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CHARACTERIZATION OF A STRUCTURALLY COMPLEX HEPTASACCHARIDE ISOLATED FROM THE PECTIC POLYSACCHARIDE RHAMNOGALACTURONAN II[†,*]

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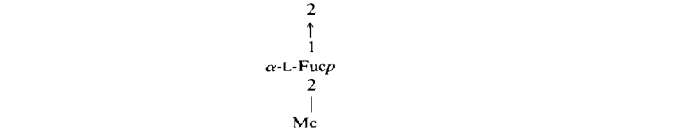
ABSTRACT

A heptasaccharide was released from the plant cell-wall, pectic polysaccharide rhamnogalacturonan II by selective acid hydrolysis of the glycosidic linkages of apiosyl residues. The heptasaccharide was purified to homogeneity by gel filtration and anion-exchange chromatography. Some of the heptasaccharide molecules were found to be mono- and some di-*O*-acetylated, but the location of the acetic ester groups was not determined. The heptasaccharide was found to have the following structure, where AceA = an aceryl (3-*C*-carboxy-5-deoxy-L-xylosyl) residue, and Api = an apiose residue.



INTRODUCTION

Rhamnogalacturonan II (RG-II) is a complex, pectic polysaccharide that is released from the cell walls of suspension-cultured, sycamore cells (*Acer pseudo-*

[†]Structure of Plant Cell-Walls, Part XIV. For Part XIII, see ref. 1.
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platanus) by the action of an endopolygalacturonase isolated from *Colletotrichum lindemuthianum*. RG-II is size-homogeneous and contains ~60 glycosyl residues[2] per molecule.

RG-II yields at least 10 different monosaccharides upon acid hydrolysis, including the unusual sugar apiose [Api; 3-*C*-(hydroxymethyl)-D-*glycero*-tetrose], and the unusual sugar derivatives 2-*O*-methylfucose, and 2-*O*-methylxylose[2]. A new branched-chain sugar, aceric acid (AceA; 3-*C*-carboxy-5-deoxy-L-xylose) has also been identified[1] as a component of RG-II. The monosaccharide constituents of RG-II are interconnected by at least 15 different glycosidic linkages[2].

The structure of intact RG-II is too complex to be characterized by conventional methods. Therefore, selective acid hydrolysis of the apiosyl glycosidic linkages of RG-II was used to generate a mixture of smaller oligosaccharides, and we now report the structural characterization of a heptasaccharide that was isolated therefrom.

EXPERIMENTAL

Isolation of RG-II. — RG-II was isolated from primary cell-walls of suspension-cultured, sycamore cells, as described[1].

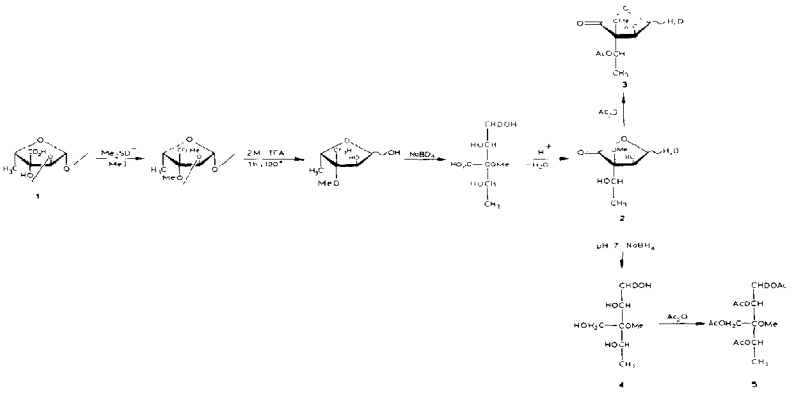
Glycosyl-composition analysis. — The glycosyl-residue composition of the heptasaccharide was determined by g.l.c. and g.l.c.–m.s. analyses after hydrolysis in 2M trifluoroacetic acid (TFA) during 2 h at 120°, reduction of the hydrolyzate with sodium borodeuteride ($NaBD_4$), and acetylation of the products[3].

Preparation of 2-O-methyl-L-fucose. — A sample of 2-*O*-methyl-L-fucose was prepared from methyl α-L-fucopyranoside (Sigma Chemical Co.). Methyl α-L-fucopyranoside (25 mg) was converted into the 3,4-*O*-isopropylidene derivative by the procedure of Lipták *et al.*[4], and this was *O*-methylated[5,6]. Acid hydrolysis of the resulting methyl 3,4-*O*-isopropylidene-2-*O*-methyl-α-L-fucopyranoside yielded 2-*O*-methyl-L-fucose.

Glycosyl-linkage composition of the heptasaccharide. — To a solution of the RG-II heptasaccharide (0.5 mg) in dimethyl sulfoxide (0.4 mL) was added 4M sodium dimethylsulfinyl anion (75 μL), and the solution was stirred for 2 h. Methyl iodide (75 μL) was added, and the solution was stirred for a further 2 h. Per-*O*-methylated carbohydrate was isolated by chromatography on a Sep-Pak C_{18} cartridge (Waters Associates), as described by Waeghe[7]. The glycosyl linkages of the neutral residues were determined by g.l.c.–m.s. after hydrolysis, reduction with $NaBD_4$, and acetylation. The glycosyl linkage of aceric acid was determined by a modified procedure. An aliquot (0.2 mg) of the per-*O*-methylated carbohydrate was hydrolyzed, and the products were reduced with $NaBD_4$, as described[8], and the solution was de-ionized by passage through a column (2 mL) of Dowex 50 (H^+) resin, and then lyophilized. This procedure yielded a mono-*O*-methyl lactone derivative (compound **2**) of aceric acid. The mono-*O*-methyl lactone **2** was carboxyl-

reduced by the procedure of Jones and Albersheim[9], yielding compound **4**, which was per-*O*-acetylated to give compound **5**; this was analyzed by g.l.c.–m.s.

Per-O-(trideuteriomethyl)ation. — A solution of the heptasaccharide-alditol (3 mg) in dimethyl sulfoxide (2 mL) was stirred for 4 h, 4M sodium dimethylsulfinyl



Scheme 1. Reaction sequence used to determine the glycosyl linkages of the aceryl residue in the heptasaccharide-alditol. [This Scheme illustrates the derivatives obtained from the 2-linked aceryl residue (1).]

anion (75 μL) was added, and the mixture was stirred overnight. Trideuteriomethyl iodide (20 μL; Stohler Isotopic Chemicals) was added, and the solution was stirred for 2 h. Sodium dimethylsulfinyl anion and trideuteriomethyl iodide were added twice more, 100 μL of trideuteriomethyl iodide being used for the final addition.

L.c.–m.s. separation of the mixture of per-O-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols generated by degradation of the aceryl residue during per-O-(trideuteriomethyl)ation of the heptasaccharide-alditol. — The mixture of per-*O*-deuteriomethylated oligomers, generated by partial degradation of the aceryl residue of the heptasaccharide, was separated by 3.52-MPa, l.c.–m.s. on a Dupont Zorbax ODS column, using a linear, 45-min gradient of 1:1 to 3:1 acetonitrile–water. All other l.c.–m.s. conditions were as described[10].

