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RESEARCH PAPER

Nativity of lignin carbohydrate bonds substantiated by biomimetic synthesis

Nicola Giummarella[1,*], Mikhail Balakshin[2,*], Sanna Koutaniemi[3], Anna Kärkönen[4,5,†] and Martin Lawoko[1,†]

[1] Fiber and Polymer Technology, Wallenberg Wood Science Center, Royal Institute of Technology, Stockholm, Sweden
[2] Department of Bioproducts and Biosystems, Aalto University, Finland
[3] Department of Food and Environmental Chemistry, University of Helsinki, Finland
[4] Department of Agricultural Sciences, Viikki Plant Science Centre, University of Helsinki, Finland
[5] Natural Resources Institute Finland, Production Systems, Plant Genetics, Helsinki, Finland

* These authors contributed equally to this work.
† Correspondence: lawoko@kth.se or anna.karkonen@luke.fi

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Abstract

The question of whether lignin is covalently linked to carbohydrates in native wood, forming what is referred to as lignin–carbohydrate complexes (LCCs), still lacks unequivocal proof. This is mainly due to the need to isolate lignin from woody materials prior to analysis, under conditions leading to partial chemical modification of the native wood polymers. Thus, the correlation between the structure of the isolated LCCs and LCCs *in situ* remains open. As a way to circumvent the problematic isolation, biomimicking lignin polymerization *in vivo* and *in vitro* is an interesting option. Herein, we report the detection of lignin–carbohydrate bonds in the extracellular lignin formed by tissue-cultured Norway spruce cells, and in modified biomimetic lignin synthesis (dehydrogenation polymers). Semi-quantitative 2D heteronuclear singular quantum coherence (HSQC)-, ^{31}P -, and ^{13}C-NMR spectroscopy were applied as analytical tools. Combining results from these systems, four types of lignin–carbohydrate bonds were detected; benzyl ether, benzyl ester, γ-ester, and phenyl glycoside linkages, providing direct evidence of lignin–carbohydrate bond formation in biomimicked lignin polymerization. Based on our findings, we propose a sequence for lignin–carbohydrate bond formation in plant cell walls.

Keywords: Benzyl ester, benzyl ether, dehydrogenation polymer; extracellular lignin, gamma (γ)-ester, lignin–carbohydrate complex, phenyl glycoside.

Introduction

Lignocellulosic biomass consists mainly of cellulose, hemicelluloses, and lignin, in addition to minor polymers which include a class of acidic polysaccharides called pectins. The polymers interact with each other through physical and chemical forces, which are not yet fully investigated. For instance, a covalent connectivity between lignin and hemicelluloses in native wood cell walls forming so-called lignin–carbohydrate complexes (LCCs) has been proposed in the literature. There is a lot of indirect evidence related to covalent linkages between lignin and carbohydrates in various lignocellulosics (Balakshin *et al.*, 2014), but still no unequivocal proof exists of their presence in the substrates *in situ*. Current criticism regarding the existence of LCCs in native plant cell walls is attributed to characterization techniques and mechanistic pathways for their

formation. On the characterization front, the challenge is to understand possible modifications that occur under mechanical and/or chemical treatments required to extract lignin from the woody matrix prior to analyses. Mechanical treatment is conventionally ball milling performed following Wiley milling as described by Bjorkman (1956).

In the past, several solvent systems with varying degrees of mildness have been used to dissolve the ball-milled wood partially or completely (Björkman, 1956). The initial ball milling step is a drawback since some modifications occur to native cell wall polymers. The extent of such modifications on polymer structure remains insufficiently understood. Another criticism is whether the ensuing dissolution with some harsh solvents imparts modifications, for example LCC formation. Several solvent classes have been used including acidic, neutral, and basic systems (Koshijima *et al.*, 1976; Eriksson and Lindgren, 1977; Lawoko *et al.*, 2005; Du *et al.*, 2013). The use of neutral solvents under common conditions (low temperature, <80 °C) should not result in any modifications. Moreover, the use of acetic acid for isolation of specific LCC preparations has not been shown to result in (additional) LCC formation (Balakshin *et al.*, 2007). However, other fractionation solvents, under the conditions used, may result in LCC modification and/or formation (Del Rio *et al.*, 2016).

Four different types of native lignin–carbohydrate bonds are proposed in the literature, namely benzyl ethers, benzyl esters, γ-esters, and phenyl glycosides (Freudenberg and Neish, 1968; Yaku *et al.*, 1976; Fengel and Wegener, 1989; Balakshin *et al.*, 2001, 2007). Among various methods for LCC analysis, 2D NMR methods have been the most revealing techniques (Balakshin *et al.*, 2001, 2014; Nishimura *et al.*, 2018, Rencoret *et al.*, 2019). Although the first LCC assignments (Balakshin *et al.*, 2001) were rather tentative, further experiments have confirmed them. In addition to new model compound studies (Miyagawa *et al.*, 2014), they were confirmed with an HMBC (heteronuclear multiple bond correlation) technique (Balakshin *et al.*, 2007), selective ^{13}C enrichment (Evtuguin *et al.*, 2005), and wet chemistry pre-treatments followed by NMR studies (Balakshin *et al.*, 2007, 2011). Moreover, rigorous NMR studies for LCC have very recently been published, showing unequivocal evidence for ether linkages between glucomannan and the lignin fraction obtained from milled wood of Japanese red pine (*Pinus densifora*) (Nishimura *et al.*, 2018) and incorporation of hydroxystilbene glucosides into Norway spruce (*Picea abies*) bark lignin (Rencoret *et al.*, 2019).

The mechanism of formation of phenyl glycosides is barely discussed in the literature. One speculative explanation relies on the acid-catalyzed addition of a phenolic hydroxyl to the reducing end of a carbohydrate moiety, by the well-known chemistry of hemi-acetal formation. If this is the case, in the acidic pH (~5–6) present in the plant cell wall (Felle, 2001), the linkage is likely to be formed. Such a scenario could exist locally in the proximity of acidic polysaccharides such as glucuronoxylan and pectins. Another possibility is transglycosylation of a phenolic end group to the reducing end of a polysaccharide catalyzed by one of the multiple types of transglycosylating enzymes. These are known to cleave and religate hemicellulose and also cellulose chains for cell wall modifications during plant development (Yaku *et al*, 1976; Fry, 1995; Simmons *et al.*, 2015). A very recent study conducted on Norway spruce (*P. abies*) bark lignin demonstrated unequivocally the incorporation of natural phenyl glycoside monomers into lignin's hydroxystilbene, supporting, for the first time, the formation of phenyl glycoside-type linkages in native wood substrates (Rencoret *et al.*, 2019). Formation of benzyl ethers and benzyl esters is based on simple nucleophile addition chemistry on an electrophilic site; C_α of a quinone methide. This intermediate is created during lignin polymerization whenever a β-radical couples to form dimers (Ralph *et al.*, 2004), and chain growth proceeds by coupling of pre-formed oligomers (Ralph *et al.*, 2004) or endwise coupling of monomers to the growing polymer (Sarkanen, 1971; Ralph *et al.*, 2004; Tobimatsu and Schuetz, 2019). Not surprisingly, when the coupling involves a β-5 or β-β bond, a favorable intramolecular addition reaction with available nucleophiles (phenol-OH and -OH in Cγ, respectively) occurs to form ring structures such as phenyl coumaran and pinoresinol structures. This favorability is due to internal trapping of the quinone methide, and is well documented by NMR studies showing the presence of such structures in lignin (Ralph *et al.*, 2004). In the case of β-O-4, the addition becomes intermolecular.

