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# STRUCTURAL FEATURES OF THE PLANT CELL-WALL POLYSACCHARIDE RHAMNOGALACTURONAN-II*†

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ABSTRACT

Rhamnogalacturonan-II (RG-II), isolated from the cell walls of suspension-cultured sycamore cells, has been further characterized. End-group analysis of RG-II showed that the polysaccharide contains about 30 glycosyl residues. Some 28 residues have been found as constituents of well characterized oligosaccharide fragments of RG-II. RG-II was treated with lithium metal dissolved in ethylenediamine to degrade the glycosyluronic acid residues. The major product was isolated, characterized, and shown to be the triglycosylalditol α-Xyl-(1→3)-α-Fuc-(1→4)-β-Rha-(1→3[1])-apiitol. This tetrasaccharide fragment of RG-II has three residues in common with a previously characterized heptasaccharide that had been derived from RG-II by partial hydrolysis with acid. RG-II was found to contain a large number of branched galactosyluronic acid residues that have not yet been identified as components of oligosaccharide fragments, although they are undoubtedly part of an octa(galactosyluronic acid) fragment generated by partial acid hydrolysis. The results of sequential partial acid hydrolysis provided evidence that, in RG-II, the extremely acid-labile 3-deoxy-D-*manno*-2-octulosonic-acid and 3-deoxy-D-*lyxo*-2-heptulosaric acid residues are attached to O-3 of 3,4-linked galactosyluronic acid residues, and that the mildly acid-labile apiofuranosyl residues are attached to O-2 of 2,4-linked galactosyluronic acid residues. These and previously published data

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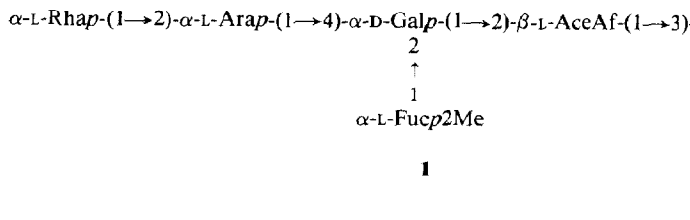
suggested that RG-II has a highly branched structure, arranged around an α-(1→4)-linked galactosyluronic acid backbone.

## INTRODUCTION

Rhamnogalacturonan-II (RG-II) is a pectic polysaccharide, having a highly defined structure and size, that is released from the cell walls of suspension-cultured sycamore cells[2] by treatment with α-(1→4)-endopolygalacturonanase isolated from *Colletotrichum lindemuthianum*[3]. RG-II has also been isolated from sycamore extracellular polysaccharides[4], the walls of suspension-cultured cells of Douglas fir[5] and rice[6], and from Pectinol-AC[1,7,8]. Pectinol-AC is a commercially available preparation of cell-wall-degrading enzymes obtained from the culture filtrate of the fungus *Aspergillus niger*. The RG-II present in Pectinol-AC is undoubtedly a fungal-enzyme-resistant residue of the plants used as a carbon source.

RG-II is a complex polysaccharide composed of at least 12 different sugars[1]. These include the unusual sugars 2-*O*-methylfucose[2], 2-*O*-methylxylose[2], apiose[2], 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid)[7], 3-deoxy-D-*manno*-2-octulosonic acid (KDO)[8], and 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA)[1]. These and other residues were shown[2] to be interconnected by about 20 different glycosidic linkages, most of which have been found in oligosaccharide fragments of RG-II, generated by mild hydrolysis with acid. These oligosaccharides are heptasaccharide **1**(ref. 9), distinguished by residues of 2-*O*-methylfucose and aceric acid, heptasaccharide **2**(ref. 10), distinguished by its fully branched rhamnosyl residue and a residue of glucuronic acid, trisaccharide **3**(ref. 10), disaccharides **4**(ref. 8) and **5**(ref. 1), distinguished by KDO and DHA residues, respectively, and an oligogalactosiduronic acid (**6**), containing

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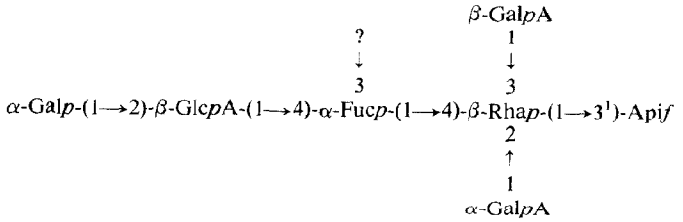


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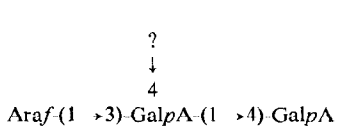

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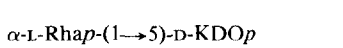

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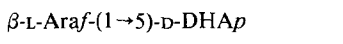

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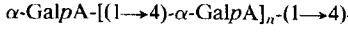


**6**

up to 8 galactosyluronic acid residues[10]. Heptasaccharide **2** contains a 4-linked fucosyl residue, whereas only 3,4-linked residues had been found[10] in RG-II, suggesting that the glycosyl residue linked to O-3 of the fucosyl residue was removed by the acid treatment.

In the study reported here, the following are discussed: (*i*) an estimate of the size of RG-II, (*ii*) the glycosyl-linkage composition of RG-II, (*iii*) the identification of 2-*O*-methylxylose as the glycosyl residue attached to O-3 of the fucosyl residue of heptasaccharide **2**, (*iv*) the probable identification of the position and glycosyl residue to which disaccharides **4** and **5** are attached in RG-II as O-3 of 3,4-linked galactosyluronic acid residues, and (*v*) the identification of the position and glycosyl residue to which at least some of the 3[1]-linked apiosyl residues are attached in RG-II as O-2 of 2,4-linked galactosyluronic acid residues.

## RESULTS AND DISCUSSION

*The size of RG-II.* — The number of glycosyl residues in RG-II was estimated by end-group analysis. RG-II was isolated from the cell walls of sus pension-cultured sycamore cells, de-esterified, and treated with endopolygalacturonanase as described[11]. The reducing-terminal residue of sycamore RG-II was reduced with $NaBD_4$ in aqueous ammonia. The reduced RG-II was methanolyzed[11,12], the products trimethylsilylated[11], and the resulting products analyzed by g.l.c (see Table I, col. 1). Aceric acid, KDO, DHA, 2-*O*-methylxylose, 2-*O*-methylfucose, and apiose

TABLE I

GLYCOSYL COMPOSITION OF SYCAMORE RG-II

| *Sugar residue* | *Normalized mole percent* | | |
|---|---|---|---|
| | *I* *By MeOH/HCl*[a] | *II* *By $H_2O$/TFA*[b] | *III* *Composite*[c] |
| Rhamnose | 17.9 | 25.3 | 12.4 |
| Fucose | 4.0 | 4.2 | 2.8 |
| 2-*O*-Methylfucose | [d] | 5.7 | 3.5 |
| Arabinose | 14.5 | 22.6 | 10.0 |
| 2-*O*-Methylxylose | | 7.9 | 4.8 |
| Apiose | | 19.7 | 12.2 |
| Galactose | 13.0 | 14.6 | 9.0 |
| Glucuronic acid | 4.6 | | 3.2 |
| Galacturonic acid | 40.9 | | 28.3 |
| C-1-Reduced galacturonic acid | 5.0 | | 3.5 |
| Aceric Acid | | | 3.5 |
| KDO | | | 3.5 |
| DHA | | | 3.5 |