Preparation of per-O-alkylated oligosaccharide-alditols from per-O-(trideuteriomethyl)ated fragments generated by degradation of the aceryl residue of the heptasaccharide-alditol. — The fractions containing material that was eluted from the Dupont Zorbax ODS column with retention times lying between 4 and 10 min (see Fig. 5) were pooled and evaporated to dryness under a stream of filtered air. The material was partially hydrolyzed by treatment with 88% formic acid for 2 h at 50°, and the formic acid was evaporated under a stream of filtered air. The sample

was reduced with $NaBD_4$, de-ionized by passage through a column (2 mL) of Dowex 50 (H^+) resin, lyophilized, and dried overnight in a vacuum oven at 40°. The sample was dissolved in dimethyl sulfoxide (0.5 mL), 4M sodium dimethylsulfinyl anion (100 μL) was added, and the solution was stirred for 4 h. Pentadeuterioethyl iodide (100 μL; Stohler Isotopic Chemicals) was added, and the mixture was stirred for 3 h. The per-*O*-alkylated oligosaccharide-alditols were isolated from the reaction mixture by chromatography on a Sep-Pak C_{18} cartridge, as described[7].

G.l.c.–m.s. of per-O-alkylated oligosaccharide-alditols derived from the heptasaccharide. — Partially-*O*-(trideuteriomethyl)ated, partially-*O*-(pentadeuterioethyl)ated, oligosaccharide-alditols were analyzed by g.l.c.–m.s. on a 15-m DBI (J & W) capillary column (0.32 mm i.d.), as described[11].

Isolation of per-O-alkylated oligosaccharide-alditols [a], [d'], and [f] (Tables II and III) by l.c. — An aliquot (2 mg) of the per-*O*-alkylated oligosaccharide-alditols derived from the heptasaccharide was separated by l.c. on a Dupont Zorbax ODS column, using a linear, 30-min gradient of 1:1 to 13:7 acetonitrile–water. Per-*O*-alkylated oligosaccharide-alditols were detected in the l.c. effluent by g.l.c.–e.i.-m.s. analysis[8] of aliquots of the column fractions.

Fast-atom bombardment–mass spectrometry (f.a.b.–m.s.). — The techniques used in f.a.b.–m.s. were as described[1].

^{1}H-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250, Fourier-transform, n.m.r. spectrometer operated at 250 MHz. Water-soluble samples were lyophilized twice from deuterium oxide (99.7 atom% D), and dissolved in deuterium oxide (99.997 atom% D). Chemical shifts of aqueous samples were assessed relative to internal 1,4-dioxane (δ 3.70). Methylated samples were dissolved in hexadeuterioacetone (99.997% D), and chemical shifts were assessed relative to internal pentadeuterioacetone (δ 2.04).

RESULTS AND DISCUSSION

Isolation of an oligosaccharide released by partial, acid hydrolysis of RG-II. — Purified RG-II (125 mg) was treated with 0.1M trifluoroacetic acid (TFA) for 24 h at 40°, and the extent of hydrolysis of glycosyl residues was determined as described[11]. Under these conditions, ~35% of the apiosidic linkages and ~5% of the 2-*O*-methylfucosidic and arabinofuranosidic linkages were hydrolyzed. There was no detectable hydrolysis of any other glycosidic linkage.

The partially hydrolyzed RG-II was applied to a heated (65°) column (1.5 × 85 cm) of Bio-Gel P-10 (200–400 mesh) that had been pre-equilibrated in 50mM sodium acetate buffer (pH 5.2). Two major peaks of carbohydrate-containing material were resolved (see Fig. 1). The first peak had approximately the same elution volume as untreated RG-II. The second peak (shaded) was pooled, de-ionized by passage through a column (1 × 6 cm) of Dowex 50 (H^+) resin, and lyophilized.

Lyophilized peak II from the Bio-Gel P-10 column was chromatographed on

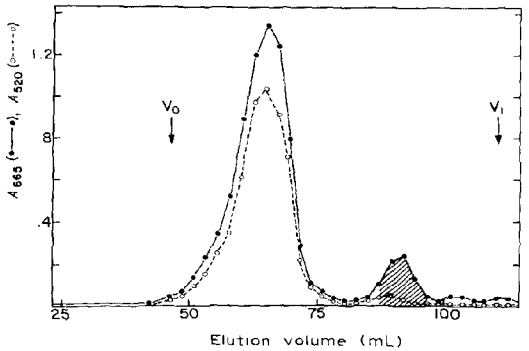


Fig. 1. Chromatography of partially hydrolyzed RG-II in a heated (65°) column (1.5 × 85 cm) of Bio-Gel P-10 (200–400 mesh). [The column was equilibrated in 50mM sodium acetate buffer (pH 5.2). Collected-fraction volume was 2.5 mL. Fractions were assayed for neutral-sugar content by the orcinol method[12] (A_{665}) and for uronic acid content by the *m*-hydroxybiphenyl method[13] (A_{520}). The shaded fractions were pooled for further purification.]

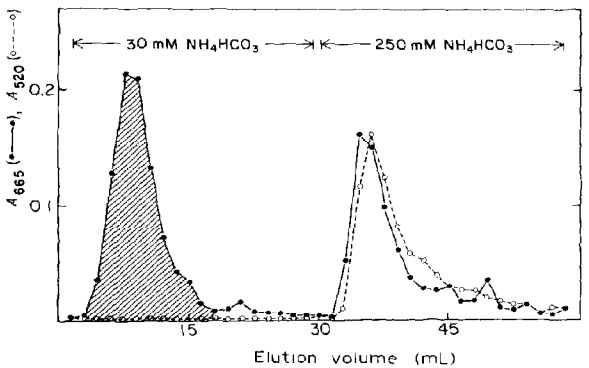
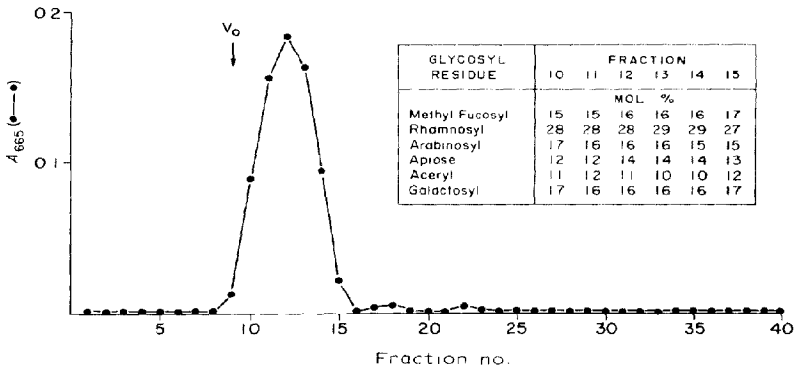


Fig. 2. Chromatography, on QAE-Sephadex, of pooled material from the Bio-Gel P-10 column. [A column (1 × 2 cm) of QAE-Sephadex Q-50-120 was equilibrated with 30mM NH_4HCO_3. After sample application, the column was eluted with 15 bed-volumes of 30mM NH_4HCO_3, followed by 15 bed-volumes of 250mM NH_4HCO_3. Collected-fraction volume was 1.5 mL. Fractions were assayed[20] as described in legend to Fig. 1.]

a column (1 × 2 cm) of QAE-Sephadex Q-50-120 that had been pre-equilibrated in 30mM NH_4HCO_3 (see Fig. 2). The column was eluted with 15 bed-volumes of 30mM NH_4HCO_3, and then with 15 bed-volumes of 250mM NH_4HCO_3. A peak of orcinol-positive[12] material (shaded region in Fig. 2) was eluted from the column by



GLYCOSYL RESIDUE	FRACTION 10	11	12	13	14	15
	MOL %					
Methyl Fucosyl	15	15	16	16	16	17
Rhamnosyl	28	28	28	29	29	27
Arabinosyl	17	16	16	16	15	15
Apiose	12	12	14	14	14	13
Aceryl	11	12	11	10	10	12
Galactosyl	17	16	16	16	16	17

Fig. 3. Chromatography, on Bio-Gel P-6, of the material that was eluted from the QAE-Sephadex column with 30mM NH_4HCO_3. [A heated (65°) column (1 × 75 cm) of Bio-Gel P-6 (100–200 mesh) was equilibrated in H_2O. Collected-fraction volume was 2.5 mL. Fractions were assayed for neutral-sugar content by the orcinol method[12]. The glycosyl-residue compositions of fractions 10–15 (inset) were determined as described in the text.]

the 30mM NH_4HCO_3. This material, which did not give a positive response in the *m*-hydroxybiphenyl assay[13] for uronic acids, was the oligosaccharide characterized from hereon. (The 250mM NH_4HCO_3 eluted material that gave positive responses in both the orcinol and the *m*-hydroxybiphenyl assays, and this material has not yet been further characterized.)

