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New features of plant-fructan structure revealed by methylation analysis and carbon-13 n.m.r. spectroscopy

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ABSTRACT

The chemical structures of inulins, levans, highly branched fructans, and fructan oligomers from plants were determined by gas-liquid chromatography of partially methylated alditol acetates and ^{13}C nuclear magnetic resonance spectroscopy of the native polymers. Results obtained by these sensitive techniques revealed that these polymers contained small proportions of linkages atypical of the expected structures; inulins were composed of primarily (2→1)-linked β-D-fructofuranosyl units, but contained measurable amounts of (2→1,2→6)-linked branch point residues and (2→6)-linked fructosyl units, and the levans possessed atypical (2→1)-linked fructofuranosyl and branch-point residues. The highly branched wheat fructans were particularly dynamic, exhibiting enrichment of (2→1)-linkages during early induction of synthesis but enrichment of (2→6) linkages upon extended periods of fructan accumulation. The techniques used here should prove valuable in determining the biological significance of these variations in structure.

INTRODUCTION

Fructans comprise major carbohydrate storage polymers in vegetative tissues of at least 36 000 species of higher plants, including 5 families of monocots and 5 families of dicots[1-3]. From chemical analysis of partially methylated fructofuranosides, Haworth and colleagues[4,5] deduced that inulins, found in dicots, consisted of contiguous (2→1)-linked β-D-fructofuranosyl units. Similar methods were used to determine that bacterial levans and the fructans of monocots were different, either being predominantly (2→6)-linked β-D-fructofuranosyl chains[6-8] or, upon more rigorous analysis, highly branched structures comprised of both (2→1)- and (2→6)-linked β-D-fructofuranosyl units terminating in sucrose[9-15]. The techniques used to deduce these features, while accurate for abundant linkages, were often not sufficiently sensitive to detect small amounts of other linkages that might have been present in the polymer. More recent studies of bacterial levans and plant fructans using ^{13}C-n.m.r.[16-20] and g.l.c.–m.s. of partly *O*-methylated alditol acetates[21-23] have demonstrated that these techniques provide sufficient sensitivity to determine both the proportion of (2→1)- and (2→6)-linked β-D-fructosyl units and

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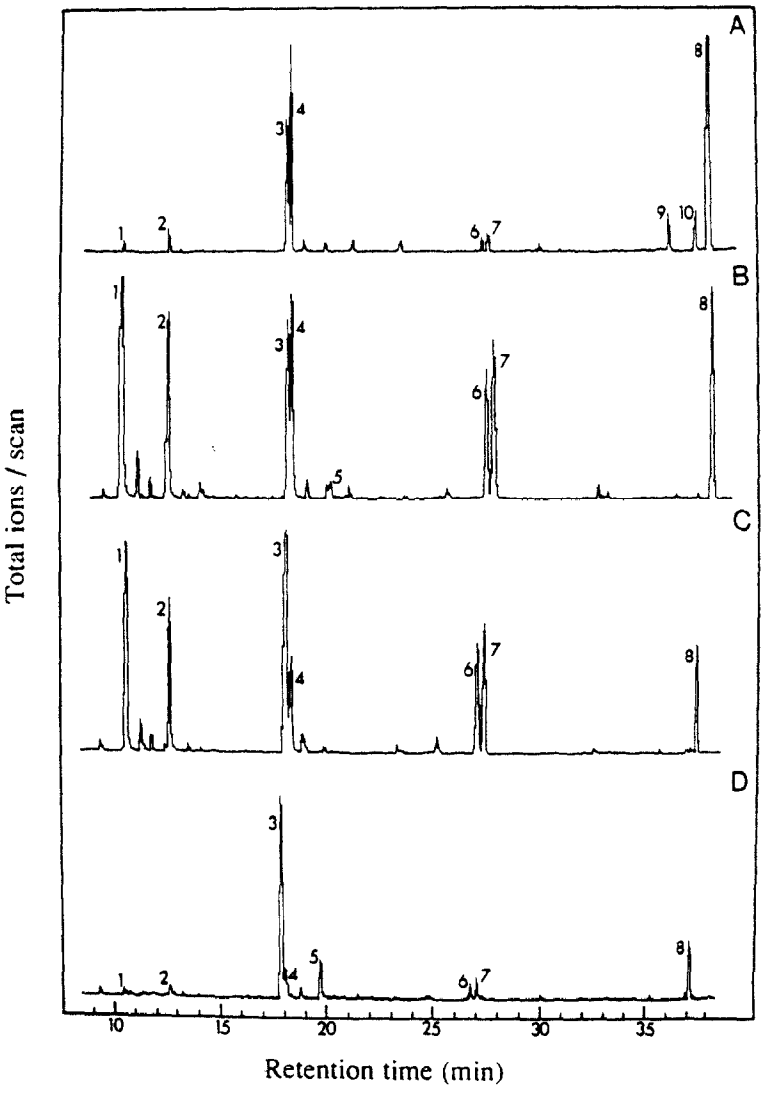