[a]Determined by analysis of the trimethylsilyl ethers of methyl glycosides[11]. [b]Determined by analysis of the alditol acetates[13]. [c]A theoretical composition, from combining columns I and II and making several assumptions (see text). [d]Not analyzed.

were not quantitated in this experiment. The reduced terminus of RG-II was found to be exclusively derived from galacturonic acid. [Similarly, the reduced terminus of Pectinol RG-II that had been carboxyl- and carbonyl-reduced prior to methylation was shown by glycosyl-linkage analysis to be derived from 4-linked galacturonic acid (data not shown)]. The C-1-reduced galacturonic acid gave two peaks, presumably resulting from a lactone and the methyl-esterified forms of C-1-reduced galacturonic acid. These two peaks, combined, accounted for 5.0 mole % of the analyzed glycosyl residues of sycamore RG-II.

The glycosyl composition of sycamore RG-II was also determined by the alditol acetate method[13] (see Table I, col. II). The relative proportions of 2-*O*-methylfucose, 2-*O*-methylxylose, and apiose were determined by this analysis, and found to be 39, 54 and 135% relative to galactose. The proportion of aceric acid was assumed to be the same as that of 2-*O*-methylfucose, as both residues were only present in heptasaccharide 1. The proportions of KDO and DHA were arbitrarily assigned as 5.0 mol % each (*i.e.*, the same as for C-1-reduced galacturonic acid). Renormalization of the combined composition data showed that the C-1-reduced galacturonic acid was 3.5 mole % of all of the known residues in RG-II (see Table I, col. III), indicating that RG-II was comprised of ~30 sugar residues; this value lies within the range initially proposed[2] for sycamore RG-II, but is roughly half of the value later suggested[9,10]. Because determination of the number of glycosyl residues in RG-II by end-group analysis required a complete knowledge of the glycosyl

composition of RG-II, and as this analysis was sensitive to small variations in the quantitation, 30 glycosyl residues can only be assumed to be an approximate num-

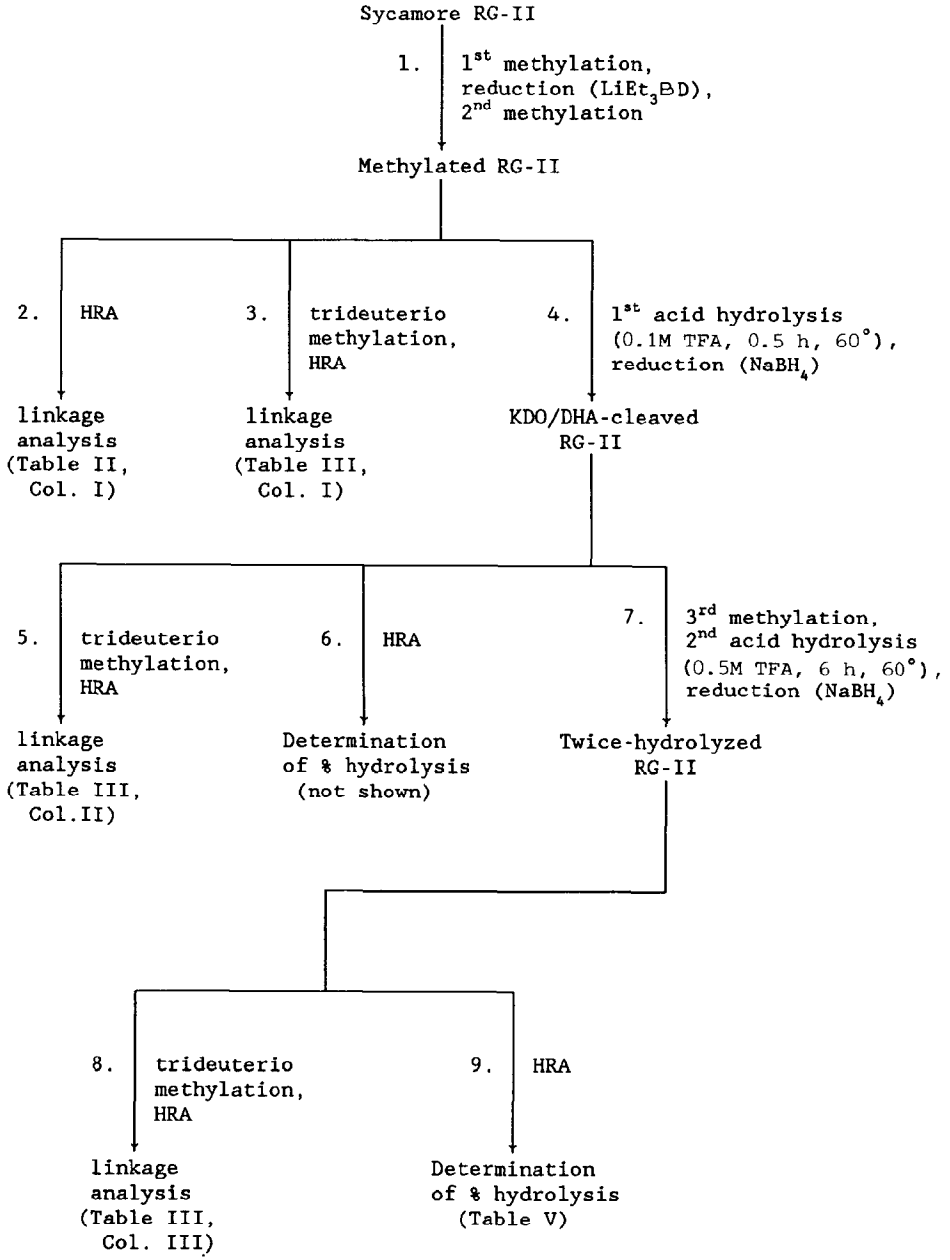



Fig. 1. Sequence of reactions applied to sycamore RG-II. HRA means that the sample was completely hydrolyzed, reduced ($NaBD_4$), and acetylated.

ber. However, the predicted molecular weight of ~4500–5500 is consistent with the elution volume of RG-II on a Bio-Gel P-10 gel-permeation column[2,8].

End-group analysis of Pectinol RG-II showed that it, too, was comprised of ~30 glycosyl residues. In addition, gel-permeation profiles of Pectinol and sycamore RG-II were identical (data not shown).