Determination of glycosyl composition, and size, of the isolated oligosaccharide. — The material that was eluted from the QAE-Sephadex column by 30mM NH_4HCO_3 (shaded region in Fig. 2) was pooled and lyophilized. This material was chromatographed in water on a heated (65°) column (1 × 75 cm) of Bio-Gel P-6, and was eluted as a symmetrical, partially included peak (see Fig. 3). Aliquots were taken from the fractions comprising the peak, and the glycosyl-residue composition was determined by g.l.c. and g.l.c.–m.s. analysis of the products of reduction with borohydride, hydrolysis (2M TFA, 1 h at 120°), reduction with borodeuteride, and acetylation. The glycosyl-residue composition was constant across the peak (see Fig. 3, inset). The glycosyl-composition data were consistent with those of a heptasaccharide. Only apiose was reducible before acid hydrolysis; this indicated that apiose was at the reducing end of the heptasaccharide, as would be anticipated from the results of the partial hydrolysis.

F.a.b.–m.s. provided strong evidence that the oligosaccharide isolated was a heptasaccharide. The negative-ion, f.a.b.–m.s. spectrum contained ions at *m/z* 1055, 1097, and 1139 that were assigned to [M − H] ions of a heptasaccharide and its mono- and di-*O*-acetylated derivatives, respectively. The results were consistent

with the glycosyl-composition data, and also indicated the presence of one and of two acetic ester groups on some of the heptasaccharide molecules.

The ^{1}H-n.m.r. spectrum of the underivatized heptasacharide in D_2O confirmed the presence of acetyl groups in some of the heptasaccharide molecules. The spectrum contained singlets, at δ 2.16, 2.12, and 2.08, having typical chemical-shifts for the methyl protons of *O*-acetyl groups[14]. Integration of these resonances showed that the proportion of *O*-acetyl groups was <1 mol per mol of heptasaccharide: the largest resonance (δ 2.16) corresponds to 0.6 mol of acetyl group per mol of heptasaccharide; each of the other two resonances corresponds to <0.3 mol of acetyl group per mol of heptasaccharide. It is probable that the *O*-acetyl groups were partially hydrolyzed by the acidic conditions used to release the heptasaccharide from the RG-II. Acidic conditions are known also to cause migration of *O*-acetyl groups[15]. No attempt has yet been made to determine the points of attachment of these substituents.

Determination of the absolute configurations of the glycosyl residues of the heptasaccharide. — The absolute configurations of all of the constituent sugars of the heptasaccharide, except apiose, were determined by the methods of Gerwig *et al.*[16,17] and Lindberg *et al.*[18]. The galactosyl residue was shown to have the D, and the rhamnosyl and 2-*O*-methylfucosyl residues, the L, configuration by the method of Gerwig *et al.* Authentic 2-*O*-methyl-L-fucose was synthesized for use in these experiments, as described in the Experimental section. The arabinosyl residue was shown to have the L configuration by the method of Lindberg *et al.*[18]. Aceric acid had previously been shown to have the L configuration[1]. It was not possible to assign the absolute configuration of apiose by the methods of Gerwig *et al.*[16,17] or Lindberg *et al.*[18], because the diastereoisomeric products were insufficiently resolved by g.l.c. The L enantiomer of apiose has not yet been found in Nature[19]. Therefore, it is assumed that the apiose in the heptasaccharide has the D configuration.

TABLE I

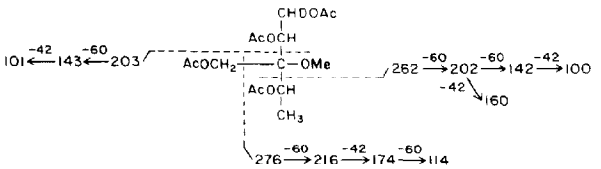
GLYCOSYL-LINKAGE COMPOSITION OF THE HEPTASACCHARIDE

Glycosyl or glycose residue	*Points of attachment of O-methyl groups*	*Glycosidic linkage deduced*	*Mol%*[a]
2-*O*-Methylfucosyl	2,3,4	terminal	17
Rhamnosyl	2,3,4	terminal	19
Rhamnosyl	2,4	3-linked	18
Arabinosyl	3,4	2-linked	19
Galactosyl	3,6	(2→4)-linked	15
Aceryl	3[b]	2-linked	6
Apiose	1,2,3,4	3[1]-linked (alditol)	5

[a]Calculated by using the effective carbon ratio[20]. [b]Determined in a separate experiment, as described in the text.

Determination of the glycosyl-linkage composition of the heptasaccharide. — The heptasaccharide was reduced with $NaBD_4$ to yield the heptasaccharide-alditol, the glycosyl-linkage composition of which was determined by per-*O*-methylation[5,6], followed by hydrolysis, reduction with $NaBD_4$, and acetylation (see Table I). The glycosyl linkage of aceric acid was determined by a modified procedure, which will be discussed separately. Pre-reduced apiose was recovered in low yield, presumably because some of the volatile tetra-*O*-methyl derivative was lost in the processing[20]. Aceric acid was partially degraded under the conditions used to methylate the heptasaccharide, and, consequently, it was also recovered in low yield. Degradation of aceric acid during the alkaline conditions of methylation became apparent from the results of experiments performed to determine the glycosyl-residue sequence of the heptasaccharide, as will be discussed later. Alkaline degradation of similar compounds has been reported[21].

The glycosyl linkage of aceric acid was determined as follows. Per-*O*-methylation of the heptasaccharide, followed by hydrolysis, reduction, and acetyla-



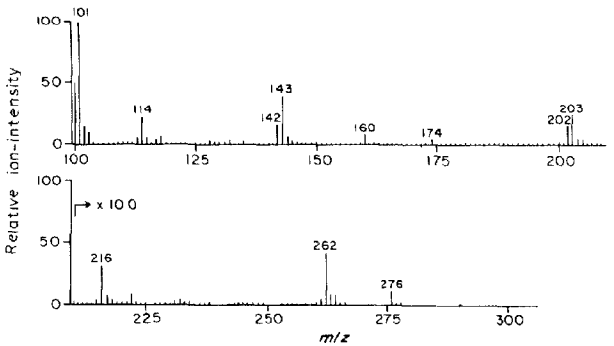


Fig. 4. E.i.-mass spectrum of compound **5** [Compound **5** is the per-*O*-acetylated, branched-chain deoxyalditol produced from the aceryl residue of the heptasaccharide by the sequence of reactions outlined in Scheme 1. Secondary fragment-ions formed by eliminations of ketene (42 mass units) or acetic acid (60 mass units) are indicated.]

tion, yielded a di-*O*-acetyl-mono-*O*-methyl lactone (compound **3**) which was identified by g.l.c.–e.i.-m.s. Because compound **3** is cyclic, it was not possible to determine the position of the *O*-methyl group by using the fragmentation patterns established for partially *O*-methylated alditol acetates[20]. Therefore, compound **2** was reduced with sodium borohydride in borate buffer[9], to yield the acyclic, carboxyl-reduced alditol (**4**). This derivative was per-*O*-acetylated, yielding compound **5**, which was analyzed by g.l.c.–m.s. The e.i.-m.s. fragmentation-pattern of compound **5** (see Fig. 4) clearly established that the aceric acid was 2-linked in the heptasaccharide.

