Carbohydrate Research, 214 (1991) 281–287
Elsevier Science Publishers B.V., Amsterdam

Structural studies of a rhamnogalacturonan from the stipules of *Musanga cercropoides*

Per-Erik Jansson, Bengt Lindberg,
Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-10691 Stockholm (Sweden)

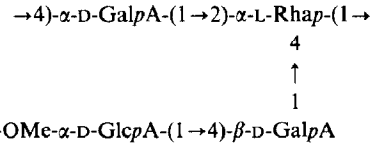
Modupe Ogunlesi,
Chemistry Department, University of Lagos, Lagos (Nigeria)

and Theophilus Orebamjo
Botany Unit, Department of Biological Sciences, Univeristy of Lagos, Lagos (Nigeria)

(Received October 2nd, 1990; accepted for publication, November 1st, 1990)

ABSTRACT

The major water-soluble polysaccharide isolated from the stipules of *Musanga cercropoides* has been investigated, using n.m.r. spectroscopy, methylation analysis, and solvolysis in liquid hydrogen fluoride as the main methods. It is concluded that the polysaccharide is of the rhamnogalacturonan type and is, at least mainly, composed of tetrasaccharide repeating-units having the following structure.



The polysaccharide further contains approximately two *O*-acetyl groups per repeating unit, which have not been located.

INTRODUCTION

The umbrella tree, *Musanga cercropoides*, is a pioneer species in secondary forests in West Africa. Its stipules, which fall off when the leaves are developed, are collected and used in food. They have medical applications[1] and, after boiling, promote bowel action and are also considered to hasten childbirth. We now report structural studies of the main polysaccharide component in the mucilage from these stipules.

RESULTS AND DISCUSSION

The fresh stipules were rinsed with water, air-dried, cursched, and defatted by extraction with light petroleum. The water-soluble material (15% of the air-dried stipules) was then extracted by exhaustive washing with water at room temperature.

0008-6215/91/$03.50

Part of this material was deproteinised by partition between phenol and water[2], and the mucilage (8% of the air-dried stipules) recovered from the aqueous phase by dialysis and freeze-drying.

Fractionation of the mucilage by chromatography on a column of DEAE-Trisacryl M, using gradient elution with aqueous sodium chloride, gave a minor and a major acidic fraction, eluted with ~0.35 and ~0.60M sodium chloride, respectively. The minor component, which on hydrolysis with acid yielded rhamnose, arabinose, xylose, mannose, glucose, and galactose, was not further investigated. The major component, $[\alpha]_D$ +96°, was eluted as a single peak, with the void volume, on chromatography on a column of Bio-Gel P-100.

Hydrolysis of the major polysaccharide (PS) gave L-rhamnose as the only neutral sugar. When the PS was carboxyl-reduced[3], sugar analysis of the product gave L-rhamnose, 4-*O*-methyl-D-glucose, and D-galactose in the proportions 1:1:2. The same result was obtained on sugar analysis of material prepared by methanolysis of the PS, followed by acetylation, and reduction with lithium borodeuteride in tetrahydrofuran. The absolute configurations of rhamnose and galactose were determined as devised by Gerwig *et al.*[4]. The 4-*O*-methylglucose was assumed to be D, as the corresponding uronic acid, 4-*O*-methyl-D-glucuronic acid, is a common component of plant polysaccharides.

Methylation analysis of the PS, with carboxyl-reduction of the methylated product, and of the carboxyl-reduced PS, gave the sugars listed in Table I, columns *A* and *B*. The results indicate that the PS is composed of L-rhamnosyl residues linked through O-2 and O-4, D-galactosyluronic acid residues linked through O-4, and terminal 4-*O*-methyl-D-glucosyluronic acid groups in the proportions ~1:2:1.

