Carbohydrate Research, 138 (1985) 109–126
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

3-DEOXY-D-*manno*-2-OCTULOSONIC ACID (KDO) IS A COMPONENT OF RHAMNOGALACTURONAN II, A PECTIC POLYSACCHARIDE IN THE PRIMARY CELL WALLS OF PLANTS*,†

WILLIAM S. YORK, ALAN G. DARVILL, MICHAEL MCNEIL, AND PETER ALBERSHEIM**
Department of Chemistry, Campus Box 215, University of Colorado, Boulder, CO 80309 (U.S.A.)
(Received August 20th, 1984; accepted for publication, September 28th, 1984)

ABSTRACT

3-Deoxy-D-*manno*-2-octulosonic acid (KDO), a sugar previously presumed to occur only as a glycosyl residue in polysaccharides produced by Gram-negative bacteria, was found to be a component of the cell walls of higher plants. In the form of the disaccharide α-L-Rha*p*-(1→5)-D-KDO, KDO was released by mild hydrolysis with acid from the purified cell wall polysaccharide rhamnogalacturonan II. KDO was shown to be present in purified cell walls of several plants, including dicots, a monocot, and a gymnosperm. Improved methods for detecting and quantitating KDO residues in polysaccharides were developed during this investigation.

INTRODUCTION

KDO (3-deoxy-D-*manno*-2-octulosonic acid) has not previously been reported to be a glycosyl constituent of any polysaccharide other than those synthesized by Gram-negative bacteria[2,3]. Although 3-deoxy-2-ketoaldonic acids have been detected in plant extracts[4,5], these aldonic acids were not fully characterized. We now present detailed evidence that KDO is a component of the primary (growing) cell walls of higher plants[6]. Unique properties of KDO make its detection difficult by the usual methods for determining the glycosyl composition of plant cell walls. However, the ketosidic linkage of KDO residues is extremely susceptible to acid hydrolysis. This property facilitated the release of a disaccharide, with KDO at the reducing terminus, from rhamnogalacturonan II (RG-II), a polysaccharide that has been purified from plant cell walls[7].

RG-II is a small, very complex pectic polysaccharide that is released from the primary cell walls of dicots[7], monocots[7], and gymnosperms[8] upon digestion with endo-1,4-α-polygalacturonase (EC 3.2.1.15). RG-II has a very unusual glycosyl composition, characterized by the presence of 2-*O*-methylfucose, 2-*O*-methyl-

*Structure of Plant Cell Walls, Part XVI. For Part XV, see ref. 1.
†Supported by U. S. Department of Energy Grant No. DE-AC02-84ER13161.
**To whom correspondence should be addressed.

0008-6215/85/$ 03.30

xylose, apiose, aceric acid[9] (3-*C*-carboxy-5-deoxy-L-xylose), and seven other sugars more typical of pectic polysaccharides. KDO, the most recently discovered constituent of RG-II, is the twelfth sugar to be found in this polysaccharide. Aceric acid was characterized[9] only recently, and has not been detected in any other polysaccharide.

Pectinol AC is a commercial preparation of enzymes secreted by the fungus *Aspergillus niger* when the fungus is grown with plant cell walls as the carbon source. This preparation has been shown[9a] to contain undigested polysaccharides of plant cell wall origin. A major component of Pectinol AC is a polysaccharide very similar to RG-II in size and glycosyl composition[7,9]. We call this polysaccharide "Pectinol RG-II". Pectinol contains a wide variety of cell-wall-degrading enzymes, and yet, Pectinol RG-II remains intact in this preparation. The resistance of RG-II to enzymic degradation may be a result of its high content of unusual glycosyl residues and linkages[7,10,11] and nonglycosidic substituents[10].

Although RG-II is resistant to degradation by fungal enzymes, the glycosidic linkages of two constituents, namely, the apiosyl[10] and 3-deoxy-D-*manno*-2-octulosylonic residues, were found to be particularly susceptible to acid-catalyzed-hydrolysis. Mild acid treatment of RG-II from sycamore cell walls has been shown to release oligosaccharides having apiose at the reducing terminus[10,11]. Also released was another small molecule that was found to contain glycosidically linked rhamnose. However, no reducing residue was detected in this molecule[11a]. We now report that the complete structure of this small molecule is α-L-Rha*p*-(1→5)-D-KDO (compound **1**).

EXPERIMENTAL

Isolation of cell walls and cell wall polysaccharides. — Cell walls were isolated, by essentially the same procedure[12], from suspension-cultured cells of sycamore (*Acer pseudoplatanus*) and Black Mexican sweet corn (culture, a gift of C. E. Green, University of Minnesota), and from tomato and soybean seedlings. Rhamnogalacturonan I (RG-I)[13], RG-II (ref. 7), and xyloglucan[14] were isolated from suspension-cultured sycamore cells, as described. A partially purified polysaccharide containing the unusual sugars characteristic of RG-II was obtained from suspension-cultured cells of Douglas fir by J. R. Thomas of this laboratory[8].

Purification of compound **1** *from Pectinol AC.* — A "carbohydrate-enriched" fraction was prepared from Pectinol AC (Corning), as described[9]. This material was loaded (10 mg/mL in 10mM imidazole · HCl, pH 7) onto a column of QAE-Q25-120 Sephadex (Sigma) equilibrated in the same buffer. Material that did not bind to the matrix was eluted with two column volumes of starting buffer; material that bound to the matrix was eluted with a stepwise gradient (1 column volume each of 0.4M, 0.6M, 0.8M, and M imidazole · HCl, pH 7). The material eluted by the starting buffer was very rich in mannose. Pectinol RG-II was eluted by the 0.4 and 0.6M buffers, which were pooled and dialyzed against distilled H_2O. The con-

