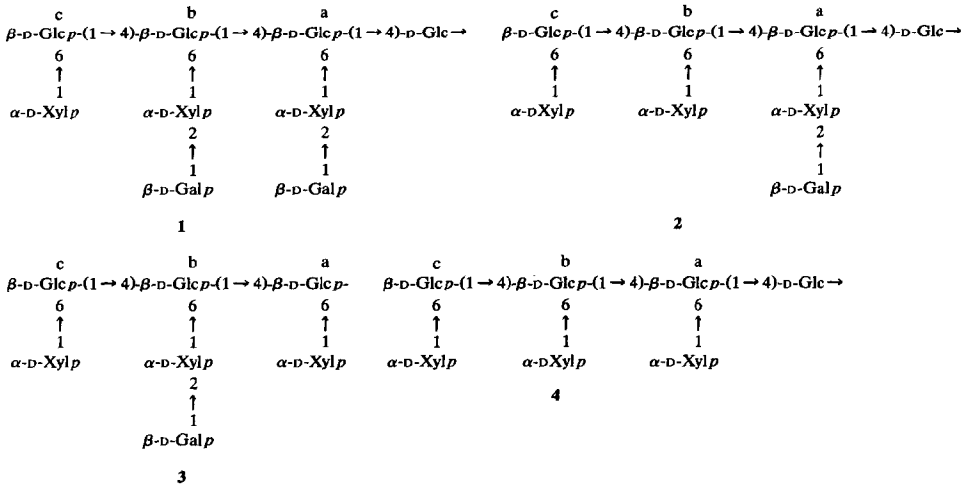


Fig 1. Bio-Gel P-2 chromatography of the oligosaccharides obtained by treatment of tamarind xyloglucan with fungal glycosyl hydrolases: (A) treatment with *endo*-(1 → 4)-β-glucanase (*Trichoderma reesei*); (B) treatment with *endo*-(1 → 4)-β-glucanase followed by β-galactosidase (*Aspergillus niger*). The octamer fraction in panel A consists of a mixture of **2** and **3** (see ref 5 and Fig. 2A). Peaks I to IX in panel B correspond to monosaccharide to nonasaccharide, respectively.

similar to the octasaccharide fraction described above, and, therefore, their structures were not unambiguously assigned.



A mixture of the four oligosaccharides obtained by *endo*-(1 → 4)-β-glucanase treatment of tamarind xyloglucan was treated with a commercial preparation of a β-galactosidase secreted by *Aspergillus niger*, and the products were separated by Bio-Gel P-2 chromatography (Fig. 1 B). β-Galactosidase treatment resulted in the conversion of the bulk of nonasaccharide **1** (peak IX) to an octasaccharide that was the most abundant product recovered (peak VIII). The octasaccharide fraction was reduced with borohydride and analyzed by ^{1}H NMR spectroscopy (Fig. 2B). It is evident from the ^{1}H NMR spectrum that only one of the two isomeric octasaccharides survived the β-galactosidase treatment. The selectivity of the β-D galactosidase for the β-D-Gal*p* residues attached to C-2 of the central α-D-Xyl residue had resulted in the conversion of nonasaccharide **1** to octasaccharide **2** and the conversion of octasaccharide **3** to heptasaccharide **4** (peak VII). The recovery of large quantities of pure **2** allowed its structure to be unambiguously determined and the ^{1}H and ^{13}C NMR spectra of its corresponding oligoglycosyl alditol **2r** to be fully assigned (see below).

A contaminating hydrolase activity present in the commercial β-galactosidase preparation partially degraded some of the oligosaccharides in the tamarind oligosaccharide mixture, leading to the formation of small (dp < 7), late-eluting oligosaccharides (Fig. 1B, peaks I to VI). ^{1}H NMR and FABMS analysis of the oligoglycaosyl alditols prepared by borohydride reduction of these oligosaccharides indicated that a contaminating hydrolase activity released the disaccharide isoprimeverose [α-D-Xyl*p*-(1 → 6)-D-Glc] from the nonreducing end of some of the oligosaccharides in the mixture. This analysis (see below) established that fractions 136–141 (peak I) contained only galactose released from **1** and **3** by β-galactosidase, while fractions 128–130 (peak II) contained only isoprimeverose. Fractions 106–110 (peak V) contained only pentasaccharide **5**, formed by the release of