Determination of the sequence of glycosyl residues in the heptasaccharide. — The formation, resolution, and characterization of overlapping per-*O*-alkylated oligosaccharide-alditol fragments constitute a general procedure for determining the glycosyl-residue sequence of a complex carbohydrate[10,11], such as the heptasaccharide. The sequencing method was modified in the present study by using deuterated alkylating reagents instead of the normal (*i.e.* nondeuterated) alkylating reagents. The reason for this modification will become evident in the following discussion. The present study was complicated by degradation of the aceryl residue during per-*O*-(trideuteriomethyl)ation of the heptasaccharide-alditol. The partial degradation of the aceryl residue made necessary an extra resolution-step, in order to recover the per-*O*-(trideuteriomethyl) derivative of the intact heptasaccharide-alditol from the mixture of per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols that resulted from this degradation.

The first step of the sequencing procedure was to reduce the heptasaccharide with $NaBD_4$, to afford the corresponding heptasaccharide-alditol, which was then per-*O*-(trideuteriomethyl)ated. As already mentioned, the aceryl residue

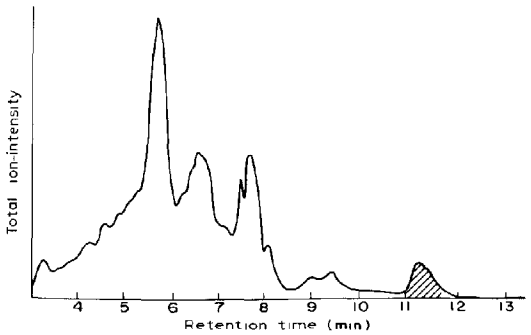


Fig. 5. Reverse-phase, l.c. elution-profile of the per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols generated by degradation of the aceryl residue during per-*O*-(trideuteriomethyl)ation of the heptasaccharide-alditol. [The profile is the e.i.-m.s., total-ion response of 3% of the effluent from the l.c. column, introduced directly into the source of the mass spectrometer. The mass spectrometer scanned from *m/z* 150 to 1000 every 3 s.]

was partially degraded during per-*O*-(trideuteriomethyl)ation of the heptasaccharide-alditol, generating a mixture of per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols; this mixture was partially resolved by l.c. on a Dupont Zorbax ODS column. The elution was monitored by chemical ionization (c.i.)-m.s. of 3% of the l.c. effluent (see Fig. 5). The remaining 97% of the l.c. effluent was collected as 0.5-mL fractions.

The material that was eluted with a retention time of 11.3 min (shaded region in Fig. 5) was found to be the per-*O*-(trideuteriomethyl) derivative of the intact heptasaccharide-alditol. This was established by the results of hydrolysis, reduction, and acetylation of aliquots of the l.c. fractions comprising the peak (data not shown), and by f.a.b.–m.s., as will be discussed later. The fractions containing the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol were combined, evaporated to dryness under a stream of filtered air, and saved for subsequent f.a.b.–m.s. and n.m.r. analyses.

Most of the features of the sequence of glycosyl residues in the heptasaccharide were elucidated by forming, and characterizing, overlapping per-*O*-alkylated oligosaccharide-alditol fragments of the heptasaccharide. The per-*O*-alkylated oligosaccharide-alditol fragments were produced by partial hydrolysis with acid, reduction, and per-*O*-(pentadeuterioethyl)ation of the mixture of per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols that resulted from degradation of the aceryl residue of the heptasaccharide (material having retention times lying between 4 and 10 min; see Fig. 5). These experiments are described next.

The fractions of eluate from the l.c. column (see Fig. 5) that were collected between 4 and 10 min were combined, and evaporated to dryness. Preliminary analyses of this material were conducted as described[11], to determine conditions for further hydrolysis to form di-, tri-, and tetra-saccharide fragments. Treatment of the per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols in the 4- to 10-min eluate with 88% formic acid for 2 h at 50° was found to yield a useful mixture of fragments.

The partially *O*-(trideuteriomethyl)ated oligosaccharide and oligosaccharide-alditol fragments generated by this partial hydrolysis were reduced with $NaBD_4$ and the products per-*O*-(pentadeuterioethyl)ated, yielding a mixture of partially *O*-(trideuteriomethyl)ated, partially *O*-(pentadeuterioethyl)ated oligosaccharide-alditols. The *O*-(pentadeuterioethyl) groups of these derivatives mark the points of attachment of other residues in the intact heptasaccharide-alditol[11]. The term "per-*O*-alkylated oligosaccharide-alditols" will be used from here on to refer to components of this mixture.

An aliquot (200 μg) of the mixture of per-*O*-alkylated oligosaccharide-alditols was resolved, and analyzed, by g.l.c.–m.s. (see Fig. 6). The structures of the per-*O*-alkylated oligosaccharide-alditol fragments were elucidated from diagnostic ions in their c.i.-mass spectra. This is illustrated in Fig. 7, in which the c.i.-mass spectrum of per-*O*-alkylated tetrasaccharide-alditol [f] is shown, and the A- and J-

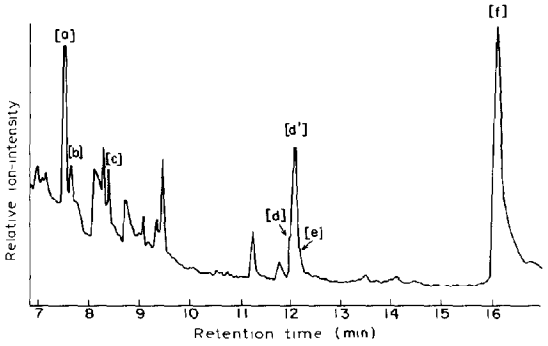


Fig. 6. G.l.c.–m.s. elution-profile of the per-*O*-alkylated oligosaccharide-alditols derived from the heptasacharide. [The per-*O*-alkylated oligosaccharide-alditols were produced by partial hydrolysis, reduction, and per-*O*-(pentadeuterioethyl)ation of the oligosaccharide fragments that resulted from partial degradation of the aceryl residue during per-*O*-(trideuteriomethyl)ation of the heptasaccharide-alditol. The mass spectrometer scanned from *m/z* 100 to 1000 every 1.5 s. Each per-*O*-alkylated fragment structurally characterized has been assigned a letter to show where it was eluted from the g.l.c. column (see Tables II, III, and IV, and Fig. 7).]

series of fragment ions, which are typical of these molecules[22], are indicated. The A- and J-series of ions, in conjunction with the glycosyl-linkage composition of the intact heptasaccharide, established, with one exception, the sequence of glycosyl residues in fragment [f]. The feature of the glycosyl-residue sequence of fragment [f] that could not be determined unambiguously from this analysis was the point of attachment, to the (2→4)-linked galactosyl residue, of the 2-*O*-methylfucosyl and the arabinosyl residues. This ambiguity was clarified in an experiment to be described later.

