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Structural studies of a heteroxylan from *Plantago major* L. seeds by partial hydrolysis, HPAEC-PAD, methylation and GC–MS, ESMS and ESMS/MS

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Abstract

The seed mucilage from *Plantago major* L. contains acidic heteroxylan polysaccharides. For further structural analysis, oligosaccharides were generated by partial acid hydrolysis and then isolated by high-pH anion-exchange chromatography (HPAEC). Each HPAEC fraction was shown by ESMS to contain one major oligosaccharide and several minor components. Partial structures of the oligosaccharides were determined using GC–MS, ESMS and ES tandem mass spectrometry (ESMS/MS). A (1→4)-linked xylan trisaccharide and (1→3)-linked xylan oligosaccharides with DP 6–11 suggested that the backbone of the heteroxylan polysaccharide consisted of blocks of (1→4)-linked and (1→3)-linked Xyl*p* residues. A (1→2)-linked Xyl*p* disaccharide and a branched tetrasaccharide were also found, revealing that single Xyl*p* residues are linked to the O-2 of some of the (1→4)-linked Xyl*p* residues in the backbone. In addition, our results confirm the presence of side chains consisting of the disaccharide Glc*p*A-(1→3)-Ara*f*.

Keywords: Plantago major L.; Heteroxylan; Oligosaccharides; Partial hydrolysis; HPAEC-PAD; GC–MS; ESMS; ESMS/MS; Methylation

1. Introduction

The leaves and seeds of *Plantago major* L. have been used for centuries in the treatment of wounds, ulcers and infections. Investigations have shown that the leaves contain a pectin and an arabinogalactan that possess anti-complementary activity [1–3]. The seeds form a mucilage in the presence of water due to polysaccharides in the seed coat. The seed mucilage polysaccharides are heteroxylans [4]. Other mucilage-forming seeds contain similar types of polysaccharides. Some examples are the seeds from *Plantago ovata* Forsk. and *Linum usitatissimum* L., which are well-known mild laxatives [5–7], and the seeds from *Plantago asiatica* L. (*P. major* var. *asiatica* Decaisne), which is used as an antiphlogistic, diuretic, antidiarrheic and as an antitussive

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agent in Japanese traditional medicine. The polysaccharides from *P. asiatica* possess anti-complementary activity and consist of a (1→4)-linked backbone with short side chains consisting of single xylose residues, α-D-Glc*p*A-(1→3)-α-L-Ara*f* and α-D-Gal*p*A-(1→3)-α-L-Ara*f* side chains linked to O-3 [8,9]. The acidic heteroxylan present in the seed mucilage of *P. major* also possesses anti-complementary activity but has a different structure than that from *P. asiatica* [4]. It consists of a (1→3)- and (1→4)-linked β-D-Xyl*p* backbone with short side chains attached to O-2 in some of the (1→4)-linked β-D-Xyl*p* residues and to O-3 of other (1→4)-linked β-D-Xyl*p* residues. The side chains consist of β-D-Xyl*p* and α-L-Ara*f* residues, α-L-Ara*f*-(1→3)-β-D-Xyl*p* and α-D-Glc*p*A-(1→3)-α-L-Ara*f*.

The sequence of the Xyl*p* residues in the heteroxylan backbone has not been determined. In the present investigation, oligosaccharides were generated from a *P. major* heteroxylan fraction by weak acid hydrolysis and then isolated by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The structures of the oligosaccharides were determined using GC–MS, ESMS and ES tandem mass spectrometry (ESMS/MS).