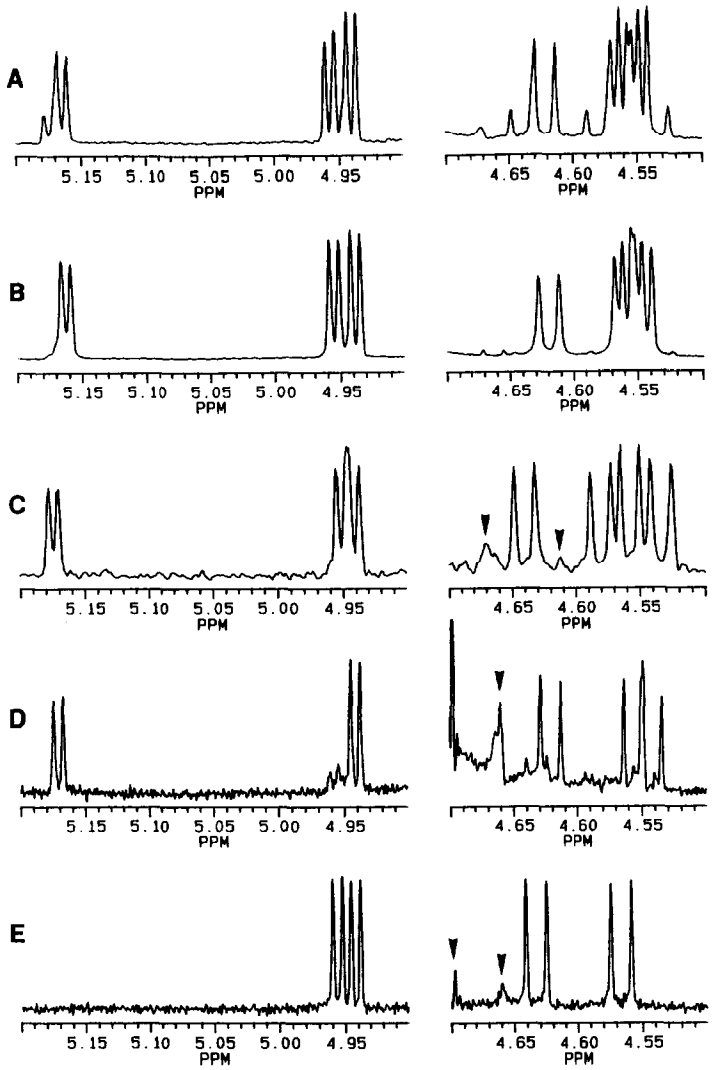
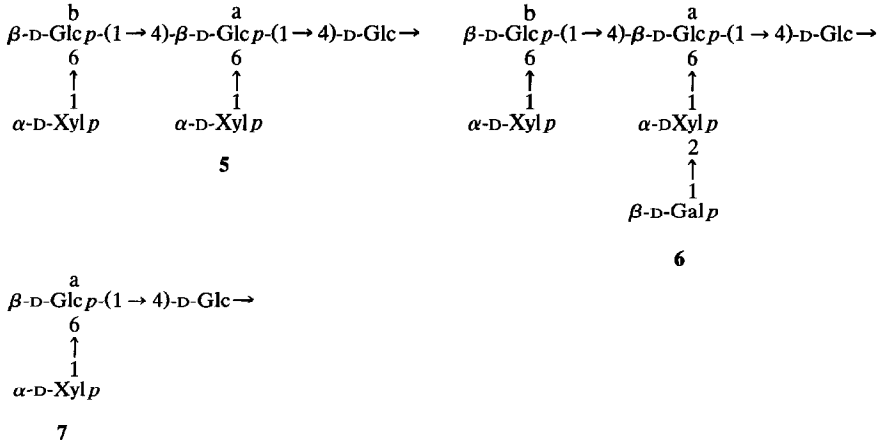


Fig. 2. Anomeric regions of the ^{1}H NMR spectra of xyloglucan oligoglycosyl alditols: (A) the mixture of **2r** and **3r** from tamarind xyloglucan (borohydride-reduced octasaccharide fraction from Fig. 1A); (B) the β-galactosidase-resistant isomer **2r** (Fig. 1B, borohydride-reduced peak VIII); (C) the β-galactosidase-susceptible isomer **3r** purified by HPAE chromatography of borohydride-reduced rapeseed xyloglucan octasaccharides; (D) the borohydride-reduced hexasaccharide **6r** fraction from tamarind xyloglucan (Fig. 1B, peak VI) containing ~15% **4r** as a contaminant; (E) the borohydride-reduced pentasaccharide from tamarind xyloglucan (**5r**, Fig. 1B, peak V). The spectral region (δ 4.7–4.9) containing the strong HDO signal has been deleted for clarity. Arrowheads indicate sidebands of the HDO signal.

isoprimeverose from **4** or release of galactose from **6**. P-2 fractions 100–104 (peak VI) contained hexasaccharide **6**, formed primarily by the release of isoprimeverose from **2** (and perhaps to some extent by release of galactose from a short-lived

intermediate $Glc_3Xyl_2Gal_2$, not detected in the mixture). Peak III and the barely detectable peak IV were not analyzed.



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A glucosidase that releases isoprimeverose from the nonreducing end of xyloglucan oligosaccharides had previously been isolated from *Aspergillus oryzae*[13], and so its presence in the *Aspergillus niger* preparation was not surprising. Although the isoprimeverose-releasing enzyme represents only a small fraction of the total glycosidase content of the enzyme preparation, its effects were observed because xyloglucan oligosaccharides **1–4** are its preferred substrates, while these same oligosaccharides (especially **2**) are quite poor substrates for the β-galactosidase. The xyloglucan oligosaccharides were treated with sufficient β-galactosidase (1.0 unit, as defined by the supplier, for 96 h) to hydrolyze more than 5000 μmol of the β-D-Gal*p* residues in a preferred substrate, such as *o*-nitrophenyl β-D-galactoside. Nevertheless, only about half of the 215 μmol of β-D-Gal*p* residues in the xyloglucan oligosaccharide mixture were hydrolyzed by the enzyme. We estimate that ~30 μmol of isoprimeverose were released, corresponding to 0.005 units of the contaminating glucosidase.

Isolation of oligoglycosyl alditol **3r** *from rapeseed xyloglucan.*—Xyloglucan was extracted[5] from defatted rapeseed hulls and treated with *endo*-(1 → 4)-β-glucanase. The enzyme-digestion products included the two isomeric octasaccharides **2** and **3**, but in a different ratio than was obtained from tamarind seed xyloglucan[5]. A mixture of **2** and **3**, prepared by Bio-Gel P-2 chromatography of the *endo*(1 → 4)-β-glucanase digestion products of rapeseed XG, was reduced with borohydride to oligoglycosyl alditols (**2r** and **3r**), which were separated by HPAE chromatography with pulsed amperometric detection (PAD). The most abundant oligoglycosyl alditol in the sample, constituting ~75% of the rapeseed octasaccharide fraction, was eluted at 7.8 min. Its structure was established as **3r** by the spectroscopic methods described below. The identity of the minor component (25% of the sample, eluted at 9.4 min) was established as **2r** by comparison of its chromatographic and spectroscopic signature to that of authentic **2r** (obtained from tamarind XG by sequential *endo*-(1 → 4)-β-glucanase and β-galactosidase treatment, see

above). A mixture of oligoglycosyl alditols **2r** and **3r** was also prepared by reduction of the tamarind xyloglucan octasaccharide fraction (no β-galactosidase treatment, Fig. 1A) and separated by HPAE chromatography. This analysis confirmed that **2r** rather than **3r** is the most abundant oligoglycosyl alditol in the reduced tamarind octasaccharide fraction, accounting for 75% of the sample.