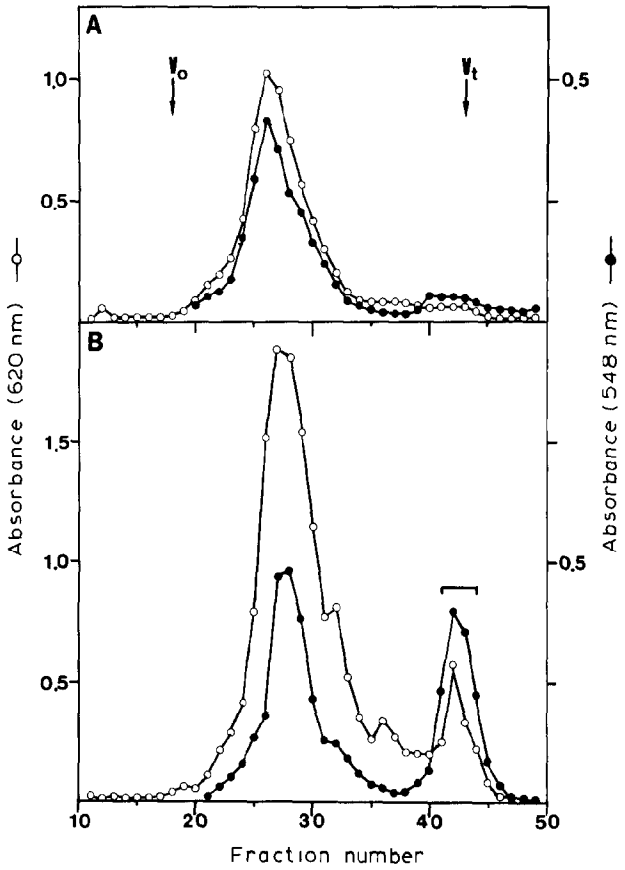


Fig. 1. Bio-Gel P-10 chromatography of native (A) and mild-acid-treated (B) RG-II from sycamore cell walls. Samples were applied to a column (25 × 1.5 cm) and eluted with 50mM NaOAc–HOAc buffer (pH 5.2). Fractions (1 mL) were collected, and assayed for hexose (—○—) and KDO (—●—) colorimetrically[15,16]. Oligosaccharide-containing fractions were pooled as indicated.

tents of the dialysis tubing were lyophilized and further fractionated by chromatography on a column of Bio-Gel P-10 in 50mM sodium acetate. The glycosyl composition and elution (from Bio-Gel P-10) of Pectinol RG-II were remarkably similar to those[7] of sycamore cell wall RG-II (see Fig. 1A). Pectinol RG-II was hydrolyzed (M acetic acid for 6 h at 40°) and the hydrolyzate applied to the P-10 column. The elution profile of carbohydrate from this column was similar to that obtained when sycamore cell wall RG-II was subjected to the same procedure (see Fig. 1B). The late-eluting fractions, which contained the oligosaccharides released by the mild acid, were pooled, passed through a column of Dowex 50 (H^+) resin, and dried under a stream of filtered air. This material was loaded (dist. H_2O) onto a column (3 mL) of anion exchange resin (BioRad-AG1 X-8, formate). Material that did not bind to the matrix was eluted with H_2O; bound material was eluted with a logarithmic gradient of 0.0 to 1.0M ammonium formate, pH 7. The eluted

carbohydrate was detected by colorimetric assays (anthrone[15] for hexose, and thiobarbituric acid[16] for KDO). The highly purified compound **1**, which was eluted at ~0.15M ammonium formate, was lyophilized. The residue was dissolved in distilled water and passed through Dowex 50 (H^+) resin, and the effluent was lyophilized again.

Gas–liquid chromatography (g.l.c.) and g.l.c.–mass spectrometry (g.c.–m.s.). — Per-*O*-trimethylsilylated methyl glycosides[17] were separated and quantitated by g.l.c. in an SP-2100, fused-silica, capillary column (Hewlett–Packard), as described[18]. Per-*O*-trimethylsilylated butyl glycosides were separated by similar techniques, except that the initial oven temperature was held for 2 min at 140°, and then increased at 1°/min to a final temperature of 240°. Per-*O*-acetylated alditol acetates were separated in a 10 m open tubular-glass capillary column coated with

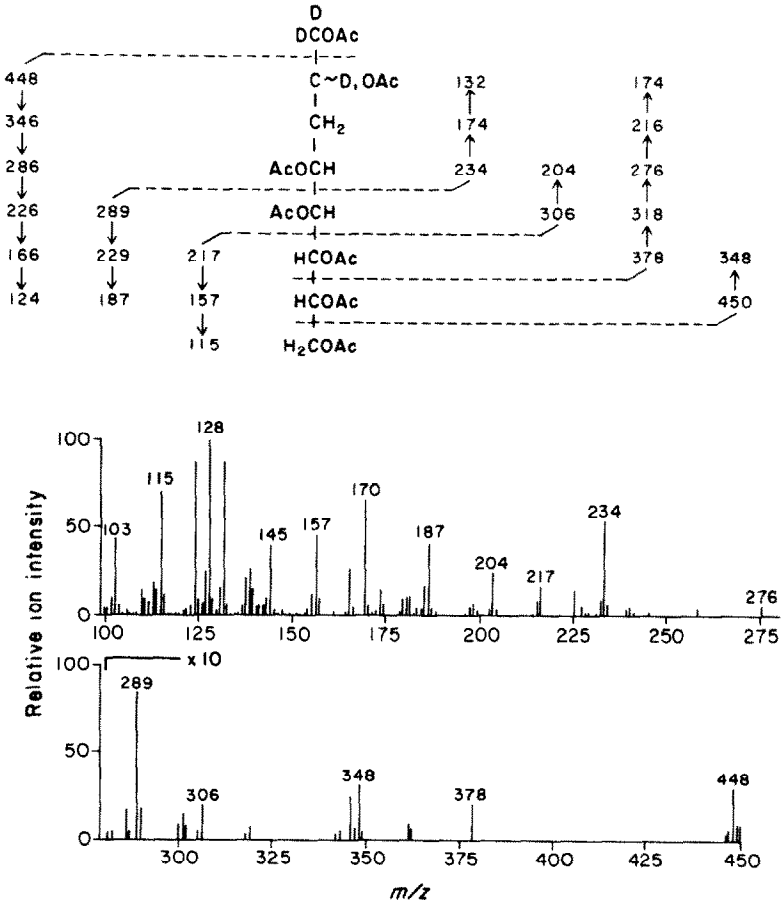
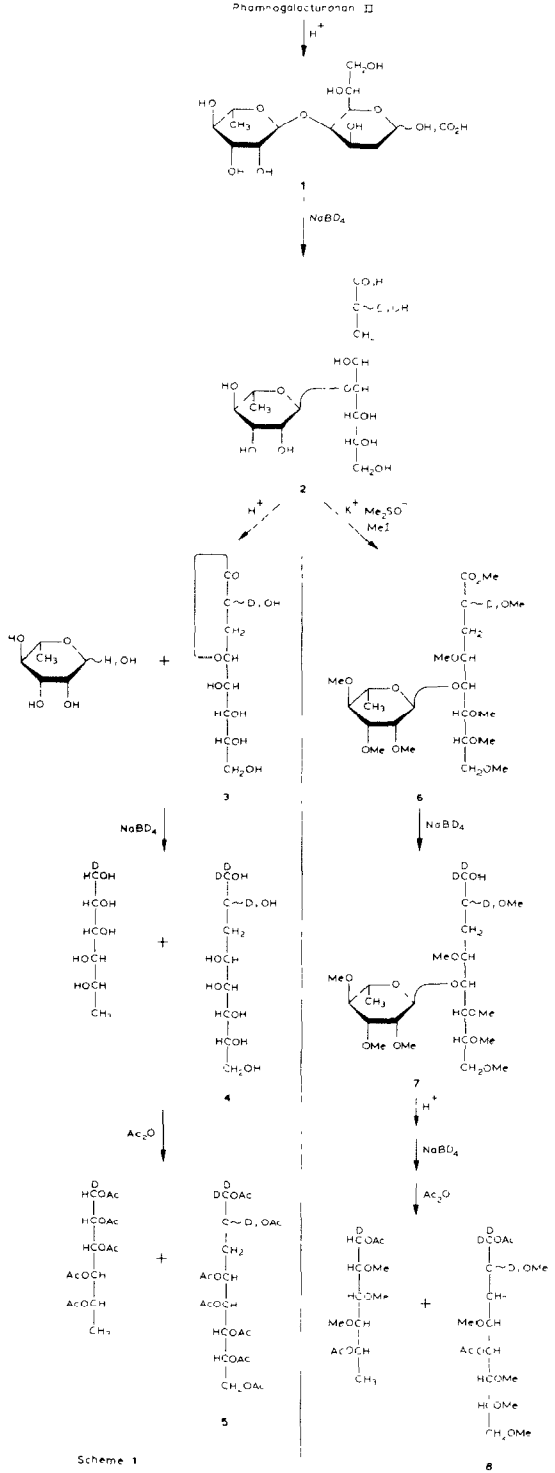