2. Results

Oligosaccharides in the acid hydrolysate were separated by HPAEC (Fig. 1). The fractions eluting before 20 min contained only monosaccharides: Fraction 1 co-eluted with xylose in HPAEC, and ESMS confirmed the presence of a pentose monomer. The fractions which were eluted after 26 min contained oligosaccharides. The monosaccharide composition of each isolated fraction was determined by total hydrolysis of the oligosaccharides with 2 or 4 M TFA depending on whether the fractions contained uronic acid residues or not. All fractions contained xylose. Arabinose and glucuronic acid were present in Fractions 9 and 10. ESMS revealed in addition to a major oligosaccharide in each fraction, the presence of several minor components. Since ESMS analysis of peaks 7 and 8 demonstrated that they were complex mixtures and the HPAEC-PAD chromatogram showed that they were quantitatively minor, no further attempts were made to determine the oligosaccharide structures present.

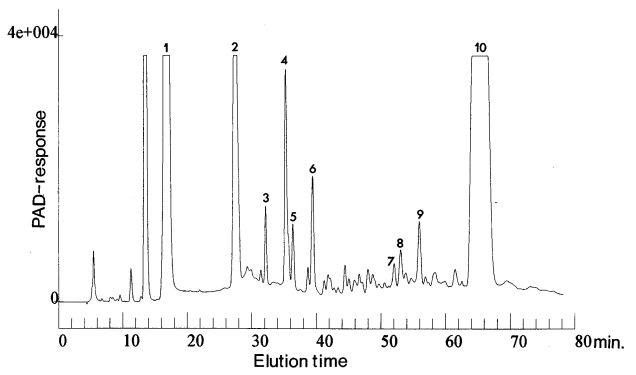

Fig. 1. Separation of oligosaccharides from the acid hydrolysate of a heteroxylan polysaccharide from the seeds of *P. major* L. using HPAEC-PAD.

The structures of the oligosaccharides were determined by combining results from total hydrolysis and HPAEC, GC–MS, ESMS and ESMS/MS (Table 1). The proposed structure of the major oligosaccharide in each isolated fraction is given in Table 2. Anomeric configurations were determined by NMR [4]. In the ES mass spectrum of HPEAC fraction 2 the most abundant ion is observed at m/z 305, which corresponds to $[M+Na]^+$ for a pentose dimer ($Pent_2$). Since composition and linkage analysis demonstrated that terminal and 4-substituted Xyl*p* are the only monosaccharides present in HPAEC fraction 2, the oligosaccharide is a (1→4)-linked Xyl*p* dimer.

The most abundant ion in the ES mass spectrum of HPAEC fraction 3 was observed at m/z 437. This ion corresponds to $[M+Na]^+$ for a trimer of pentoses. A less abundant ion was observed at m/z 305, which corresponds to $Pent_2$. Since fragmentation of oligosaccharides is seldom observed using the mild electrospray conditions applied, it was concluded that HPAEC Fraction 3 contains a mixture of $Pent_3$ and $Pent_2$. Non-reducing terminal Xyl*p* and (1→4)-linked Xyl*p* residues were found using GC–MS of the methylated alditol acetate derivatives of the oligosaccharides. Thus, the major oligosaccharide in HPAEC Fraction 3 is a trimer of (1→4)-

Table 1
The carbohydrate compositions of fractions generated from a heteroxylan polysaccharide from the seeds of *Plantago major* L. by TFA hydrolysis and HPAEC-PAD

	HPAEC fractions							
	1	2	3	4	5	6	9	10
$[M+Na]^+$ (m/z) major component	173 Pent	305 $Pent_2$	437 $Pent_3$	305 $Pent_2$	305 $Pent_2$	437 $Pent_3$	371[a], 569 HexA-Pent $Pent_4$	349 HexA-Pent
$[M+Na]^+$ (m/z) minor component(s)			305	569	437, 569	305, 701	701, 833, 965, 1097	1071[b], 1237[b] 1403[b], 1569[b] 1735[b], 1901[b]
			$Pent_2$	$Pent_4$	$Pent_{3,4}$	$Pent_{2,5}$	$Pent_{5,6,7,8}$	$Pent_{6,7,8,9,10,11}$
T-Xyl*p*		X[c]	X	X	X	X	X	X
(1→3)-Xyl*p*				X		X	(X)[d]	X
(1→2)-Xyl*p*					X			
(1→4)-Xyl*p*		X	X	X		X	X	
(1→2,4)-Xyl*p*							(X)	
(1→3)-Ara*f*							X	X
T-Glc*p*A							X	X

[a] $[M-H+2Na]^+$.
[b] $[M+Na]^+$ of per-*O*-deuteromethylated derivative.
[c] X = type of linkage present.
[d] (X) = traces of linkage detected.

linked Xyl*p* while the minor component is the (1→4)-linked Xyl*p* dimer.