TABLE I

Methylation analyses of the rhamnogalacturonan and the tetrasaccharide I obtained on solvolysis with liquid hydrogen fluoride[a]

Sugar[b]	T(1)[c]	T(2)[d]	*Mole%*		
			A	B	C
2,3,4,6-Glc	1.00	-		25	
3-Rha	1.17	1.15	30	25	30
2,3,6-Gal	1.28	-		50	
2,3,4-Glc	1.39	1.37	20		11
2,3,4-Gal	-	1.40			9
2,3-Gal	1.77	1.70	50		26
3,4-Gal	-	1.78			24

[a] Key: *A*, PS carboxyl-reduced after methylation; *B*, carboxyl-reduced PS; *C*, tetrasaccharide, carboxyl-reduced after methylation. [b] 2,3-Rha = 2,3-di-*O*-methyl-L-rhamnose, *etc.* [c] Retention time of the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an HP 54 column. Temperature program: 190° (3 min)→250° at 3°/min. [d] Same as [c] but with the program: 175° (3 min)→250° at 3°/min.

The PS gave viscous solutions and poor n.m.r. spectra. The ^{1}H-n.m.r. spectrum showed, *inter alia*, signals for *O*-acetyl groups and methyl groups of L-rhamnosyl residues in the ratio ~2:1.

In order to obtain better spectra, part of the PS was *O*-deacetylated and hydrolysed with aqueous trifluoroacetic acid under mild conditions. The ^{1}H-n.m.r. spectrum (Fig. 1) of this product, which was still polymeric, contained several signals in the "anomeric region", some of which are given by H-5 of uronic acid residues. The ^{13}C-n.m.r. spectrum contained signals for anomeric carbons at δ 103.8, 100.5, 99.8, and 99.0. In agreement with this finding, the ^{1}H-n.m.r. spectrum (Fig. 2) of the *O*-deacetylated and carboxyl-reduced PS contained signals for anomeric protons at δ 5.21 (not resolved, $\nu_{1/2}$ ~ 1 Hz, 1 H), 5.08 (not resolved, $\nu_{1/2}$ ~4 Hz, 1 H), 4.93 (J 3.7 Hz, 1 H), and 4.67 (J ~7 Hz, 1 H). The ^{13}C-n.m.r. spectrum contained signals for four anomeric carbons at δ 104.5, 100.8 (2 C), and 100.3. These results indicate that all of the sugar residues are pyranosidic, that one of the uronic acid residues is β-linked, and that the three other sugar residues are α-linked.

In order to determine the sequence of the sugar residues, the *O*-deacetylated PS was treated with liquid hydrogen fluoride at −27°, conditions under which only the rhamnosyl linkages should be cleaved[5]. The main product was isolated by chromatography on Bio-Gel P-2, and was eluted in the tetrasaccharide region. This substance, on sugar analysis with carboxyl reduction, yieded rhamnose, 4-*O*-methylglucose, and galactose in the proportions 1:1:2. Attempted reduction of the tetrasaccharide with sodium borohydride showed that it was non-reducing. Methylation analysis, with carboxyl reduction of the methylated product, gave the sugars listed in Table I, column *C*. The formation of 3,4-di-*O*-methyl-D-galactose and the fact that the tetrasaccharide is

