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HIGHLY UNEVEN DISTRIBUTION OF *O*-ACETYL GROUPS IN THE ACIDIC D-XYLAN OF *Mimosa scabrella* (BRACATINGA)

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ABSTRACT

The acidic *O*-acetyl-D-xylan of *Mimosa scabrella* contains xylose, 4-*O*-methylglucuronic acid, and *O*-acetyl groups in the molar ratios of 60:7:33 and, when subjected to controlled Smith degradation, provided xylosyl-, xylobiosyl-, xylotriosyl-, and xylotetraosyl-glycerol, as well as glycerol. Their proportions differed greatly from that which would arise from xylopyranosyl units substituted regularly with *O*-acetyl groups. In order to avoid ambiguities arising from resistance of some of the xylosyl units by substitution with 4-*O*-methylglucuronic acid, the *O*-acetylxylan was converted into a partly *O*-methylated polysaccharide having *O*-methyl groups with similar positions of substitution. Two successive Smith degradations were carried out, and the two sets of partly *O*-methylated derivatives resulting were identified by f.a.b.–m.s. The highly uneven distribution of *O*-acetyl groups along the xylan main-chain was confirmed, with the highest number of consecutive *O*-acetyl and 4-*O*-methylglucuronic acid substituents being six, and five *O*-acetyl substituents alone. 4-*O*-Methylglucuronic acid units were not substituted.

INTRODUCTION

In a recent investigation[1], we determined the location of *O*-acetyl groups on D-xylopyranosyl units in the 4-*O*-methylglucosyluronic-D-xylan of the hardwood, *Mimosa scabrella*, called bracatinga in Brazil. *O*-Acetyl migration was avoided during the isolation of the polysaccharide by the use of low temperatures and non-basic conditions. Analysis by the method of Bouveng[2,3], involving successive phenylcarbamylation, simultaneous deacetylation and methylation, de(phenylcarbamyl)ation, and hydrolysis, followed by conversion of the resulting *O*-methylaldoses into partly *O*-methylated alditol acetates, which were examined by g.l.c., indicated that the (1→4)-linked β-D-xylopyranosyl main-chain was substituted by *O*-acetyl groups at O-2 (14%), O-3 (16%), and O-2,3 (5%) (see Table I). Single-unit side-chains of 4-*O*-methyl-D-glucopyranosyluronic acid are located at O-2 on 1 in every ~9.2 units of the main chain. In the present study, the distribution

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TABLE I

POSITIONS AND PROPORTIONS OF *O*-ACETYL SUBSTITUENTS ON XYLOPYRANOSYL UNITS OF ACIDIC *O*-ACETYL-XYLANS, AS DETERMINED BY VARIOUS METHODS

Determination	*Position of O-acetyl substitution, and percentage*			
	None	*2*	*3*	*2,3-*
1[a]	65	14	15	5
2[b]	61	14	15	10
3[c]	58	15	16	11

[a]Carried out on polysaccharide prepared by using cold ethanol in the lignin removal, and Me_2NCHO in the (phenylcarbamyl)ation steps[1]. [b]Polysaccharide prepared by using refluxing ethanol containing 2-aminoethanol in the lignin removal, and Me_2SO in the (phenylcarbamyl)ation steps. [c]Same as 2, except that Me_2NCHO was the solvent used in the (phenylcarbamyl)ation steps.

of *O*-acetyl groups is determined, because its regularity or irregularity would influence the conformation of the polysaccharide in solution and in the solid state.

RESULTS AND DISCUSSION

Samples were obtained from cross sections of the wood, halfway up the trunk of an 8-year old, mature tree, and ground to a powder which was successively extracted with (*a*) benzene–ethanol in a Soxhlet apparatus, in order to remove non-polar material, and then (*b*) aqueous ammonium oxalate–ethylenediaminetetraacetic acid (EDTA) at pH 7 and 50°. Insoluble, pectin-free material was treated with aqueous chlorine at 0–5°, and then extracted with refluxing ethanol containing 3% of 2-aminoethanol, providing a product with a greatly diminished lignin content. Solubilization of desired polysaccharide with Me_2SO at 36°, followed by precipitation with an excess of ethanol, gave *O*-acetylated acidic xylan in 6% yield. It contained lignin (5%) and xylose, 4-*O*-methylglucuronic acid, and *O*-acetyl in the molar ratios of 60:7:33. Carbonyl groups, which might possibly have arisen from oxidation with the chlorine, were not detected. *O*-Acetyl migration possibly occurred during the isolation process[4], but determination of its distribution along the main chain should not be prejudiced as inter-unit migration should not take place. Furthermore, the yield of polysaccharide is much higher than the 0.4% obtained under milder conditions using cold ethanol in the lignin-removal step, and the product has a structure more representative of the component hemicellulose.