The ES mass spectrum of HPAEC Fraction 4 contains ions at m/z 305 and 569, which correspond to the $[M+Na]^+$ ions of $Pent_2$ and $Pent_4$, respectively. In the tandem mass spectrum obtained on collisional activation of $[M+Na]^+$ of per-*O*-methylated $Pent_4$ (m/z 709) from HPAEC Fraction 4, a series of sodium-cationised fragment ions is observed at m/z 215, 375, and 535. These ions are identified as Y_1, Y_2, and Y_3 ions, respectively, using the nomenclature of Domon and Costello [10], and are indicative of a linear $Pent_4$.

Composition and linkage analysis of HPAEC Fraction 4 demonstrated the presence of both (1→3)- and (1→4)-linked xylose residues. Since the dimer was the major oligosaccharide in this fraction, it is not likely to arise by further contamination with the (1→4)-linked Xyl*p* dimer from Fraction 2, but rather from a (1→3)-linked Xyl*p* disaccharide.

The ES data obtained on HPAEC Fraction 5 were indicative of yet another $Pent_2$, with m/z 305 for $[M+Na]^+$. Monosaccharide and linkage analysis of HPAEC Fraction 5 demonstrated that this dimer was Xyl*p*-(1→2)-Xyl*p*.

In the ES mass spectrum of HPAEC fraction 6 ions were observed at m/z 305, 437 and 701. These ions correspond to the $[M+Na]^+$ ions of $Pent_2$, $Pent_3$, and $Pent_5$, respectively. The most abundant ion in the spectrum was the $[M+Na]^+$ ion for $Pent_3$. In the tandem mass spectra obtained on collisional activation of $[M+Na]^+$ of per-*O*-methylated $Pent_3$ (m/z 549) and $Pent_5$ (m/z 869) from HPAEC Fraction 6, a complete series of Y_n-type fragment ions is observed, indicative of linear $Pent_3$ and $Pent_5$. The oligosaccharides present in HPAEC Fraction 6 consist of both (1→4)- and (1→3)-linked Xyl*p* residues.

The ES mass spectrum obtained from HPAEC Fraction 9 had ions at m/z 371, 569, 701, 833, 965, and 1097. These ions correspond to the $[M-H+2Na]^+$ of HexAPent and $[M+Na]^+$ of $Pent_4$, $Pent_5$, $Pent_6$, $Pent_7$, and $Pent_8$, respectively, with the two major species being HexAPent and $Pent_4$.

Prior to tandem mass spectrometry those samples that appear to contain HexA were

per-*O*-deuteromethylated, in order to differentiate based on residue masses between monomethylhexose and HexA.

The tandem mass spectrum obtained from per-*O*-deuteromethylated $Pent_4$, $[M + Na]^+$ *m/z* 739 (Fig. 2), contained Y_n-type fragment ions at *m/z* 224, 390 and 565, consistent with a linear oligosaccharide. Remarkably, fragment ions were also observed at *m/z* 207 and 373. These ions are the result of double cleavage events, *m/z* 207 corresponding to the reducing-terminal pentose which has lost three residues from two different attachment sites to leave two unsubstituted hydroxyl groups, and *m/z* 373 corresponding to the disaccharide analogue also bearing two unsubstituted hydroxyl groups, reflecting a parent ion from which two pentoses must have been lost from two different attachment sites. These ions are thus indicative of a $Pent_4$ that is branched on the reducing pentose, Pent-Pent-(Pent)Pent.

