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Characterization of an acetylated heteroxylan from *Eucalyptus globulus* Labill

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Abstract

A heteroxylan was isolated from *Eucalyptus globulus* wood by extraction of peracetic acid delignified holocellulose with dimethyl sulfoxide. Besides (1→4)-linked β-D-xylopyranosyl units of the backbone and short side chains of terminal (1→2)-linked 4-*O*-methyl-α-D-glucuronosyl residues (MeGlcA) in a 1:10 molar ratio, this hemicellulose contained galactosyl and glucosyl units attached at *O*-2 of MeGlcA originating from rhamnoarabinogalactan and glucan backbones, respectively. About 30% of MeGlcA units were branched at *O*-2. The *O*-acetyl-(4-*O*-methylglucurono)xylan showed an acetylation degree of 0.61, as determined by ^{1}H NMR spectroscopy, and a weight-average molecular weight (M_w) of about 36 kDa ($P = 1.05$) as revealed from size-exclusion chromatography (SEC) analysis. About half of the β-D-xylopyranosyl units of the backbone were found as acetylated moieties at *O*-3 (34 mol%), *O*-2 (15 mol%) or *O*-2,3 (6 mol%). Practically, all β-D-xylopyranosyl units linked at *O*-2 with MeGlcA residues were 3-*O*-acetylated (10 mol%).

Keywords: Xylan; Acetyl groups; *Eucalyptus globulus*; Structural analysis; NMR spectroscopy

1. Introduction

The heteroxylan from *Eucalyptus globulus* wood shows a chemical structure rather different from that normally found in xylans from other angiosperms.[1] Namely, about one third of the 4-*O*-methyl-α-D-glucuronopyranosyl residues in the heteroxylan from *E. globulus* is substituted at *O*-2 by terminal α-D-galactopyranose. A (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan was isolated from *E. globulus* chlorite holocellulose, by alkaline extraction, but the amount and position of acetyl groups in the β-D-xylopyranosyl backbone were not assessed. However, this information is important to complete the basic knowledge about xylans of *Eucalyptus* species as well as for a better understanding of their behaviour in the chemical processing of wood. Since xylan is a main source of acetyl groups in eucalyptus woods,[2] it plays an important role, for example, in alkali consumption during kraft pulping of *E. globulus* wood for paper production[3] or in the polysaccharide hydrolysis during hydrothermal treatment of wood.[4]

The isolation of an intact and representative xylan sample from wood is a crucial factor in the analysis of *O*-acetyl moieties in xylans. Usually, xylan is obtained by dimethyl sulfoxide extraction of a minimally modified polysaccharide complex (holocellulose) obtained from extractives-free wood by delignification with sodium chlorite.[5] The isolation of holocellulose from extractives-free sawdust by delignification with 10% peracetic acid (peracetic holocellulose) resulted in a yield of heteroxylan after dimethyl sulfoxide (Me_2SO) extraction about 4–6 times higher than that obtained after similar extraction of holocellulose resulting from traditional delignification with sodium chlorite.[6] This result was explained by a higher degree of delignification with peracetic acid under moderate conditions (85 °C; 30 min, pH 4–5) with concomitant breaking of lignin–xylan ether bonds.

In this report, the heteroxylan was isolated from *E. globulus* peracetic holocellulose by extraction with Me_2SO and thoroughly characterised by wet chemistry and NMR methods. The total amount of acetyl groups was assessed by oxalic acid hydrolysis followed by

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acetic acid determination by high-performance liquid chromatography (HPLC) and the position of *O*-acetyl substituents in heteroxylan was assigned using $^{1}H-^{1}H$ (total correlation spectroscopy (TOCSY)) and $^{1}H-^{13}C$ (heteronuclear single quantum coherence (HSQC)) NMR correlation techniques.

2. Results and discussion

2.1. Chemical analysis of heteroxylan

The heteroxylan (yield of 46% w/w based on pentosans content in *E. globulus* wood) was isolated from peracetic holocellulose by exhaustive extraction with Me_2SO followed by precipitation in 4:5:1 ethanol–methanol–water. The content of acetyl groups in the heteroxylan was 19.6% w/w (Table 1). This finding confirms that xylan is the predominant source of acetyl groups in *E. globulus* Labill (taking into account the xylan content and the total amount of acetyl groups in wood). Only a small proportion of acetyl groups (less than 7% from the total amount in wood) belongs to the pectin complex (Table 1).