*Glycosyl-linkage analysis of RG-II.* —RG-II was methylated (first alkylation), carboxyl-reduced with $LiEt_3BD$ (see Fig. 1, step 1), and remethylated[1] (second alkylation). The methylated RG-II was then fully hydrolyzed, the products reduced, and the reduction products acetylated[11] (see Fig. 1, step 2), and analyzed[1] by g.l.c. and g.l.c.-m.s. (see Table II). By this method, glycosyluronic acid residues were detected as partially methylated, partially acetylated 6,6'-dideuteriohexitols. As expected, the derivatives of terminal galactosyl residues were coeluted with those of terminal galactosyluronic acid residues, and those of 2,4-linked galactosyl residues were coeluted with those of 2,4-linked galactosyluronic acid residues. The relative proportion of each pair was determined from their e.i.- and c.i.- mass spectra by the presence of the dideuterio-labeling in the derivatives of the galactosyluronic acid residues. Derivatives of aceric acid, KDO, and DHA were not detected by this analysis.

The glycosyl-linkage analysis presented here differed from that published earlier[2]. This can be attributed to the unusual problems associated with the analysis of this labile polysaccharide, and to improved analytical techniques.

The glycosyl-linkage composition of sycamore RG-II (see Table II) showed that it was a highly branched molecule, with 22 mole % of the detected residues branched and 6 mole % doubly branched. About half of the branch points were on 4-linked galactosyluronic acid residues. The branched residues did not decrease in proportion upon further methylation, indicating that their presence in the glycosyl-linkage analysis was not due to undermethylation. The ratio of terminal residues to branch points was 1.1:1.0. The theoretical value for a large polymer is 1.0:1.0, and the expected value for a highly branched polysaccharide of ~30 glycosyl residues, such as RG-II, is 1.1:1.0.

The glycosyl-linkage analysis of Pectinol RG-II was similar to that of sycamore RG-II (see Table II). The most apparent difference was the absence of 2-linked arabinopyranosyl residues and the presence of terminal arabinopyranosyl residues in Pectinol RG-II. In sycamore RG-II, the 2-linked arabinopyranosyl residue was found in heptasaccharide **1**, where it was substituted with a terminal α-L-rhamnosyl residue. The presence of terminal arabinopyranosyl residues in Pectinol RG-II could be due to the removal of the terminal rhamnosyl residue by an enzyme secreted by *A. niger*. Significant proportions (see Table II) of 2-linked rhamnosyl and 3,4-linked galactosyl residues were found in Pectinol, but not in sycamore RG-II. These residues may have been part of a contaminant in Pectinol RG-II.

*Identification of 2-O-methylxylose as the residue attached to O-3 of the fucosyl residue of heptasaccharide* **2**. In an attempt to produce oligosaccharides whose structures overlapped those of known fragments produced by hydrolysis with dilute

TABLE II

GLYCOSYL-LINKAGE COMPOSITION OF SYCAMORE AND PECTINOL RG-II[a]

| *Sugar residue* | *Position of O-$CH_3$ groups* | *Deduced linkage* | *Mole percent* | |
|---|---|---|---|---|
| | | | *Sycamore*[b] | *Pectinol*[c] |
| Rhamnose | 2,3,4 | T[d] | 6.6 | 5.0 |
| | 3,4 | 2 | Tr[e] | 1.7 |
| | 2,4 | 3 | 5.7 | 6.3 |
| | none | 2,3,4 | 4.5 | 3.1 |
| Fucose | 2,3,4 | T[f] | 4.8 | 5.5 |
| | 2 | 3,4 | 4.5 | 2.3 |
| Arabinose | 2,3,5 | T*f* | 6.1 | 6.3 |
| | 3,4 | 2*p* | 5.0 | 0 |
| | 2,3,4 | T*p* | 0 | 4.9 |
| Xylose | 2,3,4 | T[f] | 4.5 | 4.1 |
| Apiose | 2,3 | $3^1$ | 10.9 | 10.2 |
| Galactose | 2,3,4,6 | T | 4.9 | 5.2 |
| | 3,6 | 2,4 | 5.6 | 6.5 |
| | 2,6 | 3,4 | 0 | 2.8 |
| Glucuronic acid | 3,4,6 | 2[g] | 4.3 | 6.7 |
| Galacturonic acid | 2,3,4,6 | T[g] | 10.3 | 10.0 |
| | 2,3,6 | 4[g] | 8.8 | 6.0 |
| | 2,6 | 3,4[g] | 7.3 | 7.6 |
| | 3,6 | 2,4 | 4.6 | 4.2 |
| | 6 | 2,3,4 | 1.5 | 1.5 |

[a]Residues of aceric acid, KDO, and DHA were not analyzed. [b]Eleven derivatives of less than 1 mole % each, totaling 3.8 mole %, were omitted and the data were normalized to 100 mole %. [c]No detected derivative was omitted. [d]T = a nonreducing terminal residue. [e]Tr = < 1 mole % detected. [f]Derived from a 2-*O*-methyl residue. [g]Small amounts of 6-*O*-acetyl-6,6′-dideuterio derivatives were included.

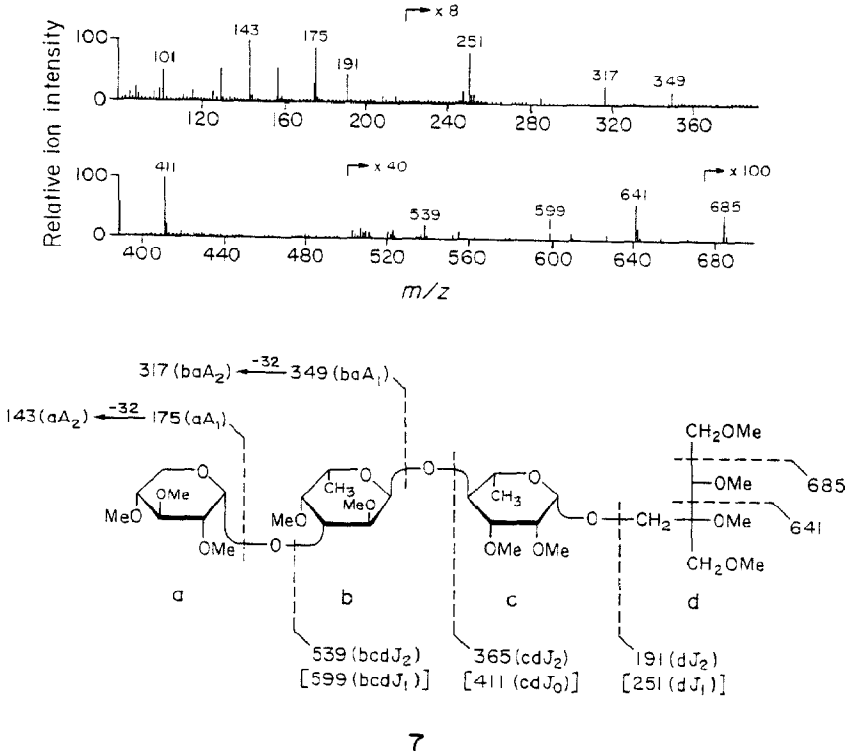
acid, sycamore RG-II was cleaved with lithium dissolved in ethylenediamine. Lithium metal, dissolved in ethylenediamine, degrades glycosyluronic acid residues[14,15] with concomitant cleavage of their glycosidic linkages. In most cases, the glycosidic linkage of the residue attached to the uronic acid residue is also cleaved, and the newly formed "reducing" end is reduced to an alditol[15].