Linkage analysis of HPAEC Fraction 9 demonstrated the presence of terminally linked and (1→4)-linked Xyl*p* residues. There were also indications for the presence of (1→3)- and (1→2,4)-linked Xyl*p* residues but no (1→3,4)-linkages. Due to the large amount of (1→4)-linked residues, the tentative structure of this tetrasaccharide is a (1→4)-linked Xyl*p* trimer with a single Xyl*p* residue attached to O-2 of the reducing end. Linkage analysis also revealed the presence of terminal Glc*p*A and (1→3)-linked Ara*f*. The dimer Glc*p*A-(1→3)-Ara*f* has been described previously [4]. Nevertheless, the presence of Glc*p*A-(1→*n*)-Xyl*p* cannot be excluded.

The ES mass spectrum obtained from underivatised HPAEC Fraction 10 very clearly demonstrated the presence of HexAPent, *m/z* 349 $[M + Na]^+$. However, in the ES mass spectrum of per-*O*-deuteromethylated fraction 10 obtained using a relatively high cone voltage of 120 V, ions were observed (in addition to the $[M + Na]^+$ from per-*O*-deuteromethylated HexAPent at *m/z* 486) at *m/z* 1071, 1237, 1403, 1569, 1735, and 1901, corresponding to $[M + Na]^+$ for $Pent_6$, $Pent_7$, $Pent_8$, $Pent_9$, $Pent_{10}$, and $Pent_{11}$, respectively (Fig. 3). Monosaccharide analysis of this fraction revealed the presence of glucuronic acid, arabinose and some xylose. Glucuronic acid was previously found only terminally linked in the original polysaccharide [4], and linkage analysis of Fraction 10 clearly demonstrated the presence of (1→3)-linked Ara*f* and terminal and (1→3)-linked Xyl*p*. Thus, we assign the isolated disaccharide as the aldobiuronic acid Glc*p*A-(1→3)-Ara*f*. This structure was also present in HPAEC Fraction 9. The polypentoses we thus assign as being composed of (1→3)-linked Xyl*p* residues.

3. Discussion

These results confirm that the original heteroxylan consists of both (1→4)- and (1→3)-linked Xyl*p* residues. The linkages may be randomly distributed along the backbone or they may exist as repeating sequences containing blocks of each linkage type having fixed sizes. The present results show that the Xyl*p* residues are in blocks, but it is not known whether the blocks have fixed sizes or not. A (1→4)-linked Xyl*p* trisaccharide and (1→3)-linked Xyl*p* oligosaccharides with DP 6–11 were found. They correspond to (1→4)-linked

Table 2
Proposed structures of oligosaccharides prepared from a heteroxylan from the seeds of *Plantago major* L.

Peak	Major oligosaccharides	Minor oligosaccharides
2	β-D-Xyl*p*-(1→4)-β-D-Xyl*p*	
3	β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*	β-D-Xyl*p*-(1→4)-β-D-Xyl*p*
4	β-D-Xyl*p*-(1→3)-β-D-Xyl*p*	
5	β-D-Xyl*p*-(1→2)-β-D-Xyl*p*	
9	β−D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p* (2↑1 β-D-Xyl*p*)	α-D-Glc*p*A-(1→3)-α-L-Ara*f*
10	α-D-Glc*p*A-(1→3)-α-L-Ara*f*	β-D-Xyl*p*-(1→3)[β-D-Xyl*p*-(1→3)]$_{4-9}$-β-D-Xyl*p*