High pH anion-exchange chromatography of oligosaccharides **2** *and* **3**.—Native (reducing) octasaccharides **2** and **3** have been separated by HPAE[14], but their elution order was not unambiguously determined due to the difficulty in differentiating between the two closely related structures. The Bio-Gel P-2 octasaccharide fractions from both rapeseed xyloglucan and tamarind seed xyloglucan (no β-D-galactosidase treatment) were therefore subjected to analytical-scale HPAE chromatography without prior borohydride reduction. The relative amounts of **2** and **3** in these mixtures was established by peak integration and spectroscopic analysis of the HPAE-purified reduced forms **2r** and **3r** (see above). The HPAE elution order of the reducing oligosaccharides, **3** (t_R = 15.9 min) followed by **2** (t_R = 16.8 min), was thus determined on the basis of peak-area ratios for the two mixtures. The retention times of the reducing oligosaccharides were significantly longer than those of the oligoglycosyl alditols, as expected[15], due to the greater acidity of the reducing glycosidic hydroxyl protons as compared to the alditol hydroxyl protons. The tendency of the glycosidic hydroxyl proton to ionize results in a higher average charge density (and stronger ionic binding to the matrix) for reducing oligosaccharides than for oligoglycosyl alditols.

Structural analysis of XG oligosaccharides.—Oligosaccharides **1** to **6** were reduced with borohydride to the corresponding oligoglycosyl alditols (**1r** to **6r**) and analyzed by ^{1}H NMR spectroscopy and FABMS. All of the oligosaccharides except **3** were obtained by sequential *endo*-(1 → 4)-β-glucanase and β-D-galactosidase treatment of tamarind xyloglucan followed by P-2 chromatography (Fig. 1B, see above). The major components of the nonasaccharide (peak IX), heptasaccharide (peak VII), disaccharide (peak II), and monosaccharide (peak I) fractions from the P-2 column were established as **1**, **4**, isoprimeverose, and galactose, respectively, by comparing the ^{1}H NMR and FABMS spectra of their borohydride-reduced forms to those of authentic standards[5]. The octasaccharide (peak VIII), hexasaccharide (peak VI), and pentasaccharide (peak V) fractions obtained by this method contained oligosaccharides which were further analyzed after their conversion to oligoglycosyl alditols by borohydride reduction. In the description of this analysis (see below), we use an abbreviated nomenclature[5,7] for substructures of the xyloglucan oligoglycosyl alditols. Glycosyl residues of the oligoglycosyl alditols are labeled with a superscript a, b, or c, depending on the proximity to the alditol moiety. Thus, backbone residues in the oligoglycosyl alditols are designated Glc^c → Glc^b → Glc^a → Glcol. The position of a side chain residue is indicated by the superscript character of the backbone residue to which the side chain is attached. For example, the galactosyl residue in **3r** is designated Gal^b as it terminates the side chain attached to Glc^b.

TABLE I

^{1}H NMR chemical shifts for compounds **2r**, **3r**, **5r** and **6r**

	H-1′	H-1	H-2	H-3	H-4	H-5	H-5$_{eq}$	H-6	H-6′
2r									
Glucitol [a]	3.72	3.78	3.93	3.90	3.94	3.96		3.75	3.87
β-Glc^a		4.620	3.431	3.670	3.645	3.85		3.975	3.901
β-Glc^b		4.560	3.398	3.671	3.735	3.822		3.897	4.010
βGlc^c		4.547	3.340	3.52	3.52	3.70		3.779	3.935
α-Xyl^a		5.163	3.674	3.835	3.71	3.58	3.73		
α-Xyl^b		4.955	3.545	3.732	3.63	3.588	3.73		
α-Xyl^c		4.939	3.542	3.735	3.61	3.55	3.714		
β-Gal^a		4.554	3.612	3.662	3.925	3.68		3.79	3.81
3r									
Glucitol	3.69	3.75	3.92	3.88	3.90	3.94		3.747	3.867
β-Glc^a		4.640	3.429	3.686	3.79	3.79		3.83	4.063
β-Glc^b		4.581	3.422	3.68	3.67	n.a. [b]		3.95	3.92
β-Glc^c		4.534	3.339	3.52	3.52	3.68		3.779	3.938
α-Xyl^a		4.951	3.544	3.678	3.62 [c]	3.57 [c]	3.73 [c]		
α-Xyl^b		5.175	3.675	3.924	3.62 [c]	3.57 [c]	3.73 [c]		
α-Xyl^c		4.940	3.541	3.740	3.62 [c]	3.57 [c]	3.73 [c]		
β-Gal^b		4.556	3.621	3.66	3.92	3.68		3.78 [c]	3.78 [c]
5r									
Glucitol	3.676	3.749	3.925	3.89	3.91	3.945		3.867	3.742
β-Glc^a		4.634	3.411	3.668	3.77	3.77		3.844	4.061
β-Glc^b		4.567	3.345	3.53	3.51	3.70		3.785	3.939
α-Xyl^a		4.956	3.546	3.67	3.63	3.578	3.735		
α-Xyl^b		4.942	4.544	3.737	3.615	3.546	3.716		
6r									
Glucitol	3.712	3.781	n.a.	n.a.	n.a.	n.a.		3.745	3.867
β-Glc^a		4.621	3.432	3.668	n.a.	n.a.		3.987	3.897
β-Glc^b		4.542	3.337	3.52	3.52	3.71		3.779	3.940
α-Xyl^a		5.170	3.673	n.a.	n.a.	n.a.	n.a.		
α-Xyl^b		4.941	3.542	3.739	n.a.	n.a.	n.a.		
β-Gal^a		4.556	3.615	3.665	3.928	n.a.		n.a.	n.a.