The formation of benzyl ethers or benzyl esters is not straightforward due to the presence of water, a nucleophile abundant in the plant cell walls especially at the beginning of lignification, very capable of performing nucleophilic attack on quinone methides. Accordingly, the product of water addition is α-hydroxylated β-O-4 structures in lignin (Ralph *et al.*, 2004). However, detection of LCCs of benzyl ether- and γ-ester types in wood isolates by 2D NMR was first reported a while ago (Balakshin *et al.*, 2001, 2007; Evtuguin *et al.*, 2005) (γ-esters; Fig. 1), with the γ-ester suggested to result from migration of the ester from the benzylic position (Balakshin *et al.*, 2001, 2007; Evtuguin *et al.*, 2005). The question remains of whether this indeed occurs in the presence of water.

The formation of lignin–carbohydrate linkages in the aqueous environment of the plant cell wall is the focus of our studies. The local concentration of water near the growing lignin chain may be low due to lignin hydrophobicity. Indeed, the literature suggests that hemicellulose or pectin is in close contact with lignin during polymerization in aqueous systems (Cathala and Monties, 2001; Cathala *et al.*, 2001; Barakat *et al.*, 2007; Li *et al.*, 2015). Such studies present a plausible explanation for how lignin–carbohydrate bonds may form in aqueous systems, and require further attention.

In this work, we investigated these questions by biomimicking LCC formation *in vivo* by using an extracellular-lignin-forming cell culture of Norway spruce (Simola *et al.*, 1992; Kärkönen *et al.*, 2002; Laitinen *et al.*, 2017) as a source of lignin, and *in vitro* by producing dehydration polymers (DHPs) in the presence of hemicellulose (xylan; Barakat *et al.*, 2007; Li *et al.*, 2015) and galacturonic acid, a constituent of pectin. Our focus was to specifically demonstrate whether certain types of LCC linkages could occur *in vivo* and/or *in vitro* or just be created exclusively during extraction/isolation procedures, such as ball milling.

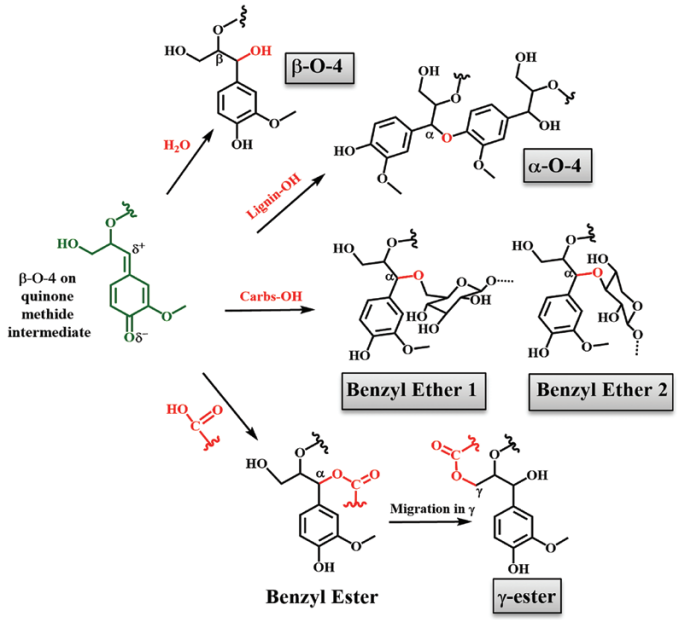

Fig. 1. Suggested formation mechanisms of α-hydroxylated β-O-4, benzyl ether, and ester bonds in native LCCs due to nucleophilic attacks in the electrophilic site (α) of β-O-4 subunits. Carbs-OH, carbohydrate -OH. (This figure is available in color at *JXB* online.)

Materials and methods

All chemicals used were of analytical grade and purchased from Sigma-Aldrich.

Tissue-culturing and treatment of extracellular lignin

Norway spruce (*Picea abies* L. Karst.) cells were maintained as a callus culture on solid nutrient medium and transferred to liquid cultures for lignin formation (Kärkönen *et al.*, 2002; Koutaniemi *et al.*, 2005). The cultures were incubated at 20 °C on a platform shaker (100 rpm, in 16 h light/8 h dark, Osram warm white, 20–50 μmol m^{-2} s^{-1}). The culture medium was collected when extracellular lignin (ECL) was clearly visible in the culture medium (~5–15 d after transfer of cells into liquid conditions). Cells were removed by filtering through Miracloth, and ECL was pelleted by centrifugation. ECL was washed with water to remove soluble compounds. Lignin-bound proteins were removed by extracting the ECL with buffered 1 M NaCl as described in Warinowski *et al.* (2016), after which the ECL was washed with water and lyophilized.

Synthetic lignin (DHP) synthesis

Synthesis of lignin (DHP) was performed as reported elsewhere (Warinowski *et al.*, 2016), with horseradish peroxidase (HRP) type VI [1 mg, 4.16×10^{-6}–5.5×10^{-6} kat coniferyl alcohol (CA)-oxidizing activity]. Both hydrogen peroxide (H_2O_2; 34 mM, 20 ml in water) and CA (prepared according to Lu and Ralph, 1998; Kim and Ralph, 2005) (34 mM, 20 ml in 50% acetone) were simultaneously injected, at a constant rate (250 μl h^{-1}) using an NE-1800 eight channel programmable syringe pump, into 50% aqueous acetone solution of beech glucuronoxylan [10% (w/v), Sigma-Aldrich] or galacturonic acid (0.2 M, Sigma-Aldrich). The pH (between 5.5 and 6) was adjusted, in the latter case, to 6.5–7 before addition of HRP to increase the concentration of carboxylate anion (pK_a ~4). As a control, DHP without any carbohydrate supplementation was produced (CA-DHP). Polymerization was carried for 20 h under slow stirring (150 rpm) at room temperature. After injection, the reaction was allowed to proceed for an additional 4 h. Synthetic lignins (DHPs) were collected by centrifugation (2000 rpm, 30 min) after removal of acetone under reduced pressure, washed twice with water, and lyophilized.