Table 1
O-Acetyl groups content in *E. globulus* wood and heteroxylan

Sample	Acetyl groups, % (w/w)
E. globulus wood	3.5
E. globulus wood (without pectins)	3.3
Heteroxylan from *E. globulus* wood	19.6

Note: The pentosans content in *E. globulus* wood was 18.1% and the content of pectic compounds was 1.2% (based on o.d. wood).

Table 2
Carbohydrate composition of *E. globulus* heteroxylan

Monosaccharide	Mol% in heteroxylan
Rha	0.75
Fuc	–
Ara	0.50
Xyl	79.1
Man	traces
Gal	5.35
Glc	3.40
MeGlcA [a]	10.9

[a] All kinds of uronosyl units were calculated as MeGlcA.

Analysis of the monosaccharide composition of the isolated heteroxylan from *E. globulus* revealed the presence of arabinose, rhamnose and glucose in addition to the expected xylose, 4-*O*-methyl-D-glucuronic acid and galactose components (Table 2). The remarkable amounts of Glc, Ara and Rha in sugar analysis can be explained by the presence of short polysaccharide fragments of different origin that, more likely, are chemically linked to the heteroxylan backbone rather than simply adsorbed during the isolation procedure. This conclusion is supported by a practically unchanged monosaccharide composition of the heteroxylan after its gradual re-precipitation from a Me_2SO solution (data not shown) and a unimodal Gaussian molecular weight distribution ($P = 1.05$, $M_w = 36{,}300$ Da) revealed by SEC analysis (Fig. 1).

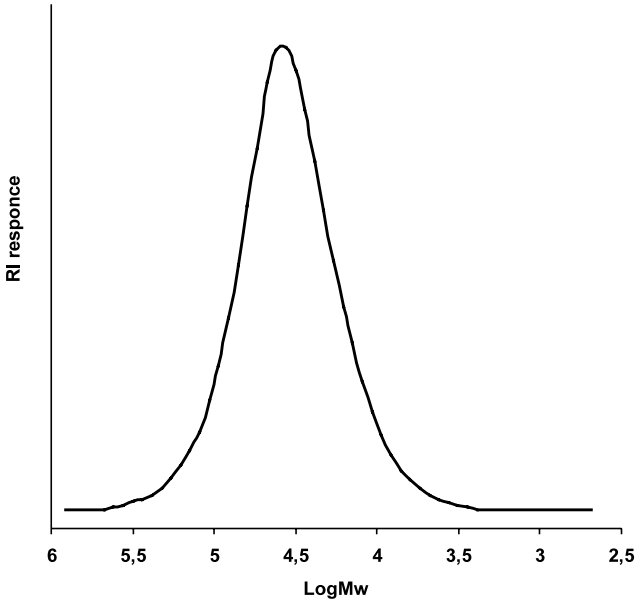


Fig. 1. SEC elution curve of *E. globulus* heteroxylan.

In order to clarify the origin of the polysaccharide fragments attached to (1→4)-linked β-D-xylopyranosyl units of the heteroxylan backbone, a linkage analysis has been carried out (Table 3). The inferred structural units [→3)-Rha*p*-(1→] and [→2)-Gal*p*A-(1→] with molar proportion equal to non-reducing end-groups (2,3,4-tri-*O*-methyl-xylose) of the heteroxylan backbone originate from the terminal structural fragment [→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→4)-D-Xyl*p*] already found in wood xylans.[7] The presence of [→2)-Gal*p*A-(1→] structural units was confirmed by 2D $^{1}H-^{1}H$ NMR (TOCSY) spectroscopy (Fig. 2). The molar ratio between 2,4-disubstituted xylose units and the sum of terminal and *O*-2 substituted 4-*O*-metylglucuronic acid residues was 1.05 (Table 3), confirming our previous conclusion that all glucuronosyl units are (1→2)-linked to the xylan backbone.[1] Surprisingly, the molar ratio between the structural units [→2)-Glc*p*A-(1→] and

Table 3
Methylation analysis of *E. globulus* heteroxylan [a]