[a] A superscript a, b, or c indicates the position of the residue in the oligoglycosyl alditol. The order of backbone residues is Glc^c → Glc^b → Glc^a → Glcol. Residues (e.g., Xyl^a) in the side chain attached to Glc^a are designated by a superscript "a", etc. [b] n.a. Indicates resonance not assigned. [c] Indicates sets of highly overlapped signals that could not be unambiguously assigned.

The ^{1}H and ^{13}C NMR spectra of oligoglycosyl alditols **2r**, **3r**, and **5r** were fully assigned (Tables I and II) by two-dimensional techniques, and the ^{1}H NMR spectra of **6r** was partially assigned. These ^{13}C chemical shift assignments, along with those previously assigned[5] for **4r** and **7r**, are presented in Table II using internal 1,4-dioxane, δ 67.4 (ref 16), as the chemical shift reference. Homonuclear COSY[17] and TOCSY[18] spectra of the compounds were recorded, and proton resonances were assigned by virtue of their scalar-coupling patterns and connectivity, and by comparison to the previously assigned spectra of related oligoglycosyl alditols[5]. The observed homonuclear scalar coupling constants (data not shown)

TABLE II

^{13}C NMR chemical shifts for compounds **2r**, **3r**, **4r**, **5r**, and **7r**

Residue	C-1	C-2	C-3	C-4	C-5	C-6
2r						
Glucitol	63.2	73.0	70.3	80.7	72.1	62.9
β-Glc[a]	103.1	73.7	75.0 [b]	80.5	74.4	67.7
β-Glc[b]	103.4	73.5	74.9 [b]	80.1	74.1	66.9
β-Glc[c]	103.7	73.8	76.4	70.3	75.1	66.8
α-Xyl[a]	99.7	80.9	72.9	70.1	62.0 [b]	
α-Xyl[b]	99.7	72.3	73.8	70.3	62.3 [b]	
α-Xyl[c]	99.1	72.3	73.8	70.3	62.0	
β-Gal[a]	105.3	71.9	73.4	69.4	75.9	61.9
3r [c]						
Glucitol	63.1	72.9	70.1	80.7	72.1	62.7
β-Glc[a]	103.6	73.7	75.1	80.1	n.a. [d]	66.9
β-Glc[b]	103.6	73.7	75.1	80.9	n.a.	67.5
β-Glc[c]	104.6	73.9	76.5	70.3	n.a.	66.7
α-Xyl[a]	100.3	72.4	73.4	70.2	62.0	
α-Xyl[b]	99.9	80.9	74.7	70.2	62.0	
α-Xyl[c]	99.5	72.4	74.0	70.2	62.0	
β-Gal[b]	105.8	72.0	73.7	69.5	75.8	61.7
4r						
Glucitol	63.3	73.2	70.2	80.6	72.0	62.9
β-Glc[a]	103.2	73.9	74.9	79.7	74.2	67.0
β-Glc[b]	103.3	73.5	74.9	80.3	74.2	67.0
β-Glc[c]	103.8	73.9	76.4	70.3	75.2	66.9
α-Xyl[a]	100.1	72.3	74.0	70.2	62.4	
α-Xyl[b]	99.7	72.3	73.9	70.3	62.5	
α-Xyl[c]	99.2	72.3	73.9	70.4	62.1	
5r						
Glucitol	63.2	73.1	70.2	80.7	72.0	62.8
β-Glc[a]	103.2	73.9	74.9	79.9	74.2	67.0
β-Glc[b]	103.6	73.9	76.4	70.4	75.1	66.8
α-Xyl[a]	100.0	72.3	73.9	70.3	62.4	
α-Xyl[b]	99.1	72.3	73.9	70.3	62.1	
7r						
Glucitol	63.2	73.1	70.2	80.7	72.0	62.8
β-Glc	103.5	74.1	76.5	70.1	75.2	66.7
α-Xyl	99.2	72.3	74.0	70.2	62.0	

[a] Superscripts a, b, or c indicate the position of the residue in the oligoglycosyl alditol.
[b] Signal overlap in the two-dimensional experiments made it impossible to unambiguously distinguish these pairs of nuclei. [c] Assignment of ^{13}C-resonances for **3r** were based solely on the 2D HSQC spectrum recorded at 600 MHz (accuracy of measurement ±0.3 ppm). [d] n.a. Indicates that the resonance was not assigned.

were completely consistent with those of related xyloglucan oligoglycosyl alditols[5] and with the assignments (Tables I and II) presented herein. One-bond heteronuclear scalar connectivities were visualized by single-quantum coherence spectroscopy (HSQC)[19,20] or by two-dimensional inverse DEPT[21]. Examination of the

HSQC or inverse DEPT spectra allowed nearly all of the ^{13}C resonances of **2r**, **3r**, and **5r** to be assigned. In addition, strongly coupled ^{1}H systems (such as those for the alditol moieties) that could not be assigned by the homonuclear techniques were assigned on the basis of heteronuclear connectivities detected by HSQC. Correlations between the chemical shifts of anomeric proton resonances and specific oligoglycosyl alditol substructures are described below. Diagnostic signals (i.e., crosspeaks) involving nonanomeric resonances were also observed in the two-dimensional spectra and were correlated with specific structural features of the oligoglycosyl alditols, but are not discussed due to space limitations.