Fig. 1. Chromatograms reconstructed from total ion counts (m/z 50 to m/z 300) from 1-s scans from g.l.c.–e.i.m.s. of partially methylated alditol acetates from several fructans. A, Chicory inulin; B, induced wheat-leaf fructan; C, mature-leaf fructan; D, tall fescue fructan. Peaks identified were: 1, *t*-Fru (1,3,4,6-Me_4-Man-ol + 1,3,4,6-Me_4-Glc-ol); 2, *t*-Glc (2,3,4,6-Me_4-Glc-ol); 3, 1- and 6-linked Fru (3,4,6-Me_3-Man-ol and 1,3,4-Me_3-Man-ol); 4, 1- and 6-linked Fru (3,4,6-Me_3-Glc-ol and 1,3,4-Me_3-Glc-ol); 5,6-linked Glc (2,3,4-Me_3-Glc-ol); 6 and 7, 1,6-linked Fru branch point residue (3,4-Me_2-Man-ol and 3,4-Me_2-Glc-ol, respectively); 8, *myo*-inositol hexaacetate (internal standard); 9 and 10, mannitol and glucitol hexaacetate, respectively, resulting from slight undermethylation of some of the inulin.

the degree of branching of complex fructans. As a framework for future studies of the biosynthesis and degradation of complex fructans of higher plants, we compared these new methodologies in a study of the structures of some inulins and levans and the highly branched fructans of wheat.

RESULTS

Methylation analyses. — The 3,4,6- and 1,3,4-tri-*O*-methyl mannitol and glucitol acetate derivatives from (2→1)- and (2→6)-linked fructofuranosyl units, respectively, yield identical fragmentation patterns upon e.i.–m.s. The basis for determination of linkage is the asymmetry introduced by reduction of the hydrolytically formed, partially *O*-methylated fructoses at C-2 with sodium borodeuteride. Separation of the 3,4,6-Me_3 and 1,3,4-Me_3 derivatives of glucitol is theoretically possible, but they were essentially unresolved on the capillary column we used; and the corresponding mannitol derivatives are actually identical molecules. However, the 1,3,4-Me_3 derivatives from (2→6)-linked fructan yield ions of *m/z* 162 and 190 whereas the 2,3,6-Me_3 derivatives from (2→1)-linked fructan yield *m/z* 161 and 189. Lindberg *et al.*[21] and Hancock *et al.*[22] relied on this asymmetry to deduce the linkage structure of bacterial levans, inulin, and other plant fructans.

In both reports, the authors recognized that the reduction of fructose derivatives should yield both the mannitol and glucitol epimers, but neither the di- (nonreducing terminal), tri- (interior), nor tetra-*O*-acetyl (branch point) pairs of epimer derivatives were resolved chromatographically. However, these derivatives can be resolved on a highly polar capillary column (Fig. 1A–D). Either the mannitol or the glucitol di- or tri-*O*-methyl derivatives may be the favored reduction products, depending on the structure of the fructan. Partial *O*-methylation of the (2→6)-linked β-D-fructofuranosyl units in the cereal fructans, then hydrolysis and reduction, yields a single predominant epimer of 2,5,6-tri-*O*-acetyl-2-deuterio-1,3,4-tri-*O*-methylhexitol (Fig. 1C,D). Because every partially methylated mannitol acetate derivative precedes the comparable glucitol derivative on this type of column[24], we presume that formation of the mannitol derivative was favored. On the other hand, (2→1)-linked fructofuranosyl units yielded slightly more of the 1,2,5-tri-*O*-acetyl derivatives of glucitol than those of mannitol (Fig. 1A). Branched fructofuranosyl units also displayed a slight tendency to form the putative glucitol di-*O*-acetyl derivative (Fig. 1A–D).