The e.i.-mass spectrum of fragment [f] also illustrates the value of using deuterated alkylating agents for the sequence analysis of the heptasaccharide. For example, the A_1 and A_2 ions having *m/z* 198 and *m/z* 163 could arise only from a terminal rhamnosyl residue. These ions were only distinguishable from the A_1 and A_2 ions arising from the terminal 2-*O*-methylfucosyl residue (*m/z* 195 and *m/z* 160) because the heptasaccharide was per-*O*-(trideuteriomethyl)ated. Had nondeuterated alkylating reagents been used, the A_1 and A_2 fragment-ions of both of these residues would have had *m/z* 189 and *m/z* 154, respectively, and, thus, the terminal rhamnosyl and the terminal 2-*O*-methylfucosyl residues could not have been distinguished.

Three per-*O*-alkylated disaccharide-alditols and three per-*O*-alkylated trisaccharide-alditols were identified by g.l.c.–m.s. analysis, in addition to per-*O*-alkylated tetrasaccharide-alditol [f]. These per-*O*-alkylated oligosaccharide-alditols are shown in Tables II and III, and the pertinent e.i.-m.s. ions are listed. The

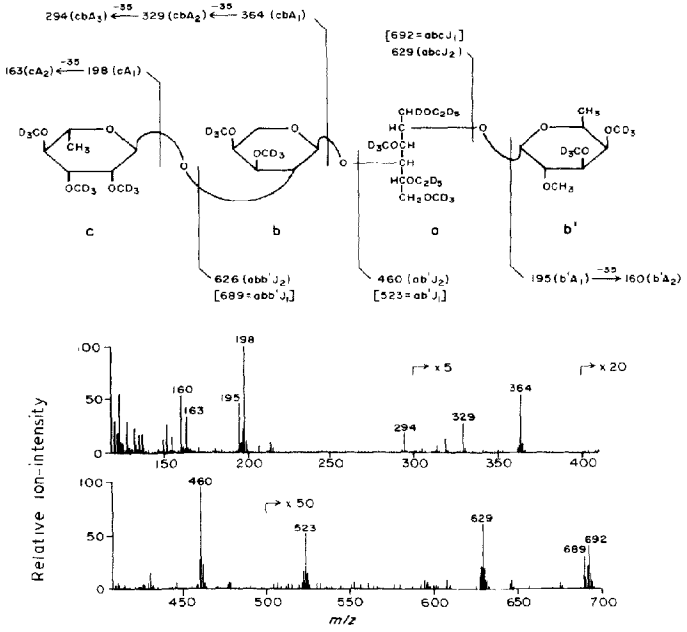


Fig. 7. E.i.-mass spectrum of per-*O*-alkylated tetrasaccharide-alditol [f]. (The g.l.c.–m.s. conditions are described in the legend to Fig. 6.)

use of pentadeuterioethyl iodide in the second alkylation step resulted in a unique molecular weight for each of the per-*O*-alkylated oligosaccharide-alditols derived from the heptasaccharide. In fact, alkylation with deuterated reagents allowed the unequivocal identification, from mass-spectral data, of the derivative of each glycosyl residue, simplifying the sequence analysis.

Fragments [d] and [d'] (see Table III) had the same sequence of glycosyl residues, and differed only in the number of *O*-(pentadeuterioethyl) groups on the alditol moiety. Each fragment had *O*-(pentadeuterioethyl) groups at O-1 and O-5 of the alditol, showing that the galactosyl residue was reduced after the partial hydrolysis. Fragment [d] had a third *O*-(pentadeuterioethyl) group, marking the point of attachment of the 2-*O*-methylfucosyl residue in the original heptasaccharide. Fragment [d'] had an *O*-(trideuteriomethyl) group instead of a third *O*-(pentadeuterioethyl) group. The ethylation pattern indicated that fragment [d'] was de-

TABLE II

DIAGNOSTIC IONS FROM EI-MS OF PARTIALLY *O*-(TRIDEUTERIOMETHYL)ATED, PARTIALLY *O*-(PENTADEUTERIOETHYL)ATED DISACCHARIDE-ALDITOLS DERIVED FROM THE HEPTASACCHARIDE

Oligosaccharide	*Fragment*[a]	*Electron-impact mass-spectral fragment-ions* [m/z *(relative abundance)*]			
		aJ_2	aJ_1	bA_1	bA_2
Et→3Rha→3[1]Api	[a]	204(100)	283(26)	214(74)	163(21)
Rha→2Ara→	[b]	236(84)	299(28)	198(100)	163(33)
Et→4Gal→[b]	[c]	299(17)	362(4)	195(100)	160(67)
2 ↑ Fuc 2 | Me					

[a]See Fig. 6 for location of fragment on g.l.c. trace. [b]The point of attachment of the 2-*O*-methylfucosyl and arabinosyl residues to the (2→4)-linked galactosyl residue was determined in a separate experiment.

rived from a hexasaccharide molecule from which the 2-*O*-methylfucosyl residue was absent.

The presence of fragment [d'] in the mixture of per-*O*-alkylated oligosaccharide-alditols is explained by hydrolysis of some of the glycosidic linkages of 2-*O*-methylfucosyl residues in the partial hydrolysis that released the heptasaccharide from RG-II. The rates of hydrolysis determined for apiosidic and 2-*O*-methylfucosidic linkages support this interpretation. Under the hydrolysis conditions used to release the heptasaccharide from RG-II, 35% of the glycosidic linkages of apiosyl residues was hydrolyzed, and 5% of the glycosidic linkages of 2-*O*-methylfucosyl residues was also hydrolyzed. Hydrolysis of 2-*O*-methylfucosidic linkages would have generated hexasaccharide molecules lacking the 2-*O*-methylfucosyl residue. Removal of the 2-*O*-methylfucosyl residue from the heptasaccharide would have had little effect on its radius of gyration and on its charge-to-mass ratio. Therefore, it is not surprising that the hexasaccharide was eluted along with the intact heptasaccharide during chromatography on Bio-Gel P-10, QAE-Sephadex, and Bio-Gel P-6.

Per-*O*-alkylated tetrasaccharide-alditol [f] (see Table III and Fig. 7) and per-*O*-alkylated disaccharide-alditol [a] (see Table II) define the sequence of six of the glycosyl residues in the heptasaccharide. No per-*O*-alkylated oligosaccharide-alditol containing aceric acid was characterized in this experiment. This was as expected, because the per-*O*-alkylated alditols were generated from the mixture of per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols that resulted from degradation of the aceryl residue during per-*O*-(trideuteriomethyl)ation of the heptasaccharide-alditol. The 2-linked aceryl residue

TABLE III

DIAGNOSTIC IONS FROM E.I.-M.S. OF PARTIALLY *O*-(TRIDEUTERIOMETHYL)ATED PARTIALLY *O*-(PENTADEUTERIOETHYL)ATED TRI- AND TETRA-SACCHARIDE-ALDITOLS DERIVED FROM THE HEPTASACCHARIDE