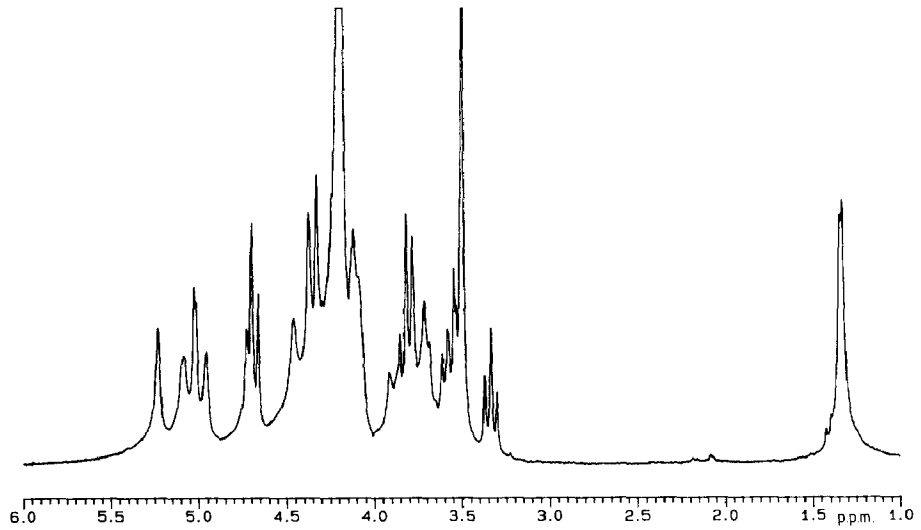


Fig. 1. ^{1}H-N.m.r. spectrum of the *O*-deacetylated, partially depolymerised PS.

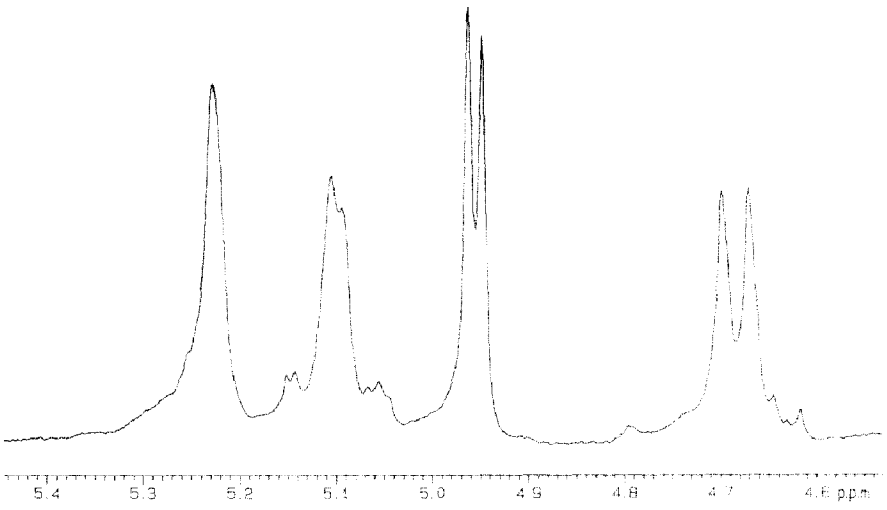
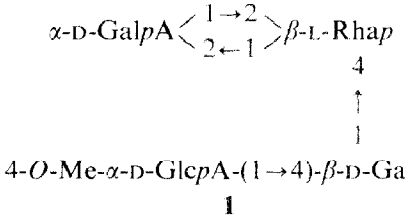


Fig. 2. Low-field part of the ^{1}H-n.m.r. spectrum of the carboxyl-reduced PS.

non-reducing demonstrate that the sequence of sugar residues is that given in structure **1**, with a dianhydride component.



4-*O*-Me-α-D-Glc*p*A-(1→4)-β-D-Gal*p*A

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In structure **1**, the L-rhamnosyl residue has to be β-linked for steric reasons, which is also evident from the n.m.r. spectra. The ^{1}H-n.m.r. spectrum (Fig. 3) thus contains signals for four anomeric protons at δ 5.25 (J 3.6 Hz, 1 H), 5.02 (J 3.7 Hz, 1 H), 4.85 (J ~1 Hz, 1 H), and 4.79 (J 7.9 Hz, 1 H), and that at δ 4.85 is given by the β-L-rhamnopyranosyl residue. The signals for the anomeric carbons appear at δ 104.1, 100.7, 95.5, and 92.1. The torsional angles at the glycosidic linkages in the dianhydride moiety differ from those for ordinary oligosaccharides, which may explain the low values of some of the chemical shifts observed. The signals given by all protons of the four sugar residues in the tetrasaccharide were assigned from COSY spectra (Table II). The chemical shifts of the resonances of the dianhydride moiety coincide well with those given by Komalavilas and Mort[7]. It is evident, from the chemical shifts and coupling constants, that the terminal 4-*O*-Me-D-Glc*p*A ($J_{1,2}$ 3.7, $J_{3,4}$ 8.8 Hz) is α-linked and that one of the two D-Gal*p*A residues ($J_{1,2}$ 7.3, $J_{3,4}$ 3.7 Hz) is β-linked. That this is the residue in the side chain was demonstrated by a NOESY spectrum which, *inter alia*, showed