Inulins produced mostly tri-*O*-acetyl derivatives that yielded ions of *m/z* 190 and *m/z* 161 as primary fragments, hence these derivatives were from (2→1)-linked β-D-fructofuranosyl units. After correction for ^{13}C spillover and $NaBD_4$ purity, small but significant amounts of *m/z* 189 and *m/z* 162 were detected, suggesting that (2→6)-linkages were also present (Fig. 1A; Table I). Pairs of tetra-*O*-acetyl derivatives indicative of (2→1,2→6)-linked branch point residues were also observed (Fig. 1A; Table I). Because the amounts of (2→6)-linked fructosyl units detected are indeed small, these results should be regarded as tentative. Nevertheless, the proportions of *m/z* 189 were always higher in the mannitol derivative than the glucitol derivative (not shown), and, with the virtual absence of undermethylation, detection of the branch point residues is unequivocal (Fig. 1A). Both the (2→6)-linked and the branched residues were ignored or not considered in earlier reports, perhaps because of their low abundance. Degrees of polymerization of the inulins, based on the ratio of the integrals of the fructosyl and glucosyl derivatives, were about 20 for both the dahlia tuber and chicory root polysaccharides (Table I).

TABLE I

Comparison of linkage structures of inulins and other fructans

Alditol products[a]	*Inulins*		*Other fructans*					
	Chicory root[b]	*Dahlia tuber*[c]	*Tall fescue leaves*[b]	*Wheat*				
				Induced leaves[b]	*Mature leaves*[b]	*Mature stem*[c]	*Immature stem*[c]	*Inflorescence*[c]
	(values in each column are molar ratios)[d]							
t-Fructosyl units:								
1,3,4,6-Me_4-Man-ol	1.3	0.6	1.2	2.2	2.9	1.9	4.9	6.9
1,3,4,6-Me_4-Glc-ol	tr	tr	tr	tr	tr	tr	tr	tr
t-Glucosyl units:								
2,3,4,5-Me_4-Glc-ol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1-Substituted fructosyl units:								
3,4,6-Me_3-Man-ol	6.2	7.4	0.8	0.7	0.5	0.1	0.9	1.8
3,4,6-Me_3-Glc-ol	7.6	9.7	0.4	0.5	0.8	0.3	0.2	2.1
6-Substituted fructosyl units:								
1,3,4-Me_3-Man-ol	0.2	0.1	40.6	1.2	2.3	1.6	2.6	9.4
1,3,4-Me_3-Glc-ol	0.2	0.1	n.d.	tr	tr	0.1	0.4	0.8
6-Substituted glucosyl units:								
2,3,4-Me_3-Glc-ol	tr	tr	3.2	tr	tr	tr	tr	tr
1,6-Disubstituted fructosyl units:								
3,4-Me_2-Man-ol	0.6	0.6	1.4	0.8	2.1	0.4	1.9	2.9
3,4-Me_2-Glc-ol	0.9	1.1	2.6	1.0	1.8	0.5	2.2	3.6
Ratio 6-linked/1-linked	0.03	0.01	32.7	0.29	1.9	5.7	1.4	2.6
D.p.	18.0	20.6	47.9	7.6	11.4	5.9	14.1	28.5

[a] Listed according to the types of units furnishing them. 1,3,4,6-Me_4-Man-ol = 2,5-di-*O*-acetyl-2-deuterio-1,3,4,6-tetra-*O*-methyl-D-mannitol, etc. [b] *O*-Methylated according to method A. [c] *O*-Methylated according to method B. Response factors, relative to terminal glucosyl units, for derivatives of fructosyl nonreducing terminal units (0.66), internal units (1.07), and branch-point units (1.12) were calculated from total ion counts from derivatives of 1-kestose, 6-kestose, nystose, and bifurcose standards[25]. [d] Based on 1 glucosyl residue per molecule; tr = trace amounts (less than 0.05); n.d. = not detected.

In contrast, the tri-*O*-acetyl derivatives from the fructofuranosyl units of tall fescue fructan yielded mostly ions of *m/z* 189 and *m/z* 162, showing that the polymer was mainly a (2→6)-β-D-fructan (Table I). Nevertheless, (2→1)-linked and (2→1,2→6)-linked branch point units were also observed (Fig. 1D). A substantial amount of 6-substituted glucose was also detected, indicating that the neokestose series of oligomers was also present (Table I). The fructans of tall fescue had d.p. of about 50 (Table I).