Oligosaccharide	*Fragment*[a]	*Electron-impact mass-spectral fragment-ions* [m/z (*relative abundance*)]							
		aJ_2	aJ_1	abJ_2	abJ_1	cbA_1	cbA_2	cA_1	cA_2
Rha→2Ara→4Gal→[b] 2 | Et	[d]	362(2)	299(13)	465(4)	528(1)	364(8)	329(2)	198(100)	163(37)
Rha→2Ara→4Gal→[b]	[d']	346(2)	283(21)	449(2)	512(3)	364(6)	329(5)	198(100)	163(38)
		$ab'J_2$	$ab'J_1$	abJ_2	abJ_1	$b'A_1$	$b'A_2$	bA_1	bA_2
Ara→4Gal→[b] 2 2 | ↑ Et Fuc 2 | Me	[e]	460(4)	523(1)	465(3)	528(1)	195(100)	160(63)	200(63)	165(50)
		$ab'J_2$	$ab'J_1$	cbA_1	cbA_2	cA_1	cA_2	$b'A_1$	$b'A_2$
Rha→2Ara→4Gal→[b] 2 ↑ Fuc 2 | Me	[f]	460(5)	523(1)	364(9)	329(5)	198(100)	163(34)	195(43)	160(53)

[a]See Fig. 6 for location of fragment on g.l.c. trace. [b]The point of attachment of the 2-*O*-methylfucosyl and arabinosyl residues to the (2→4)-linked galactosyl residue was determined in a separate experiment.

was the only other residue in the intact heptasaccharide, and, therefore, had to be situated between the (2→4)-linked galactosyl residue and the 3-linked rhamnosyl residue; this was confirmed by f.a.b.–m.s., as described later. Each of the per-*O*-alkylated oligosaccharide-alditol fragments identified was consistent with the glycosyl-residue sequence deduced for the heptasaccharide.

Determination of the points of attachment of the 2-O-methylfucosyl and arabinopyranosyl residues to the (2→4)-linked galactosyl residue. — The point of attachment of the 2-*O*-methylfucosyl and the arabinopyranosyl residues to the (2→4)-linked galactosyl residue was determined by partial hydrolysis of the underivatized heptasaccharide with acid, followed by glycosyl-linkage analysis of the hydrolyzate. This experiment is described next.

An aliquot (0.2 mg) of the underivatized heptasaccharide was treated with 2M TFA for 30 min at 80°. The extent of hydrolysis of the arabinosidic linkage, de-

TABLE IV

DETERMINATION OF THE POINT OF ATTACHMENT OF THE ARABINOSYL AND THE 2-*O*-METHYLFUCOSYL RESIDUES TO THE (2→4)-LINKED GALACTOSYL RESIDUE

Positions of O-methyl groups on the methylated galactosyl residue	*Glycosidic linkage deduced*	*Observed area (%)*	*Predicted area (%)*[a]	
			If 2-O-methylfucosyl residue is linked to O-2 of the (2→4)-linked galactosyl residue	*If 2-O-methylfucosyl residue is linked to O-4 of the (2→4)-linked galactosyl residue*
2,3,4,6	terminal	21		
2,3,6	4-linked	51	42	13
3,4,6	2-linked	16	13	42
3,6	(2→4)-linked	11		

[a]Calculated, based on 34% hydrolysis of arabinosidic linkages and 63% hydrolysis of 2-*O*-methylfucosidic linkages.

termined as described[11], was found to be 34%, and the hydrolysis of the 2-*O*-methylfucosidic linkages was determined to be 63%.

An identical aliquot of the heptasaccharide was partially hydrolyzed by using the same conditions. This sample was then reduced with $NaBD_4$, per-*O*-methylated, fully hydrolyzed with 2M TFA during 1 h at 120°, reduced with $NaBD_4$, and per-*O*-acetylated. This treatment generated a mixture of terminal, 2-linked, 4-linked, and (2→4)-linked galactosyl residues (see Table IV). The 4-linked galactosyl residues listed in Table IV resulted from hydrolysis of the glycosidic linkage to O-2 of the (2→4)-linked galactosyl residue, whereas the 2-linked galactosyl residues resulted from hydrolysis of the linkage to O-4 of the (2→4)-linked galactosyl residue. The observed ratio (51:16) of 4-linked to 2-linked galactosyl residues established that the 2-*O*-methylfucosyl residue was glycosidically linked to O-2, and the arabinosyl residue to O-4, of the (2→4)-linked galactosyl residue.

F.a.b.–m.s. of the per-O-(trideuteriomethyl)ated heptasaccharide-alditol. — The proposed sequence of glycosyl residues in the heptasaccharide was confirmed by positive-ion, f.a.b.–m.s. of the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol (see Fig. 8). This material had been isolated by l.c. (retention time of 11.3 min; see Fig. 5), as already described. Ions corresponding to $[M + H]^+$ (*m/z* 1349) and $[M + Na]^+$ (*m/z* 1371) were observed in the f.a.b.–mass spectrum. The A series of fragment ions, resulting from fragmentation from the nonreducing termini of the molecule, was also observed, as indicated in Fig. 8. These fragment ions confirmed the glycosyl-residue sequence of the heptasaccharide, and provided definitive evidence for the location of the aceryl residue.

Determination of the ring forms of the glycosyl residues of the heptasaccharide. — Glycosyl residues usually exist in furanoid or pyranoid forms. The apiosyl and aceryl residues of the heptasaccharide can form only furanoid rings. Methylation analysis of the heptasaccharide (see Table I) established the pyranoid

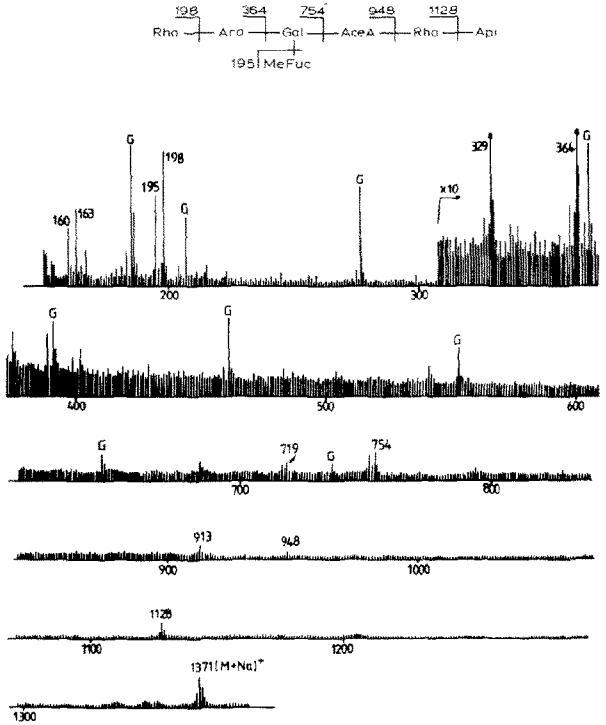


Fig. 8. Positive-ion, f.a.b.-mass spectrum of the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol. [The signals labeled "G" represent glycerol polymers generated from the glycerol matrix on the f.a.b. target. The per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol was isolated by l.c. (retention time, 11.3 min; see Fig. 5).]

ring-forms of the 2-*O*-methylfucosyl, arabinosyl, and the two rhamnosyl residues of the heptasaccharide, because the partially *O*-methylated alditol acetates derived from these residues had methyl groups on O-4.