Carbohydrate and lignin compositional analyses

The sugar composition of the ECL was determined after acidic methanolysis (Bertaud *et al.*, 2002; Appeldoorn *et al.*, 2010) incubating 1 mg of lyophilized ECL with 1 ml of 2 M HCl in dry methanol for 5 h at 100 °C. Samples were then neutralized with pyridine, dried under a stream of air, and further hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. The samples were again dried under a stream of air and dissolved in water. The monosaccharides were analyzed using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with the ICS-3000 system (Dionex) equipped with a CarboPac PA1 column (4×250 mm, Dionex), as previously reported (McKee *et al.*, 2016).

The lignin content in the ECL preparation was analyzed by Klason lignin analysis as described by Effland (1977), together with acid-soluble lignin (TAPPI, 1991). The former was determined gravimetrically after acid hydrolysis of the ECL, whereas the latter was characterized by UV spectroscopy of the hydrolysate at 205 nm using an absorptivity of 128 l cm^{-1} g^{-1}. All experiments were carried out in duplicate.

Size exclusion chromatography

Molecular weight distributions and polydispersity of the lignin samples were investigated by size exclusion chromatography (SEC) dissolving ~5 mg of lyophilized samples in 2 ml of DMSO+0.5% LiBr (w/w) solution. After filtration of the samples through 0.45 μm PTFE filters, analysis was performed with SEC 1260 Infinity (Polymer Standard Services, Germany). The equipment consisted of an isocratic pump (G1310B), a micro degasser (G1379B), and a standard autosampler (G1329B). The detection system included a UV detector (G1314B) in series with a refractive index detector (G1362A). The mobile phase was DMSO+0.5% LiBr set to a constant flow rate of 0.5 ml min^{-1} for a total run time of 65 min. The injection volume was 100 μl. The separation system consisted of a PSS GRAM Precolumn, and PSS GRAM 100 Å and PSS GRAM 10 000 Å analytical columns thermostated at 60 °C and connected in series. For standard calibration, pullulan standards with nominal masses of 708, 337, 194, 47.1, 21.1, 9.6, 6.1, 1.08, and 0.342 kDa were used.

NMR analysis

Quantitative ^{31}P-NMR analysis was performed as reported before (Granata and Argyropoulos, 1995). In brief, an accurately weighed amount of lignin (~30 mg) was phosphorylated using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP). All chemical shifts reported are relative to the reaction product of water with Cl-TMDP, which gives a sharp signal in dimethylformamide/pyridine/$CDCl_3$ (v_{ratio}: 1/1/4) at 132.2 ppm.

For HSQC-NMR, a 10–15% sample solution was prepared in deuterated DMSO-d_6. The experiments were carried out with a Bruker pulse program 'hsqcetgpsi', and spectra were acquired with the following parameters: size of flame ionization detector 1024, pulse 9.2 mm, number of dummy scans 16, spectral width 13 ppm, and a relaxation delay of 1.5 s. The number of scans was set to 120 which led to a run time of 14 h. The unsubstituted carbon 2 of aromatic groups was used as an internal standard for quantifications as described elsewhere (Sette *et al.*, 2011). The central DMSO (δ_C/δ_H=39.5/2.5 ppm) was used as an internal reference.

On CA-DHP, the original method by Zhang and Gellerstedt (2007) adopted by Balakshin *et al.* (2011) for LCC studies, which applies to both ^{13}C and 2D HSQC analyses, was used. The ^{13}C-NMR experiments were carried out with the Bruker pulse program 'zgig' with 90° pulse width using an acquisition time of 1.4 s and a relaxation delay of 1.7 s. Chromium(III) acetyl acetonate (2 mg) was added to the DHP solution to provide complete relaxation of all nuclei. A total of 24 000 scans were collected.

A spectrum of the ECL was acquired at 25 °C using a Shigemi NMR microtube. Quantitative ^{13}C-NMR spectra were acquired at a sample concentration of 30% in DMSO-d_6 on a Bruker AVANCE 500 MHz spectrometer equipped with a 5 mm QNP probe using an inverse-gated proton-decoupling sequence under the conditions described earlier (Capanema *et al.*, 2004). Chromium(III) acetyl acetonate (0.016 M) was

added to the NMR tube prior to quantitative ^{13}C-NMR acquisition to provide complete relaxation of all nuclei. Acquisition parameters included a 90° pulse width, a relaxation delay of 1.7 s, and an acquisition time of 1.2 s. A total of 20 000 scans were collected.

2D HSQC-NMR spectra were acquired at a sample concentration of ~10% on a Bruker Avance III 950 MHz spectrometer equipped with a cryo-platform and a Bruker 5 mm ID CPTCI (^{1}H/^{13}C/^{15}N/D) cryoprobe with an Z-Axis Gradient spectrometer. The acquisition parameters were: 24 transient scans per block were acquired using 2000 data points in the F2 (^{1}H) dimension for an acquisition time of 72 ms and 512 data points in the F1 (^{13}C) dimension for an acquisition time of 5.36 ms and for a total experiment time of 4 h 20 min. The 2D data set was processed with 2000×2000 data points using Qsine function in both dimensions.

Results and discussion

We aimed to circumvent the relatively harsh conditions used to isolate LCCs from plant materials in order to determine if they could possibly form during lignin biosynthesis at cell wall proximate conditions. The first strategy we adopted was to analyze ECL formed by tissue-cultured Norway spruce cells *in vivo*. The second approach was a modified version of the classical production of synthetic lignin *in vitro*, also referred to commonly as DHPs. In the modification, the cell wall polysaccharide glucuronoxylan was included in the synthesis. An additional experiment was done with inclusion of galacturonic acid monomers, building blocks of pectin, for reasons discussed later. The aim was to mimic the mechanisms of formation of native LCCs in the cell wall. Both of the adopted strategies produced lignins which were easy to isolate. These were recovered through simple centrifugation, washed with water, and freeze-dried.

In terms of structural analysis, synthetic lignins, so-called DHPs, differ from native or isolated lignins, such as milled wood lignin (MWL), by a lower content of β-O-4 bonds. In DHP lignins, dehydrodimerization reactions of CA, resulting from coupling of at least one of the monolignols at its β-position, are over-represented due to the fact that monolignol radicals preferentially couple with each other rather than cross-couple with dimers or oligomers (Ralph, 2004). The resulting synthetic lignin is, therefore, a condensed polymer with low β-O-4 content.