The highly branched fructans from field-grown wheat plants that had accumulated fructans for long periods of time also were mostly (2→6)-linked (Fig. 1C), but considerable amounts of (2→1)-linked fructosyl units were observed in addition to the (2→1,2→6)-linked branch-point residues (Table I). In contrast, fructans formed quickly after induction by chilling contained mostly (2→1) rather than (2→6) linkages in addition to the numerous branch point residues (Fig. 1B; Table I). The size of the fructans varied substantially depending on the tissue from which they were extracted. The d.p. of the polysaccharide from the inflorescence was about 29, while the value for that from young, immature stems was about 14, and for that from older, mature stems about 6. No further size fractionation was performed, so these values represent the averages of samples of polydisperse polysaccharides. Several of the oligomers were purified by gel-permeation and reverse-phase chromatography, and their structures were deduced as described in the companion paper[25].

Carbon 13 nuclear magnetic resonance spectroscopy. — ^{13}C-N.m.r. spectroscopy was used to verify the linkages deduced by g.l.c.–m.s., and in the process a more complete tabulation was made of the chemical shifts of the carbons of the D-fructofuranosyl units of the (2→6)-linked fructans and branched grass fructans. The chemical shifts of the carbons of linked fructosyl units were compared to those deduced for fructose, sucrose, bacterial and plant levans, and inulin[16–20]. Our results indicate that all of the fructosyl units were β-D-linked and in the furanose form (Table II).

Inulins from chicory and fructan from tall fescue each yielded six major proton-decoupled fructosyl signals, and the chemical shifts listed here (Table II) are consistent with values reported previously[16–20]. The signals of the CH_2OH groups also have been assigned unambiguously through deuterium labeling experiments[16]. Literature values of the resonances for fructofuranosyl units of grass levans have confirmed that most of the fructosyls are (2→6)-linked, but minor resonances were also observed and not fully characterized[16–18]. Two unusual features of the fructofuranosyl residues are the small changes in chemical shift displayed upon linkage compared to the changes shown by well-studied α and β glycopyranosides, and the effect of linkage on C-1, which induces a shift in resonance upfield rather than downfield[17–20]. Also in contrast to hexopyranosides, linkage to either C-1 in (2→1)-linked inulin or C-6 in (2→6)-linked fescue "phlein" results in downfield rather than upfield shifts of the adjacent carbons, and the magnitudes of the shift changes are greater than for the linked carbons themselves. Minor changes in chemical shift were also observed for some signals due to C-1 through C-4, most of which could be attributed to the carbons of the terminal units. The stable chair forms of pyranoses are essentially unaltered by linkage, whereas the furanose ring is puckered and more susceptible to changes in conformation. Hence, linkage results in

TABLE II

Carbon-13 n.m.r. chemical shifts of the fructofuranosyl moieties of sucrose, inulin, and other fructans[a]

Carbon atoms	*Sucrose (terminal)*	*Chicory inulin (2→1)-*	*Tall fescue fructan (2→6)-*	*Wheat leaf fructan (mixed)*
C-1	**64.07**	**63.55** 63.17 (0.16)	**62.61** 63.11 (0.18)[d] 63.75 (0.09)[e]	**63.23**[b,c] 62.82 (0.91)[d,e]
C-2	**106.38**	**105.88** 106.34 (0.10)[e]	**106.91** 106.50 (0.10) 106.33 (0.07)[e]	**106.36**[e] 106.19 (0.65)[c] 106.91 (0.47)[b] 105.95 (0.28)[d]
C-3	**79.11**	**79.67** 79.99 (0.25) 79.46 (0.24)[e]	**78.99** 79.40 (0.17)[c] 79.71 (0.10)[d]	**79.54**[b,d] 79.32 (0.65)[b,c] 79.13 (0.56)[c]
C-4	**76.71**	**76.97** 76.65 (0.08)[e]	**77.91** 77.30 (0.13)[d,e]	**77.11**[c] 78.04 (0.54)[b] 77.47 (0.42)[c,d]
C-5	**84.07**	**83.75**	**82.99** 83.84 (0.16)[d,e]	**83.03**[b,c] 83.79 (0.95)[d,e]
C-6	**65.11**	**64.82**	**66.10** 65.17 (0.18)[c] 64.89 (0.16)[c,d]	**66.12**[b] 65.06 (0.92)[c] 64.94 (0.59)[d] 65.35 (0.52)[c]

[a] Values for the largest signals are in bold type and, except for wheat fructan, indicate the chemical shift for the principal linkage group denoted in the legend. Minor signals are listed in order of relative abundance (in parentheses). Probable linkage groups, either fully resolved or overlapped, are indicated where possible. [b] (2→6)-Linked fructosyl unit. [c] (2→1,2→6)-Linked branch residue. [d] (2→1)-Linked fructosyl unit. [e] Unlinked, terminal fructosyl unit.

marked changes in the chemical shifts of carbons adjacent to the linked carbon[19].