Methylated residue	Structural units deduced	Relative abundance (mol%)
Xyl-2,3,4	Xyl*p*-(1→	1.0
Xyl-2,3	→4)-Xyl*p*-(1→	136.0
Xyl-3	→2,4)-Xyl*p*-(1→	16.8
Xyl-2	→3,4)-Xyl*p*-(1→	traces
Glc-2,3,4 (D_2)	Glc*p*A-(1→	10.8
Glc-3,4 (D_2)	→2)-Glc*p*A-(1→	5.7
Gal-3,4 (D_2)	→2)-Gal*p*A-(1→	1.0
Gal-2,3,4,6	Gal*p*-(1→	2.8
Gal-2,3,6	→4)-Gal*p*-(1→	2.8
Gal-2,3	→4,6)-Gal*p*-(1→	1.4
Gal-2,4	→3,6)-Gal*p*-(1→	1.0
Gal-2,3,4	→6)-Gal*p*-(1→	1.0
Glc-2,3,4,6	Glc*p*-(1→	1.2
Glc-2,3,6	→4)-Glc*p*-(1→	2.4
Glc-2,3	→4,6)-Glc*p*-(1→	1.0
Glc-2,3,4	→6)-Glc*p*-(1→	0.5
Rha-3	→2,4)-Rha*p*-(1→	2.0
Rha-2,4	→3)-Rha*p*-(1→	1.0
Ara-2,3,5	Ara*f*-(1→	2.4

[a] Several partially methylated residues of small abundance originating presumably from the terminal units were not identified.

[Gal*p*-(1→] (Table 3) was very far from 1 as reported previously for the (2-*O*-α-D-galactopyranosyl-4-*O*-methyl-α-D-glucurono)-D-xylan from *E. globulus*.[1] At the same time, several polysubstituted galactopyranose (Gal*p*), rhamnopyranose (Rha*p*), glucopyranose (Glc*p*) and terminal arabinofuranose ([Ara*f*-(1→]) structural units were detected, which, according to the balance of products in methylation analysis, cannot be linked to Xyl*p* residues of the backbone (Table 3). The results obtained were interpreted in terms of an improved conservation of the native heteroxylan complex isolated by soft extraction with Me_2SO when compared to the alkali-extracted heteroxylan isolated in our previous work.[1] Thus, when the heteroxylan was extracted from chlorite holocellulose (obtained after eight 1 h-treatments of wood sawdust at 80 °C) by 10% aqueous potassium hydroxide followed by several purification steps involving dissolution in Fehling solution,[1] the labile galactan linked at *O*-2 of the MeGlcA residues was degraded, originating in the detected residual terminal α-D-Gal*p*. This proposal is confirmed by a recent study on xylo-oligosaccharides from *E. globulus* using wet chemistry methods, where MALDI-TOF MS revealed a decrease in the Gal*p* and Ara*f* content in heteroxylan fractions during hydrothermal and alkaline treatment of wood, with the simultaneous formation of relatively stable oligomers $(Xyl p)_n$-MeGlc*p*A-Hex ($n = 3–5$).[8] However, it is still difficult to conclude if all terminal α-D-Gal*p* units linked at *O*-2 to MeGlcA in the heteroxylan originated from degraded galactan or if part of them were originally present in the xylan as terminal units.

The substitution pattern in Gal*p*, Rha*p* and Ara*f* units, inferred from methylation analysis (Table 3), was very similar to that found for rhamnoarabinogalactan isolated from beech wood[9] and having a highly ramified backbone at *O*-6 constituted by [→4)-Gal*p*-(1→] units. According to a long range proton correlation observed in the TOCSY spectrum (Fig. 2), most of the terminal Ara*f* units should be present in rhamnoarabinogalactan oligomers as α-anomers (α-L-Ara*f*). The long-range proton correlation in β-L-Ara*f* units was not observed.