Previous studies on lignin interunit linkage of ECL have shown that the relative composition of interunit linkages is intermediate to that in MWL and in DHPs (Koutaniemi *et al.*, 2005; Warinowski *et al.*, 2016). The main contributors making ECL, rather than DHP, more similar to MWL were both the relatively high content of β-O-4 and the noticeable presence of dibenzodioxocin structures (Warinowski *et al.*, 2016) which cannot arise from monolignol dehydrodimerization reactions (Ralph *et al.*, 2004). However, DHPs have been used for fundamental studies aimed at understanding mechanisms of lignification (Terashima *et al.*, 1996; Cathala and Monties, 2001; Cathala *et al.*, 2001; Kärkönen *et al.*, 2002; Ralph *et al.*, 2004; Koutaniemi *et al.*, 2005; Li *et al.*, 2015; Warinoski *et al.*, 2016; Tobimatsu and Schuetz, 2019).

Mechanisms for the formation of the lignin–carbohydrate ethers and esters have been proposed (Fig. 1) but, to our knowledge, not experimentally shown.

Investigating if LCCs form at cell wall proximate conditions

Analysis of ECL

Unlike extracted wood lignins, the ECL released by the suspension-cultured cells of Norway spruce (Simola *et al.*, 1992; Kärkönen *et al.*, 2002; Koutaniemi *et al.*, 2005; Warinowski *et al.*, 2016) can be isolated in an unchanged state. The obvious benefit is overcoming any kind of extraction under harsh conditions, since ECL can be isolated from the culture medium just by centrifugation, and washed with water. The spruce cell culture, originating from 1985, has been used to study the structure and biosynthesis of lignin (Simola *et al.*, 1992; Brunow *et al.*, 1993, 1998; Kärkönen *et al.*, 2002, 2014; Koutaniemi *et al.*, 2005, 2007, 2015; Warinowski *et al.*, 2016; Laitinen *et al.*, 2017). The NMR analyses conducted revealed that the structure of ECL, with relatively higher levels of β-O-4 and dibenzodioxocin than in DHPs, is quite similar to MWL as a sign that polymerization occurred by the coupling of monolignol radicals to a growing polymer by so-called 'end-wise' polymerization (Ämmälahti and Brunow, 2000; Koutaniemi *et al.*, 2015; Warinowski *et al.*, 2016). In the present analysis, molecular weight distribution showed that the degree of polymerization of the soluble fraction of ECL in DMSO was 7–8 (mol. wt=1500 Da) with quite a narrow distribution ($Đ_M$=1.4) as shown in Supplementary Fig. S1 at *JXB* online).

ECL was shown to contain carbohydrates (~17% w/w), especially pectic sugars (Warinowski *et al.*, 2016), making this material an excellent substrate to evaluate lignin–carbohydrate bonds. We have investigated the presence of LCCs in the ECL. Carbohydrate analysis indicated the presence of various sugars being bound to ECL, with arabinose, galactose, and galacturonic acid especially abundant (Table 1), giving an indirect indication of interactions between lignin and pectin, which contains all the named sugars. Xylose, mannose, and glucose were also present, although in small amounts (Table 1). The dominance of arabinose, galactose, and galacturonic acid is consistent with studies that showed that these sugars were associated with lignin formed at early stages of plant development (Balakshin *et al.*, 2007; Rencoret *et al.*, 2011). ECL therefore seems to mimic the early-deposited lignins in the cell wall.

Another unique feature for the ECL, unveiled by both HSQC- and ^{31}P-NMR, was the relatively high (20%) content of *para*-hydroxyphenyl hydroxyphenyl (H) units (Fig. 2), which has also been noted before (Brunow *et al.*, 1993; Viljamaa *et al.*, 2018). H units were considerably more abundant than in MWL preparations, which consist predominantly of guaiacyl units (G units) with trace amounts of H units (Capanema *et al.*, 2004). An increased proportion of H units has also been reported for lignin formed at early stages of lignification (Whiting and Goring, 1982; Terashima and Fukushima, 1988; Rencoret *et al.*, 2011), and in lignins formed under stress, such as in compression wood (Timell, 1982), further reinforcing the argument for ECL mimicking the early-deposited lignin in plant cell walls. In addition to the Norway spruce cell culture lignin used in the present experiment, other conifer cell culture lignins have been observed to have elevated H unit contents (Eberhardt *et al.*, 1993; Lange *et al.*, 1995).

Table 1. *Sugar unit composition and lignin content of ECL*

Monosaccharide content (%)								
Fuc	Ara	Rha	Gal	Glc	Xyl	Man	GalA	GlcA
0.7±0.1	46.6±1.1	1.5±0.2	31.1±0.9	4.7±0.1	0.9±0.1	2.1±0.1	10.2±0.5	2.2±0.3
Lignin content (%)								
Klason	ASL							
68.7±0.8	1.8±0.2							

The values are expressed as relative % (w/w). Sugar composition was analyzed by HPAEC-PAD after acid methanolysis followed by TFA hydrolysis. ASL, acid-soluble lignin.

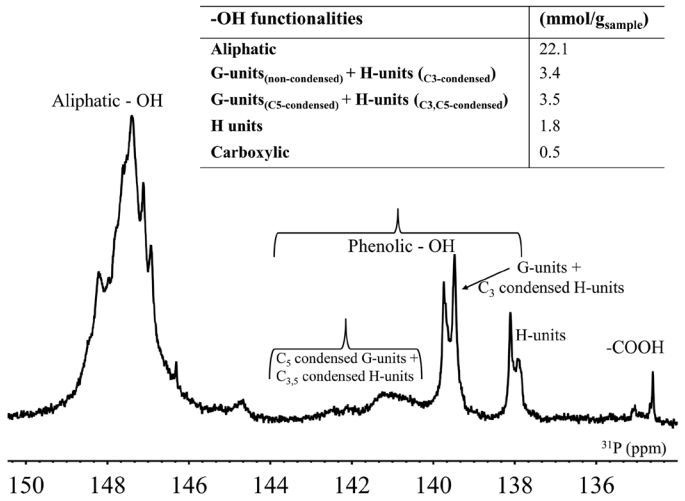

Fig. 2. ^{31}P NMR spectra of ECL and quantification of hydroxyl functionalities.

For the HSQC studies, solubility of ECL in DMSO-d_6 was ~70%. HSQC analysis of interunit linkages showed the expected structural outcome with the dominance of β-O-4 structures (35% of C_9 units; Table 2) and condensed structures with resinol (β-β) and phenylcoumaran (β-5) accounting together for 40% of all interunit linkages. Dibenzodioxin (DBDO) structures were also present.