In the present study, the positions of *O*-acetyl substitution were also determined by using the Bouveng procedure, but it was found that Me_2SO could be used as solvent in the (phenylcarbamyl)ation step in addition to Me_2NCHO. Using each solvent, the product was simultaneously deacetylated and methylated, and the product de(phenylcarbamyl)ated, hydrolyzed with acid, reduced with aqueous borohydride, and acetylated. Resulting partly *O*-methylated alditol acetates were examined by g.l.c.–m.s., and the location of *O*-acetyl groups in the xylan main-

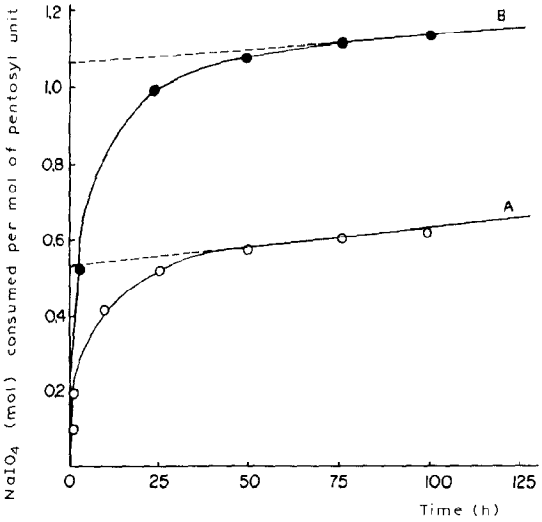

Fig. 1. Periodate uptake of (A) partly *O*-acetylated acidic xylan, and (B) *O*-deacetylated acidic xylan.

chain was found to be similar (see Table I). Also, polysaccharide prepared *via* the sequence involving 2-aminoethanol contained a higher proportion of di-*O*-acetylated xylopyranosyl units. Those of 4-*O*-methylglucuronic acid are not acetylated, as the mixture of *O*-methylaldoses obtained during the Bouveng procedure contained, according to paper chromatography, only 4-*O*-methylglucuronic acid, and not the di- and tri-*O*-methyl derivatives.

On periodate oxidation, the *O*-acetylated polysaccharide in its acidic form consumed 0.53 mol/mol of xylosyl unit, in contrast with the sodium form of *O*-deacetylated material, which consumed 1.06 mol/mol. Because the oxidation still continued after 100 h, possibly because of free-radical decomposition[5], the aforementioned values were derived by extrapolation to zero time (see Fig. 1). As the Bouveng analysis indicated that 58–61% of the xylosyl units are not acetylated (see Table I), it was confirmed that these were cleaved by periodate. Confirmation of these values was obtained by periodate oxidation of the *O*-acetylated polysaccharide, followed by sodium borohydride reduction, hydrolysis with strong acid, borohydride reduction, and acetylation. G.l.c. examination of the resulting mixture of acetates of xylitol and glycerol showed a molar ratio of 19:31. It is of interest that, when submitted to the same procedure, *O*-deacetylated xylan indicated a molar ratio of 3:17, with xylitol only arising from (1→4)-linked xylopyranosyl residues substituted at O-2 by those of 4-*O*-methylglucuronic acid. Thus, this uronic acid comprises 13% of the total anhydroaldosyl units, a value that is close to the 11% indicated by the 3-phenylphenol method.