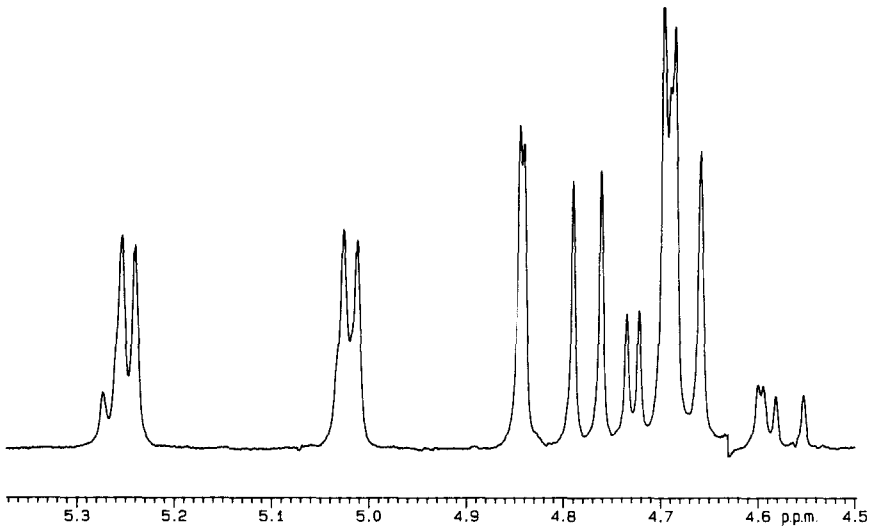


Fig. 3. Low-field part of the ^{1}H-n.m.r. spectrum of the tetrasaccharide.

TABLE II

Chemical shifts in the ^{1}H-n.m.r. spectrum of the tetrasaccharide **1**

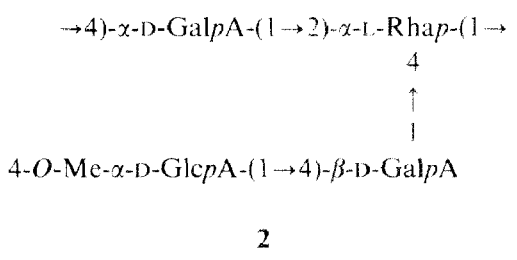
Sugar residue	*Chemical shift*[a] (δ)					
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
→2)-α-D-Gal*p*A-(1→	5.25 (3.1)	3.88	4.71	4.35	4.71	
α-D-Glc*p*A-4Me-(1→	5.02 (3.7)	3.52	3.80 (8.8)	3.33	4.70	
→4)-β-L-Rha*p*-(1→	4.85 (1.5)	4.21	3.96	3.72	3.45	1.41
2 ↑						
→4)-β-D-Gal*p*A-(1→	4.79 (7.3)	3.57	3.81 (3.7)	4.35	4.35	

[a] *J* values (Hz) in parentheses.

n.O.e. contacts between H-1 of 4-*O*-Me-α-D-Glc*p*A and H-4 of β-D-Gal*p*A, and between H-1 of β-D-Gal*p*A and H-4 of β-L-Rha*p*.

The formation of 2,3,4-tri-*O*-methyl-D-galactose in the methylation analysis of the tetrasaccharide may be due to partial elimination of the terminal 4-*O*-methyl-D-glucosyluronic acid residue during the methylation. The formation of tetrasaccharide **1** on solvolysis with liquid hydrogen fluoride nevertheless demonstrates that the PS is, at least mainly, composed of tetrasaccharide repeating-units having the structure **2**. Rhamnogalacturonans with the same linear backbone as **2** are common in plants[6], but they generally have complicated and non-regular structures. The finding of a polysaccharide belonging to this group, but with a simple, essentially regular structure, was therefore unexpected.

