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(2-*O*-α-D-Galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan from *Eucalyptus globulus* Labill

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Abstract

An unusual heteroxylan composed of galactosyl, 4-*O*-methyl-glucuronosyl and xylosyl residues with molar ratio 1:3:30 was isolated from the wood of *Eucalyptus globulus* Labill. The results of linkage analysis, supported by data of ^{1}H, 2D ^{1}H–^{1}H COSY and ^{13}C NMR spectroscopy, revealed that the polysaccharide is a (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan with a (1→4)-linked β-D-xylopyranosyl backbone branched at O-2 by short side chains composed of terminal 4-*O*-methyl-α-D-glucuronic acid and of 4-*O*-methyl-α-D-glucuronic acid substituted at O-2 with α-D-galactose.

Keywords: (2-*O*-α-D-Galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan; *Eucalyptus globulus* Labill; Structural analysis

1. Introduction

Recently, considerable attention has been focused on the wood of *Eucalyptus* species as a rapid growing source of fiber material for pulp and paper industry. *Eucalyptus globulus* is one of the more distributed and widely used *Eucalyptus* species in the world [1].

It was suggested more than 30 years ago [2] that the xylan from *E. globulus* has a typical structure for xylans of hardwoods with (1→4)-linked xylose backbone and single residues of glucuronic acid, attached to O-2 of the xylose residues. However, our current trials on analysis of oligosaccharide fractions from the industrial streams (black liquor) of kraft pulping of *E. globulus* showed, with sufficient reliability, the existence of oligomeric fragments constituted of galactosyl and glucuronosyl residues linked to the xylan backbone [3]. This interesting fact was one of the reasons to re-examine the structure of the heteroxylan from *E. globulus* in an effort to find the explanation for the structure of these oligosaccharides.

In this paper, the results of structural investigations of the heteroxylan isolated from *E. globulus* wood are presented.

2. Experimental

Materials.—A 12-year-old *E. globulus* tree of a clone plantation in northern Portugal was supplied by RAIZ (Paper and Forest Research Institute, Aveiro, Portugal). The wood sample used in this work was representative of the whole tree.

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Isolation and purification of heteroxylan.—Air-dried wood sawdust (40 mesh), pre-extracted for 12 h with 1:2 EtOH–toluene in a Soxhlet extractor, was delignified by a modification of the chlorite method [4]. The acidic heteroxylan was extracted from the washed and air-dried holocellulose by 2 M KOH soln [5]. The recovery of the heteroxylan from alkaline soln was made by acidification to pH 5.5 with AcOH and addition of abs EtOH (1 vol). The crude xylan (19.9% of o.d. wood) was purified further by two fractional precipitations with EtOH from a soln in 2% KOH and by three precipitations with the Fehling soln as the copper complex [6].

Carbohydrate and linkage analysis.—Neutral sugars were released by Saeman hydrolysis (by dispersing of sample (3–5 mg) in 0.2 mL 72% H_2SO_4 at 20 °C followed by dilution to 1 M H_2SO_4 (2.2 mL water) and hydrolysis for 2.5 h at 100 °C) and analyzed as their alditol acetates by GC [7]. Uronic acids were determined colorimetrically with *m*-phenylphenol by a modification [8] of the method of Blumenkrantz and Asboe-Hansen [9]. An absolute configuration of sugar residues was determined by synthesis of the corresponding glycosides with optically active 2-butanol, followed by silylation and analysis of products by GC [10,11].

The polysaccharide was methylated by a modification of the Hakomori method [12], as described elsewhere [13], or by a modification of the method of Ciucanu and Kerek [14] and Isogai et al. [15]. To ionize the hydroxyl groups, the soln of the heteroxylan in Me_2SO was treated with sodium methylsulfinylmethanide (according to Hakomori) or with NaOH pellets (according to the latter procedure). The methylation has been carried out by methyl iodide in Me_2SO followed by carboxyl-reduction of methylated polysaccharide by $LiAlD_4$ in THF, as described elsewhere [16,17], to identify the uronic acid derivatives. The methylated polysaccharide was hydrolyzed with aq 90% formic acid for 1 h at 100 °C and then with 2 M trifluoroacetic acid for 1.5 h at 120 °C, reduced by $NaBD_4$ and then converted into partially methylated alditol acetates (PMAA). The PMAA were separated by GC on a DB-1 capillary column and identified by GC–MS [18,19] on a Hewlett–Packard 5890 series II chromatograph, equipped with a quadrupole HP 5970 mass selective detector (EI, 80 eV), using helium as the carrier gas. The column program temperature was 150–225 °C, 3 °C/min; injector temperature: 230 °C; detector temperature: 250 °C.