The observation that the *O*-acetylated polysaccharide did not give a blue color with iodine in the presence of concentrated aqueous calcium chloride, in

TABLE II

ANALYSIS OF FRACTIONS ISOLATED FOLLOWING CONTROLLED SMITH DEGRADATION OF ACIDIC *O*-ACETYL XYLAN

Fraction and % yield[a]	$[\alpha]_D^{25}$ *(degrees)*	R_{Xyl}	*Ratio Xyl to Glyc*[b]	*F.a.b.–m.s. (m/z)*		*Fragments obtained on methylation analysis, (mol %)*		
				H^+	Na^+	*Me-Xyl*	*2,3-Me_2-Xyl*	*2,3,4-Me_3-Xyl*
A, 47	−36	1.00	1.0:1	225	247	tr.[c]	—	10.0
B, 23	−39	0.60	2.0:1	—	379	tr.	50.6	49.4
C, 16	−37	0.27	3.0:1	—	511	tr.	64.0	36.0
D, 14	−28	0.11	4.0:1	621	643	2.8	70.3	26.8

[a]Weight percentage, following preparative paper chromatography. [b]Glyc, glycerol. As determined by g.l.c. of derived alditol acetates, following hydrolysis. [c]tr., trace.

TABLE III

^{13}C-N.M.R. SIGNALS OF OLIGOSACCHARIDES OBTAINED FROM ACIDIC *O*-ACETYLXYLAN ON CONTROLLED SMITH DEGRADATION[a]

Fraction	*β-D-Xylopyranosyl signals (δ)*					*Glycerol signals (δ)*	
	C-1[b]	*C-2*	*C-3*	*C-4*	*C-5*	*C-1,C-3*	*C-2*
A, Xyl-Glyc	103.66	74.15	76.64	70.22	66.10	62.50 61.81	81.68
B, Xyl_2-Glyc	103.53 (C-1^1) 102.88 (C-1^2)	74.08 74.75	76.62 77.52	70.23 73.61	66.24 63.94	62.35 61.87	81.74
C, Xyl_3-Glyc	103.55 (C-1^1) 102.84 (C-1^2) 102.65 (C-1^3)	74.05 74.70	76.63 77.43	70.19 73.77	66.21 63.99	62.21 61.82	81.70
D, Xyl_4-Glyc	103.52 (C-1^1) 102.85 (C-1^2) 102.65 (C-1^3,C-1^4) 101.98, tr.[c]	74.06 74.70 73.78 75.34	76.64 77.43 77.03	70.20 73.70	66.22 63.98	62.31 61.84	81.71

[a]Determined on solutions in D_2O. [b]C-1^1, C-1^2, C-1^3, and C-1^4 designate C-1 of successive xylopyranosyl units, beginning with the unit attached to glycerol. [c]tr., trace.

contrast with *O*-deacetylated material, shows that it does not contain runs of 6 or more xylosyl units lacking *O*-substituents[6,7].

In order to determine the distribution of substituents in the *O*-acetylated polysaccharide, it was subjected to a controlled Smith degradation. Paper-chromatographic examination gave rise to spots corresponding to glycerol and others with mobilities, compared with that of xylose (R_{Xyl}), of 1.00, 0.60, 0.27, and 0.11. They were called Fractions A, B, C, and D, respectively, on isolation from the paper chromatograms, and had weight ratios of 1.00:0.49:0.33:0.30. The possibility

that they were a homologous series of compounds with the formula β-D-Xyl*p*-[(1→4)-β-D-Xyl*p*]$_{0-3}$-(1→2)-glycerol was confirmed as follows. The ratios of xylose to glycerol formed on acid hydrolysis, and of the acetates of 2,3-di- and 2,3,4-tri-*O*-methylxylitol obtained on methylation analysis (see Table II), served as confirmation. Each fraction was pure, with the exception of D, which contained 3% of di-*O*-substituted units. The results of positive-ion f.a.b.-m.s. of Fractions A to D agreed with attributed structures, each one giving rise to appropriate M + 1 (proton) and M + 23 (sodium) ion peaks, using glycerol–H_2O as the solvent (see Table II). Absent were peaks corresponding to 2-hydroxyethylidene derivatives of each compound[8].