Fig. 2. Mass spectrum and proposed fragmentation of 1,2,4,5,6,7,8-hepta-*O*-acetyl-3-deoxy-1,1,2-trideuterio-octitol (compound **5**). Primary fragment-ions can decay[25] by loss of acetic acid (mol. wt. 60), *O*-deuterioacetic acid (61), ketene (42), and acetic anhydride (102), to form daughter ions.

SP-2330 (Supelco); an initial temperature of 210° was held for 2 min, and then increased by 4°/min to a final temperature of 250°, which was maintained for 5 min in order to elute the relatively nonvolatile, per-*O*-acetylated derivative of KDO. G.c.–m.s. (see Fig. 2) was performed as previously described[19,20], except that real time selected-ion monitoring was not needed.

Quantitation of KDO by g.l.c. of a per-O-acetylated alditol. — A method for the analysis of KDO by g.l.c. of a per-*O*-acetylated alditol derivative of KDO was developed. The reactions involved in this method are outlined in the left half of Scheme 1. Samples (0.1 to 1.0 mg) were lyophilized in glass tubes having Teflon-lined screwtops. Aqueous acetic acid (1%, 1 mL) was added, and the sealed tube was heated for 2 h at 100°. Aqueous acetic acid was removed by evaporation at 40° under a stream of filtered air. In order to remove the last traces of acetic acid (which otherwise would interfere with the subsequent reduction), toluene (~200 μL) was added and, after vortex mixing, evaporated, as before. When the residue appeared to be completely dry, a solution of alkaline sodium borodeuteride (150 μL of 10 mg $NaBD_4$ per mL of M NH_4OH) was added, and allowed to react for 1 h. The reaction was quenched with acetic acid (50 μL) and borate was removed as its methyl ester by alternately adding and evaporating methanolic acetic acid, as described[21]. The remaining glycosidic linkages were fully hydrolyzed with 2M trifluoroacetic acid (TFA) for 1 h at 121°. Aqueous TFA was evaporated (using toluene) in the same way in which aqueous acetic acid was removed after the first hydrolysis step; all but traces of the TFA must be removed at this step. A freshly prepared, aqueous solution of $NaBD_4$ (10 mg/mL) was added in 100-μL aliquots until further addition did not produce significant effervescence. The reaction was quenched after 1 h with acetic acid, and borate was removed as before. The resulting alditols were acetylated with Ac_2O (100 μL) and pyridine (100 μL) for 25 min at 121°. The contents of the tube were partitioned between CH_2Cl_2 and H_2O (1 mL each). The organic phase was transferred to a clean tube with a Pasteur pipet, and the CH_2Cl_2 was evaporated under a stream of filtered air. The residue was dissolved in acetone (50 to 500 μL), and 1 μL was injected into the gas–liquid chromatograph. Retention times and response factors for individual sugars were determined by injecting similarly prepared samples of known amounts of the respective sugars.

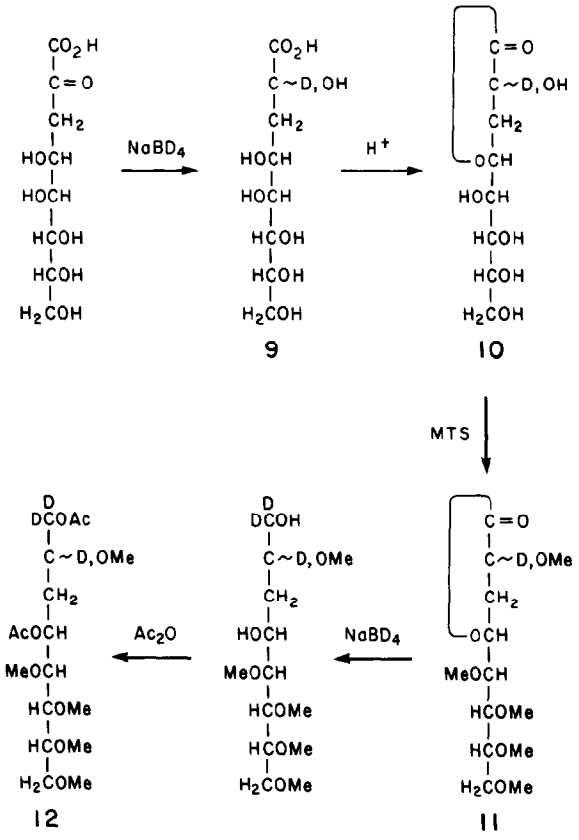
Formation of partially O-acetylated, partially O-methylated KDO alditols. — Oligosaccharides (produced by mild acid hydrolysis of RG-II and purified cell walls) were pre-reduced and per-*O*-methylated, as described[2]. KDO residues at the reducing terminus were reduced at C-2, and methyl-esterified at C-1 by this procedure (see right half of Scheme 1). In order to reduce C-1 of the KDO residue of these per-*O*-methylated oligosaccharides, they were dissolved in absolute ethanol (100 μg in 350 μL). A freshly prepared solution of aqueous $NaBD_4$ (150 μL of 10 mg/mL in distilled H_2O) was added, and, after 1 h, the reaction was quenched with acetic acid (50 μL). Borate was removed as already described. The resulting, partially *O*-methylated oligosaccharide-alditols were hydrolyzed, the products reduced, and



Scheme 1. Formation of 1,1,2-trideuterio derivatives of KDO from RG-II Compounds **2** through **8** are drawn with ambiguous stereochemistry at C-2 of the KDO residue, because the reduction that results in the formation of compound **2** is not stereospecific.

the reduction products *O*-acetylated, as described[21], and, in this way, partially *O*-acetylated, partially *O*-methylated alditols were formed. These products were analyzed by g.c.–m.s. The KDO released from plant cell wall polysaccharides by mild acid treatment was converted into 1,5-di-*O*-acetyl-3-deoxy-1,1,2-trideuterio-2,4,6,7,8-penta-*O*-methyloctitol by this procedure (see later).