The carboxyl groups of sycamore RG-II were converted into the proton form by treatment with a cation-exchange resin. The RG-II was dissolved in a small amount of water; ethylenediamine was then added, followed by lithium metal. (Note: attempts to cleave dry RG-II dissolved in ethylenediamine were frequently unsuccessful, with no degradation of glycosyluronic acid residues.) After 30 min, the reaction was quenched with water, and the products were isolated[15].

The resulting oligosaccharide alditols were methylated[16] and the products separated by h.p.l.c. on a C-18 reversed-phase column. The structures of the methylated oligoglycosylalditols isolated were determined by h.p.l.c.–c.i.-m.s., g.l.c.–e.i.-m.s., $^1$H-n.m.r. spectroscopy, and glycosyl-linkage analysis.

Analysis of the most abundant oligoglycosylalditol (**7**) by h.p.l.c.–c.i.-m.s. gave an ion at *m/z* 731 (M + 1) that was consistent with a per-*O*-methylated triglycosylalditol containing two pentosyl and two deoxyhexosyl residues. G.l.c.–e.i.-m.s. analysis of **7** showed intense ions at *m/z* 143 ($aA_2$)[17], 175 ($aA_1$), 191 ($dJ_2$), 251 ($dJ_1$), 317 ($baA_2$), 349 ($baA_1$), and 411 ($cdJ_0$) that established the sequence as pentose → 3-deoxyhexose → deoxyhexose → pentitol (see Fig. 2). The intense ion at *m/z* 411 demonstrated that the 'b' residue (see Fig. 2) was 3-linked[18], and this was confirmed by the low abundance of the ion at *m/z* 425 ($cdJ_1$); a more intense ion at *m/z* 425 would have been expected had residue 'b' not been 3-linked[18].

Compound **7** was hydrolyzed, the sugars reduced ($NaBD_4$), the alditols acetylated, and the acetates analyzed by g.l.c. and g.l.c.-m.s.[11] Four major peaks were observed in approximately equimolar ratios, with the retention times and mass spectra expected for 1,5-di-*O*-acetyl-1-deuterio-2,3,4-tri-*O*-methylxylitol, 1,3,5-tri-*O*-acetyl-1-deuterio-2,4-di-*O*-methylfucitol, 1,4,5-tri-*O*-acetyl-1-deuterio-2,3-di-*O*-




**7**

Fig. 2. Electron-impact mass spectrum and proposed fragmentation of **7**.

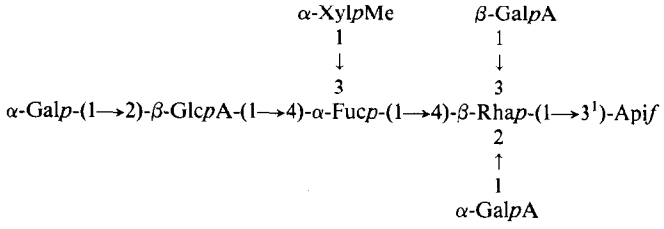
methylrhamnitol, and 3[1]-*O*-acetyl-1,2,3,4-tetra-*O*-methylapiitol, the products expected from terminal xylosyl, 3-linked fucosyl, 4-linked rhamnosyl, and 3[1]-linked apiitol residues, respectively. Thus, the glycosyl sequence was shown to be Xyl-(1→3)-Fuc-(1→4)-Rha-(1→3[1])-apiitol.

$^1$H-N.m.r. spectroscopy of **7** showed three signals with the chemical shifts expected for anomeric protons. The most upfield signal ($\delta$ 4.79, $J_{1,2}$ 1.7 Hz) was assigned to a $\beta$-rhamnosyl residue on the basis of its chemical shift (H-1 axial) and the small $J_{1,2}$ values, showing that H-2 was equatorial (*manno* configuration). Two signals, at $\delta$ 5.46 ($J_{1,2}$ 3.7 Hz) and 5.21 ($J_{1,2}$ 3.6 Hz), were assigned to the fucosyl and xylosyl residues, respectively, of **7**. These chemical shifts indicated that both anomeric protons were equatorial (*i.e.*, $\alpha$ for these residues). The signal at $\delta$ 5.46 was assigned to the fucosyl residue with the aid of a 2-D COSY n.m.r. spectrum, which showed that this signal was "connected" *via* spin–spin coupling to the upfield resonance at $\delta$ 1.18 (H-6 of this 6-deoxy residue). Compound **7** was therefore shown to be methylated $\alpha$-Xyl-(1→3)-$\alpha$-Fuc-(1→4)-$\beta$-Rha-(1→3[1])-apiitol.

Triglycosylalditol **7** is the product that was expected from octasaccharide **8**, after lithium cleavage and methylation. The two terminal galactosyluronic acid residues and the 2-linked glucosyluronic acid residue of **8** would be degraded during the lithium treatment. If the apiosyl residue was attached to a lithium-degradable residue, triglycosylalditol **7** would be recovered after lithium treatment and methylation.

The glycosyl linkages of the terminal xylosyl residues of methylated RG-II are acid-labile. A re-examination of the formolysis conditions used[10] to characterize heptasaccharide **2** showed that virtually all of the terminal xylosyl residues were cleaved (data not given). Because all of the xylosyl residues of RG-II were shown[2] to be terminal, and substituted at O-2 with methyl ether groups, the unknown residue attached to heptasaccharide **2** has now been shown to be a terminal, 2-*O*-methyl-$\alpha$-xylosyl residue. Octasaccharide **8** has been isolated from acid-hydrolyzed rice RG-II and structurally characterized[6], and it has been isolated from acid-hydrolyzed Pectinol RG-II, and partially characterized (data not shown).

Other oligoglycosylalditols released by lithium treatment of RG-II were derived from fragments that had previously been characterized, and are thus not fully characterized here. These included Rha-(1→3[1])-Apiitol and Rha-(1→2)-




**8**

Ara*p*-(1→4)-Fuc-(1→2)-Galactitol, derived from heptasaccharide **1**, with cleavage of the aceric acid residue and the residue to which the apiosyl residue was attached, and Rha-(1→5)-KDOitol, derived from disaccharide **4**.

*The points of attachment of oligosaccharides* **1**, **4**, **5**, and **8**. — To determine the residues to which oligosaccharides **1**, **4**, **5** and **8** were attached, two sequential, partial acid hydrolyses were performed on methylated sycamore RG-II. The glycosidic linkages of the derivatized KDO and DHA residues (of disaccharides **4** and **5**, respectively) could be cleaved under extremely mild conditions. Somewhat harsher conditions were then used to cleave some of the methylated apiosyl residues (of oligosaccharides **1** and **8**). After each partial acid hydrolysis, the newly produced hydroxyl groups were labeled by realkylation with trideuteriomethyl iodide. Subsequent glycosyl-linkage analyses showed the relative proportions of each derivative (by g.l.c.) and the locations and proportions of the trideuteriomethyl groups (by g.l.c.-e.i.- and -c.i.-m.s., respectively). These experiments were performed as follows.