Structures of the reduced octasaccharides.—We have described[5,7] many correlations between the chemical shifts of certain signals in the ^{1}H NMR spectra of xyloglucan oligoglycosyl alditols and specific details of their chemical structures. Most of these correlations were firmly established on the basis of thorough analysis of highly purified oligoglycosyl alditols. However, the analysis[5] of **2r** and **3r** had been performed on mixtures of the two components. These analyses indicated that the H-1 resonance ($\delta \sim 5.16$) of a Xyl^{a} residue substituted at C-2 with a terminal β-Gal*p* residue is slightly upfield of the H-1 resonance ($\delta \sim 5.17$) of a Xyl^{b} substituted at C-2 with terminal β-Gal*p*. The ^{1}H NMR spectrum of the highly purified, borohydride-reduced, β-galactosidase-resistant octasaccharide from tamarind (Fig. 1B, peak VIII) includes an α-anomeric proton resonance at δ 5.163 suggesting that its terminal galactosyl residue is attached to Xyl^{a}. The ^{1}H NMR spectrum of the oligoglycosyl alditol prepared by HPAE chromatography ($T_R = 7.8$ min, see above) of the reduced rapeseed octasaccharide fraction includes a signal at δ 5.175, suggesting that its terminal galactosyl residue is attached to Xyl^{b}. The glycosyl sequence of these oligoglycosyl alditols, and thus the validity of the correlations upon which these structural assignments were based, has now been unambiguously confirmed by FABMS (see below).

Negative-ion FABMS of the purified borohydride-reduced octasaccharides indicated a molecular weight of 1226 (i.e., $GalGlc_3Xyl_3Glcol$) for both samples. Alditol-end fragment ions[5] at *m/z* 637 (GalXylGlcGlcol) and *m/z* 931 ($GalXyl_2Glc_2Glcol$) in the spectrum of the reduced, β-galactosidase-resistant tamarind octasaccharide indicated that the terminal Gal residue was attached to Xyl^{a} as in structure **2r**. Conversely, alditol-end fragment ions at *m/z* 475 (XylGlcGlcol) and *m/z* 931 ($GalXyl_2Glc_2Glcol$) in the spectrum of the reduced rapeseed octasaccharide indicated that the terminal β-Gal residue was attached to Xyl^{b} as in structure **3r**.

Complementary sequence information was obtained by positive-mode FABMS of the per-*O*-acetylated oligoglycosyl alditols, which indicated a molecular weight of 2234 for both samples, consistent with the composition $GalXyl_3Glc_3Glcol$. The positive-ion spectrum of the reduced, per-*O*-acetylated, β-galactosidase-resistant octasacharide from tamarind xyloglucan included high abundance, nonreducing end fragment ions[5] at *m/z* 547 (XylGlc and GalXyl) and *m/z* 1051 (Xyl_2Glc_2). Fragment ions at *m/z* 1339 were present in very low abundance in this spectrum,

and could be attributed to a low-probability, double-cleavage event[5] leading to an ion with the composition Glc_3Xyl_2. These spectral features indicated that the β-Gal residue was *not* attached to Xyl^b or Xyl^c in the β-galactosidase-resistant octasaccharide. The fragment ion observed at *m/z* 1843 ($GalXyl_3Glc_3$) is consistent with attachment of the terminal-Gal residue to Xyl^a (structure **2r**) in the β-galactosidase-resistant octasaccharide. Conversely, the high abundance of fragment ions at *m/z* 547 (XylGlc and GalXyl) and *m/z* 1339 ($GalXyl_2Glc_2$) and low abundance of ions at *m/z* 835 (GalXylGlc) and *m/z* 1051 in the positive-ion spectum of the reduced, per-*O*-acetylated rapeseed octasaccharide indicated that the β-Gal residue was attached to Xyl^b in this molecule (structure **3r**).

Structures of the reduced penta- and hexa-saccharides.—The structure of the borohydride-reduced pentasaccharide (P-2 fraction V, Fig. 1B), from tamarind XG treated with *endo*-(1 → 4)-β-glucanase and galactosidase, was assigned as **5r** on the basis of the chemical shifts of diagnostic resonances[5] in its ^1H NMR spectrum. Only two α-anomeric signals were observed in the ^1H NMR spectrum of reduced fraction V (Fig. 2E, Table I), having chemical shifts corresponding[5] to H-1 of a terminal α-Xyl*p* residue (i.e., Xyl^b, δ 4.942) attached to C-6 of a unbranched β-Glc*p* residue and H-1 of a terminal α-Xyl*p* residue (i.e., Xyl^a, δ 4.956) attached to a (4 → 6)-linked β-Glc*p* residue. The signal at δ 4.634 corresponds[5] to H-1 of a (4 → 6)-linked β-Glc*p* residue (Glc^a) attached directly to the alditol, and the resonance at δ 4.567 is due[5] to H-1 of another backbone β-Glc*p* residue (Glc^b). The four glycosyl residues (two α and two β) in the chemical environments that were deduced from the ^1H NMR spectrum can combine with a glucitol residue in only one arrangement (structure **5r**), which was confirmed by FABMS (see below).

Structure **6r** was assigned to the main component of the borohydride-reduced P-2 fraction VI (Fig. 1B). The ^1H NMR spectrum of this sample (Fig. 2D) contains a signal (δ 4.941) corresponding[5] to H-1 of a terminal α-Xyl*p* residue (Xyl^b) linked to C-6 of an unbranched β-Glc*p* residue. The other α-anomeric signal (δ 5.170) in the spectrum corresponds[5] to H-1 of an α-Xyl*p* residue having a β-Gal*p* substituent at C-2. The chemical shift of this resonance is approximately midway between that of H-1 of the 2-linked α-Xylp residue in **2r** (δ 5.163) and H-1 of the 2-linked α-Xylp residue in **3r** (δ 5.175), which is consistent with the unique position of the β-Gal*p*-(1 → 2)-α-Xyl*p* side chain of **6r**. This side chain is linked to C-6 of a β-Glc*p* residue with an alditol aglycon (as in **2r**) and an unbranched, 6-linked β-Glc*p* substituent at C-4 (as in **3r**). Three β-anomeric signals (δ 4.542, 4.556, and 4.621) in the ^1H NMR spectrum of fraction VI correspond[5] to a (4 → 6)-linked β-Glc*p* residue (Glc^a) glycosidically linked to the alditol, a terminal β-Gal*p* residue, and another backbone β-Glc*p* residue (Glc^b), respectively. Again, these five glycosyl residues in the chemical environments that are indicated by the chemical shifts of their anomeric protons can only be combined with a glucitol residue in one arrangement (structure **6r**), which was confirmed by FABMS.