Minor resonances from fructosyl carbons have been recognized before but were attributed to the non-reducing terminal units, even though the signals from corresponding glucosyl carbons were barely detectable and certainly not stoichiometric with the minor fructosyl resonances[18]. Chemically, the fructans from tall fescue analyzed by g.l.c.–m.s. were comprised predominantly of (2→6)-linked fructofuranosyl units, but with smaller yet significant amounts of (2→1)-linked and (2→1,2→6)-linked, branch-point furanosyl residues (Fig. 1; Table I). We propose that these minor resonances result from the substitution at C-1 in either the (2→1)-linked or (2→1,2→6)-linked, branch-point residues in addition to being derived from terminal fructosyl units (Table II). This proposal is supported further by our analysis of wheat fructan. Wheat fructans are also enriched in (2→6) linkages, but they were of smaller mol.wt. and exhibited a greater degree of branching (Table I). This complexity of structure yielded several changes in

chemical shifts for the anomeric carbons, the linked and unlinked primary CH_2OH carbons, and the secondary carbinol carbons (Table II). Because wheat fructans have four distinct types of fructosyl units, namely, (2→6)-linked, (2→1)-linked, (2→1,2→6)-linked branch-points, and the corresponding unsubstituted terminal units, several of the minor signals were resolved, and found to more closely match the minor resonances found in tall fescue fructans (Table II). The greater complexity of the C-3 and C-4 signals indicated that the four types of units should be differentiated, but numerous minor resonances broaden the signals considerably, and the extent to which this broadening reflects additional complexity of oligomeric structure is not clear. The signals for the anomeric carbons of various fructosyl units of acetylated 1-kestose, 6-kestose, neokestose, and their related oligomers were well resolved by ^{13}C-n.m.r.[26] Forsythe *et al.*[26] showed further that the signals of C-2 of each (2→1)-linked fructosyl unit of nystose and each terminal fructosyl unit of neokestose were also resolved, indicating that the neighboring sugars exert marked influence on the anomeric carbons[25]. We also observed multiple resonances for the anomeric carbons of the α-D-glucosyl terminal units of the wheat fructan oligomers (characterization as nonreducing terminal units confirmed by methylation analysis, data not shown). We have now purified and characterized specific tetra-, penta-, and hexa-meric components of the wheat fructan which should provide reasonable model compounds to study these subtle aspects of structure[25].

DISCUSSION

G.l.c.–e.i.m.s. and ^{13}C-n.m.r. spectroscopy provide methodologies sensitive enough to detect relatively small proportions of "unusual" linkages and branches in what were once considered homopolymers. It is important to note that inulins contain very small amounts of (2→6) linkages and (2→1,2→6)-linked branch-point units in addition to the expected (2→1) linkages, and the fructans of grasses contain (2→1) linkages and sometimes exhibit considerable branching in addition to the typical (2→6)-linked structure. The atypical linkages may result from the involvement of multiple enzyme activities in the synthesis of a single highly branched polymer, or may signal the presence of two kinds of branched fructans, one having a (2→1)-linked "inulin" and the other a (2→6)-linked "phlein" backbone[10–14]. It is important to consider that fructans are dynamic structures whose size is modulated diurnally depending on the physiological state of the plant[27]. Hence, enzymic synthesis of either (2→1) or (2→6) linkages and branch points could be of high fidelity whereas enzymic degradation of the branch points may not discriminate between the (2→1) and the (2→6) linkage, so that atypical linkages are introduced into the polymer structure upon hydrolysis of the branch point[23]. An enzyme specific for (2→1)-β-D-fructan was purified from barley[28], and this enzyme was used *in vitro* to establish unequivocal chemical structures for several oligomeric fructans[25]. This methodology could well be incorporated into studies of fructan dynamics to investigate the evolution of the complex polymer structure.