TABLE III

GLYCOSYL-LINKAGE ANALYSES OF METHYLATED SYCAMORE RG-II BEFORE AND DURING SEQUENTIAL, PARTIAL ACID HYROLYSES, REDUCTIONS, AND REALKYLATIONS

| *Residue and linkage* | *Mole percent* | | |
|---|---|---|---|
| | *I* *unhydrolyzed*[a] | *II* *1st hydrolysis*[a] | *III* *2nd hydrolysis*[a] |
| T-Rha | 9.3[b] | 9.4[b] | 10.2[b] |
| 3-Rha | 3.1 | 2.7 | 1.4 |
| 2,3,4-Rha | 4.0 | 3.4 | 3.1 |
| T-Fuc | 5.1 | 5.2 | 4.1 |
| 3,4-Fuc | 4.6 | 4.1 | 3.7 |
| T-Ara*f* | 5.2 | 5.4 | 3.2 |
| 2-Ara*p* | 5.1 | 5.0 | 4.5 |
| T-Ara*p* | 0 | 0 | 0.7[b] |
| T-Xyl | 4.8 | 3.8 | 3.1 |
| 3'-Api*f* | 11.8 | 10.2 | 8.7 |
| T-Gal | 4.8 | 5.2 | 6.2 |
| 2,4-Gal | 5.3 | 5.8 | 2.1 |
| 2-GlcA | 4.5 | 4.7 | 5.5 |
| T-GalA | 11.3 | 11.8 | 15.5[b] |
| 4-GalA | 8.6 | 12.2[b] | 14.9[b] |
| 2,4-GalA | 4.5 | 3.7 | 2.6 |
| 3,4-GalA | 6.3 | 1.1 | 0.4 |
| 2,3,4-GalA | 1.6 | 1.3 | 1.1 |
| 2-GalA | 0 | 1.1[b] | 0.9[b] |
| 5-KDOitol | 0 | P[b,c] | P |
| 5-DHAitol | 0 | P[b] | P |
| 2,4-Galitol | 0 | 0 | 2.1[b] |
| 4-Galitol | 0 | 0 | P[b] |

[a]See Fig. 1 for source of sample. [b]Includes some trideuteriomethylated products; see Table IV. [c]P = present, but not quantitated.

TABLE IV

INTRODUCTION OF TRIDEUTERIOMETHYL GROUPS ON METHYLATED SYCAMORE RG-II BEFORE AND DURING SEQUENTIAL, PARTIAL ACID HYDROLYSES, AND REDUCTIONS

| *Residue and linkage* | *Position of O-$CD_3$ group* | *Mole percent* *I unhydrolyzed* | *II 1st hydrolysis* | *III 2nd hydrolysis* |
|---|---|---|---|---|
| T-Rha[a] | none | 6.7 | 6.7 | 9.1 |
| | 3 | 2.6 | 2.7 | 1.1 |
| T-Arap | 2 | [b] | | 0.7 |
| T-GalA[a] | none | | | 13.9 |
| | 2 | | | 1.6 |
| 2-GalA[a] | none | | 0 | 0.7 |
| | 4 | | 1.1 | 0.2 |
| 4-GalA[a] | none | | 7.7 | 11.9 |
| | 2 | | | 2.2 |
| | 3 | | 4.5 | 0.8 |
| 5-KDOitol | 2,6 | | P[c] | |
| 5-DHAitol | 2,6 | | P | |
| 2,4-Galitol | 1,5 | | | 2.1 |
| 4-Galitol | 1,2,5 | | | P |

[a]Only a portion of these residues contained trideuteriomethyl groups. [b]Residues were only entered in this Table if some of the derivative contained a trideuteriomethyl group. [c]P = present, but not quantitated.

*Identification of unsubstituted hydroxyl groups in methylated RG-II.* — Before the 3-deoxy-2-keto residues could be selectively cleaved in order to identify their points of attachment, a control experiment had to be performed to determine whether there were any unsubstituted hydroxyl groups in methylated RG-II. Therefore, unhydrolyzed, methylated (carboxyl-reduced) sycamore RG-II was trideuteriomethylated (third alkylation; see Fig. 1, step 3). This labeled any unsubstituted hydroxyl group that was exposed because of incomplete alkylation, or cleavage during the second alkylation. Subsequent glycosyl-linkage analysis (see Table III, col. I) showed that the relative proportion of 3-linked rhamnosyl residues dropped from 5.7 mole %, after the second alkylation (see Table II), to 3.1 mole %, after the third alkylation (see Table III, col. I). Terminal rhamnosyl residues rose from 6.6 mole % (see Table II) to 9.3 mole % (see Table III, col. I). This increase in terminal rhamnosyl residues after the third alkylation was largely accounted for by trideuteriomethyl groups, found on O-3 of 2.6 mole % of the terminal rhamnosyl residues (see Table IV, col. I). Small proportions of trideuteriomethyl groups were also found

on O-6 of 6,6'-dideuteriohexosyl residues, due to incomplete methylation during the second alkylation (data not shown).

These data confirmed the observation[9] that some of the aceric acid residues are cleaved during methylation as aceric acid is attached to O-3 of the internal rhamnosyl residue of **1**. Following the first alkylation and glycosyl-linkage analysis, a fully acetylated derivative of aceric acid was found (data not shown), indicating that at least some of the aceric acid residues were not methylated. This was not surprising, because significant proportions of apiosyl and galactosyl residues were also not methylated during the first alkylation[1]. After the second alkylation and glycosyl-linkage analysis (see Table II), no derivative of aceric acid was found, suggesting that the aceric acid residue had been altered either during the second alkylation or during the glycosyl-linkage analysis. The appearance of trideuteriomethyl groups on O-3 of some of the terminal rhamosyl residues after the third alkylation showed that a portion of the glycosidic linkages of the aceric acid residues had been cleaved during the second alkylation, leaving an exposed hydroxyl group on C-3 of the previously 3-linked rhamnosyl residue; it was this hydroxyl group that was trideuteriomethylated during the third alkylation.

The modified aceric acid residue apparently remained as an aglycon of the 2,4-linked galactosyl residue of **1**, as partial acid hydrolysis with 0.1M TFA for 0.5 h, at 60°, (first hydrolysis, see later) and reduction, followed by trideuteriomethylation and glycosyl-linkage analysis, did not reveal any 2,4-di-*O*-acetyl-3,6-di-*O*-methyl-1,5-di-*O*-(trideuteriomethyl)galactitol (see Table III, col. II), the derivative expected from 2,4-linked galactosyl residues that had been glycosidically cleaved, C-1-reduced with $NaBH_4$, and trideuteriomethylated. Subsequent harsher, partial acid hydrolysis with 0.5M TFA for 6 h at 60°, (second hydrolysis, see later) and reduction, followed by trideuteriomethylation and glycosyl-linkage analysis, did reveal significant proportions of this derivative (see Table III, col. III), showing that at least some of the glycosidic linkages of the galactosyl residue attached to the modified aceric acid had been cleaved.