The partially *O*-methylated alditol acetate derived from the galactosyl residue of the heptasaccharide was 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylgalactitol (see Table I). This derivative could have arisen from a (2→4)-linked galactopyranosyl residue or from a (2→5)-linked galactofuranosyl residue. This ambiguity was resolved in the experiment that revealed the point of attachment of the

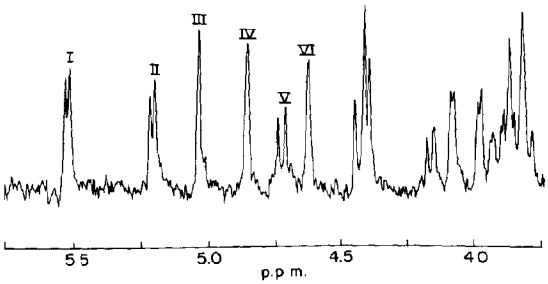


Fig. 9. Spectral region from 3.75 to 5.75 p.p.m. of the ^{1}H-n.m.r. spectrum of the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol in $CDCl_3$. [Chemical shifts were assessed relative to internal hexadeuterioacetone (δ 2.04). The resonances arising from the six anomeric protons have been designated I–VI (see Table V). The per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol was isolated by l.c. (retention time, 11.3 min; see Fig. 5).]

arabinosyl and 2-*O*-methylfucosyl residues to the galactosyl residue. In that experiment, the heptasaccharide was partially hydrolyzed, reduced, and per-*O*-methylated. The resulting fragments were then completely hydrolyzed, reduced, and acetylated. One of the partially *O*-methylated alditol acetates resulting from this series of reactions was 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol (see Table IV). The methyl group on O-4 of this derivative established that the galactosyl residue in the heptasaccharide was in the pyranoid ring-form[23].

TABLE V

^{1}H-N.M.R. CHEMICAL-SHIFTS AND COUPLING CONSTANTS OF THE ANOMERIC PROTONS OF THE PER-*O*-(TRIDEUTERIOMETHYL)ATED HEPTASACCHARIDE-ALDITOL AND OF DERIVED PER-*O* ALKYLATED OLIGOSACCHARIDE-ALDITOLS

Per-O-(trideuteriomethyl)ated heptasaccharide-alditol			*Per-O-alkylated oligosaccharide-alditol*			*Assignment*
Resonance[a]	*Chemical shift*[b] *(p.p.m.)*	$J_{1,2}$ *(Hz)*	*Fragment*[c]	*Chemical shift*[b] *(p.p.m.)*	$J_{1,2}$ *(Hz)*	
I	5.53	4				α-D-galactosyl
II	5.21	4	[f]	5.21	4	2-*O*-methyl-α-L-fucosyl
III	5.04	1	[d']	5.34	1	α-L-rhamnosyl (terminal)
			[f]	5.18	1	
IV	4.87	2				β-L-aceryl
V	4.73	7	[d']	4.61	7	α-L-arabinosyl
			[f]	4.70	7	
VI	4.64	1	[a]	4.70	1	β-L-rhamnosyl (3-linked)

[a]See Fig. 9. [b]Assessed relative to internal hexadeuterioacetone at δ 2.04. [c]See Tables II and III.

Determination of the anomeric configurations of the glycosyl residues of the heptasaccharide. — The anomeric configurations of the glycosyl residues of the heptasaccharide were determined by ^{1}H-n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum of the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol (see Fig. 9) contained resonances from the six anomeric protons. Four of those resonances were assigned to specific glycosyl residues of the heptasaccharide, with the assistance of the ^{1}H-n.m.r. spectra of the per-*O*-alkylated oligosaccharide-alditol fragments [a], [d'], and [f] (see Table V). These fragments were isolated by l.c. of an aliquot (2 mg) of the mixture of per-*O*-alkylated oligosaccharide-alditols, and were detected by g.l.c.–e.i.-m.s. of aliquots of the column fractions.

The ^{1}H-n.m.r. spectrum of per-*O*-alkylated disaccharide-alditol [a] contained an anomeric resonance, from the anomeric proton of the 3-linked rhamnosyl residue, at δ 4.70 with J 1 Hz (see Table V). A small (0–2 Hz) coupling constant is typical of a rhamnopyranosyl anomeric resonance[24], and the chemical shift indicated that the anomeric proton of the 3-linked rhamnopyranosyl residue was axial. Therefore, the 3-linked rhamnopyranosyl residue had the β-anomeric configuration.

It should be noted that the chemical shift of the anomeric-proton resonance of a particular glycosyl residue may differ when the residue is situated in different oligosaccharide-alditols. For example, the chemical shift of the β-rhamnosyl residue was δ 4.64 for the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol and δ 4.70 for fragment [a]. This was as expected, because the anomeric proton was not in an identical chemical environment in the two oligosaccharide-alditols.

The ^{1}H-n.m.r. spectrum of per-*O*-alkylated trisaccharide-alditol [d'] contained a resonance at δ 5.34, with a coupling constant of <1 Hz, and another resonance, at δ 4.61, with J 7 Hz. These resonances arose from the anomeric protons of the terminal rhamnopyranosyl residue and the arabinopyranosyl residue. The resonance having J 7 Hz could not have arisen from a rhamnopyranosyl residue, because H-1 and H-2 of both α- and β-rhamnopyranosyl residues are in *gauche* orientation, and exhibit[24] coupling constants of <2 Hz. Therefore, the resonance at δ 4.61, J 7 Hz, was assigned to the 2-linked arabinopyranosyl residue. The chemical shift and coupling constant of this resonance indicated that the 2-linked arabinopyranosyl residue had the α-anomeric configuration. The resonance at δ 5.34, with $J<1$ Hz, must have arisen from the terminal rhamnopyranosyl residue. The chemical shift indicated that the anomeric proton of this residue was equatorial, and that the residue had the α-anomeric configuration.

The ^{1}H-n.m.r. spectrum of per-*O*-alkylated tetrasaccharide-alditol [f] contained resonances arising from the anomeric protons of the 2-*O*-methylfucopyranosyl residue, the terminal rhamnopyranosyl residue, and the arabinopyranosyl residue. Two of the resonances corresponded to those identified in the spectrum of per-*O*-alkylated trisaccharide alditol [d']. Therefore, the third resonance (δ 5.21, J 4 Hz) must have arisen from the anomeric proton of the 2-*O*-methylfucopyranosyl residue. The chemical shift and coupling constant of this reso-

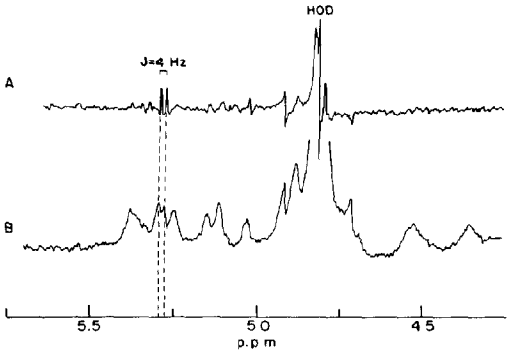


Fig. 10. ^{1}H-N.m.r., n.O.e.-difference spectrum of the underivatized heptasaccharide in D_2O. [A: spectral region from 4.25 to 5.75 p.p.m. of a n.O.e.-difference spectrum obtained with presaturation of the methoxyl protons of the 2-*O*-methylfucosyl residue. B: spectral region from 4.25 to 5.75 p.p.m. of the normal, Fourier-transform spectrum.]

nance indicated that the 2-*O*-methylfucopyranosyl residue had the α-anomeric configuration[25].

The assignments of the anomeric configurations of the 2-*O*-methylfucopyranosyl and the arabinopyranosyl residues were confirmed by a nuclear Overhauser effect (n.O.e.)-difference, n.m.r. experiment[26,27] that was performed with underivatized RG-II heptasaccharide in D_2O. The anomeric region of the ^{1}H-n.m.r. spectrum of the underivatized heptasaccharide (trace B, Fig. 10) was more complex than the same region of the spectrum of the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol. There were two causes for most of the increased complexity of the former spectrum: the heptasaccharide was not reduced to the heptasaccharide-alditol; therefore, some of the heptasaccharide molecules had α-apiose, whereas others had β-apiose, at their reducing termini. Some of the heptasaccharide molecules also contained *O*-acetyl groups. The inductive effect of an *O*-acetyl group can cause the resonance of a nonanomeric proton to appear in the anomeric region of the spectrum.