A sample of 1,4-di-*O*-acetyl-3-deoxy-1,1,2-trideuterio-2,5,6,7,8-penta-*O*-methyloctitols (mixture of D-*glycero*-D-*talo* and D-*glycero*-D-*galacto* isomers) was synthesized (see Scheme 2) as follows. KDO (Sigma, 1 mg) was reduced at C-2 (250 μL of 10 mg/mL $NaBD_4$ in M NH_4OH) for 1 h. The reaction was quenched with acetic acid (50 μL), and the solution passed through Dowex 50 (H^+) resin to remove sodium ions. Borate was removed as its methyl ester as usual. In order to form a lactone, the KDO derivative (compound **9**) was dissolved in 2M TFA (250 μL) and heated for 5 min at 121°. TFA was thoroughly removed by evaporation (as



Scheme 2

Scheme 2. Synthesis of 1,4-di-*O*-acetyl-3-deoxy-1,1,2-trideuterio-2,5,6,7,8-penta-*O*-methyloctitol from KDO. Compounds **9** through **12** are drawn with ambiguous stereochemistry at C-2, because the reduction that results in the formation of compound **9** is not stereospecific.

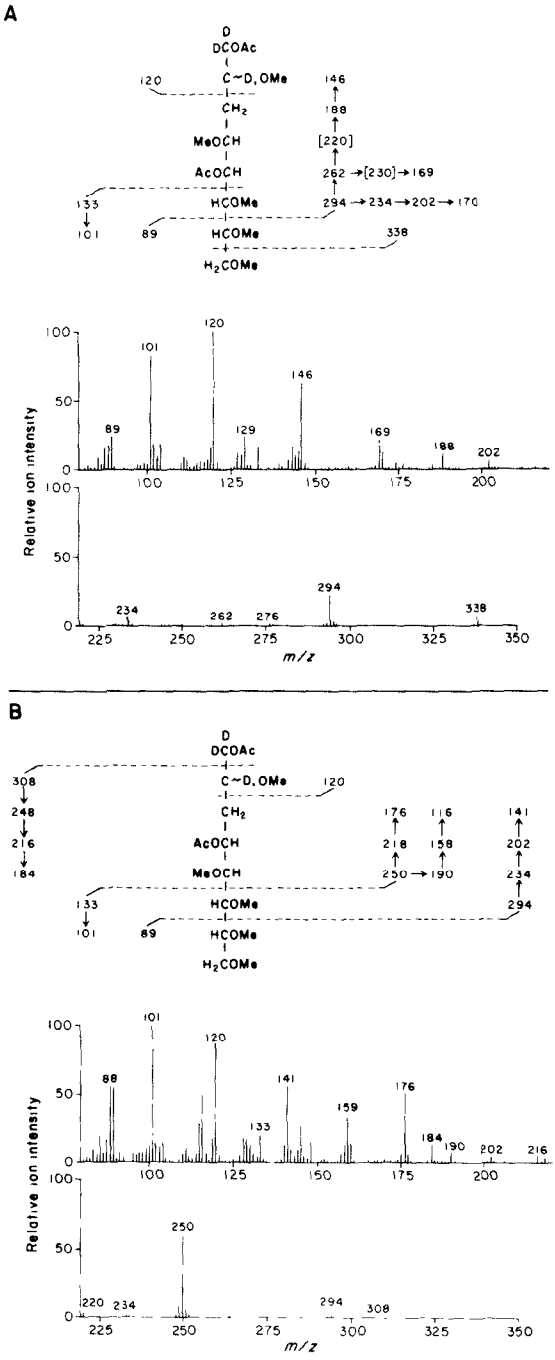


Fig. 3 Mass spectra and proposed fragmentation of 1,5-di-*O*-acetyl-3-deoxy-1,1,2-trideuterio-2,4,6,7,8-penta-*O*-methyloctitol (compound **8**) (A) and 1,4-di-*O*-acetyl-3-deoxy-1,1,2-trideuterio-2,5,6,7,8-penta-*O*-methyloctitol (compound **12**) (B). Primary fragment ions can decay[25] by loss of methanol (mol. wt 32), acetic acid (60), *O*-deuterioacetic acid (61), and ketene (42), to form daughter ions

described in the previous section), and the residue was dried *in vacuo* over P_2O_5. The lactone was then dissolved in trimethyl phosphate (Sigma, 250 μL) and per-*O*-methylated[22] by adding methyl trifluoromethanesulfonate (MTS, 25 μL), and then 2,6-di-*tert*-butylpyridine (25 μL). The mixture was stirred for 1 h, the reaction quenched with buffer (1 mL of M sodium acetate, pH 4.75), and loaded immediately onto a SEP-PAK (Waters) octadecyl-silica cartridge (prewashed with 20 mL of abs. EtOH, followed by 10 mL of distilled H_2O). The loaded cartridge was washed with buffer (10 mL of 100mM sodium acetate, pH 4.75) and purged with air (5 mL). The expected product (**11**), which was eluted from the cartridge with 4 mL of absolute ethanol, is base-labile and quite volatile. Because of the volatility of **11**, ethanol was only partially removed by evaporation under filtered air (final volume, 1.0 mL). In order to reduce C-1 of **11**, aqueous $NaBD_4$ (10 mg/mL in distilled H_2O) was added to the ethanol solution of **11** in five 100-μL aliquots. After 2 h, the reaction was quenched with acetic acid (100 μL), and borate was removed as usual. The residue was acetylated with Ac_2O (100 μL) for 3 h at 100° (with residual sodium acetate as the catalyst). The reaction was quenched with H_2O (1 mL) plus solid Na_2CO_3 (50 mg). CH_2Cl_2 (1 mL) was added, and the contents of the tube were mixed thoroughly. After the phases had separated, the organic phase was transferred to a clean tube, and evaporated under a stream of filtered air. The residue was dissolved in acetone and analyzed by g.c.–m.s. The two major products gave e.i.-mass spectra (see Fig. 3B) consistent with the structure 1,4-di-*O*-acetyl-3-deoxy-1,1,2-trideuterio-2,5,6,7,8-penta-*O*-methyloctitol (mixture of D-*glycero*-D-*galacto* and D-*glycero*-D-*talo* forms).