The Glc*p* structural units detected (Table 3) belong, more likely, to glucan oligomers. These also may be linked to α-D-glucopyranosyluronic residues at *O*-2. Indirect evidence for such possibility is the fact that a small misbalance between the structural units [→2)-Glc*p*A-(1→] and [Gal*p*-(1→] (molar ratio 1.22) was found during the methylation analysis in our previous work[1] together with the simultaneous presence of small amounts of Glc*p* residues even in highly purified heteroxylan. Supposing a ramification degree of the galactan and glucan backbone not very different, as inferred from the relative amounts of detected terminal [Gal*p*-

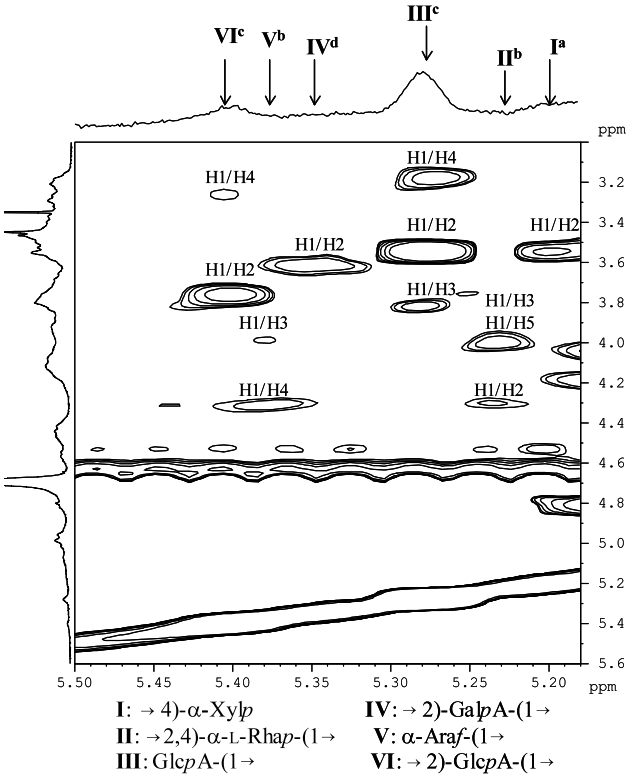


Fig. 2. TOCSY spectrum (D_2O, 25 °C) of the heteroxylan from *E. globulus* showing the anomeric region of uronosyl residues (assignments were made using the literature data: (a) [10]; (b) [11]; (c) [1]; (d) inferred from Refs. 1 and 12).

(1→] and [Glc*p*-(1→] structural units (Table 3), it may be suggested that roughly 70% of the D-glucopyranosyluronic residues are substituted at *O*-2 by Gal*p* and about 30% by Glc*p* units. Thus, based on the above considerations, it may be proposed that the *O*-2 position of the D-glucopyranosyluronic residue in *E. globulus* heteroxylan is the linking point with other polysaccharides in the cell wall, namely, with the rhamnoarabinogalactan and glucan. However, this statement should be confirmed by more detailed investigation, which is in progress.

2.2. Analysis of acetyl moieties in heteroxylan

The quantification and distribution patterns of *O*-acetyl groups in the structural units of heteroxylan backbone were assessed employing ^{1}H NMR spectroscopy. The proton resonances were assigned using known literature data,[13–15] which were confirmed and completed by homonuclear (TOCSY) and heteronuclear (HSQC) correlation NMR experiments. Basic ^{1}H and ^{13}C signals in *E. globulus* xylan are presented in Table 4. The ^{1}H NMR spectrum of the heteroxylan with the designation of regions used for the integration of protons from different structural fragments is shown in Fig. 3. The quantification of different β-D-Xyl*p* units possessing *O*-acetyl groups was made using a methodology very similar to that as described elsewhere.[14] Thus, non-acetylated, 3-*O*- and 2-*O*-acetylated internal xylose residues, and 4-*O*-methyl-D-glucopyranosyluronic residues were assessed based on their anomeric proton resonances, whereas the amounts of 2,3-di-*O*-acetylated and 3-*O*-acetylated MeGlc*p*A *O*-2 substituted internal xylose residues were estimated based on H-3 resonances in the corresponding structures (Fig. 3). The total balance of acetyl groups was checked by integration of its CH_3– moieties at 2.05–2.30 ppm. The results on the distribution of acetyl groups per 100 xylose residues are presented in Table 5. The degree of substitution (DS) of xylopyranose residues by acetyl groups was found to be 0.61, which is very close to values reported for the heteroxylan from aspen (*Populus tremula*)[14] and about 30% higher than that detected in heteroxylans from birch and beech woods.[15] Thus, approximately half of the D-xylose residues in the backbone possesses at least one acetyl group. About 35% of acetyl groups in the *E. globulus* heteroxylan are attached at *O*-2 and about 65% are linked at *O*-3 of the Xyl*p* residues in the backbone: 39% in Xyl-3Ac, 10% in Xyl-2,3Ac and 16% in Xyl-3Ac-GlcA units (Table 5). As it follows from the relative molar proportions of acetylated xylose units (Table 5), practically all β-D-Xyl*p* units substituted at *O*-2 by MeGlc*p* residue should be 3-*O*-acetylated. The same conclusion was reached recently in a study on heteroxylan from *P. tremula*.[14]