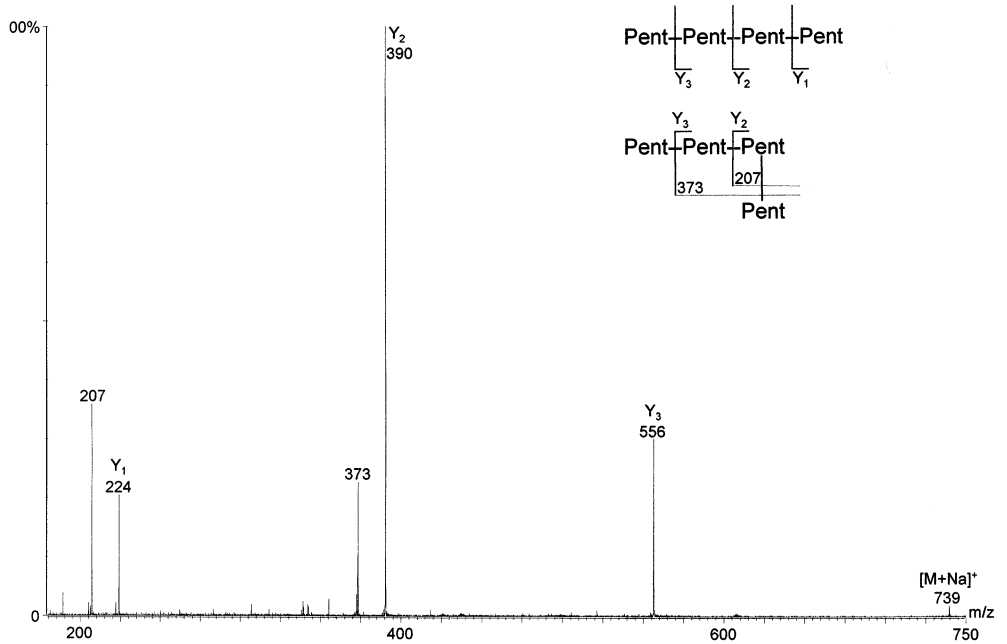

Fig. 2. Positive ES tandem mass spectrum of $[M + Na^+]$ of per-*O*-deuteromethylated $Pent_4$ oligosaccharides in HPAEC fraction 9.

blocks consisting of two Xyl*p* residues and (1→3)-linked blocks having up to ten residues. The presence of larger blocks in the original polysaccharide cannot be ruled out. Due to the (1→3)-/(1→4)-linked Xyl*p* ratio that was about 1:0.8 in the original polysaccharide [4] and the finding of blocks consisting of up to ten (1→3)-linked Xyl*p* residues in the present study, (1→4)-linked blocks containing more than two residues probably exist.

Since no (1→2)-linked Xyl*p* residues were detected in the original heteroxylan, the (1→2)-linked Xyl*p* disaccharide isolated must have been formed from side chains on the xylan backbone. Both the (1→2,4)- and (1→3,4)-linked Xyl*p* residues that were previously found in the original polysaccharide are assumed to originate from (1→4)-linked residues in the backbone, and the short side chains of Xyl*p* and Ara*f* residues, Ara*f*-(1→3)-Xyl*p* and Glc*p*A-(1→3)-Ara*f*, are attached to O-2 and O-3 [4]. Thus, the reducing-end Xyl*p* in the disaccharide from HPAEC fraction 5 is from the backbone while the non-reducing end is a substituent linked to O-2 of the backbone. This type of Xyl*p* substitution was confirmed by the demonstration of the branched tetrasaccharide that was detected in HPAEC Fraction 9.

The disaccharide Glc*p*A-(1→3)-Ara*f* originates from side chains attached to O-2 or O-3 of the (1→4)-linked Xyl*p* residues in the backbone [4].

From the present and previous investigations, the structure of the *P. major* seed heteroxylan can be summarized as follows: the xylan backbone consists of blocks of (1→4)-linked and (1→3)-linked Xyl*p* residues, respectively. The sizes of the blocks may vary, but the largest blocks isolated contain two (1→4)-linked residues and ten (1→3)-linked residues. Single Xyl*p* residues are linked to O-2 of some (1→4)-linked Xyl*p* residues in the backbone. Glc*p*A-(1→3)-Ara*f* and Ara*f*-(1→3)-Xyl*p* side chains are also present in addition to single Ara*f* residues linked to O-2 or O-3 of the (1→4)-linked Xyl*p* backbone residues.