The glycosyl sequences the oligoglycosyl alditols (compounds **5r** and **6r**) formed by borohydride-reduction of P-2 fractions V and VI, respectively, were confirmed

by FABMS. Their molecular weights were established by negative-ion FABMS as 770 (Xyl_2Glc_2Glcol) for **5r** and 932 ($GalXyl_2Glc_2Glcol$) for **6r**. The high abundance of the m/z 637 ion (GalXylGlcGlcol) and *low* abundance of the m/z 475 ion (XylGlcGlcol) in the negative-ion FAB mass spectrum of **6r** indicated that the terminal β-Gal$p$ residue in **6r** was attached to Xyl^a. The negative-ion FAB mass spectrum of **5r** included abundant ions at both m/z 475 (XylGlcGlcol) and m/z 637 ($XylGlc_2Glcol$). Complementary glycosyl sequence information for these oligoglycosyl alditols was obtained by positive-ion FABMS of their per-*O*-acetylated derivatives, which indicated molecular weights of 1442 (Xyl_2Glc_2Glcol) for **5r** and 1730 ($GalXyl_2Glc_2Glcol$) for **6r**. The high abundance of nonreducing end fragment ions at m/z 547 (XylGlc and GalXyl) 1339 ($GalXyl_2Glc_2$) and low abundance of ions at m/z 1051 (Xyl_2Glc_2) in the positive-ion FAB mass spectrum of per-*O*-acetylated **6r** confirmed that its galactosyl residue was attached to side chain a. Abundant fragment ions at m/z 547 (XylGlc) and 1051 (Xyl_2Glc_2) in the positive-ion FAB mass spectrum of per-*O*-acetylated **5r** were consistent with its proposed structure.

The spectral data obtained in this study, when combined with previously described data[5,7], allowed additional correlations between xyloglucan oligoglycosyl alditol structure and proton chemical shifts to be deduced. For example, the H-1 resonance of the (4 → 6)-linked β-Glc^a is relatively easy to identify in the ^{1}H NMR spectra of xyloglucan oligoglycosyl alditols, especially if COSY or TOCSY data are available. The chemical shift of this resonance is shifted upfield (−0.005 to −0.013 ppm) by substitution of α-Xyl^a with a β-Gal residue at C-2 but downfield (0.009 to 0.015 ppm) by substitution of α-Xyl^b with a β-Gal residue at C-2. This situation is typical for the β-anomeric resonances of xyloglucan oligoglycosyl alditols, which often exhibit more complex dependence on the nature and location of side-chain structures than do the α-anomeric resonances[5].

Effect of residual borate on NMR spectra.—Reduction of xyloglucan oligosaccharides with borohydride eliminates the complicating effects of anomeric equilibria at the reducing end and therefore results in simplification of their ^{1}H and ^{13}C NMR spectra[5]. In addition, the FAB mass spectra of underivatized and per-*O*-acetylated xyloglucan oligoglycosyl alditols are significantly easier to interpret than those of the unreduced oligosaccharides[5,6]. However, traces of borate not completely removed during cleanup of the reduced samples form complexes with the oligoglycosyl alditols, leading to anomalies in the NMR spectra. Borate appears to selectively interact with side chains containing galactosyl (and fucosyl) residues (see below) producing chemical shift effects in the all of the residues of the side chain to which borate is esterified.

Specific chemical shift effects of the interaction of oligoglycosyl alditol **2r** with borate were examined by recording ^{1}H NMR spectra of highly purified **2r** in the presence of various additives (Fig. 3). The spectrum recorded in the absence of additives (Fig. 3A) included signals at δ 5.164 (H-1 of 2-linked αXyl^a) and δ 4.622 (H-1 of β-Glc^a). Addition of ~2 μL (35 μmol) of acetic acid-d_1 brought the pD