EXPERIMENTAL

Plant material. — Fructans were extracted from the inflorescences and stems of developing wheat (*Triticum aestivum* L. cv. Dobroudja). The winter wheat plants from field plots near Ft. Collins, CO were harvested 12 days prior to anthesis, and stems (minus leaf sheaths) and maturing inflorescences were excised and frozen in liquid N_2. Fructans were also induced by chilling wheat seedlings to 10° and keeping them 4 days under photosynthetically active light at 350 μE $m^{-2}s^{-1}$. Tissues were homogenized in aqueous 90% v/v ethanol, samples of the insoluble material were then extracted with water at 65° for thirty minutes to remove fructans, and the extract was lyophilized. Samples of the extract were analyzed for fructans by a furanose-specific anthrone method[29,30], and the molecular weights of the fructans were estimated by chromatography using a 1.5-cm × 85-cm column of Sephadex G-50 (Sigma Chemical Co.) with 0.1M NaCl containing 16 mg L^{-1} chlorhexidine as eluate. Leaves of a single tall fescue genotype (*Festuca arundinaceae* Schreb.) were collected from plants grown in a greenhouse at Purdue University. Fructans from these leaves were obtained essentially the same way except these tissues were preextracted with hot aqueous 92% ethanol and the residue was resuspended in water at ambient temperature; fructans were obtained by ethanol precipitation from concentrates of this water extract. The pre-extraction with aqueous ethanol removed low molecular weight oligomeric fructans (d.p. 3 to 6), and this may have contributed to the large apparent molecular weight recorded here (Table I) as compared to that reported by Volenec[31]. Inulins from roots of chicory (*Cichorium intybus* L.) and tubers of dahlia (*Dahlia variabilis* L.) were from Sigma.

Chemical analyses. — For structural determinations, lyophilized materials (1 to 3 mg) were dried over P_2O_5 in a vacuum desiccator and *O*-methylated according to Hakomori[32], but with technical refinements (method A)[23]. The inulins, tall fescue fructans, and wheat leaf fructans were also O-methylated with the aid of lithium methylsulfinylmethanide, formed by addition of *n*-butyllithium directly to the suspension of carbohydrate in Me_2SO (method B)[24,33]. The *O*-methylated fructans were hydrolyzed in 1 mL of M CF_3COOH containing 1 μmol of *myo*-inositol for 30 min at 70°. One mL of *tert*-butyl alcohol was added after cooling, and the solvents were evaporated at 30° in a stream of N_2. Sugars were reduced with sodium borodeuteride and acetylated as described by Blakeney *et al.*[34], but volumes of reagents were adjusted so that reactions could be processed in 1-dram vials having screw caps lined with poly(tetrafluoroethylene)[24]. Derivatives were separated in a 30-m × 0.2-mm vitreous silica WCOT capillary column coated with 3% SP-2330 (Supelco), temperature-programmed from 180° to 240° at 2 deg min^{-1}. Aliquots of 1 to 2 μL were injected with a split-ratio of about 1:50 into a helium flow of 1.5 mL min^{-1}. Injector and m.s. interface were at 260°. E.i.–m.s. was performed either with a Finnigan/MAT 9610 gas chromatograph coupled to a Finnigan/MAT 4021 quadrupole mass spectrometer interfaced with a Finnigan/MAT 2100C INCOS data system, or a Hewlett–Packard 5890 gas chromatograph coupled to a Hewlett–Packard quadrupole mass spectrometer Model 21 (MSD). Spectra were obtained at 70 eV and a source temperature of 160°. Ratios of peaks at *m/z* 190 to *m/z*

189 and *m/z* 161 to *m/z* 162 were determined in duplicate, and sometimes triplicate samples; these fragments contain 8 carbons, and hence each value was corrected for a natural abundance of ^{13}C of 8 × 1.11% (ref. 35) and judged empirically by comparison with other derivatives reduced with $NaBH_4$. The purity of the $NaBD_4$ (98%) was also reckoned into the correction.

^{13}C-N.m.r. spectroscopy. — Fifty to 200 mg of wheat or tall fescue fructan or 50 mg of inulin was dissolved in about 2 mL of deuterium oxide at ambient temperature. Spectra were accumulated at 50.31 MHz with a Varian XL 200A n.m.r. spectrometer system, using a sweep width of 16 000 Hz (320 p.p.m.), a recycle time of 2 s, and a 1 μs (70°) pulse. Broad-band waltz proton decoupling was achieved by irradiating the sample with 5 watts of r.f. power centered at a frequency 7 p.p.m. downfield from the proton resonance of Me_4Si, and having a bandwidth of 52 p.p.m. The resulting time-domain signals were processed with an exponential (2 Hz) filter prior to Fourier transformation.

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