When focused to lignin–carbohydrate linkages in the ECL, the spectra showed, in addition to typical lignin substructures (Fig. 3), the existence of small sugar signals assigned to arabinoglucuronoxylan (Fig. 4). The carbohydrate signals have also been detected in studies on MWL (Lundquist *et al.*, 1977; Björkman, 1956, 1957; Balakshin *et al.*, 2011). Interestingly, absence of the signals assigned to galactose and galacturonic acid, sugars abundant in pectins, was striking, since these were among the most abundant sugar units in the ECL (Table 1). Furthermore, primary alcohol-based benzyl ethers (BE1) (δ_C/δ_H=80.1–81.2/4.21–4.68 ppm) were detected in line with previous works (Toikka *et al.*, 1998; Toikka and Brunow, 1999). These seemed, however, to be mainly lignin–lignin linkages (αOAlk), since the amount of C6 sugars that would contribute to these was small, or were absent. A small part of this signal could, however, be due to benzyl ether linkage to C5 in arabinose, since weak signals from arabinose were observed. Benzyl ethers to a secondary alcohol (BE2), presumably due to C2 or C3 hydroxyls of xylose in arabinoglucuronoxylan, unfortunately overlapped with spirodienone structures in lignin, if present. The amount of spirodienone structures only can be quantified from the signal of CH-β at 79.4/4.11 ppm (Capanema *et al.*, 2005; Zhang *et al.*, 2006; Balakshin *et al.*, 2011); the former was a factor of two higher, strongly suggesting the presence of the benzyl ether (BE2) linkage with xylan. In addition, phenyl glycosides were detected, consistent with recent studies (Miyagawa *et al.*, 2014; Rencoret *et al.*, 2019) (δ_C/δ_H=99–101/5.1–5.2; Fig. 4), suggesting a phenyl glycoside linkage towards glucose.

From the ECL studies conducted here, therefore, the presence of LCC benzyl ethers and phenyl glycosides was corroborated by the presence of signals in the spectra from xylose and arabinose. Interestingly, the carbohydrate composition of the original ECL sample (Table 1) was not reflected in the spectra (Fig. 4). The spectra suggest the presence of xylose with the bulk of the ECL that was soluble in the NMR solvents used. These data also suggest that the insoluble fraction, which accounted for ~30% of the ECL, contained pectic substances detected in the methanolysis analysis (Table 1). As pectins are

Table 2. *Percentage of lignin interunit linkages and LCCs detected by NMR in the extracellular lignin (ECL), in the synthetic lignin produced with horseradish peroxidase (CA-DHP), and in DHPs produced with HRP in the presence of galacturonic acid (GalA-DHP) or methylglucuronoxylan (10%, Xylan$_{10}$-DHP)*

Lignin interunit linkages (relative % of C_9 units)	ECL	CA-DHP	CA-DHP[a]	GalA-DHP	Xylan$_{10}$-DHP	Water phase
β-O-4	35	32	30	11	15	9
β-5	21	24	20	35	19	10
β-β	20	18	16.4	25	20	7
DBDO	4	2	2.6	–	4	–
SD	2	2	2	–	–	–
CA[b]	15	20	26	27	20	50
LCC						
BE$_1$	(4.5)	–	–	–	–	–
BE$_2$	2	–	–	–	–	1
Best	–	–	–	3	–	1
GE[b]	–	–	–	–	–	3
PG	D	–	–	–	–	–

Water phase: supernatant of the Xylan$_{10}$-DHP production mixture.
Abbreviations used are given in Fig. 3
–, not detected; D, detected but not quantified, (), γ-O-4 structures.
[a] Quantified with ^{13}C NMR-HSQC.
[b] Very relative values due to the difference in response factor for γ-CH_2 versus aromatic ^{13}C/^{1}H,

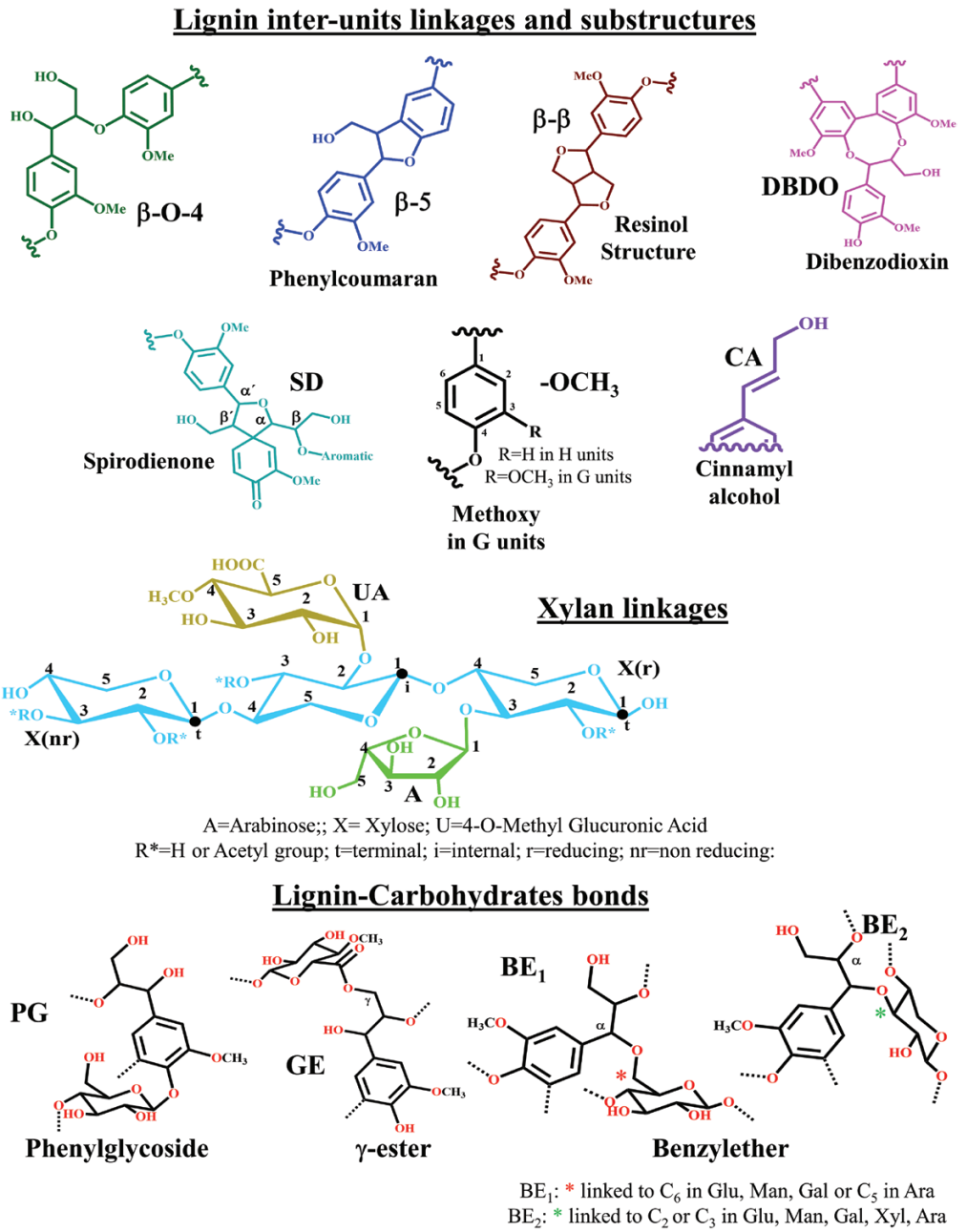

Fig. 3. Main interunit linkages and end groups in lignin, xylan linkages, and lignin–carbohydrate bonds identified in the 2D HSQC-NMR spectra shown in Figs 5 and 6.

largely constituted of galacturonic acid, it is likely that lignin-carbohydrate esters, if present, were enriched in the fraction that could not be analyzed due to the solubility issues.