*Determination of the points of attachment of the 2-ketosidic linkages.* — Knowledge that the free hydroxyl group on C-3 of some of the terminal rhamnosyl residues was the only site for significant trideuteriomethylation of methylated RG-II caused us to proceed with an experiment to locate the points of attachment of the particularly acid-labile ketosidic bonds of disaccharides **4** and **5**. Methylated sycamore RG-II was hydrolyzed under very mild, acidic conditions (first hydrolysis, with 0.1M TFA for 0.5 h at 60°), and reduced with $NaBH_4$ (see Fig. 1, step 4). Treatment with $NaBH_4$ reduced the newly formed carbonyl groups of the hydrolyzed residues. This served two functions: first of all, it protected the derivatized 3-deoxy-2-ketoses, KDO and DHA, from degradation during the harsher acid-hydrolysis conditions used for glycosyl-linkage analysis (without carbonyl reduction, no derivatives of KDO or DHA were found by glycosyl-linkage analysis). Secondly, each residue whose glycosidic linkage was cleaved by the first hydrolysis was labeled at C-1 (or C-2 of ketoses) with an H atom; these residues were therefore distinguishable from

those whose linkages were hydrolyzed later (during glycosyl-linkage analysis), as the later-hydrolyzed residues were subsequently labeled at C-1 with a D atom.

Methylated RG-II that had been subjected to the first acid hydrolysis and reduced with $NaBH_4$ was then treated in three different ways. A portion was trideuteriomethylated (see Fig. 1, step 5). Glycosyl-linkage analysis of this material (see Table III, col. II) showed that the composition of the galactosyluronic acid residues of the hydrolyzed material was different from that of the unhydrolyzed control (see Table III, col. I). The relative proportion of 3,4-linked galactosyluronic acid residues dropped from 6.3 mole % in the unhydrolyzed sample to 1.1 mole % after the first acid hydrolysis. The relative proportion of 4-linked galactosyluronic acid residues rose from 8.6 to 12.2 mole %, and a small proportion (1.1 mole %) of 2-linked galactosyluronic acid residues appeared after the first hydrolysis. In addition, the derivatives of 5-linked, reduced KDO and DHA were now detected. Trideuteriomethyl groups (see Table IV, col. II) were found on O-3 of terminal rhamnosyl residues, O-4 of 2-linked galactosyluronic acid residues, O-3 of 4-linked galactosyluronic acid residues, and O-2 and O-6 of 5-linked, C-2-reduced KDO and DHA residues. The trideuteriomethyl group on O-3 of the rhamnosyl residues was expected, as it was found on this atom after trideuteriomethylation of unhydrolyzed, methylated RG-II (see earlier). The trideuteriomethyl groups on O-2 and O-6 of C-2-reduced KDO and DHA were also expected, as the ketosidic linkages of these residues are known to be cleaved by the conditions of the first acid hydrolysis[1]. The trideuteriomethyl groups on O-3 of 4-linked and O-4 of 2-linked galactosyluronic acid residues showed that these were the points of attachment of the residues cleaved during the first acid hydrolysis.

A second portion of the methylated RG-II that had been hydrolyzed and reduced was analyzed to determine the extent of hydrolysis of each glycosyl residue[19]. The sample was completely hydrolyzed with 2M TFA for 1 h at 120°, reduced with $NaBD_4$, the alditols acetylated, and the acetates analyzed by g.l.c.–e.i.-m.s. (see Fig. 1, step 6). Residues that had been reduced with $NaBH_4$ (cleaved during the first, partial acid hydrolysis) were distinguished from those that had been reduced with $NaBD_4$ (hydrolyzed during the complete acid hydrolysis) by integration of the appropriate ions in the e.i.-mass spectra. For instance, for terminal residues, the ions containing C-1 and C-2 (cleavage between C-2 and C-3), $m/z$ 117 for H-reduced alditols and 118 for D-reduced alditols, were used.

The first acid-hydrolysis conditions resulted in hydrolysis of 7% or less of each glycosidic linkage (data not shown), an insignificant proportion. All of the ketosidic linkages of the derivatized KDO and DHA residues were cleaved under these conditions, as further hydrolysis under the same conditions resulted in no increase in the relative proportions of 5-linked KDO or DHA (data not shown). Because the only known glycosidic linkages cleaved to significant extents were those of KDO and DHA, this trideuteriomethyl-labeling experiment provided evidence that these residues (and thus, disaccharides **4** and **5**) were attached to O-3 of 3,4-linked galactosyluronic acid residues. However, it is also possible that a small proportion of KDO,

DHA, or an unidentified acid-labile substituent was attached to O-4 of a 2,4-linked galactosyluronic acid residue.

*Determination of the points of attachment of the apiosidic linkages.* — The third portion of the methylated RG-II that had been subjected to the first acid hydrolysis and reduced with $NaBH_4$ was remethylated (third alkylation), partially acid-hydrolyzed a second time with 0.5M TFA for 6 h at 60°, and reduced with $NaBH_4$ (see Fig. 1, step 7). This material, hydrolyzed twice with acid, was analyzed in two ways. A portion was trideuteriomethylated (see Fig. 1, step 8). Glycosyl-linkage analysis of this portion (see Table III, col. III) showed an increase in the relative proportions of terminal and 4-linked galactosyluronic acid residues, as well as the appearance of the derivative expected from 2,4-linked galactitol (discussed earlier). These changes were accompanied by a decrease in the relative proportions of terminal arabinofuranosyl, 3'-linked apiofuranosyl, and 2,4- and 3,4-linked galactosyluronic acid residues. Trideuteriomethyl groups were introduced only on hydroxyl groups freed during the second partial acid hydrolysis. These were mostly found on O-3 of terminal rhamnosyl residues (due to further cleavage of the aceric acid residue of heptasaccharide **1**), 2,4-linked galactitol residues (due to cleavage of the galacto-groups on O-3 of 4-linked galactosyluronic acid residues (see **3**) and the disappearance of some of the 5-linked DHAitol (see **3**; data not shown). Cleavage of 19% of later) and 2,4-linked galactosyluronic acid residues was the attachment point of most of the glycosidic bonds cleaved during the second partial acid hydrolysis.