GPC analysis.—The GPC analysis of isolated polysaccharide, dissolved in *N*,*N*-dimethylacetamide (DMAC) containing 0.5% LiCl (w/v), was performed on two Plgel 10 μm MIXED B 300 × 7.5 mm columns protected by a Plgel 10 μm pre-column (Polymer Laboratories Ltd., UK) using a PL-GPC 110 system (Polymer Laboratories) [20]. The columns, guard column and injection system were maintained at 70 °C. The eluent (0.5% w/v LiCl in DMAC) was pumped at a flow rate of 0.9 mL/min. The GPC columns were calibrated using pullulan reference materials (Polymer Laboratories).

^1H NMR spectroscopy.—1D ^1H NMR spectra (300 MHz) were recorded at 50 °C on a Bruker AMX 300 spectrometer. The dried polysaccharide (4–5 mg) was dissolved in D_2O (0.7 mL) and sodium 3-(trimethylsilyl)propionate-d_4 was used as internal standard (δ 0.000). Relaxation delay was 1 s, r.f. pulse angle 30°, and about 2000 pulses were collected.

2D ^1H–^1H COSY spectroscopy was performed at 50 °C using a standard COSY sequence (90° pulse, relaxation delay 1 s).

^{13}C NMR spectroscopy.—^{13}C NMR spectra (75.47 MHz) were recorded at 50 °C on a Bruker AMX 300 spectrometer with a soln of the polysaccharide (40 mg/mL) in Me_2SO-d_6 and 1% Me_4Si as internal standard (δ 0.000). The relaxation delay was 2.5 s, r.f. pulse angle 90°, and about 16,000 pulses were collected.

3. Results and discussion

An examination of the monosaccharide composition of *E. globulus* after complete hydrolysis with the sulfuric acid solution (Table 1) showed that xylose and glucuronic acid (present as 4-*O*-methylglucuronic acid, as suggested from NMR data given below) are the

Table 1
Carbohydrate composition of *E. globulus* wood and isolated heteroxylan (%)

Monosaccharide residue	Wood	Crude heteroxylan	Purified heteroxylan
Ara	0.54	0.73	
Xyl	20.76	80.95	85.18
Man	1.80	0.16	
Gal	2.22	6.05	2.70
Glc	68.54	0.59	traces
4-*O*-Me-GlcA	6.14	11.52	12.12

main constituents of non-cellulose polysaccharides. The crude polysaccharide, isolated from the alkaline 2 M potassium hydroxide solution, contained mainly xylose, 4-*O*-methylglucuronic acid and galactose together with minor proportions of other sugars. The further careful purification of the crude polysaccharide by graded precipitation with ethanol and via the copper complex revealed that the polysaccharide is composed of xylose, 4-*O*-methylglucuronic acid and galactose with a molar ratio of 30:3:1, respectively. Thus, even after the purification process, galactose was present in the polysaccharide, being certainly included in the structure of the heteroxylan. The GPC analysis of the heteroxylan, carried out in the LiCl–DMAC solvent system, showed the homogeneity of the isolated polysaccharide without impurities of other polymeric carbohydrates (Fig. 1). The average molecular weight of the heteroxylan (MW_p), obtained using pullulan calibration standards, was 25,600. To prove the existence of a heteroxylan with such an unusual structure, the linkage analysis and ^1H and ^{13}C NMR investigations of the isolated polysaccharide have been carried out.

Linkage analysis of the heteroxylan.—The purified heteroxylan was subjected to methylation by two different procedures: a modification of the Hakomori method and a modification of the method of Ciucanu and Kerek, and Isogai et al. Results obtained using both procedures were in close agreement.