Interestingly, the signals from 4-O-methylglucuronic acid groups were relatively stronger in ECL (Fig. 4) than those observed in wood-extracted xylans (Fig. 5c, d; Supplementary Fig. S2). However, the quantification of various carbohydrate signals from the HSQC spectra is inaccurate due to different response factors of different $^{13}C/^{1}H$ carbohydrates and, therefore, their quantitative comparison is very challenging. In the context of lignin–carbohydrate bond types, it could be argued that the detected phenyl glycosides are due to non-LCC metabolites naturally present in the cell cultures. However, coniferin β-glucosidase activity has been detected in the culture medium of spruce cells, whereas no coniferin was observed to be present (Kärkönen *et al.*, 2002). More recently, the same group detected several types of glycosylated oligolignols in the culture medium of both lignin-forming and non-lignin-forming cultures (Laitinen *et al.*, 2017). According to the earlier analysis, coniferin was not detected in the medium, but was detected intracellularly in cells that did not produce extracellular lignin due to H_2O_2 scavenging. Thus, although the extracellular lignin had been thoroughly washed with water before the present NMR analysis, it is possible that small signals assigned to phenyl glycoside could have originated from trace amounts of the low molecular weight metabolites still present in the samples. Alternatively, incorporation of such oligolignol glucosides into ECL may occur through nucleophilic attack

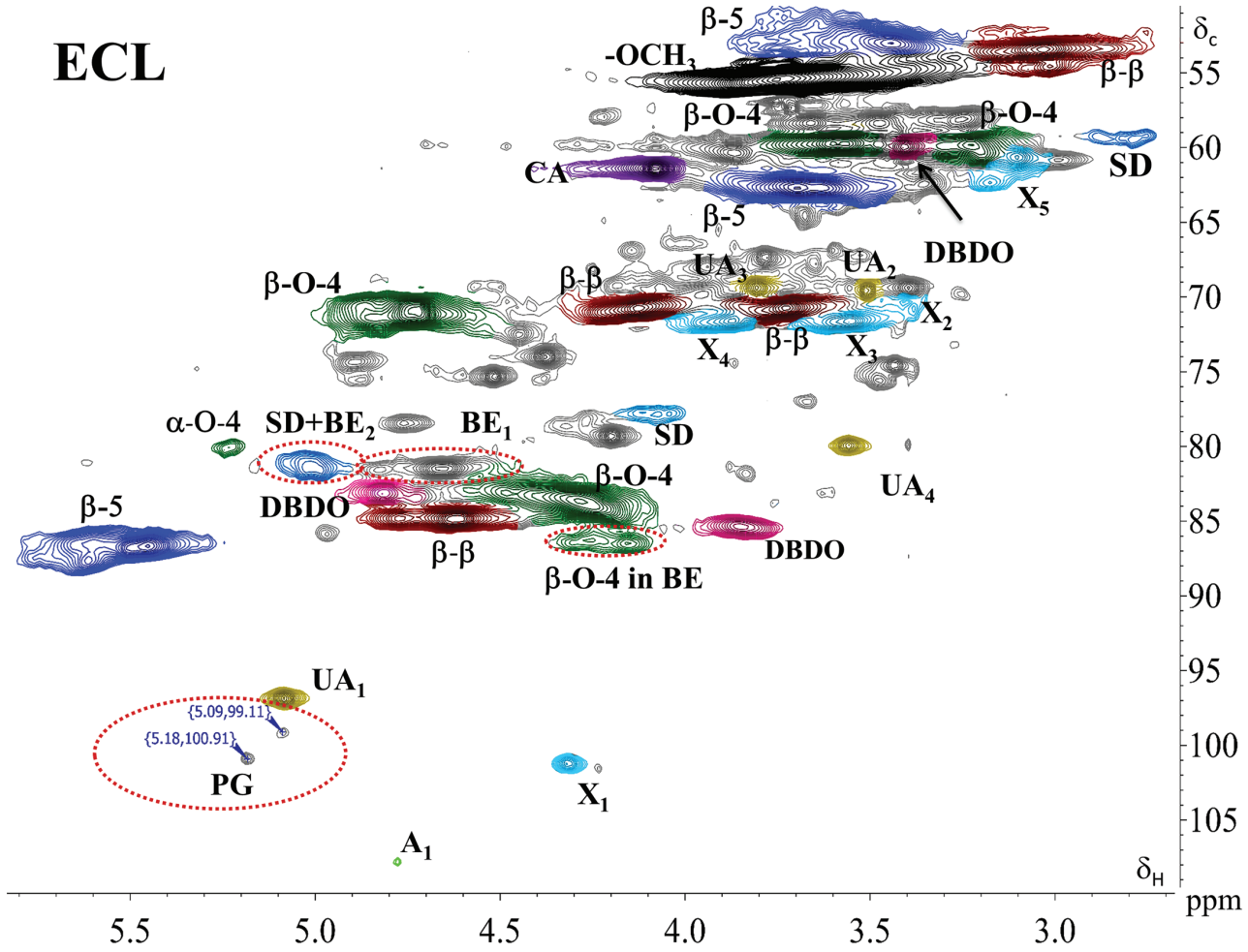

Fig. 4. HSQC spectrum in DMSO-d_6 of ECL. Color and abbreviations used are given in Fig. 3.

to quinone methide intermediates during ECL polymerization. Sugar glycosides, however, normally resonate at a higher proton field (<4.6 ppm) which is not in the area we have assigned to the phenyl glycosides. Glycosides to uronic acids, on the other hand, appear in a lower field on proton (>4.8 ppm) but a higher field on carbon (<100 ppm), which also lie outside our assignment. Furthermore, the absence of any signals related to pectin rules out the possibility that the cross-peak assigned to the anomeric peak in phenyl glycoside overlaps with, for instance, the internal anomeric signals between galacturonic acid and rhamnose appearing at 99.8/5.02 ppm (Balakshin *et al.*, 2007, 2011).

The chemical shift values for phenyl glycosides have very recently obtained additional support by a comprehensive study of Rencoret *et al.* (2019) which has confirmed the HSQC assignments with an HMBC technique. The authors reported incorporation of hydroxystilbene glucosides into lignin of Norway spruce bark.