Modification of thiobarbituric acid assay for KDO. — The colorimetric assay for KDO described by Karkhanis *et al.*[16] was modified in order to avoid generating solutions containing high concentrations of $NaAsO_2$ and Me_2SO. By replacing the $NaAsO_2$ with Na_2SO_3 (2% in 0.5M HCl), the potential hazard presented by this procedure was significantly lessened. The sulfite solution was prepared fresh daily. In order to determine exactly how much sodium sulfite was needed to quench the periodate, aliquots (50 μL) of this solution were added at the appropriate time to one of the water controls. When the brown color disappeared, it was assumed that enough sulfite had been added to react completely with the periodate. The amount of sulfite thus determined was then added to the other samples in the assay, including unknowns, standards, and the remaining water controls. The color of the final reaction mixture was stabilized by the addition of Me_2SO to the hot solution of chromophore, as described[16]. This procedure gave a linear response for KDO standards.

^{1}H-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded as described[9].

RESULTS AND DISCUSSION

Isolation of compound **1** *from RG-II.* — RG-II isolated from the cell walls of suspension-cultured sycamore (*Acer pseudoplatanus*) cells[7] was subjected to gel

permeation chromatography on Bio-Gel P-10, before and after treatment with M HOAc for 6 h at 40°. When untreated RG-II was applied to the column, KDO, as detected by a modified (see Experimental) thiobarbituric acid assay, and RG-II, as detected by the anthrone assay (see Fig. 1A), were co-eluted. When acid-treated RG-II was applied to the column, ~50% of the thiobarbituric acid-positive material was eluted at the volume characteristic of small oligosaccharides (see Fig. 1B). The rest of the thiobarbituric acid-positive material was co-eluted with the major, hexose-containing peak. The elution volume of the main peak changed slightly when RG-II was treated with acid. The major component of the oligosaccharide fraction obtained by mild acid treatment of RG-II was shown (see next) to be compound **1**.

Isolation of compound **1** *from Pectinol AC.* — A "carbohydrate-enriched" fraction was prepared from Pectinol AC, as described[9]. A purified rhamnogalacturonan similar to sycamore cell wall RG-II in size and glycosyl composition was isolated from this fraction by ion exchange and gel permeation chromatography (see Experimental). This polysaccharide was called Pectinol RG-II. The results of mild acid treatment and subsequent gel permeation chromatography of Pectinol RG-II were similar to those already described for sycamore cell wall RG-II.

The oligosaccharide fraction obtained by mild acid hydrolysis of Pectinol RG-II was subjected to anion exchange chromatography (see Experimental), in order to obtain a highly purified preparation of the disaccharide that was shown to be compound **1**. Pectinol RG-II was a convenient source of the relatively large amounts of this disaccharide needed for developing the analytical techniques (described later) used to determine its structure and to confirm its presence in the cell walls of higher plants.

Release of compound **1** *from purified plant cell walls.* — Purified cell walls of suspension-cultured sycamore cells, soybean seedlings, tomato seedlings, and suspension-cultured maize cells were treated with mild acid (1 g of cell walls suspended in 100 mL of M acetic acid) for 18 h at 50°. The insoluble residue was removed by centrifugation (10,000 × *g* for 15 min) and the supernatant liquor was filtered (Whatman GF/C). The concentration of acetic acid in the filtrate was decreased to <3% by dilution with water, and the aqueous acid was then removed by evaporation at 40° under diminished pressure. The soluble fraction thus obtained from sycamore cell walls was subjected to gel permeation chromatography on Bio-Gel P-10. The broad, oligosaccharide-containing peak that was eluted from the P-10 column was pooled, passed through Dowex 50 (H^+) resin, and the effluent evaporated to dryness under a stream of filtered air. The mild-acid-solubilized fractions from other plant cell walls were passed through Dowex 50 (H^+) resin (omitting the gel permeation step) and dried. Compound **1** was shown (see later) to be a component of the material solubilized by mild acid treatment of each of the plant cell walls examined.

Detection of 3-deoxy-2-octulosonic acid by methanolysis and trimethylsilylation. — The purified disaccharide isolated from Pectinol RG-II and the oligosac-

charides released by mild acid from plant cell walls were methanolyzed and the products trimethylsilylated, as described[17]. An authentic sample of KDO (Sigma) was similarly treated. Gas–liquid chromatography of the derivatives of authentic KDO resulted in six major peaks (r.t. 9.53, 10.89, 14.10, 15.22, 15.93, and 16.06 min). Analysis of the disaccharide isolated from Pectinol RG-II resulted in a chromatogram that included the six peaks characteristic of KDO. This chromatogram also included two peaks corresponding to the per-*O*-trimethylsilylated methyl glycosides of rhamnose. Furthermore, this analysis was used to confirm the presence of KDO in all of the mild-acid-solubilized, plant cell wall fractions, although only four of the six derivatives diagnostic for KDO were completely resolved from other components of these complex samples by the g.l.c. conditions used.

The retention times and relative amounts of the products of methanolysis and trimethylsilylation are strongly influenced by the stereochemistry of the sugars subjected to this treatment[17]. Therefore, stereoisomers are distinguished by this technique. The retention times and relative amounts of KDO derivatives of plant cell wall origin were the same as those of the authentic 3-deoxy-D-*manno*-2-octulosonic acid. This indicated that KDO in the cell wall of higher plants is in the *manno* configuration. However, this technique gave no information about the absolute configuration (D *vs.* L) of the KDO in plant cell walls (see later).

*Quantitation of KDO by formation of per-*O*-acetylated alditols.* — One method[23] for analyzing KDO by g.l.c. results in the formation of a per-*O*-acetylated derivative of KDO that is reduced at C-2. This method usually results in an underestimate of the amount of KDO, as KDO is degraded by the harsh conditions used for hydrolysis. Therefore, we have developed a technique that uses g.l.c. of per-*O*-acetylated alditols to quantitate more accurately KDO and neutral sugars simultaneously.

Converting a 3-deoxyoctulosylonic residue (a ketosidically linked KDO residue) within a polymer into a reducing 3-deoxyoctulosonic acid residue (see Scheme 1, compound **1**, for example) by mild acid hydrolysis causes[3] only limited degradation of the KDO. Carbon-2 of the reducing KDO residue can then be reduced by alkaline $NaBD_4$ (see Scheme 1; compounds **2–8** are actually diastereoisomeric pairs of products, due to the nonstereospecific nature of the reduction that affords compounds **2**). The remaining glycosidic linkages can be hydrolyzed completely without significantly degrading the reduced KDO derivative, which is relatively stable in acid. The reduced derivative of KDO is converted into a lactone during hydrolysis (forming **3**). This lactone can be reduced with sodium borodeuteride under neutral conditions (giving **4**). Acetylation of **4** produces a volatile derivative (compound **5**) that can be separated from other per-*O*-acetylated alditols and quantitated by g.l.c. Although the procedure actually affords a diastereoisomeric pair of products (1,2,4,5,6,7,8-hepta-*O*-acetyl-3-deoxy-D-*glycero*-D-*talo*-octitol and 1,2,4,5,6,7,8-hepta-*O*-acetyl-3-deoxy-D-*glycero*-D-*galacto*-octitol), the two diastereoisomers were not separated by the chromatographic conditions used.