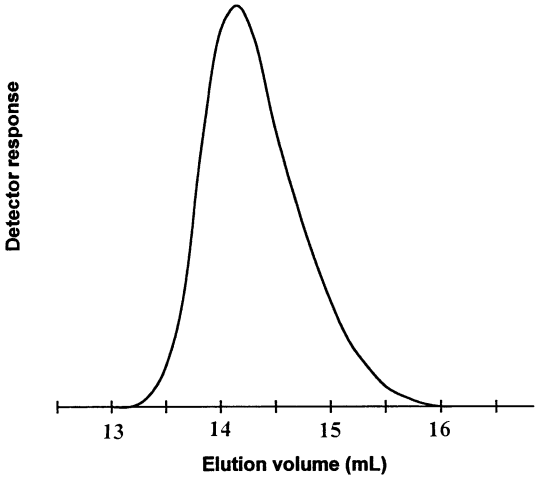


Fig. 1. GPC curve of purified (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan in the LiCl–DMAC solvent system.

The linkage analysis revealed the presence of derivatives of six partially methylated sugars (Table 2). The ratio between terminal residues and branched units (0.92) and the good correlation with the direct sugar analysis of the native heteroxylan showed that no undermethylation had occurred. Xylose was essentially present as (1→4)-linked units: 88% being 4-substituted (2,3-di-*O*-methylxylose), 11% carrying also a substituent at the O-2 position (3-*O*-methyl-xylose) and 1% consisting of non-reducing end-groups (2,3,4-tri-*O*-methyl-xylose). The substitution at O-3 or substitution at both the O-2 and O-3 positions was not detected.

The structural localization of 4-*O*-methylglucuronic acid was achieved by carboxyl-reduction of the methylated polysaccharide. The

Table 2
Results of linkage analysis (mol %) of heteroxylan

Residue	Position of *O*-methyl groups	Relative mole ratio	Structural units deduced
Xyl	2,3,4	1	Xyl*p*-(1→
Xyl	2,3	80.3	→4)-Xyl*p*-(1→
Xyl	3	10.3	→2,4)-Xyl*p*-(1→
Glc	2,3,4	7.1	Glc *p* A-(1→
Glc	3,4	3.3	→2)-Glc*p* A-(1→
Gal	2,3,4,6	2.7	Gal*p*-(1→

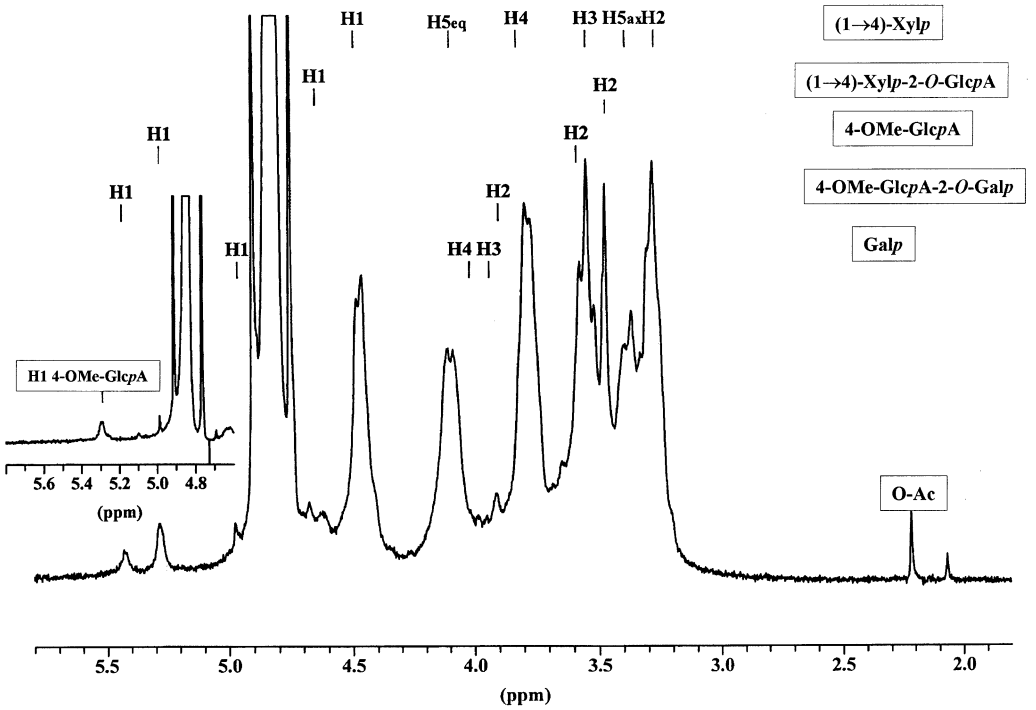


Fig. 2. 1D ^{1}H NMR spectrum of (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan in D_2O (referenced to internal 3-(trimethylsilyl)propionate-d_4). The anomeric region of 4-OMe-Glc*p*A of the model birch xylan (Sigma reagent) is shown in the upper left corner.