The n.O.e.-difference, n.m.r. experiment was designed to discriminate between the anomeric protons of the 2-*O*-methylfucosyl and arabinosyl residues by taking advantage of the methyl group on O-2 of the fucosyl residue. The experiment entailed presaturating, at δ 3.5, the methoxyl protons of the 2-*O*-methylfucosyl residue of the underivatized heptasaccharide. Under the conditions of the experiment, any protons that were within ~400 pm of the presaturated protons would experience a slight (1–5%) enhancement in signal intensity. The n.O.e. enhancement is extremely distance-dependent, diminishing with increasing distance as R^{-6} (ref. 27). Trace A in Fig. 10 shows the anomeric region of the n.O.e.-differ-

ence spectrum. The n.O.e.-difference spectrum was generated by obtaining free-induction decays with and without presaturation of the methoxyl protons of the 2-*O*-methylfucosyl residue, and then performing a Fourier transformation on the difference between the free-induction decays. The resonance at δ 5.3, with $J_{1,2}$ 4 Hz, which was the only anomeric resonance with significant n.O. enhancement, was attributed to the anomeric proton of the 2-*O*-methylfucosyl residue. The result confirmed the assignment of this resonance that had been made on the basis of ^{1}H-n.m.r. analysis of fragments [d'] and [f].

Two resonances of the ^{1}H-n.m.r. spectrum of the per-*O*-(trideuteriomethyl)ated heptasaccharide-alditol (δ 5.53, J 4 Hz; and δ 4.87, J 2 Hz) were not assigned by ^{1}H-n.m.r. analysis of the per-*O*-alkylated alditols [a], [d'], and [f]. These resonances were attributed to the anomeric protons of the (2→4)-linked galactopyranosyl residue and the 2-linked acerofuranosyl residue, because these were the only glycosyl residues whose anomeric resonances were not identified in the foregoing experiments. A 2-Hz coupling constant is smaller than that usually observed with either anomer of a galactopyranosidic residue, but is typical of that observed when H-1 and H-2 are *trans* on a furanoid ring[25-28]. Therefore, the resonance at δ 4.87 was attributed to the 2-linked acerofuranosyl residue in the β configuration. The resonance at δ 5.53, with J 4 Hz, was in a typical range of chemical shift, with a coupling constant typical for an α-galactosidic residue, and therefore, this resonance was assigned to a (2→4)-linked-α-galactosyl residue. Thus, the anomeric configuration of each glycosyl residue of the heptasaccharide was determined.

GENERAL DISCUSSION

The heptasaccharide released from RG-II has the structure shown in the Abstract. All aspects of the primary structure have been elucidated, except the points of attachment of the *O*-acetyl groups. The heptasaccharide contains six different glycosyl residues, including aceric acid, a hitherto-unobserved glycosyl residue. The complexity of this heptasaccharide is unprecedented among structures that have thus far been elucidated for primary cell-wall constituents; this finding reinforces our conclusion that RG-II is a well defined, structurally complex polysaccharide.

The glycosyl-linkage composition of RG-II is compared to that of the heptasaccharide in Table VI. The value given in Table VI for the 2-linked aceryl residue is an estimate, because this residue cannot be quantitated reliably after methylation. The glycosyl residues that comprise the heptasaccharide are present in approximately equal numbers in intact RG-II, and, with the exception of the 4-linked galactosyluronic acid residues, are the most abundant glycosyl residues of RG-II. The data in Table VI also reveal that a molecule of RG-II probably contains more than one heptasaccharide unit.

Three heptasaccharide units would account for ~30%, and four heptasac-

TABLE VI

COMPARISON OF THE GLYCOSYL-LINKAGE COMPOSITION OF INTACT RG-II TO THAT OF THE HEPTASACCHARIDE

Glycosyl residue	*Linkage*	*Number per molecule[a] of RG-II*	*Number per molecule of heptasaccharide*
Galactosyluronic acid	4-linked	12	
	terminal	2	
	3,4-linked	1	
Rhamnosyl	terminal	4	1
	3-linked	4	1
	2,4-linked	1	
	3,4-linked	1	
	2,3,4-linked	1	
Arabinosyl	2-linked (pyranose)	4	1
	terminal (furanose)	2	
Galactosyl	2,4-linked	5	1
	terminal	2	
	3-linked	1	
Apiosyl	3^1-linked	4	1
Aceryl	2-linked	(4)[b]	1
Fucosyl	3-linked	2	
	3,4-linked	1	
2-*O*-Methylfucosyl	terminal	3	1
2-*O*-Methylxylosyl	terminal	2	
Glucosyluronic acid	2-linked	2	
Glucosyl	4-linked	2	
	Total	60	

[a]The values given for intact RG-II were calculated by using the data of Darvill *et al.*[2]. [b]The value given for the 2-linked aceryl residue is an estimate, because this residue cannot be quantitated reliably after methylation.

charide units for >40% of the glycosyl residues of RG-II. Release of the heptasaccharide(s) does not significantly change the P-10 elution volume of the rest of the molecule (unpublished results). This suggests that the radius of gyration of RG-II is not significant changed by release of the heptasaccharide, and implies that the heptasaccharide units may be side chains on a 4-linked, galactosyluronic acid-rich backbone. RG-II is considered to be linked to other cell-wall polysaccharides by such a 4-linked, galactosyluronic acid chain[2].

The structure of the heptasaccharide provides the first information about the locations of the 2-*O*-methylfucosyl and apiosyl residues in the primary cell-wall of dicots. Methylfucose has been long recognized as a minor constituent of pectic polysaccharides[29,30], and was identified[2] as a component of RG-II. The heptasaccharide is the first plant-cell-wall oligosaccharide characterized that contains a 2-*O*-methylfucosyl residue.

Apiose-containing polysaccharides from monocots have been partially

characterized. The best characterized of these is the apiogalacturonan of *Lemna*[31,32], which consists of a chain of 4-linked α-D-galactosyluronic acid residues, with apiobiose units attached to O-2 or O-3 of some of the galactosyluronic acid residues[31,32]. Zosterin, an apiose-containing polysaccharide isolated from *Zosteraceae*[33], is structurally more complex than the *Lemna* apiogalacturonan, but is degraded by pectinase to a molecule very similar to the *Lemna* apiogalacturonan[34]. The apiosyl residue in the RG-II heptasaccharide occurs in a chain with other neutral glycosyl residues, the first such example. It is possible that the apiosyl residues of RG-II are glycosidically linked to 4-linked galactosyluronic acid residues. If this is correct, RG-II could be structurally related to zosterin and to apiogalacturonans.

The heptasaccharide molecule has many hydrophobic functional groups: four of the glycosyl residues are deoxy sugars and contain methyl groups instead of hydroxymethyl groups at C-5; the arabinosyl residue is in the pyranoid ring-form and contains no hydroxymethyl group; and the heptasaccharide has one *O*-methyl group and at least two *O*-acetyl groups. The hydrophobicity and structural complexity of the heptasaccharide are important, because recent evidence indicates that the interactions between carbohydrates and their protein receptors are governed by hydrophobic bonding[35,36]. These properties of the heptasaccharide are made more significant by recent reports that oligosaccharide fragments of the cell walls function as regulatory molecules in plant–pathogen interactions and in plant growth and development[37,38].

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