Modified dehydrogenation polymer (DHP) synthesis

Due to the lack of detection of lignin–carbohydrate esters in the ECL studies described above, further investigations were required in order to determine if they could form under other conditions. The working hypothesis was that if the esters were present in the ECL, they possibly escaped detection due to their low concentration in the NMR solvent-soluble polymer. Considering the low concentration of xylan in the DMSO-soluble ECL, synthesizing lignin (DHPs) in the presence of galacturonic acid or glucuronoxylan to increase possible formation of ester linkages to galacturonic acid/glucuronic acid was worth investigating.

First, DHP was synthesized from CA in 50% acetone using commercial HRP and H_2O_2 (CA-DHP; Fig. 5a, b). For the analysis, two NMR quantification methods for the common lignin interunit bonds were applied (Table 2). In the ^{13}C NMR-HSQC method described by Zhang and Gellerstedt (2007), a cluster quantification of signals with similar T_2-relaxation ^{13}C-NMR spectra is performed using the aromatic carbon signals as an internal reference. The quantitative values are then applied to HSQC, where the peaks are better resolved, to allow more specific interunit quantitation. The second method utilizes only HSQC using the C_2 of aromatics as internal references based on an earlier observation that the signal of G-2 CH in softwood native lignins (or G2+S2,6/2 in hardwood ones) is close to 100/Ar (Zhang and Gellerstedt, 2007; Balakshin and Capanema, 2015). Although the second method is less precise and allows only relative comparison for CH_2 moieties (such as CA and γ-esters), it was used for the other DHP samples because of the shorter NMR running time.

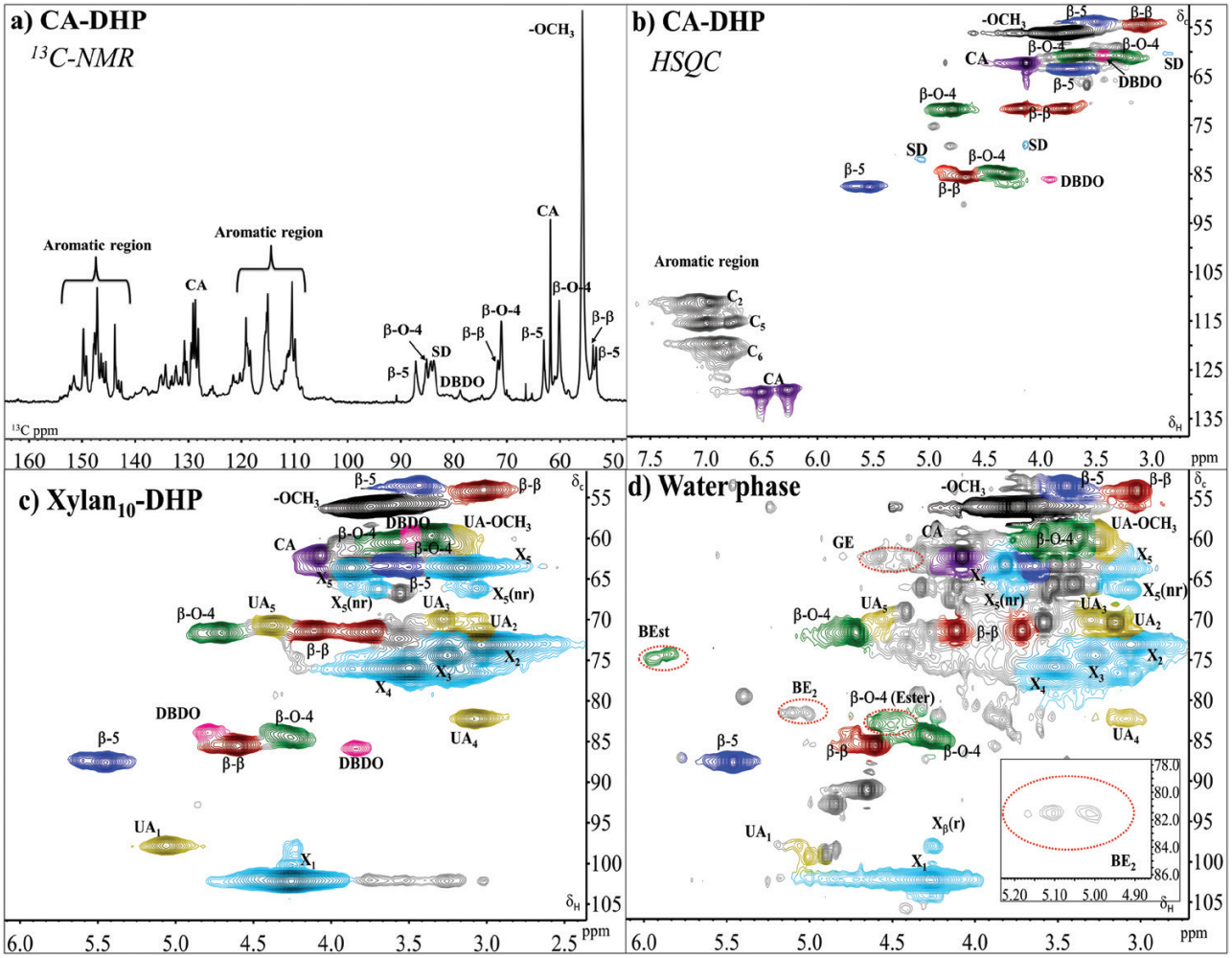

Fig. 5. Expanded (a) ^{13}C spectrum and (b) HSQC spectrum of the CA-DHP. HSQC spectrum of (c) DHP produced in the presence of 10% methylglucuronoxylan ($Xylan_{10}$-DHP), and (d) supernatant of the $Xylan_{10}$-DHP production mixture. All spectra were run in DMSO-d_6. Presence and absence of characteristic LCCs are marked by red, dotted circles. Colors and the abbreviations used are given in Fig. 3.

We next investigated the formation of esters by including a conjugate base of a simple acid—the sodium salt of galacturonic acid—to the DHP production mixture (GalA-DHP). This strategy, adopted to use the conjugate base instead of the acid, was to increase the concentration of the carboxylate nucleophile, so that esters, if formed, would be detectable. In the presence of galacturonic acid, formation of a benzyl ester was observed (Supplementary Fig. S3), evidenced in $C_\alpha H_\alpha$ correlation in α-esterified β-O-4 appearing at 74.3/5.96 ppm and corresponding $C_\beta H_\beta$ at 82.5/4.6 ppm [β-O-4(Ester) peak]. No γ-ester was detected, indicating that uronosyl migration (see Fig. 1) did not occur.