The ^{13}C-n.m.r. spectra of Fractions A to D were consistent with attributed structures, the number and relative sizes of C-1 signals of xylopyranosyl units at δ 102.65 to 103.7 indicating the number of units present (see Table III). Their relatively low field[9], and the specific rotations are consistent with the β-D-configuration. The magnitudes of these signals can also be related to those of single-unit glycerol moieties at δ 81.7 (substituted O-2) and at δ 61.8 and 62.3–62.5 (C-1 and C-2). Other signal assignments were made with the aid of model compounds[10–12]. The occurrence of a small proportion of branched structure in Fraction D is reflected in minor carbohydrate signals at δ 102.2, 73.8, and 75.3. Signals of lignin were not detected, but its presence was indicated by u.v. absorption at 280 nm. The foregoing results show that there is an irregular pattern of substitution, with up to 4 successive substituents, quite different from those that would be expected from a regular distribution that would give glycerol and xylosyl- and xylobiosyl-glycerol, expected from a molar ratio of xylose to *O*-acetyl of 3:2.

As 4-*O*-methylglucuronic units at O-2 of the xylan main-chain would also render the units resistant to periodate oxidation, these results only indicated the number of successive, substituted units. Furthermore, fractions running slower than Fraction D were not isolated, and the number of successive, substituted xylopyranosyl units could be higher. Also, the possibility exists that periodate oxidation might be incomplete due to inter-residual hemiacetal formation[13]. Thus, a more accurate determination of location of substituents was carried out 2 successive, controlled Smith degradations on a polysaccharide material whose *O*-acetyl groups had been replaced by *O*-methyl groups. The mixture of partly *O*-methylated glycerols, obtained after 1 Smith degradation (Fraction E), is analogous to Fractions A to D in terms of successive xylosyl units. Fraction E was examined by positive-ion f.a.b-m.s. using both glycerol–H_2O and 1,4-dithioerythritol–1,4-dithiothreitol (Cleland's reagents) as solvents. Detected were ion peaks with *m/z* values of xylosylglycerols having 1 and 2 *O*-methyl groups, xylobiosylglycerols with 2, 3, and 4 *O*-methyl groups, xylotriosylglycerols with 3, 4, 5, and 6 *O*-methyl groups, xylotetraosylglycerols with 4 and 6 *O*-methyl groups, and xylohexaosylglycerols with 6 and 7 *O*-methyl groups. Thus, the highest number of successive *O*-acetyl and 4-*O*-methylglucuronic acid substituents together is 6.

The product obtained following 2 Smith degradations (Fraction F) was also

submitted to f.a.b.–m.s., and was found to contain fragments having masses consistent with xylosylglycerols with 1 and 2 *O*-methyl groups, xylobiosylglycerols with 2, 3, and 4 *O*-methyl groups, xylotriosylglycerols with 3, 4, 5, and 6 *O*-methyl groups, xylotetraosylglycerol with 4-*O*-methyl groups, and xylopentaosylglycerol with 7 *O*-methyl groups. As the numbers of these groups correspond to those of the *O*-acetyl groups in the original polysaccharide, the largest number of successive xylopyranosyl units having *O* acetyl substituents is 5.

It may be observed that xylotetraosylglycerol, with 6 *O*-methyl groups, formed after 1 Smith degradation, disappears after the second. Also, xylohexaosylglycerols with 6 and 7 *O*-methyl groups disappear, the latter probably to form xylopentaosylglycerol having 7 *O*-methyl groups.

EXPERIMENTAL

General methods. — Analyses were carried out to determine the contents of xylose[14], 4-*O*-methylglucuronic acid[15], *O*-acetyl[16], and lignin[17]. In lignin determinations, the sample (20 mg) was treated with 5M NaOH (5 mL) for 2 h at 105°, and the suspension was then centrifuged. The supernatant liquor was diluted in order to measure absorbance at 280 nm, using coniferyl alcohol as the standard. Positive f.a.b. mass spectra of oligosaccharides were recorded, using H_2O–glycerol and 1,4-dithiothreitol–1,4-dithioerythritol as solvents.