4. Methods

Material.—Polysaccharides were isolated from the seeds of *P. major* L. by water extraction at 50 °C. Fractions were obtained by ion-exchange chromatography of the crude extract on a DEAE-Sepharose fast flow (Pharmacia) column which was eluted with a NaCl gradient (0–1 M). The fraction subjected to further analysis was eluted from the column with 0.84–0.90 M NaCl and designated C [4].

Weak acid hydrolysis.—Fraction C (25 mg) was treated for 1 h with 0.1 M TFA at 100 °C. The hydrolysate was cooled and evaporated under reduced pressure at 40 °C, washed with methanol and concentrated to dryness. The washing with methanol was repeated several times for removal of the reagent. The hydrolysate was then dissolved in distilled water and fractionated by HPAEC-PAD.

Separation of oligosaccharides by HPAEC-PAD.—Oligosaccharides were separated by HPAEC carried out on an LC-system (Dionex Corporation, Sunnyvale CA) equipped with a semi-preparative Carbo Pac PA-1 (Dionex) column (9 × 250 mm), coupled to a Spectra System AS 3500 autosampler (Thermo Separation Products). Detection was carried out using pulsed amperometric detection (PAD-II, Dionex).

A 100 μL sample was injected, and the column was eluted at 1.0 mL/min with a gradient composed of 100 mM NaOH (E1) and 100 mM NaOH/1000 mM sodium acetate (NaOAc) (E2) using the following program: 0–5 min 98% E1 and 2% E2, 5–60 min 98–80% E1 and 2–20% E2, 60–100 min 80% E1 and 20% E2.

NaOH (100 mM) was prepared from a 50% solution NaOH (T.J. Baker, The Netherlands) to minimize the carbonate content in the final eluent. NaOAc (100 mM) was prepared using solid NaOAc (Riedel-de Haen, Germany) and MilliQ water. The eluents were degassed by flushing with helium and pressurized continuously using an eluent degas module (EDM-2, Dionex). The eluted fractions were desalted on-line using a Carbohydrate Membrane Desalter (CMD, Dionex) that was eluted with 75 mM sulfuric acid at ≥ 7 mL/min with the SRC-1 power supply (Dionex) at the highest setting (500 mA).

Monosaccharide analysis.—Each peak isolated by HPAEC-PAD was hydrolyzed with 2 or 4 M TFA at 100 °C for 4 h. The hydrolysates (25 μL) were applied to HPAEC-PAD analysis on a CarboPac PA-10 guard and analytical column (Dionex) and eluted with deionized, degassed water at a flow rate of 1.5 mL/min. Postcolumn addition of 300 mM NaOH was performed to aid detection. After 27 min, the column was regenerated with 200 mM NaOH.

Uronic acids were analyzed under the same experimental conditions as used for the separation of oligosaccharides as described above.

Methylation and GC–MS.—The isolated oligosaccharides were methylated using NaOH in Me_2SO [11,13]. The partially methyl-