occurrence of 2,3,4-tri-*O*-methyl-glucose indicated that part of the 4-*O*-methylglucuronic acid (68%) is in a terminal position, attached to the xylan backbone. It is evident that a part of 4-*O*-methylglucuronic acid (32%) must be linked at O-2 due to the presence of 3,4-di-*O*-methylglucose. The molar ratio between 2,4-disubstituted xylose and the sum of terminal and substituted at O-2 4-*O*-methylglucuronic acid (0.99) suggested that all glucuronosyl units should be attached to the polysaccharide main chain through O-2 of xylose residues.

The galactose was observed only in non-reducing end positions like 2,3,4,6-tetra-*O*-methyl-galactose. The good correlation between the molar content of galactose and substituted at O-2 4-*O*-methylglucuronic acid suggested that all galactosyl residues should be linked to glucuronosyl moieties as the terminal units in the short side-chains. The galactose is present, probably as the α-pyranoside form, as was found for galactoxylans from *Gramineae* [21–23].

^{1}H NMR spectroscopy.—The ^{1}H NMR analysis (Fig. 2) revealed a group of prominent proton signals at 4.46, 4.09, 3.77, 3.54, 3.37 and 3.28 ppm, corresponding to H-1, H-5_{eq}, H-4, H-3, H-5_{ax} and H-2, respectively, of non-substituted internal (1→4)-linked β-D-xylopyranosyl residues [24–26]. The signals at 4.63 and 3.47 ppm were assigned to H-1 and H-2, respectively, of β-D-xylopyranosyl units substituted at O-2. The presence of terminal α-D-galactopyranosyl residues was supported by resonances at 4.98, 3.94 and 4.09 ppm, corresponding to H-1, H-3 and H-4, respectively [27].

The proton resonance at 5.28 ppm was assigned to H-1 of 4-*O*-methyl-α-D-glucuronic acid [28], suggesting that glucuronosyl units are essentially methylated at O-4. The resonance at 5.43 ppm is normally absent in hardwood xylans [29] and was assigned to H-1 of 4-*O*-methyl-α-D-glucuronic acid substituted at O-2. The ratio between these two peaks (2.1) is in a good agreement with results of linkage analysis. Thus, the substitution at O-2 of 4-*O*-methylglucuronic acid residues leads to displacement of H-1 resonance to downfield by about 0.17 ppm. The 2D ^{1}H–^{1}H COSY spectrum of the isolated heteroxylan (Fig. 3) showed that H-1 signals of non-substituted

(5.28 ppm) and substituted at O-2 (5.43 ppm) glucuronosyl residues have correlation cross-signals from H-2 protons at 3.60 [25] and 3.92 ppm, respectively. Hence, the substitution at O-2 of glucuronosyl residue leads to displacement of H-2 chemical shift to downfield by about 0.32 ppm. This displacement was attributed to the deshielded H-2 protons in the carbohydrate pyranosyl cycle substituted at O-2 (the usually reported range of displacement is 0.20–0.35 ppm [25,30]).

The characteristic signal at 2.22 ppm showed (Fig. 2) that the isolated heteroxylan was slightly acetylated [31], although the bulk of acetyl groups is expected to be lost during the alkaline extraction [4].

^{13}C NMR spectroscopy.—The ^{13}C NMR spectrum of the purified native heteroxylan (Fig. 4) was interpreted taking into account data from methylation analysis of the isolated heteroxylan and literature assignments [32–34]. The most prominent signals were assigned