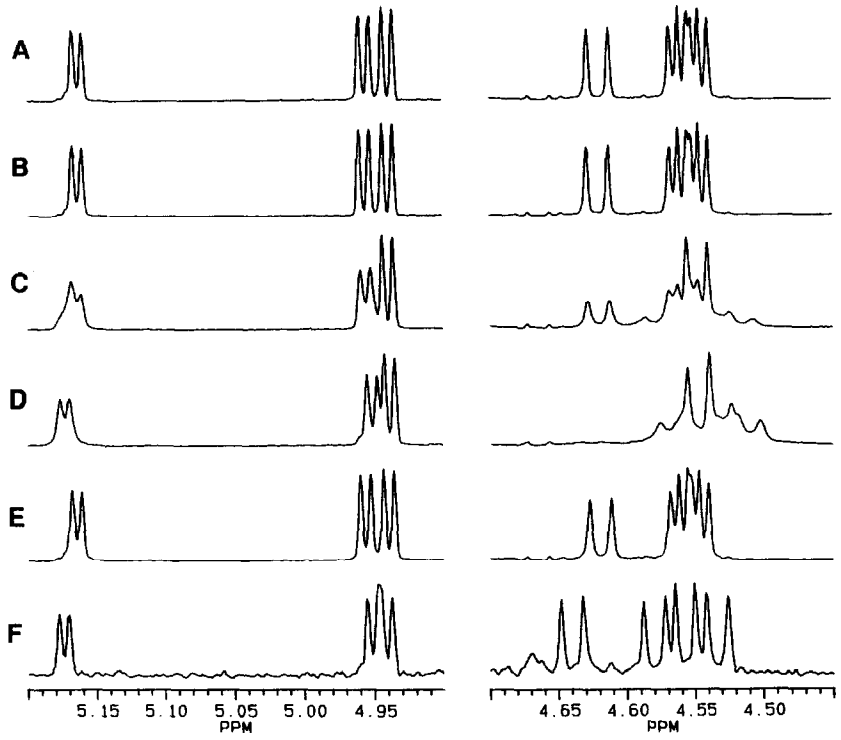

Fig. 3. Effects of borate and pD on the ^{1}H NMR spectrum of **2r**. Various components were added sequentially to a solution of highly purified **2r** in 0.5 mL of D_2O: (A) no additives; (B) acetic acid-*d* (2 μL, 35 μmol), pD ~ 4; (C) Na_2CO_3 (25 μmol) and $Na_2B_4O_7$ (5 μmol), pD 7.5; (D) additional Na_2CO_3 (35 μmol), pD ~ 9; (E) acetic acid-d_1 (8 μL, ~ 280 μmol), pD ~ 5; (F) purified **3r** with no additives, for comparison. Approximate pD values were measured with pH paper, and are not corrected for the different ionization constants for the protonated and deuterated forms of the pH indicator.

to ~ 4, but had no effect on the carbohydrate signals in the spectrum (Fig. 3B). Subsequent addition of 25 μmol of Na_2CO_3 and 20 μequiv of borate (5 μmol of $Na_2B_4O_7$) brought the pD to ~ 7.5, and resulted in a broadening and splitting of the H-1 resonance of the α-Xyl^a residue and a broadening of the H-1 resonance of β-Glc^a (Fig. 3C). Further adjustment of the pD to ~ 9 with Na_2CO_3 resulted in a single (broad) signal at δ 5.174 (Fig. 3D) for α-Xyl^a H-1, thus appearing to complete the transformation of the magnetic environment for this nucleus that was observed at pD 7. This result is consistent with a pD-dependent shift[22] in the equilibrium concentrations of the free and borate-complexed forms of **2r**. In addition, the β-Glc^a resonance at δ 4.622 was completely missing at pD 9 as a result of extreme line broadening and/or chemical shift effects. Finally, adjustment of pD to ~ 5 by addition of acetic acid-d_1 caused the borate complexes to dissociate and the original appearance of the spectrum to be reestablished (Fig. 3E).

The chemical shifts of signals in the α-anomeric region ($4.9 < \delta < 5.3$ of the spectrum of **2r** in the presence of alkaline borate (Fig. 3D) are nearly identical to

those in the spectrum of purified, borate-free **3r** (Fig. 3F). Although the two can be distinguished on the basis of signals in the β-anomeric region ($4.4 < \delta < 4.7$), the complete exclusion of borate–carbohydrate complexes from the sample is generally required for unambiguous results. Experiments involving the formation of borate complexes with mixtures of **2r** and **3r** (obtained from rapeseed and tamarind xyloglucan) were performed (data not shown) in order to demonstrate that the correlations that we have observed between the chemical shifts of ^{1}H NMR resonances and substructures of these oligoglycosyl alditols were not based on artifacts that resulted from the presence of traces of borate. These experiments showed that, under conditions where borate complexes did not form (i.e., no borate and/or low pD), the spectra contained the diagnostic signals for both components in the expected ratios, and that formation of borate complexes resulted in chemical shift and line-broadening phenomena similar to those described above for homogeneous **2r**.

Borate can be removed by passing the oligoglycosyl alditols through a mixed-bed ion-exchange resin, as previously suggested[5], but the anion-exchange phase of these resins tend to bind carbohydrate, resulting in unacceptably low recoveries for samples that contain only small amounts of material. Samples dissolved in D_2O can be titrated to pD < 6 with acetic acid-d_1 in order to prevent the formation of borate complexes (see above). Alternatively, the sample can be passed through a strong cation-exchange resin in the H^+ form, followed by repeated additions and evaporations of methanol to remove borate as its trimethyl ester. Care must be taken, however, to insure that the sample does not contain the salts of nonvolatile strong acids, because the acidic residue remaining after cation-exchange will destroy the oligoglycosyl alditol. Sulfuric acid, which is formed from sulfate salts commonly found in enzyme preparations used to release the oligosaccharides from the polymer, is particularly destructive. Sulfate salts are often coeluted with oligosaccharides during Bio-Gel P-2 chromatography, in spite of their significant difference in size, due to processes such as solvation of ions and ionic interactions with the matrix.

Effects of temperature variation.—Certain proton resonances in the ^{1}H NMR spectra of xyloglucan oligoglycosyl alditols exhibit significant temperature-dependent chemical shift effects, and so comparative analysis of these spectra requires careful temperature control. For example, the chemical shift of H-1 of the (2-linked) αXyl^a in the ^{1}H NMR spectrum of **2r** varies from δ 5.167 to 5.160 in the temperature range 296 to 302 K. Structure-dependent chemical shift effects were unambiguously distinguished from temperature-dependent chemical shift effects by recording spectra after adjusting the sample temperature to a point slightly above the ambient room temperature such that the chemical shift of the (highly temperature-dependent) HDO resonance was 4.75 ± 0.01 ppm, relative to internal acetone at 2.225 ppm. This corresponded to a setting of 298 to 300 K on the temperature control unit of the Bruker AM 500 spectrometer.