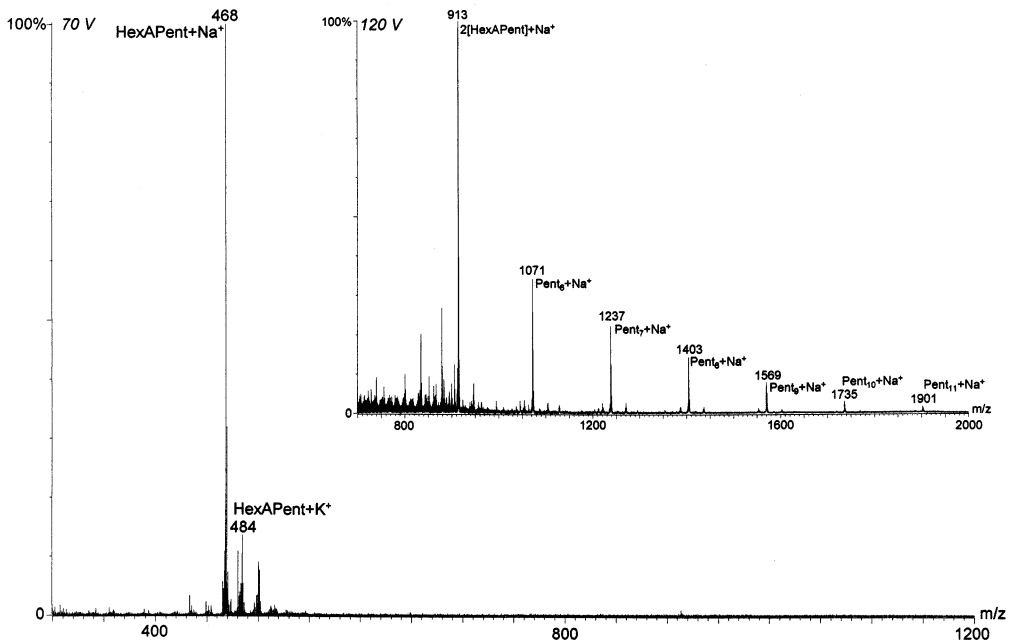

Fig. 3. Positive ES mass spectrum of per-*O*-deuteromethylated HPAEC fraction 10 using a cone voltage of 70 V. Insert: positive ES mass spectrum of per-*O*-deuteromethylated HPAEC fraction 10 using a cone voltage of 120 V. The ion observed at m/z 913 is the singly charged dimeric cluster of HexAPent.

ated alditol acetates were analyzed by GC–MS. The gas chromatograph was fitted with a split–splitless injector used in the split mode and a Supelco fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm. The column was inserted directly into the ion source of the mass spectrometer. The injector temperature was 250 °C and the detector temperature was 300 °C. The column temperature was 80 °C at the time of injection; after 5 min temperature was increased with 30 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then 30 °C/min to 300 °C at which it was kept for 25 min. Helium was the carrier gas with a flow rate of 0.9 mL/min. EI mass spectra were obtained using Fisons Instruments MD800 Mass Selective Detector 5970 with a Fisons Instruments GC8000 series (8065) gas chromatograph.

ESMS.—Oligosaccharides were dissolved in 100 μL methanol/water (1:1, v/v, 1% HCOOH). Positive-mode electrospray mass spectra were obtained using a VG Platform II single quadrupole mass spectrometer. Aliquots (10 μL) were infused into a mobile phase of methanol/water (1:1, v/v, 1% HCOOH) and introduced into the electrospray source at a flow rate of 5 μL/min. Spectra were scanned at a speed of 8 s for m/z 200–2000, with a cone voltage of approx 70 V, and recorded and processed using the MassLynx software, version 2.0. Mass calibration was performed by multiple-ion monitoring of per-*O*-acetylated maltooligosaccharides derived from corn syrup.

ESMS/MS.—To elucidate the monosaccharide sequence and branching patterns, the oligosaccharides were per-*O*-methylated to enhance their mass spectrometric sensitivity and to direct mass spectrometric fragmentation.

The oligosaccharides were per-*O*-methylated using a modification of the method of Ciucanu and Kerek [11] as described in Ref. [12].

Positive ES tandem mass spectra were obtained on a Micromass Q-TOF hybrid tandem mass spectrometer equipped with a Z-Spray sample introduction system and gold coated glass capillaries in a nanospray ionization source. Cone voltage and skimmer off-set were set at approximately 70 and 0 V, respectively, with a capillary voltage of 2700 V. Argon was used as collision gas with a collision energy of 60 eV for the sodium-cationized compounds. 10% of the native sample was used for methylation. The per-*O*-methylated products were dissolved in 100 μL methanol:water (1:1) and 1 μL of the sample was introduced into the glass capillary. Spectra were acquired with the Tof analyzer over a mass range that is dependent on the molecular mass of the analyte, data were integrated every 2.3 s, and processed using the MassLynx software, version 3.0.

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