TABLE I

SUGAR COMPOSITION (NORMALIZED MOLE %) DETERMINED BY GAS–LIQUID CHROMATOGRAPHY OF ALDITOL ACETATES[a]

Sample	*Sugar*											
	MeFuc[b]	*Rha*	*Fuc*	*MeXyl*[b]	*Ara*	*Xyl*	*Api*[b]	*AceA*[b]	*Man*	*Gal*	*Glc*	*KDO*
Rhizobium leguminosarum 128C53 LPS[c] (prep 3)	0	27 1	33.8	0	0	0	0	0	22 7	5 8	1.0	9.6
Purified α-L-Rha*p*-(1→5)-D-KDO from Pectinol	T[d]	52 6	0	T[d]	T[d]	0	T[d]	T[d]	T[d]	T[d]	T[d]	47.4
Mixed Oligosaccharides Released from RG-II by Mild Acid	T[d]	48.3	T[d]	T[d]	10.1	0	T[d]	T[d]	0	4.4	T[d]	37.3
Material Solubilized from Cell Walls by Mild Acid												
Sycamore[e]	2.3	23 3	1.9	0.5	34.0	T[d]	8.9	0.9	1.7	8 6	8.3	9.4
Tomato	1.0	9.1	0.6	0 8	19 1	T[d]	9.0	0.9	7.5	23.8	25.8	2 3
Soybean	1 5	12.6	1.4	0.8	48.3	T[d]	6.0	2.3	4.6	12.0	5.9	4 7
Corn	0.6	4.3	0.6	0.3	54.9	T[d]	8.5	0.4	1 2	11 7	15.3	2.3
Cell Wall Polysaccharides												
Rhamnogalacturonan I	0	11.2	3.2	0	56.6	1.3	0	0	0	26.4	1.3	0
Rhamnogalacturonan II	5 8	28.1	5.1	6.4	21.8	T[d]	7 4	4.2	T[d]	15.7	2.3	3.1
Xyloglucan	0	0	6.8	0	1.6	38 1	0	0	0.7	7.7	45 1	0
Partially Purified RG-II from Douglas Fir	2.2	21 4	2.1	2.5	36.6	T[d]	4.5	1.8	2.1	22.5	3 3	1.2

[a]See Experimental section. [b]MeFuc = 2-*O*-methyl-L-fucose, MeXyl = 2-*O*-methylxylose, Api = apiose, and AceA = aceric acid[8] (3-*C*-carboxy-5-deoxy-L-xylose). [c]Compare to data of Carlson *et al.* [24] (expressed here as normalized mole %): Rha, 25.6; Fuc, 37.6; Man, 20 6; Gal, 5.2; Glc, 2.1; and KDO, 8.9. Samples also contained a small proportion of GlcNAc. [d]T = trace (<0.1%). [e]Mild-acid-released sycamore cell walls were chromatographed on P-10, selecting for small oligosaccharides, before this experiment

We have employed these reactions to develop a standardized assay (see Experimental) to detect and quantitate KDO in biological samples. Use of this assay confirmed the results obtained by methanolysis and trimethylsilylation (see earlier). However, the results obtained by g.l.c. of per-*O*-acetylated alditols were more readily interpreted. Quantitation of KDO by this method was reproducible and agreed with quantitation obtained by other techniques[24] (see Table I, *Rhizobium leguminosarum* LPS).

Various samples were analyzed by g.l.c. of per-*O*-acetylated alditol derivatives (see Table I). All samples containing a component having a g.l.c. retention time equal to that of a similarly derivatized sample of authentic KDO were analyzed by g.c.-m.s. In each case, the mass spectrum of the suspected KDO derivative was indistinguishable from that of the authentic KDO derivative (see Fig. 2).

Gas–liquid chromatography of per-*O*-acetylated alditol derivatives indicated that KDO was present in untreated RG-II, the oligosaccharide fraction released by mild acid hydrolysis of sycamore cell wall RG-II, the highly purified disaccharide obtained by mild acid hydrolysis of Pectinol RG-II, and the oligosaccharides solubilized by mild acid hydrolysis of cell walls from every flowering-plant species examined (see Table I). In addition, analysis of a partially purified polysaccharide obtained from cell walls of suspension-cultured Douglas fir cells demonstrated the presence of all of the sugars characteristic of RG-II, including KDO, and thus confirmed that RG-II is also a component of the cell walls of gymnosperms[8]. KDO was consistently found in association with the unusual sugars characteristic[7] of RG-II. The only cell wall polysaccharide examined that contained detectable amounts of KDO was RG-II (see Table I).

Methylation analysis of fractions containing KDO. — Per-*O*-methylation of oligosaccharides having KDO at the reducing terminus can be performed successfully if the keto group (C-2) of this residue is reduced before the *O*-methylation is performed[2] (see right half of Scheme 1). Therefore, fractions having low molecular weights and containing KDO, which were obtained by mild acid treatment of whole cell walls (four species) and of RG-II, both from cell walls and Pectinol AC, were reduced with $NaBD_4$ and then per-*O*-methylated[2,10]. The products obtained by this procedure were analyzed by gas–liquid chromatography–electron impact mass spectrometry (g.l.c.–e.i.m.s.). Plots of the fragmentation ion diagnostic for terminal rhamnosyl residues (*m/z* 189) featured, in every case, a dominant double peak, both components of which gave essentially identical spectra. These spectra were indistinguishable from that published by Schmidt and Jann[2] for the deuterium-reduced, per-*O*-methylated disaccharide α-L-Rha*p*-(1→5)-D-KDO (see Scheme 1, compound **6**). The double peak that was eluted from the g.l.c. column corresponded to the two diastereoisomeric products that are formed on reduction of C-2 of the KDO residue. The retention times of the components of this double peak were the same for all of the samples analyzed by this method. The presence of rhamnose and KDO in all of these samples, along with the detection of *O*-methyla-

tion products having the same retention time and mass spectrum as compound **6**, indicated that all of these samples contained the disaccharide α-L-Rha*p*-(1→5)-D-KDO.