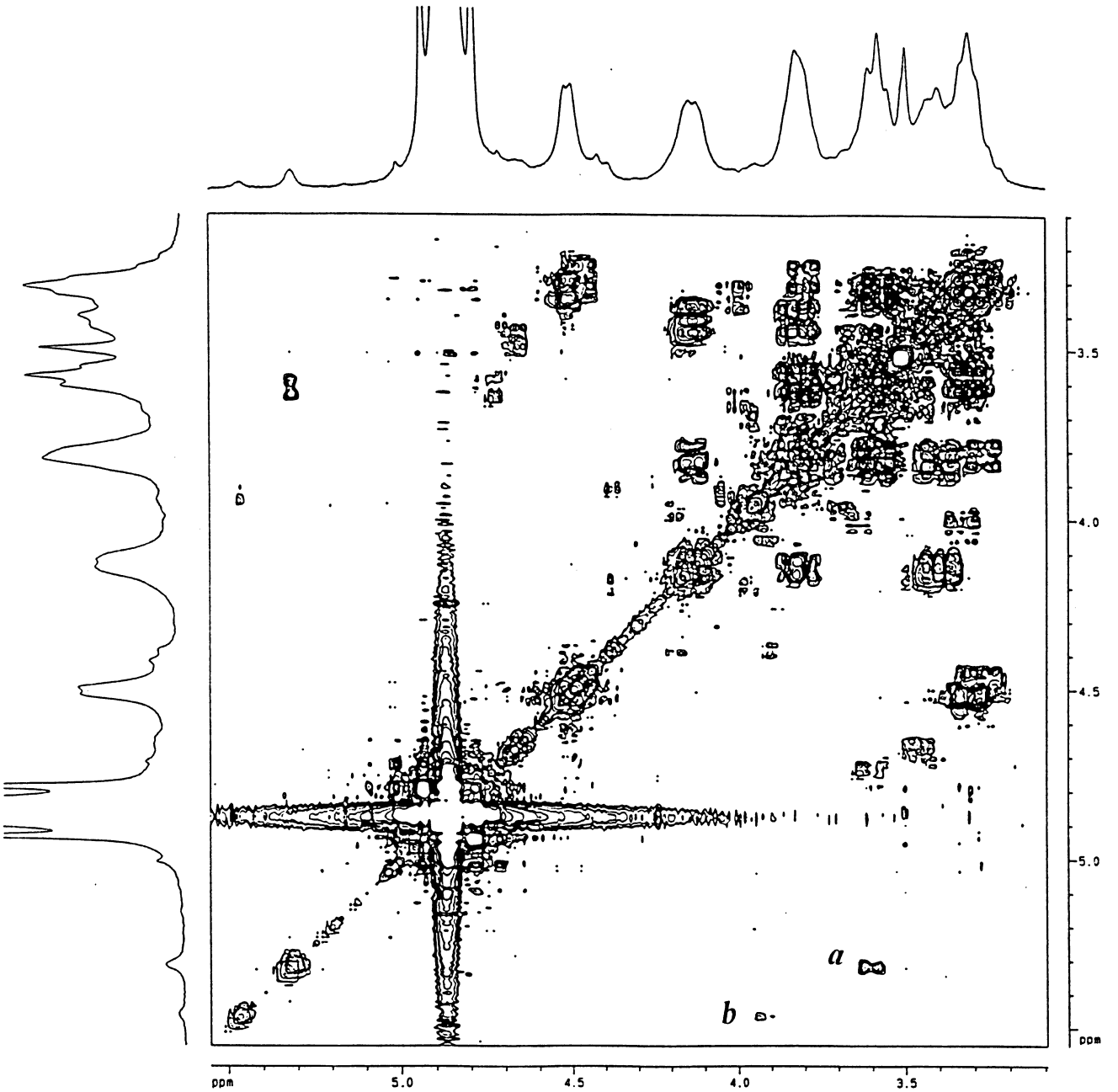


Fig. 3. 2D $^1H-^1H$ COSY spectrum of (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan in D_2O. The cross-peaks labeled are the correlations between H-1 and H-2 protons of non-substituted (a) and substituted at O-2 (b) 4-*O*-methylglucuronic acid.