A second portion of the twice-hydrolyzed material was analyzed to determine the amount of cleavage of each residue during the second acid hydrolysis (see Fig. 1, step 9), as already described. The cleaved residues (see Table V) can be matched with the labeled hydroxyl groups (see Table IV, col. III). Cleavage of 7% of the terminal rhamnosyl residues resulted in a trideuteriomethyl group on O-2 of the terminal arabinopyranosyl residue (see **1**). Cleavage of 17% of the terminal fucosyl residues resulted in a trideuteriomethyl group on O-2 of 4-linked galactitol (see 1). Cleavage of 31% of the terminal arabinofuranosyl residues resulted in trideuteriomethyl groups on O-3 of 4-linked galactosyluronic acid residues (see **3**) and the disappearance of some of the 5-linked DHAitol (see **3**; data not shown). Cleavage of 19% of the terminal xylosyl residues should have resulted in a trideuteriomethyl label on O-3 of a 4-linked fucosyl residue (see **8**), but its derivative would have been coeluted with those of the terminal galactosyl and galactosyluronic acid residues, and would have had similar m.s. fragment-ions; thus, the small proportion of this derivative could not be detected.

The g.l.c.–e.i.-m.s. method for determining the percent hydrolysis could not distinguish whether the 33% cleavage of the 2,4-linked galactosyl and galactosyluronic acid residues was of the galactosyl residues or the 6,6'-dideuterio-labeled galactosyl residues (derived from galactosyluronic acid residues). However, g.l.c.–c.i.-m.s. analysis showed that a large proportion, if not all, consisted of cleavage of the galactosyl residue. This cleavage could be correlated with the appearance of the 3-*O*-(trideuteriomethyl)-labeled, terminal rhamnosyl residue and of the 2,4-linked

TABLE V

RESIDUES PARTIALLY HYDROLYZED, AND EXPECTED HYDROXYL GROUPS PRODUCED, FOLLOWING THE SECOND PARTIAL ACID HYDROLYSIS OF METHYLATED RG-II

| *Residue and linkage* | *Percent hydrolysis*[a] | *Expected attachment*[b] | *Oligosaccharide* |
|---|---|---|---|
| T-Rha | 7 | (2)-Ara*p*<br>(5)-KDO | **1**<br>**4** |
| 3-Rha | 4 | (3[1])-Api | **1** |
| 2,3,4-Rha | 1 | (3[1])-Api | **8** |
| T-Fuc | 17 | (2),4-Gal | **1** |
| 3,4-Fuc | 4 | 2,3,(4)-Rha | **8** |
| T-Ara*f* | 31 | (3),4-GalA<br>(5)-DHA | **3**<br>**5** |
| 2-Ara*p* | 7 | 2,(4)-Gal | **1** |
| T-Xyl | 19 | (3),4-Fuc | **8** |
| 3[1]-Api*f* | 24 | ? | **1,8** |
| 2-GlcA | 1 | 3,(4)-Fuc | **8** |
| T-Gal | 3[c] | (2)-GlcA | **8** |
| T-GalA | | (2),(3),4-Rha | **8** |
| 2,4-Gal | 33[c] | (2)-AceA | **1** |
| 2,4-GalA | | ? | **6** |
| 4-GalA | 7 | ? | **6** |
| 3,4-GalA | 6 | ? | **6** |

[a]Expressed as a percentage of each residue, *i.e.*, 7% of all terminal rhamnosyl residues were hydrolyzed. [b]Number in parentheses is the hydroxyl group expected to be produced during hydrolysis. [c]Neutral residues not distinguished from 6,6′-dideuterio-labeled residues.

galactitol (*i.e.*, cleavage of the aceric acid in heptasaccharide **1**).

The only significant cleavage not accounted for was that of the 3[1]-linked apiosyl residues (24%), which are present at the reducing termini of oligosaccharides **1** and **8**. The only significant proportions of trideuteriomethyl labels remaining were on O-2 of the terminal and 4-linked galactosyluronic acid residues. Because no 2-linked galactosyluronic acid residues were found in sycamore RG-II (see Table II), this residue must have originally been 2,4-linked, with the substituent on O-4 cleaved during the first partial acid hydrolysis and then methylated (third alkylation). We

concluded that the apiosyl residues of **1** or **8**, or of both, are attached to O-2 of 2,4-linked galactosyluronic acid residues.

## CONCLUSIONS

All of the known glycosyl residues of sycamore RG-II (see Table II) have now been found in isolated oligosaccharides. The 4-, 2,4-, and 3,4-linked galactosyluronic acid residues of RG-II are undoubtedly parts of the linear $\alpha$-(1→4)-linked oligogalactosiduronic acid (**6**) of up to 8 residues found by Melton *et al.*[10]. The procedure used in that study did not locate branch points. It is likely that oligogalactosiduronic acid **6** is the backbone of RG-II. The ability of endopolygalacturonanase to release RG-II from sycamore cell-walls and the identification of galacturonic acid at the reducing terminus of RG-II support this hypothesis.

The high proportion of branched galactosyluronic acid residues in sycamore RG-II (see Table II) provides evidence that the backbone is highly branched. We have educed evidence that the apiose-containing oligosaccharides **1** and **8** are side chains and are attached to O-2 of 2,4-linked galactosyluronic acid residues of the backbone. The data do not preclude the possilibity that one of the terminal galactosyluronic acid residues of octasaccharide **8** was originally a 2,4-linked galactosyluronic acid residue having an apiosyl residue attached to O-2, and another acid-labile substituent on O-4. However, the likelihood of this is very small. The 3-deoxy-2-ketose-containing disaccharides **4** and **5** had been shown[1] to be terminal side-chains. Evidence has been obtained that disaccharides **4** and **5**, and the terminal arabinofuranosyl residue[10] of **3** are attached to O-3 of the 3,4-linked galactosyluronic acid residues of the backbone.

The glycosyl-linkage analysis of sycamore RG-II (see Table II) shows that oligosaccharides **1** and **8** occur in approximately equal proportions. If RG-II is a structurally homogeneous polysaccharide, its small size (~30 residues) limits the number of oligosaccharides **1** and **8** to one each per molecule. The proportion of the arabinofuranosyl side-chain (see **3**) and of the two 3-deoxy-2-ketose-containing disaccharide side-chains (**4** and **5**) in sycamore RG-II are difficult to determine, as it is difficult to quantitate the 3-deoxy-2-ketoses, and the terminal rhamnosyl and arabinofuranosyl residues of these side-chains are each found in two places. However, the low proportions of the terminal rhamnosyl and arabinofuranosyl residues and of 3,4-linked galactosyluronic acid residues suggest that the **4**, **5**, and arabinofuranose side-chains are not present in proportions greater than one each per molecule.

The complete structure of RG-II is not yet known. However, for the first time, the data presented here allow us to propose a model (already discussed) that describes how the various oligosaccharide fragments of RG-II are interconnected. It is only because RG-II, as isolated, has a well-defined structure that it has been possible to structurally characterize oligosaccharide fragments containing all of the glycosyl constituents of RG-II, and it is only because of the well-defined structure of RG-II

that a model of the primary structure of the entire molecule can be proposed. The model of RG-II will allow experiments to be designed to determine its complete structure.