From the analyses of ECL (Fig. 4) and DHP produced in the presence of galacturonic acid (GalA-DHP; Supplementary Fig. S3), clear evidence of benzyl ethers was still lacking, since the BE2 signals, if present, were overlapping with the signal of CH-α' at 81.7/5.09 in spirodienone and BE1 with lignin–lignin ethers (Balakshin *et al.*, 2011). One way to solve the first inconvenience was to synthesize lignin at a high concentration of xylan, in order to increase the probability of BE2 formation, and then compare the volume integrals of the two signals of spirodienone (C_α'/H_α' and C_β/H_β at 81.7/5.09 and at 79.4/4.11 ppm, respectively). Thus, DHP was produced in a 10% solution of polymeric beech methylglucuronoxylan. Both the precipitate ($Xylan_{10}$-DHP) and the supernatant were collected, analyzed by HSQC, and the signals compared with those in the references, namely CA-DHP (Fig. 5b) and commercial methylglucuronoxylan (Supplementary Fig. S2).

In the $Xylan_{10}$-DHP (Fig. 5c), no evidence of LCC was found, suggesting either that the high molar mass fraction of the xylan and the DHP co-precipitated due to physical interactions, or that lignin–carbohydrate bonds were below the detection limit. Interestingly, the amount of DBDO interunit linkages detected in the $Xylan_{10}$-DHP produced with high (~5 μkat) CA-oxidizing activity of HRP were at the same level as those in the ECL (Table 2). These interunit linkages were considered to form with a high oxidation rate of the polymer (Warinowski *et al.*, 2016). The observation that DHP produced in the presence of xylans contains elevated levels of DBDO is of interest, since the reference DHPs produced with the same enzyme activity, but without carbohydrate supplementation, contained fewer of these units (Table 2). Due to water solubility of the xylan, ~90–95% of the xylan ended up

in the water phase (supernatant; Fig. 5d), together with the unreacted CA (giving a relative value for 50% of all aromatic signals; Table 2). HSQC analysis of this fraction also revealed the presence of some dimeric/oligomeric lignin fractions (mol. wt ~300 Da, by SEC analysis), with interunit linkages consisting of phenylcoumaran, resinol, and β-O-4 structures, all present at roughly the same level (~10%).

Interestingly, both benzyl esters (δ_C/δ_H=74.3/5.96 ppm) and γ-esters (δ_C/δ_H=62.3/4.58 ppm) (Li and Helm, 1995) were detected in the water phase, with the latter markedly more abundant, at 3% relative to lignin interunit linkages (Table 2).

Native acyl groups in lignin (e.g. ferulate, *p*-coumarate, and benzoate) (Ralph *et al.*, 2004) as well as acetyl on the carbohydrate backbone could overlap in the same region of γ-esters (δ_C/δ_H: 62–65/4.0–4.5 ppm) (Balakshin *et al.*, 2011). However, this was not the case for the analyzed substrate (supernatant of $Xylan_{10}$-DHP) where only CA and deacetylated beech methylglucuronoxylan (Supplementary Fig. S2) were adopted. Furthermore, the presence of C_β/H_β cross-signal at δ_C/δ_H: 82.5/4.6 ppm [β-O-4(Ester); Fig. 5d] which is a diagnostic peak of the presence of ester in the γ-position of the β-O-4 lignin structure, is further evidence of the presence of γ-ester. In addition, in a similar work where the DHP approach was adopted as a tool to selectively functionalize lignin, the cross-peak assignment of benzyl ester (δ_C/δ_H=74.3/5.96 ppm; Fig. 5d) has been thoroughly demonstrated by HMBC and HSQC-TOCSY, showing multiple bond correlations both within the same spin system and between aromatic and $^{13}C/^{1}H$ carbohydrates (Giummarella *et al.*, 2018).

In addition, BE2s to xylan were detected (Fig. 5d, and the expanded region in the bottom right corner). This conclusion was reached due to the absence of signals assigned to spirodienone from C_β'/H_β' at 59.6/2.80 ppm and the signal of $C_\beta H_\beta$ at 79.4/4.11 ppm (see Fig. 1 for difference between β and β' in the spirodieneone structure) meaning that the signal at 81.6/5.02 ppm where C_α/H_α should be is actually the BE2 in xylan. In softwood arabinoglucuronoxylan, formation of BE1 via primary alcohol in arabinose has been detected (Balakshin *et al.*, 2011). However, in the case of beech xylan where arabinose is absent, the only possibilities are the BE2 type.

A molecular weight analysis by SEC (Fig. 6) showed a dramatic decrease in the elution volume, corresponding to a higher hydrodynamic volume of both DHP produced in the presence of 10% methylglucuronoxylan and the supernatant of the $Xylan_{10}$-DHP experiment as compared with the DHP produced without hemicellulose supplementation (CA-DHP; Fig. 6). The results support that xylan was attached to DHP, resulting in a higher molar mass when compared with DHP produced without any hemicellulose supplementation.

Conclusion

In the present work, we investigated the possibilities of lignin–carbohydrate bond formation at the proximate cell wall conditions. ECL lignin from the Norway spruce cell culture was used as one research material. In addition, synthetic lignins, classically referred to as DHPs, were modified by inclusion of galacturonic acid or water-soluble xylan in the synthesis step, to form derivatives of synthetic lignins (GalA-DHP and $Xylan_{10}$-DHP, respectively). Altogether, four types of lignin–carbohydrate bonds, namely benzyl ethers, benzyl esters, γ-esters, and phenyl glycosides, were detected. Thus, experimental evidence through direct linkage studies by HSQC-NMR showed possibilities of lignin–carbohydrate bonds being formed in the plant cell walls rather than formed during the isolation procedure. Results from SEC studies further support the above conclusion.

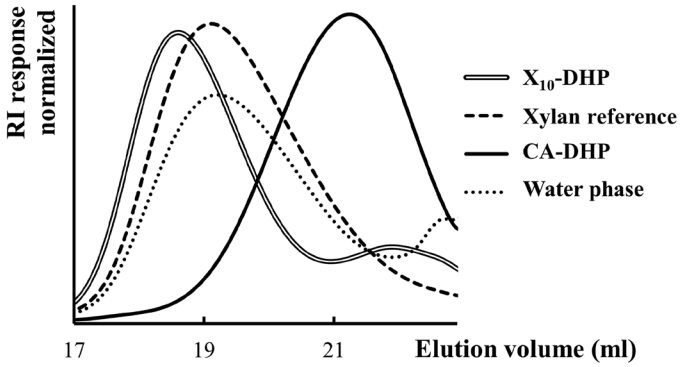

Fig. 6. SEC chromatograms in DMSO+0.5% LiBr of $Xylan_{10}$-DHP and its water phase (supernatant) fractions compared with both DHP produced without hemicellulose supplementation (CA-DHP) and beech xylan used as a reference.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Analysis by size exclusion chromatography (SEC) in DMSO+0.5% LiBr of Norway spruce extracellular lignin (ECL).

Fig. S2. Expanded HSQC spectrum of beech methylglucuronoxylan.

Fig. S3. Expanded HSQC spectrum of DHP produced with HRP in the presence of galacturonate (GalA-DHP).

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