Assigning O-5 as the point at which the rhamnosyl residue is attached to the KDO residue was based on comparison of the published[2] and experimentally obtained mass spectra of the disaccharide. We obtained additional evidence that the assigned structure, α-L-Rha*p*-(1→5)-D-KDO, was correct. Methylated oligosaccharide samples obtained by mild acid treatment of RG-II (sycamore and Pectinol) were reduced at neutral pH with $NaBD_4$. This resulted in the reduction of the methyl ester function (C-1) of the KDO residue (see Scheme I, compound **7**). Hydrolysis, reduction, and per-*O*-acetylation of **7** resulted in the formation of partially *O*-acetylated, partially *O*-methylated alditols. Analysis[25] of these derivatives by g.c.–m.s. indicated, as expected, that the rhamnosyl residues were terminal and in the pyranoid form. The diastereoisomeric pair of partially *O*-methylated 3-deoxyalditol acetates derived from KDO was eluted from the DB 1 (J & W) fused-silica capillary column as two peaks, although the compounds were eluted as a single peak when chromatographed on a Supelco SP-2330 fused-silica capillary column. The mass spectra (see Fig. 3A) of the two diastereoisomers were indistinguishable, and were consistent[26] with that of compound **8**, *i.e.*, a 1,5-di-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctitol. This was the derivative expected from 5-linked KDO. However, one fragment-ion (*m/z* 234), detected in significant abundance, could theoretically[25] have originated from either a derivative of 4-linked KDO or a derivative of 5-linked KDO. This ion could have been formed by α-elimination[25] of acetic acid from the primary ion (*m/z* 294) formed by fragmentation between C-6 and C-7 of compound **9** (the derivative of 5-linked KDO). This ion could also have been formed by β-elimination[25] of acetic acid from a primary ion (*m/z* 294) resulting from fragmentation between C-6 and C-7 of a 1,4-di-*O*-acetyl-3-deoxy-2,5,6,7,8-penta-*O*-methyloctitol, compound **12**, which would be the product expected if the KDO were 4-linked. However, the absence of a significant signal at *m/z* 250 was evidence against presence of this derivative of 4-linked KDO (see later).

Further to establish that the plant cell wall oligosaccharide contained 5-linked, not 4-linked KDO, compound **12** (the derivative expected from 4-linked KDO) was synthesized (see later). The mass spectra and g.l.c. retention times of the diastereoisomeric pair of synthetic 1,4-di-*O*-acetyl KDO derivatives were different from those of the diastereoisomeric pair of products obtained by derivatization of the KDO residue from plant-cell-wall oligosaccharides. This analysis confirmed that the plant cell wall oligosaccharide contained 5-linked KDO.

The procedure used to synthesize compound **12** is illustrated in Scheme 2. KDO was reduced at C-2 with $NaBD_4$ to yield **9**, a diastereoisomeric pair. The reduced derivative of KDO was dissolved in acid to facilitate the formation of a lactone. The major product was, as expected, the 1,4-lactone **10** (see later). The lactone was per-*O*-methylated by using methyl trifluoromethanesulfonate (MTS), a reagent that effectively *O*-methylates carbohydrates in the absence of a strong

base[22]. Use of this reagent permitted per-*O*-methylation of the sugar without opening the lactone, thus protecting O-4 from methylation. The per-*O*-methylated lactone **11** was reduced under neutral conditions, and the product acetylated. The diastereoisomeric pair of products (**12**) was separated both on DB-1 and SP-2330 fused-silica capillary columns, giving two indistinguishable mass spectra (see Fig. 3B). As expected, a strong signal at *m/z* 250 was present in the mass spectra of these 1,4-di-*O*-acetyl derivatives. The high abundance of this ion (produced by fragmentation between C-5 and C-6) is partially due to its stability; it cannot undergo β-elimination without first rearranging, because the β-carbon atom (C-3) is unsubstituted. The synthesis of a 1,4-di-*O*-acetyl derivative as the major product of the reactions outlined in Scheme 2 indicated that KDO that has been reduced at C-2 forms a 1,4-lactone at low pH.

Chemical ionization mass spectrometry[19] (isobutane) of both the synthetic 1,4-di-*O*-acetyl KDO derivatives (compounds **12**) and the cell wall-derived 1,5-di-*O*-acetyl KDO derivatives (compounds **8**) produced spectra consistent with the structures just proposed. These spectra featured signals at 384 (M + 1), 324 (M + 1 − 60), 352 (M + 1 − 32), and 422 (M + 39), as expected[19] for di-*O*-acetyl-3-deoxy-penta-*O*-methyloctitols.

^{1}H-N.m.r. spectroscopy of compound **1**. — The ^{1}H-n.m.r. spectrum was obtained for the highly purified compound **1** isolated from Pectinol RG-II. The spectrum included only one major signal in the anomeric region (at 5.19 p.p.m.), which indicated that the rhamnosyl residue is in the α configuration[2]. The disaccharides released from plant cell walls and from cell wall RG-II were reduced, and the products per-*O*-methylated (see earlier). Products were then detected that had mass spectra and retention times identical to those of compound **6** (that had been prepared, *via* compound **1**, from Pectinol RG-II). The disaccharide β-L-Rha*p*-(1→5)-D-KDO would almost certainly give rise to derivatives having retention times different from those of compound **6**. We conclude that the rhamnosyl residue in the cell wall-derived, rhamnosyl-KDO disaccharide is also in the α configuration.

It has been reported[3] that, in aqueous solutions of ammonium KDO, the ^{1}H-n.m.r. signal arising from the equatorial proton attached to C-3 of the sugar occurs at slightly higher magnetic field than that arising from the axial proton on C-3. This is unusual, as equatorial protons typically afford signals at lower magnetic field than do otherwise equivalent axial protons[27,28]. Other exceptions to this rule have been found[29]. We observed this unusual phenomenon in the ^{1}H-n.m.r. spectrum of compound **1**. The proton attached to C-3 (of the KDO residue of compound **1**) that was strongly coupled to both H-4 (*J* 12 Hz) and to the other proton attached to C-3 (*J* 12 Hz) gave rise to a multiplet at δ 2.05. This coupling pattern is characteristic of an axial methylene proton[27,28]. The other proton attached to C-3 was weakly coupled to H-4 (*J* 4 Hz) and, as just stated, was strongly coupled to the aforementioned methylene proton. The coupling pattern in this case is characteristic of an equatorial methylene proton[27,28], but the multiplet arising from this proton nevertheless occurs at higher magnetic field (δ 1.75).

Determination of the absolute configuration of KDO and rhamnose. — Syca-

more RG-II and compound **1** (obtained by mild acid treatment of Pectinol RG-II) were treated[30] with HCl in (+)-*sec*-butanol in order to form (+)-*sec*-butyl glycosides, which were per-*O*-trimethylsilylated as described[30]. Authentic L-rhamnose and 3-deoxy-D-*manno*-2-octulosonic acid (D-KDO) were similarly treated with both (+)- and (−)-*sec*-butanol. The two major products obtained after treating authentic D-KDO with (+)-*sec*-butanol had g.l.c. retention times (30.00 and 30.47 ±0.02 min) distinguishable from those of the two major products obtained after treating authentic D-KDO with (−)-*sec*-butanol (r.t. 29.91 and 30.58 ±0.02 min). When either RG-II or compound **1** from Pectinol RG-II was treated with (+)-*sec*-butanol, products were formed having retention times equal to those of products formed when D-KDO was treated with (+)-*sec*-butanol (*i.e.*, 30.00 and 30.47 min ±0.02 min). These results indicated that the 3-deoxyoctulosonic acid present in RG-II and in Pectinol RG-II is in the same configuration as the standard (*i.e.*, D-*manno*). The rhamnose in these molecules was shown by this technique[30] to be in the L configuration.