The proposed backbone of RG-II is a repeat of α-(1→4)-linked galactosyluronic acid residues having branches on O-2 or O-3, or both, of most of the residues of the backbone. As a rule, plant cell-wall polysaccharides have a regular backbone with a repeat unit of one or two glycosyl residues and most have side-chains[20]. Although the number and complexity of the side-chains of RG-II are unique, its architecture is typical of other pectic polysaccharides. For example, pectic material from lemon peel was found[21] to have side-chains attached to O-3 of the backbone residues, and an apiogalacturonan isolated from *Lemna* was shown[22,23] to have a 4-linked galactosyluronic acid backbone with side-chains of apiobiose attached either to O-2 or O-3 of some of the backbone residues.

The complexity of RG-II is unique. No other known polysaccharide contains as many different sugars. Of the 12 different sugars known in RG-II, aceric acid has been found in Nature only as a component[7] of RG-II, DHA has been found in Nature only as a component[1] of RG-II (and possibly in the lipopolysaccharide of a Gram-negative bacterium[24]), and KDO has been found in plants only as a component[8] of RG-II. This is remarkable, particularly as RG-II contains only ~30 glycosyl residues! No other known polysaccharide is so highly branched, 22% of the residues being singly branched and 6% doubly branched. Even the side-chains of RG-II are branched, and yet, RG-II is found in a wide variety of plants. The unusual sugars characteristic of RG-II have been found in every angiosperm[1,2,8] and in the one gymnosperm[5] that has been investigated by this laboratory. A preliminary investigation obtained evidence of a pectic polysaccharide similar to RG-II in the cell walls of Bracken fern root-callus[25]. RG-II isolated from the cell walls of rice (a monocot) was shown[6] to be identical in all known aspects to RG-II isolated from the cell walls of sycamore (a dicot); rice and sycamore are evolutionarily distant angiosperms. Thus, the structure of RG-II is highly conserved.

## EXPERIMENTAL

*Preparation of RG-II* — Pectinol[1] and sycamore[11] RG-II were isolated and purified as described. Sycamore RG-II was de-esterified, treated with endopolygalacturonanase a second time[11], and further purified by gel-permeation chromatography on a Bio-Gel P-10 column[11].

*The size of RG-II.* — The reducing terminus of RG-II (1 mg) was reduced with $NaBD_4$ (0.5 mL, 10 mg/ml in M $NH_4OH$) for 3 h at room temperature.

The reaction was quenched with HOAc, the sodium ions were removed with Dowex cation-exchange resin, and the borate ions by repeated codistillation with MeOH. The dried, reduced RG-II was treated with M HCl in MeOH for ~16 h at 80°, and the products were isolated as described[11,12]. The products were trimethylsilylated[11], and analyzed by g.l.c. on a DB-1 fused-silica capillary column (0.25 mm x

30 m), programmed from 160 to 200° at 1°/min and then to 260° at 10°/min, with 2-min inital and 5-min final hold-periods. The derivatives of C-1-reduced galacturonic acid were eluted at 22.8 and 26.2 min. Response factors were calculated from sugar standards derivatized as already described. Alditol acetates were prepared as described[13], and analyzed on an SP-2330 fused-silica capillary column (0.25 mm × 30 m) operated isothermally at 235°.

*Treatment of sycamore RG-II with lithium dissolved in ethylenediamine.* — A solution of sycamore RG-II (24 mg) in water was eluted through a column (2-mL bed-volume) containing Dowex 50W-X12 ($H^+$) resin. The eluate was lyophilized, and the residue dissolved in 120 μL of water. Ethylenediamine (12 mL) was added, and the solution was sonicated for 15 min. Lithium metal was added and, with rapid stirring, the mixture turned blue, indicating the start of the reaction. The blue color was maintained for 30 min before the reaction was quenched with water, and the products (9 mg) isolated as described[15]. To assure complete reduction of the reducing termini, the products were reduced with $NaBH_4$ as already described. The products were methylated[16], and the methylated oligoglycosylalditols isolated from the mixture by chromatography on a 1-mL C-18, reversed-phase cartridge[11]. Individual methylated oligoglycosylalditols were purified by chromatography on an IBM C-18, reversed-phase, l.c. column, eluted at 1 mL/min with a gradient of aqueous MeCN, from 30% MeCN at the start to 40% at 30 min and 85% at 60 min. Approximately 3% of the effluent was monitored by c.i.-m.s. on an H.P. 5985 mass spectrometer equipped with a direct-liquid-introduction interface. The remainder of the column effluent was collected as 0.3-mL fractions. Triglycosylalditol 7 was eluted at ~38% MeCN, and gave the spectral data discussed in the Results and shown in Fig. 2. The $^1$H-n.m.r. spectrum of 7 in acetone-$d_6$ was recorded with a Bruker AM 250 MHz spectrometer, using acetone-$d_5$ as an internal standard (δ 2.04).

*Partial acid hydrolyses of methylated RG-II.* — RG-II from sycamore and Pectinol were methylated, carboxyl-reduced, and remethylated[1] (Fig. 1, step 1).

Partial acid hydrolyses of methylated sycamore RG-II were carried out in a heating block, using the conditions indicated, with 0.5 mL of trifluoroacetic acid, in screw-capped tubes. The aqueous acid was removed by evaporation with a stream of filtered, dry air at room temperature. Reductions with $NaBD_4$ or $NaBH_4$ were performed as already described, except that the reactions were carried out in 0.5M $NH_4OH$ dissolved in aqueous 50% EtOH, and the reaction mixture was eluted with 100% EtOH through an ~2-mL bed-volume column packed with Dowex cation-exchange resin. Realkylations with methyl iodide or trideuteriomethyl iodide were conducted as described[1], and the products were isolated[11] by chromatography on a 1-mL C-18 cartridge.

Glycosyl-linkage analysis of methylated material consisted of hydrolysis with 2M TFA for 1 h at 120°, reduction with $NaBD_4$, and acetylation with $Ac_2O$ for 3 h at 120°, as described[11]. The partially methylated, partially trideuteriomethylated alditol acetates were analyzed by g.l.c. on an SP-2330 fused-silica column (0.25 mm × 30 m) programmed from 150° to 235° at 3°/min with initial and final holds of 2 and

10 min, and by g.l.c.-m.s. using the same column.

Derivatives of glycosyl residues differing only by 6,6′-dideuterio labeling were coeluted. Their relative proportions were determined by integrating the equivalent ions in the e.i.-mass spectrum and the M + 1 − HOAc ion in the c.i.-mass spectrum (methane as reagent gas). For instance, 1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-galactitol, derived from terminal galactosyl residues was coeluted with 1,5-di-*O*-acetyl-1,6,6′-trideuterio-2,3,4,6-tetra-*O*-methylgalactitol, derived from terminal galactosyluronic acid residues. The relative proportion of each derivative was determined by integration of the e.i. fragment-ions, *m/z* 205 and 207, arising from carbon atoms 3–6 of the alditols, and by integration of the c.i. ions 264 and 266 (M + 1 − HOAc). The e.i.- and c.i.-m.s. methods gave very similar values. The same m.s. methods were used to quantitate the relative proportion of a trideuteriomethyl-labeled derivative that was coeluted with its nonlabeled analog. The percent hydrolysis was determined as described[19]. Molar ratios were calculated by using the appropriate effective-carbon response factors[26].

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