Lindberg and Rosell[16] suggested previously the possibility of an acetyl group migration in xylopyranose units of the xylan backbone under the weakly acidic conditions of the chlorite holocellulose isolation. Thus, the acidity of the reaction medium in the holocellulose isolation procedure may be an important factor influencing distribution patterns of acetyl groups in xylan. It was verified that the abundance and positions of acetyl groups in heteroxylan was maintained when the pH of

Table 4
Proton and carbon chemical shifts (δ, ppm) in *E. globulus* heteroxylan structural units

Structural unit	Assignments					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	
					ax	eq
Xyl (isol.)	4.48/102.8	3.30/73.8	3.57/74.5	3.79/77.4	3.40/64.0	4.10/64.0
Xyl (Xyl-Ac)	4.42/103.7	3.22/73.8	3.54/74.5	3.76/77.4	3.38/64.0	4.05/64.0
Xyl-3Ac	4.58/102.5	3.49/72.0	4.98/76.3	3.93/76.5	3.48/63.8	4.13/63.8
Xyl-2Ac	4.67/101.0	4.68/74.5	3.80/72.5	3.86/77.2	3.44/63.8	4.16/63.8
Xyl-2,3Ac	4.79/100.4	4.82/74.6	5.14/76.0	4.05/78.2	3.53/63.8	4.20/63.8
Xyl-2GlcA	4.66/101.9	3.52/77.4	3.70/73.3	3.84/77.2	n.d./n.d.	n.d./n.d.
Xyl-3Ac-2GlcA	4.73/102.0	3.69/76.0	5.05/76.1	3.98/77.0	3.48/63.7	4.14/63.7
MeGlcA	5.28/98.8	3.56/72.3	3.82/73.3	3.18/83.2	n.d./n.d.	[a]
MeGlcA-2Gal	5.42/98.2	3.77/75.9	3.83/n.d.	3.22/83.1	n.d./n.d.	[a]
GalA-2Rha	5.36/n.d.	3.62/n.d.	n.d./n.d.	n.d/n.d.	n.d./n.d.	[a]

The following designations are used: Xyl (isol.), non-acetylated Xyl*p* in the backbone isolated from other acetylated Xyl*p* units; Xyl (Xyl-Ac), Xyl*p* linked with neighbouring acetylated Xyl*p*; Xyl-3Ac, 3-*O*-acetylated Xyl*p*; Xyl-2Ac, 2-*O*-acetylated Xyl*p*; Xyl-2,3Ac, 2,3-di-*O*-acetylated Xyl*p*; Xyl-3Ac-2GlcA, MeGlcA 2-*O*-linked and 3-*O*-acetylated Xyl*p*; MeGlcA-2Gal, Gal*p* 2-*O*-linked MeGlcA; GalA-2Rha, Rha*p* 2-*O*-linked Gal*p*A. n.d., non determined.

[a] Not relevant.