GENERAL DISCUSSION

KDO was found to be a component of the cell walls of three dicots, a monocot, and a gymnosperm. This eight-carbon acidic sugar was released from a highly purified cell wall pectic polymer, RG-II, as well as from whole cell walls, by very mild hydrolysis with acid. KDO was always found at the reducing end of the disaccharide α-L-Rha*p*-(1→5)-D-KDO. This disaccharide was also released from Pectinol RG-II.

We consider that the isolation of a unique, KDO-containing disaccharide from the cell walls of several plant species, including cell walls purified from aseptically cultured plant cells of two species and from a highly purified, plant cell wall, pectic polymer, establishes that KDO is not a product of a contaminating micro-organism, but is an integral component of the cell walls of higher plants. The co-elution, from Bio-Gel P-10, of KDO and RG-II is further evidence supporting this conclusion. All of the cell walls from which the KDO-containing disaccharide was isolated also contained the unique sugars characteristic of RG-II. KDO was *not* detected in RG-I or xyloglucan, two other purified plant cell wall polysaccharides. Furthermore, a search of the literature revealed that 3-deoxy-2-ulosonic acids had previously been detected in plant extracts[4,5].

Improved techniques for detecting and quantitating KDO were developed during this investigation. One procedure[16] for the colorimetric determination of KDO (in which a periodate-oxidation product of KDO forms a chromophore in the presence of thiobarbituric acid) poses a potential health hazard, because solutions containing high concentrations of sodium arsenite and Me_2SO are generated. Manipulating many such samples could be dangerous. Replacing the highly toxic sodium arsenite with the less toxic sodium sulfite (see Experimental) significantly lowers the potential hazard involved in performing these assays.

KDO has not been routinely detected by g.l.c. analysis of its per-*O*-acetylated

alditol, because KDO is significantly degraded when subjected to the harsh conditions typically used for acid hydrolysis. Mild treatment with acid and subsequent reduction of the carbonyl group at C-2 of the reducing KDO residue results in a product that can withstand strong conditions of hydrolysis. The monomeric derivative of KDO released by this hydrolysis can then be lactonized, the lactone reduced at C-1, and the product acetylated. This procedure allows good quantitation of internal 3-deoxyoctulosyl constituents of polymers, as evidenced by the results obtained when samples having known compositions were analyzed.

ACKNOWLEDGMENTS

The authors gratefully acknowledge isolation by Jerry R. Thomas of cell wall polysaccharides from Douglas fir and thank Leigh Kirkland for her editorial assistance.

REFERENCES

1 J. M. LAU, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 137 (1985) 111–125.
2 M. A. SCHMIDT AND K. JANN, *Eur. J. Biochem.*, 131 (1983) 509–517.
3 F. M. UNGER, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 323–388.
4 F. DOWNS AND W. PIGMAN, *Methods Carbohydr. Chem.*, 7 (1976) 233–240.
5 W. GIELEN, *Z. Naturforsch., Teil B*, 23 (1968) 1598–1601.
6 W. S. YORK, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, in W. M. DUGGER AND S. BARTNICKI-GARCIA (Eds.), *Structure, Function, and Biosynthesis of Plant Cell Walls*, Waverly Press, Baltimore, 1984, pp. 498–500.
7 A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Plant Physiol.*, 62 (1978) 418–422.
8 J. R. THOMAS, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol. (Suppl.)*, 72 (1983) 59.
9 M. W. SPELLMAN, M. MCNEIL, A. G. DARVILL, P. ALBERSHEIM, AND K. HENRICK, *Carbohydr. Res.*, 122 (1983) 115–129.
9a J. R. THOMAS, W. S. YORK, AND M. W. SPELLMAN, unpublished results.
10 M. W. SPELLMAN, M. MCNEIL, A. G. DARVILL, P. ALBERSHEIM, AND A. DELL, *Carbohydr. Res.*, 122 (1983) 131–153.
11 L. D. MELTON, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, unpublished results.
11a M. W. SPELLMAN, unpublished results.
12 K. W. TALMADGE, K. KEEGSTRA, W. D. BAUER, AND P. ALBERSHEIM, *Plant Physiol.*, 51 (1973) 158–173.
13 M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 66 (1980) 1128–1134.
14 W. D. BAUER, K. W. TALMADGE, K. KEEGSTRA, AND P. ALBERSHEIM, *Plant Physiol.*, 51 (1973) 174–187.
15 Z. DISCHE, *Methods Carbohydr. Chem.*, 1 (1962) 477–512.
16 Y. D. KARKHANIS, J. Y. ZELTNER, J. J. JACKSON, AND D. J. CARLO, *Anal. Biochem.*, 85 (1978) 595–601.
17 R. E. CHAMBERS AND J. R. CLAMP, *Biochem. J.*, 125 (1971) 1009–1018.
18 M. G. HAHN, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 68 (1981) 1161–1169.
19 M. MCNEIL AND P. ALBERSHEIM, *Carbohydr. Res.*, 56 (1977) 239–248.
20 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
21 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
22 P. PREHM, *Carbohydr. Res.*, 78 (1980) 372–374.
23 G. A. ADAMS, C. QUADLING, AND M B. PERRY, *Can. J. Microbiol.*, 13 (1967) 1605–1613.
24 R. W. CARLSON, R. E. SANDERS, C. NAPOLI, AND P. ALBERSHEIM, *Plant Physiol.*, 62 (1978) 912–917.

25 J. LONNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41–106.
26 H. B. BORÉN, P. J. GAREGG, B. LINDBERG, AND S SVENSSON, *Acta Chem. Scand*, 25 (1971) 3299–3308.
27 E. B. BROWN, W. S. BREY, AND W. WELTNER, *Biochim. Biophys. Acta*, 399 (1975) 124–130
28 R. U LEMIEUX AND J D. STEVENS, *Can. J. Chem.*, 44 (1966) 249–262.
29 A. NICHON, M. A. CASTLE, R. HARADA, C. E. BERKOFF, AND R. O. WILLIAMS, *J. Am. Chem. Soc.*, 85 (1963) 2185–2186
30 G. J GERWIG, J P. KAMERLING AND J. F. G VLIEGENTHART, *Carbohydr. Res.*, 62 (1978) 349–357