EXPERIMENTAL

Xyloglucan oligosaccharides.—Tamarind seed xyloglucan (200 mg, prepared by EtOH precipitation as previously described[5]) was incubated (96 h, ambient temperature) in buffer (100 mL, 50 m*M* HOAc–NaOAc, pH 5.2) containing *endo*-(1 → 4)-β-glucanase (20 units) from *Trichoderma reesei* (Megazyme Australia, Inc.). β-Galactosidase (EC 3.2.1.23) from *Aspergillus niger* (Sigma Chemical Co., Cat. No. G 3522, 1 unit) was added to an aliquot consisting of 90% of the *endo*-(1 → 4)-β-glucanase (EC 3.2.1.4) digest which had been acidified (pH 4.5) with glacial HOAc, and the solution was incubated for 96 h. Enzymes and salts were removed from the two aliquots (±β-galactosidase) by passing the solutions through a column of Amberlite MB-1 (10 and 2 mL, respectively). The eluates were lyophilized, dissolved in ~2 mL of H_2O, and separated into size classes by high-resolution Bio-Gel P-2 chromatography (Fig. 1), as had been described[5]. Xyloglucan oligosaccharides were also prepared by *endo*-(1 → 4)-β-glucanase digestion of xyloglucan purified from rapeseed hulls[5].

Oligoglycosyl alditols.—Oligosaccharides (0.5–3.0 mg) were reduced in aq NH_4OH (1 M, 250 μL) containing $NaBH_4$ (10 mg/mL). The borohydride was quenched with glacial HOAc added dropwise until no further effervescence was observed, and the solution was passed through AG 50W-X4 (H^+, Bio-Rad) cation-exchange resin. The solvent was evaporated and borate was removed by repeated addition and evaporation of CH_3OH (1 mL aliqouts).

High-pH anion-exchange chromatography (HPAE).—Oligosaccharides or oligoglycosyl alditols were dissolved (1–10 μg/μL) in H_2O and injected (20 μL) onto a Dionex CarboPac PA1 column (4 × 250 mm) equilibrated in 100 mM NaOH, containing 50 mM NaOAc (solution *A*). Separation was accomplished by elution with solution *A* for 1 min, followed by a 20-min linear gradient elution starting with solution *A* and ending with 100 mM NaOH containing 100 mM NaOAc (solution *B*), and finally isocratic elution with solution *B* for 20 min. All steps were carried out at a flow rate of 1 mL/min with pulsed amperometric detection.

FAB-mass spectrometry.—FAB-mass spectra were recorded with a VG Analytical ZAB-SE mass spectrometer operating at low resolution (1:1000) with an accelerating voltage of 8 kV. Underivatized oligoglycosyl alditols (1 μL of a ~10 μg/μL solution in H_2O) were mixed on the probe tip with 3-amino-1,2-propanediol (2 μL, Aldrich) for negative-ion FABMS. Oligoglycosyl alditols (100–500 μg) were per-*O*-acetylated with a mixture of trifluoroacetic anhydride and HOAc (ref 23), dissolved (10 μg/μL) in CH_3OH, and ~1 μL of this solution was mixed on the probe tip with thioglycerol (3-mercapto-1,2-propanediol, 2 μL, Aldrich) for positive-ion FABMS. The nominal masses reported herein were calculated from the observed monoisotopic exact masses of resolved isotopomers or from the chemical masses of unresolved high-mass ion clusters using the CARBOMASS[4] software developed in this laboratory.

NMR spectroscopy.—Hydroxyl protons of the oligoglycosyl alditols were ex-

changed with deuterons, samples (0.5–5 mg) were dissolved in D_2O (0.5 mL), and NMR spectra were recorded, at ~299 K (HDO line at δ 4.75 ± 0.01 relative to internal acetone at δ 2.225), with a Bruker AM 500 NMR spectrometer, except for the two-dimensional spectra of **3r**, which were recorded with a Bruker AMX 600 spectrometer. Double-quantum filtered {^{1}H, ^{1}H} COSY[17] spectra and 2D ^{1}H TOCSY[18] (HOHAHA) spectra were obtained under previously described[5] conditions.

{^{1}H, ^{13}C} HSQC spectra were recorded using the pulse sequence of Bodenhausen and Ruben[19] using composite 180° proton pulses (e.g., $90_x180_y90_x$) and two 3 ms spin-lock purge pulses[20] to eliminate the resonances of protons attached to ^{12}C. Typical aquisition parameters for {^{1}H, ^{13}C} HSQC spectroscopy at 500 MHz included a spectral width of 1250 Hz (2.5 ppm) in 2048 data points for the ^{1}H dimension (t_2), a spectral width of 7200 Hz (57.25 ppm) in 128 data points, zero-filled to 512 data points for the ^{13}C dimension (t_1), and a relaxation delay of 1 s between each of the 128 transients per t_1 increment. ^{13}C Decoupling during signal aquisition was accomplished with the GARP pulse sequence[24] (~4 watts power generated with the Bruker BFX-5 pulse amplifier).

{^{1}H,^{13}C} Inverse DEPT correlation spectroscopy was performed using a modification of the pulse sequence of Bendall et al.[21] with proton presaturation via WALTZ-16 during the relaxation delay. Chemical shift labeling in t_1 was accomplished by moving the first 90° ^{13}C-pulse incrementally into the presaturation period, which was kept constant (2.0 s), and phase-sensitive spectra were obtained by incrementing the phase of this pulse by 90° for each t_1 (i.e., a time proportional phase increment, TPPI[25]). Other parameters were comparable to those used for recording HSQC spectra, except that the data for the ^{1}H dimension consisted of 1024 data points. The pulse angle θ of the first proton pulse was set to 45°, giving all positive signals[21]. This approach has the advantage of permitting spectral editing based on the multiplicity of ^{13}C-attached protons[21] and provided high quality heteronuclear correlation spectra for **2r**. Nevertheless, its lack of sensitivity relative to that of HSQC made it innappropriate for the other oligoglycosyl alditols, which were only available in small quantities. Proton pulses for the TOCSY, HSQC and inverse DEPT experiments were generated by the decoupler.

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