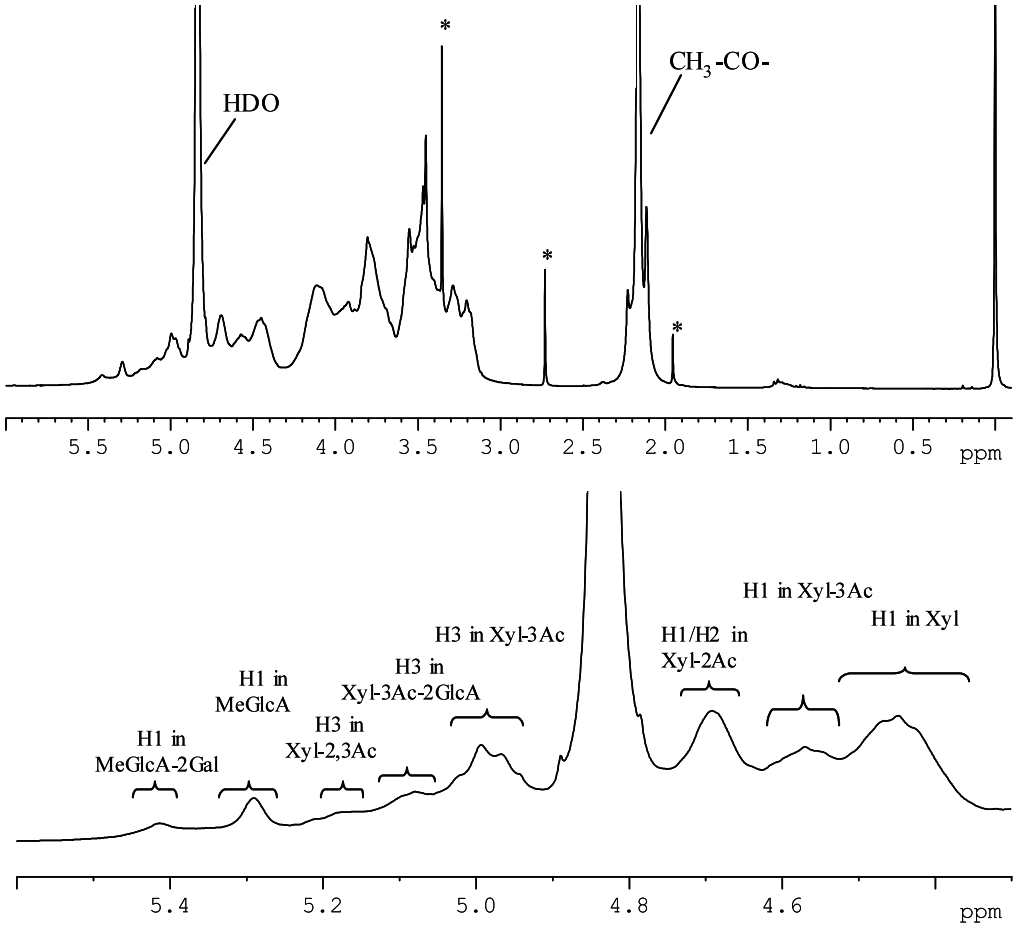


Fig. 3. ^{1}H NMR spectrum of *O*-acetyl-(4-*O*-methylglucurono)xylan from *E. globulus* (top image) and the expanded region of anomeric protons (bottom image). The designations are the same as presented in Table 4. Solvent impurities are marked by asterisk.

Table 5
Relative content in acetyl groups in structural units of *E. globulus* heteroxylans

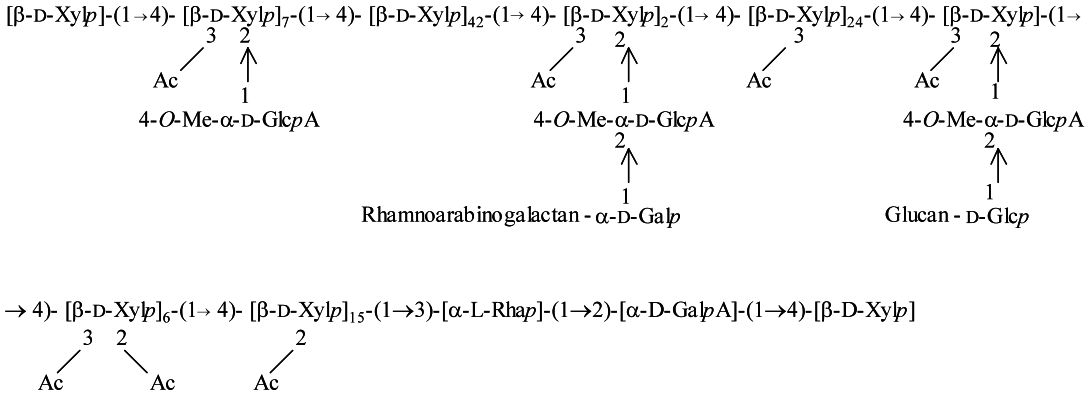
Structural fragment and short designation	Relative abundance (per 100 Xyl*p* units)	
	Xylan from peracetic holocellulose	Xylan from chlorite holocellulose
→4)-β-D-Xyl*p*-(1→ (**Xyl**)	45	44
→4)[3-*O*-Ac]-β-D-Xyl*p*-(1→ (**Xyl-3Ac**)	24	22
→4)[2-*O*-Ac]-β-D-Xyl*p*-(1→ (**Xyl-2Ac**)	15	14
→4)[3-*O*-Ac][2-*O*-Ac]-β-D-Xyl*p*-(1→ (**Xyl-2,3Ac**)	6	8
→4)[4-*O*-Me-α-D-Glc*p*A-(1→2)][3-*O*-Ac]-β-D-Xyl*p*- (1→ (**Xyl-3Ac-2GlcA**)	10	12
4-*O*-Me-α-D-Glc*p*A-(1→ (**MeGlcA**)	7	8
→2)-4-*O*-Me-α-D-Glc*p*A-(1→ (**MeGlcA-2Gal**)	3	4