Preparation of O-*acetylated acidic xylan.* — Meal (130 g) was prepared by grinding a section of the trunk from halfway up an 8-year-old *M. scabrella* tree, and was extracted with 2:1 benzene–ethanol in a Soxhlet apparatus (55–60°). A further extraction was carried out with aqueous 0.5% ammonium oxalate–0.25% EDTA (800 mL × 3), pH 7.0, at 50°. The residue was washed with water, added to water cooled to 0–5°, and suspended by the action of bubbling chlorine for 15 min. Following filtration and drying, lignin was removed by extraction for 5 min with refluxing ethanol (1 L) containing 3% of 2-aminoethanol. The meal was isolated by filtration, and then resubjected to treatment with chlorine followed by 2-aminoethanol in refluxing ethanol. The residue was extracted with Me_2SO (1 L) for 14 days at 36°. Extracted polysaccharide was precipitated by addition to an excess of ethanol, isolated, and dissolved in water. Impurities were removed by centrifugation, and the pH of the supernatant liquor was adjusted to 2 with hydrochloric acid; the mixture was dialyzed, concentrated to a small volume, and the concentrate added to an excess of ethanol. The precipitated partly *O*-acetylated acidic xylan (7.8 g; 6% yield) was isolated by centrifugation. Examination of a solution in D_2O by ^{13}C-n.m.r. spectroscopy did not reveal, in the low-field region, signals (other than those of carboxyl and *O*-acetyl) that might arise from carbonyl groups formed by oxidation with the aqueous chlorine.

Deacetylation of O-*acetylated acidic xylan.* — The xylan (100 mg) was dissolved in 0.1M NaOH (10 mL) and, after 1 h at 25°, the solution was adjusted to neutrality with AcOH, dialyzed, and lyophilized.

Interaction of O-*acetylated and* O-*deacetylated polysaccharides with aqueous iodine.* — Each polysaccharide sample (10 mg) in water (10 mL) was diluted with 3.7M calcium chloride containing 0.1% of potassium iodide and 0.05% of iodine in order to form solutions with polysaccharide at concentrations of 1 to 10 mg%. After 30 min, the absorbance at 610 nm was found to be proportional to the concentration of deacetylated polysaccharide, and practically zero in the case of *O*-acetylated polysaccharide.

Location of O-*acetyl groups in individual units of acidic xylan.* — This was carried out by a modification[3] of the method of Bouveng[2], two separate experiments being carried out in the (phenylcarbamyl)ation step, in which Me_2SO and Me_2NCHO were employed as solvents. Resulting mixtures of partly *O*-methylated alditol acetates were examined by g.l.c.–m.s.[1] with a Model 4000 Finnegan unit, interfaced with an Incos 2300 Data System, and equipped with a fused-silica capillary column (0.25 mm i.d. × 30 m) coated with DB-210. Electron-impact spectra were obtained repetitively every 2 s, scanning from mass 40 to 420. Injections were made in the split mode at 50° and the column was then programmed rapidly (40°/min) to 195° (hold). The carrier gas was helium (linear velocity, 35 cm/s).

In the Bouveng procedure, a mixture of partly *O*-methylated aldoses was obtained with methylated positions corresponding to those of *O*-acetyl groups in the original polysaccharide. This was examined on paper chromatograms in 1:5:3:3 (v/v) benzene–butanol–pyridine–water (solvent *A*) and 9:2:2 (v/v) ethyl acetate–acetic acid–water (solvent *B*), with *p*-anisidine hydrochloride spray reagent. In solvent *B*, recognizable spots were detected corresponding to xylose, 4-*O*-methylglucuronic acid (R_{Xyl} 1.12), and mono- (R_{Xyl} 1.53) and di-*O*-methylxylose (R_{Xyl} 2.05). A slower-moving, trace component, possibly a free or partly *O*-methylated aldobiouronic acid, was also detected. In solvent *A*, xylose, and mono- (R_{Xyl} 1.42) and di-*O*-methylxylose (R_{Xyl} 1.81) were detected, and the 4-*O*-methylglucuronic acid component was retarded (R_{Xyl} 0.25). No di- and tri-*O*-methyl derivatives of glucuronic acid were detected.

Quantitative periodate oxidation of O-*acetylated and* O-*deacetylated acidic xylans.* — The *O*-acetylated xylan was dissolved in water, aqueous sodium metaperiodate was added to a final molarity of 0.05, and the consumption of oxidant was monitored at intervals up to 100 h at 25° in the dark (see Fig. 1). In the case of the *O*-deacetylated material, it was insoluble initially in the reaction mixture, but after 24 h it had dissolved.