A dianhydride corresponding to **1**, but without the side chain, has been obtained on hydrogen-fluoride solvolyses of other rhamnogalacturonans[7], and also on methanolysis of a related polysaccharide[8]. In the former investigation[7], the presence of *O*-acetyl groups at positions 3 of the galactosyluronic acid residues were demonstrated.



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EXPERIMENTAL

General methods. — These, including n.m.r. spectroscopy, were the same as previously described[9].

Isolation of the major polysaccharide component. — Fresh stipules were collected during the dry season (January–March) from the ground under a tree at the campus of the University of Lagos. They were air-dried at room temperature, and then crushed, and a sample (20 g) was extracted with light petroleum. The defatted material (18.7 g) was stirred with water (300 mL) at room temperature for 20 h, then centrifuged, and the supernatant solution was freeze-dried. The extraction was repeated five times, giving, in total, 3.0 g of crude mucilage. This material (500 mg) was partitioned between phenol and water[2], and the aqueous phase dialysed and freeze-dried, to give the protein-free mucilage (277 mg).

This material (63 mg) in 10mM phosphate buffer of pH 6.2 (45 mL) was added to the top of a column (2.8 × 34 cm) of DEAE-Trisacryl M previously equilibrated with the same buffer. The column was irrigated, first with the buffer (520 mL) and then with a linear gradient (0→M) of sodium chloride in the buffer (380 mL). A minor fraction (6.7 mg) was eluted at ~0.36M, and the major fraction (39.3 mg) at ~0.60M, sodium chloride. The products were recovered by dialysis and freeze-drying.

The major fraction (18 mg) was dissolved in 5.0mM sodium acetate buffer of pH 6 (5 mL) and fractionated on a column (2.8 × 9.0 cm) of Bio-Gel P-100, which had been equilibrated and was irrigated with the same buffer. The fractionation was monitored using a differential refractometer, and the whole material was eluted with the void volume.

Part of the product was *O*-deacetylated by treatment with 0.1M aqueous sodium hydroxide (25 mL) containing sodium borohydride (30 mg), followed by conventional work-up.

Part of the *O*-deacetylated material was treated with 0.5M trifluoroacetic acid at 70° for 2 h, followed by chromatography on a column of Bio-Gel P-2. The main fraction, eluted in the void volume, was used for n.m.r. studies.

Carboxyl reduction of the polysaccharide. — This was performed as devised by Taylor *et al.*[3], and the procedure was repeated once.

Sugar analysis. — The material (~ 1 mg) in dry methanol (1.5 mL) to which acetyl chloride (200 μL) had been added, in a Teflon-lined screw-cap vial, was kept at 80° for 16 h. After cooling, the contents were neutralised with silver carbonate, centrifuged, concentrated, and acetylated by treatment with acetic anhydride–pyridine. A solution of the acetylated material in dry tetrahydrofuran (2.5 mL) containing lithium borohydride (25 mg) was kept at 70° for 2 h. After conventional work-up, the product was hydrolysed by treatment with aqueous 2M trifluoroacetic acid at 120° for 2 h, and the products were reduced with sodium borohydride, acetylated, and analysed by g.l.c.

Methylation analysis. — This was performed as previously described[10,11]. Methylated products containing uronic acid residues were carboxyl-reduced, using lithium borohydride in tetrahydrofuran, before they were hydrolysed.

Solvolysis with liquid hydrogen fluoride. — The *O*-deacetylated PS (40 mg) was dissolved in anhydrous hydrogen fluoride (2 mL) and kept at −27° for 30 min. Diethyl ether (5 mL) was added and the hydrogen fluoride–ether complex evaporated under reduced pressure at room temperature. The product was fractionated on a column of Bio-Gel P-2, using 50mM acetate buffer of pH 5.2 as irrigant. The major component (23 mg) was isolated by treatment with Dowex 50 (H^+) resin and freeze-drying.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swedish National Board for Technical Development and by a maintenance grant (to M.O.) from the International Program in the Chemical Sciences (Uppsala University). We thank Mrs. Helena Liedgren for help with n.m.r. spectroscopy and Mr. O. Alabi for technical assistence.

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