the peracetic acid aqueous solution (10%) used for the holocellulose isolation varied between 3.5 and 5.5 (data not shown). Moreover, no major difference in positions and in the abundance of acetyl group in heteroxylans was detected when the holocellulose was obtained from wood, either by conventional chlorite method at pH 4.0 or by delignification with peracetic acid at pH 3.5 (Table 5). This means, that no significant difference in migration of acetyl groups in the heteroxylan backbone occurred during these holocellulose isolation procedures. Small variations in the abundance of particular *O*-acetyl substituents in xylans isolated from two holo-

celluloses may be tentatively explained by differences in their morphological origin. However, results on acetyl group determination in xylan isolated by Me_2SO extraction from peracetic holocellulose seems to be more reliable since the yield of the former one was about five times higher when compared to the yield of xylan isolated by the same manner from chlorite holocellulose (46 against 10%).

3. Conclusions

Based on the experimental results obtained from methylation analysis and NMR studies, the empirical structure of the *E. globulus* *O*-acetyl-(4-*O*-methylglucurono)xylan ramified with galactan and glucan side-chains may be proposed as below:



No significant difference in the migration of acetyl groups and their amounts in xylan units of the heteroxylan backbone were detected during the holocellulose isolation from *E. globulus* wood by peracetic acid treatment at pH 3.5–5.5 and by conventional treatment with sodium chlorite.

4. Experimental

4.1. Materials

A representative *E. globulus* wood sample from a 12-years old tree of a clone plantation near Aveiro (Portugal) was supplied by RAIZ (Portuguese Forest and Paper Research Institute). Wood sawdust (40–60 mesh) was extracted with 2:1 $C_6H_5CH_3$–EtOH for 6 h in a Soxhlet extractor. The pentosans content was determined via the furfural index according to Tappi standard T 223. The pectins content was determined by sawdust reflux with 1% NH_4 citrate soln during 1 h.[5]

4.2. Isolation of heteroxylan

The holocellulose was obtained from extractives-free sawdust by delignification with 10% peracetic acid at pH 3.5 (adjusted with aq NaOH). Typically, 2.0 g of o.d. sawdust was treated with a peracetic acid soln (100 cm^3) at 85 °C during 30–35 min in an Erlenmeyer under constant stirring. After the delignification, the flask content was quickly cooled, diluted two times with distilled water and filtered off using a porous glass filter (porosity 100). The holocellulose was successively washed with warm water and with 1:1 C_3H_6O–EtOH. The holocellulose (72–73% yield) was air-dried. The acidic heteroxylan was isolated from peracetic holocellulose by extraction with Me_2SO (HPLC grade) under nitrogen atmosphere at 50 °C (1:60 hollocellulose-to-Me_2SO ratio) for 12 h under stirring. After the extraction, the residual insoluble holocellulose was filtered off using a porous glass filter (porosity 100) and washed with small portions of distilled water (1:2 Me_2SO extract-to-water ratio). The aq Me_2SO extract of heteroxylan was precipitated with an excess (about ten times) of 4:5:1 EtOH–MeOH–water acidified by HCOOH (to check the homogeneity of the heteroxylan, in a separate experiment, the precipitation was performed with 3:3:4 EtOH–MeOH–water and the precipitate and the supernatant were analysed for carbohydrate composition). The complete precipitation of heteroxylan was accomplished in 12 h at 4 °C. The heteroxylan was isolated by centrifugation, washed four to five times with dry MeOH and quickly dried under diminished pressure at room temperature. Alternatively, the heteroxylan was isolated by Me_2SO extraction as described above for chlorite holocellulose.[5]