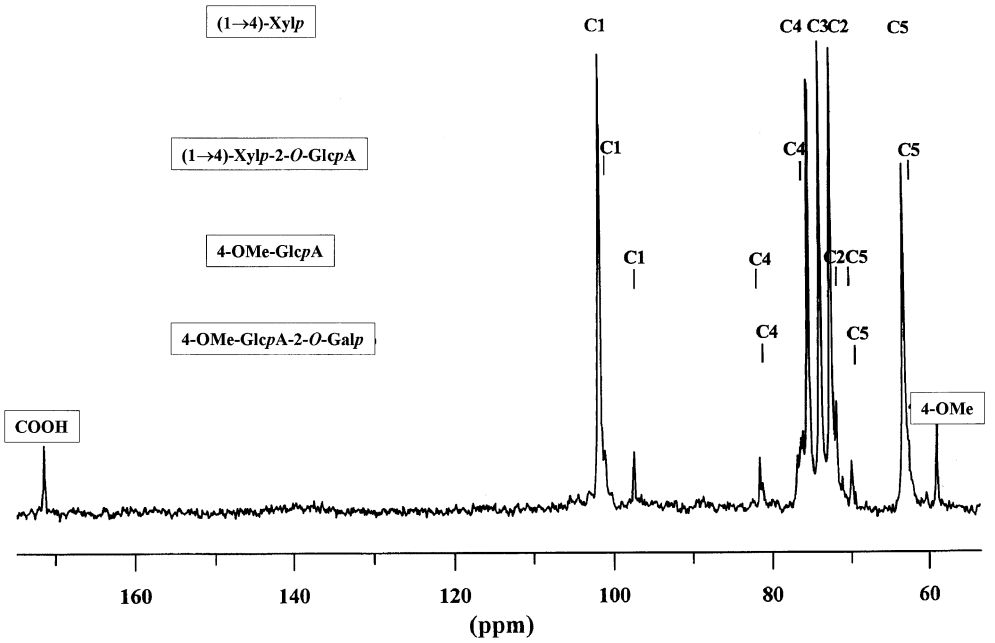


Fig. 4. ^{13}C NMR spectrum of (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan in Me_2SO-d_6 (referenced to internal Me_4Si).

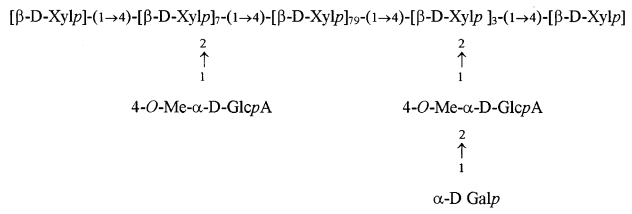
to internal (1→4)-linked β-D-xylosyl residues. The resonances at 101.7, 75.4, 73.9, 72.5 and 63.2 ppm correspond to C-1, C-4, C-3, C-2 and C-5, respectively, of the internal units of the main xylan chain. The branching of the xylan side-chains at O-2 of the main-chain units is indicated by the set of signals for 2-substituted xylose at 101.1, 76.1 and 60.4 ppm corresponding to C-1, C-4 and C-5, respectively, of the 2,4-disubstituted xylosyl residues. It can be inferred from the ratio between signals for C-5 (as well as for C-1) of internal (1→4)-linked xylopyranosyl residues and 2,4-disubstituted xylopyranosyl residues, that every ninth residue of the xylan backbone is branched at O-2. This is in a good agreement with the results of linkage analysis of the heteroxylan (Table 1).

Signals at 97.4, 81.6, 71.8 and 69.9 ppm were assigned to C-1, C-4, C-2 and C-5 of 4-*O*-methyl-α-D-glucuronic acid, respectively. Signals at 81.0 and 69.4 were assigned to C-4 and C-5 of 4-*O*-methyl-α-D-glucuronic acid substituted at O-2, as previously shown by methylation analysis. This proposition is supported by the ratio between C-4 of 4-*O*-methylglucuronic acid and substituted 4-*O*-methylglucuronic acid signals (2.2) and by the ratio between C-4 of all 4-*O*-methylglucuronic acid (non-substituted plus substituted) and (1→4)-linked xylose signals (8.4). These figures are in good agreement with the results of methylation analysis, where ratios of 2.3 and 8.2, respectively, were obtained. Thus, about one in three 4-*O*-methylglucuronic acid units of xylan is substituted at C-2 position.

The single resonances at 59.0 and 171.4 ppm correspond to 4-*O*-methyl- and carboxyl groups, respectively, of glucuronic acid. The ratio between these peaks, 0.94, as well as the ratio of 1.11 between peaks of carbon in the OMe and C-4 in glucuronic acid (non-substituted and substituted), suggests that each of the glucuronic acid units in the xylan carries a methyl substituent at O-4, corroborating data based on ^1H NMR analysis.

Conclusions.—Considering the methylation analysis data and the results of ^1H and ^{13}C NMR spectroscopy, it can be concluded that the heteroxylan isolated from *E. globulus* Labill is a (4-*O*-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan with a β-(1→4)-xylan backbone branched at O-2 and short side chains composed of terminal 4-*O*-methyl-α-D-glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid substituted at O-2 with α-D-galactose. Summarizing all data, the fol-

lowing structure for the isolated heteroxylan can be proposed:



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