Periodate oxidation of O-*acetylated and* O-*deacetylated acidic xylans, followed by successive sodium borohydride reduction, strong acid hydrolysis, borohydride reduction, and acetylation.* — Samples were oxidized with 0.05M sodium periodate for 120 h, and an excess of sodium borohydride was added and, after 24 h, decomposed with acetic acid. The solution was dialyzed, lyophilized, the product hydrolyzed with M sulfuric acid for 5 h at 100°, the solution cooled, the acid neutralized with barium carbonate, the suspension filtered, and the filtrate treated with sodium borohydride. The product was acetylated, and examined by g.l.c.[18] in a column

(0.15 cm i.d. × 2 m) of 3% (w/w) of OV-225 on Gas Chrom Q (100–200 mesh), programmed from 120 to 190° (4°/min, then hold), using nitrogen as the carrier gas (40 mL/min). Acetates of glycerol and xylitol were detected as products from *O*-acetylated and *O*-deacetylated polysaccharides in ratios of 19:31 and 17:3, respectively.

Isolation of oligosaccharides formed on Smith degradation of O-acetylated acidic xylan. — The polysaccharide (1.0 g) was treated with aqueous 0.05M sodium periodate (200 mL) for 160 h, and the product converted into polyalcohol with sodium borohydride, as already described. It was partly hydrolyzed in water (100 mL), whose pH had been preadjusted to 2 with sulfuric acid, by heating for 40 min at 100°; the solution was cooled, the acid neutralized ($BaCO_3$), the suspension filtered, the filtrate de-ionized with resins, and evaporated, and the product examined on a paper chromatogram (solvent *A*) showing, with the $AgNO_3$–NaOH reagent, mainly glycerol (R_{Xyl} 1.6), with other spots at R_{Xyl} 1.00, 0.60, 0.27, and 0.11. Each of these four components was isolated *via* chromatography on Whatman No. 1 filter paper (solvent *A*), the appropriate bands being eluted with water; each eluate was concentrated to a small volume, an excess of ethanol was added, and the precipitates which formed were removed. The supernatant liquors gave rise respectively to Fraction A: (60 mg), $[\alpha]_D^{25}$ −36° (*c* 0.9, water); Fraction B: (29 mg), $[\alpha]_D^{25}$ −39° (*c* 0.4, water); Fraction C: (20 mg), $[\alpha]_D^{25}$ −37° (*c* 0.3, water); and Fraction D: (18 mg), $[\alpha]_D^{25}$ −28° (*c* 0.3, water).

Characterization of oligosaccharides. — Fractions A to D were each hydrolyzed with M TFA for 5 h at 100°, cooled, and evaporated to a syrup, which was examined by paper chromatography in solvent *A*, and spots detected by the $AgNO_3$–NaOH dip method. Xylose and glycerol, thus identified, were quantitated by conversion into a mixture of acetates of xylitol and glycerol by successive sodium borohydride reduction and acetylation, followed by g.l.c. examination on OV-225 (for methodology, see quantitation of similar mixtures). The ratios are presented in Table II.

Methylation analysis of Fractions A to D was carried out as follows. Each sample was methylated twice by the method of Hakomori[19] and the products hydrolyzed by the procedure of Saeman[20]. The hydrolyzate was converted into a mixture of partly *O*-methylated alditol acetates, which was examined by g.l.c. on a conventional column of OV-225 at 170° using nitrogen as the carrier gas (40 mL/min). Ratios of components are presented in Table II.

^{13}C-N.m.r. spectra of Fractions A to D and *O*-acetylated acidic xylan were recorded with a Bruker AM-360-WB spectrometer in the Fourier-transform mode, using D_2O (2 mL) as solvent contained in a tube (10 mm diam. × 20 cm) maintained at 33°. The spectral width was 20 000 Hz, the acquisition time 0.85 s, and the pulse width 23 μs. Chemical shifts are expressed in δ (see Table III) relative to the resonance of Me_4Si, obtained in a separate experiment.

Smith degradation of partly O-methylated polysaccharide. — Partly *O*-methylated polysaccharide, which was an intermediate in the Bouveng procedure, was

submitted to 2 successive Smith degradations under conditions already described, to give Fractions E and F respectively.

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