4.3. Carbohydrate analysis

The heteroxylan was subjected to Saeman hydrolysis (treatment with 72% H_2SO_4 at 20 °C (3 h) followed by 1.5 h hydrolysis with dilute 1 M H_2SO_4 at 100 °C) and neutral monosaccharides were determined as alditol acetate derivatives by gas chromatography.[17] Uronic acids were determined colorimetrically with *m*-phenylphenol[18] by a modification of the method described by Blumenkrantz and Asboe-Hansen.[19]

4.4. Linkages analysis

The heteroxylan was methylated by two consecutive treatments using alkali-metal hydroxide method[20] in the modification proposed by Isogai et al.[21] The absence of acetyl and free hydroxyl groups in the methylated glycan was controlled by FTIR. The heteroxylan methylation was followed by a carboxyl group reduction with $LiAlD_4$ in THF to identify the uronic acid moieties.[22] Methylated heteroxylan was hydrolysed with 90% HCOOH at 105 °C for 1.5 h followed by hydrolysis with 0.15 M H_2SO_4 at 105 °C for 6 h.[23] After the neutralisation, partially methylated monosaccharides were reduced with $NaBH_4$ and acetylated.[23] Partially methylated alditol acetates were analysed by GC–FID (Varian model 3350) and identified by GC–MS (Trace GC 2000 series coupled with Finnigan Trace MS mass spectrometer) using a DB-1 capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were exactly the same as reported previously.[1]

4.5. Analysis of acetyl groups

O-Acetyl groups of wood and of heteroxylan were hydrolysed with 0.5 M oxalic acid for 1 h at 155 °C.[24] Acetic acid in the hydrolyzate (internal reference propionic acid) was analysed by HPLC on a Gilson HPLC system (Pump Model 305/306 equipped with dynamic mixer Model 811C; detector—fast scanning Chrom-A-Scope Bar Spec operating at 230 nm wavelength; column—5 μm C18 Spherosorb 250 × 4.6 mm; eluent—95:5 water–MeCN, 0.8 mL/min).

4.6. Size-exclusion chromatography (SEC)

The xylan sample was dissolved in a small amount of 10% LiCl solution in *N*,*N*-dimethylacetamide (DMAC) at 70–80 °C and further diluted with DMAC to a xylan concn of about 0.4% (4 mg/mL). The SEC analysis has been carried out on two PLgel 10 μm MIXED B 300 × 7.5 mm columns protected by a PLgel 10 μm pre-column (Polymer Laboratories, UK) using a PL-GPC 110 system (Polymer Laboratories). The columns, injector system and the detector (RI) were maintained at 70 °C during the analysis. The eluent (0.1 M LiCl soln in DMAC) was pumped at a flow rate of 0.9 mL/min. The analytical columns were calibrated with pullulan standards (Polymer Laboratories) in the range 0.8–100 kDa.

4.7. ^{1}H NMR spectroscopy

^{1}H NMR spectra were recorded in D_2O (25 °C) on a Bruker AMX 300 spectrometer operating at 300.13 MHz. Sodium 3-(trimethylsilyl)propionate-d_4 was used as internal standard (δ 0.00). Relaxation delay was 12 s, r.f. 90°-pulse width of 10.2 μs and about 1000 pulses were collected.

4.8. 2D NMR studies

The 2D ^{1}H–^{1}H TOCSY spectrum ($\tau_{mix} = 0.050$ s) was acquired using the MLEVST standard pulse program. A spectral width of 2185 Hz was employed in both dimensions. The relaxation delay was 1.5 s. For each FID, 128 transients were acquired, the data size was 1024 in $t_1 \times 512$ in t_2. The phase sensitive ^{1}H-detected HSQC spectrum was acquired over a $F1$ spectral weight of 12,000 Hz and a $F2$ width of 2000 Hz with a 2048 × 1024 matrix and 128 transients per increment. The delay between scans was 2 s and the delay for polarization transfer was optimized for $^1J_{CH}$ 150 Hz.

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