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ORIGINAL

A possible explanation for the structural inhomogeneity of lignin in LCC networks

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Abstract Lignin has a very complex structure, and this is partly due to the monomers being connected by many different types of covalent bonds. Furthermore, there are multiple covalent bonds between lignin and polysaccharides in wood, and it is known that the structure of lignin covalently bound to the hemicellulose xylan is different to lignin bound to the hemicellulose glucomannan. Here, synthetic lignin (DHP) is synthesized at different pH and it is shown that lignin made at lower pH has a structure more similar to the lignin bound to xylan, i.e., having higher relative content of β-O-4 ethers. It is hypothesized that xylan due to its carboxylic acids forms a locally lower pH and thus "direct" the lignin structure to have more β-O-4 ethers. The biological significance of these results is discussed.

Introduction

True wood, i.e., secondary xylem produced by plants with secondary growth, such as trees and bushes, is among the most common biological tissues on earth (Sjöström 1993). It carries out several important functions for the plant, i.e., being a mechanical support allowing trees to develop impressive dimensions, performing transport of water and dissolved minerals from the root up to photosynthetic tissues (leafs, needles, etc.), and storing nutrition during resting seasons, i.e., winter (Kim et al. 2016). Chemically, wood consists mainly of the polysaccharides cellulose and

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hemicelluloses, and the aromatic polymer lignin (Sjöström 1993). The latter polymer is in many ways an oddity within the group of biomolecules, being racemic, lacking well-defined primary structure, and most likely being branched (Boerjan et al. 2003). The explanation for these unusual properties is that the lignin monomers, the *monolignols*, are polymerized by an uncatalyzed radical–radical coupling reaction from resonance stabilized radicals, directly or indirectly generated by enzymatic oxidation (Fig. 1) (Freudenberg 1959; Westermark 1982). In this way, several forms of ethers and carbon–carbon bonds are created (Freudenberg 1959). The by far most common the β–O-4 bond is also the most chemical reactive one, and the other bonds, ethers and carbon–carbon bonds such as 4-O-5, 5-5 and β-β bonds are often called *condensed bonds,* although they are not formed by condensations (Henriksson 2009).

Thus, the formation of lignin reminds more of a radical polymerization of a synthetic polymer, such as polyethylene, than of the controlled condensation synthesis of proteins and polysaccharides, and this explains the "random" element in the lignin structure. Furthermore, during the formation of the β–O-4 bond, water or other hydroxyl-carrying molecules can be incorporated in the lignin (Fig. 1) (Freudenberg and Harkin 1960). If the hydroxyl group is located on a carbohydrate, an ether or ester linkage between lignin and polysaccharides can be created (Fig. 2).

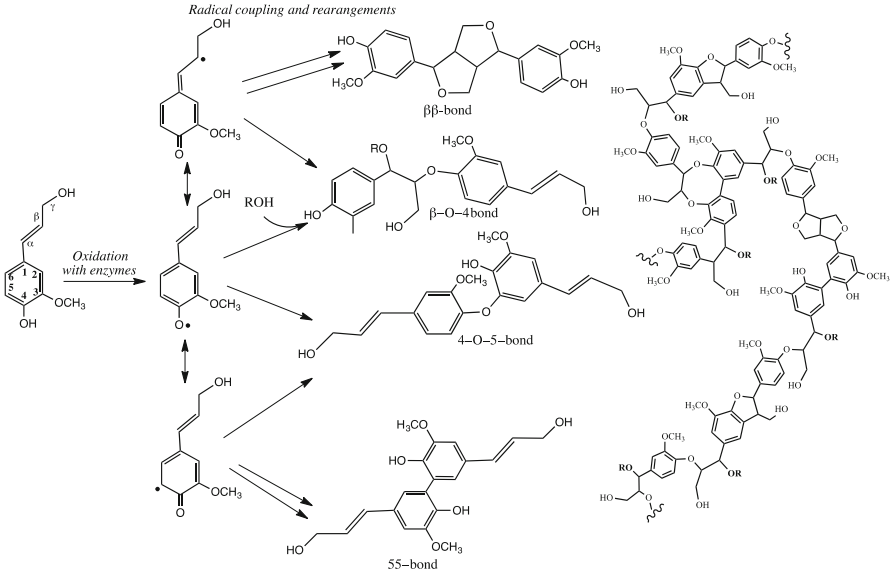

Fig. 1 Lignin and lignin formation. On the left coniferyl alcohol, the dominating monolignol in softwood, and also common in hardwood, is shown. The nomenclature system is explained in the structure of the monolignol. Oxidation of the monolignols generates stable radicals with several resonance structures (not all are shown here). The radicals can form different covalent bonds by combinations of different radicals of which examples are shown. Note that during the formation of the most common intermonolignol bond, the β-O-4 bond, ROH is added to the structure. This can be water (R=H) or a carbohydrate hydroxyl (R=carbohydrate residue). In the latter case, a covalent bond between lignin and polysaccharides is created. On the *right*, a part of a hypothetical lignin structure is shown. Possible covalent bonds to polysaccharides are marked as "**OR**"

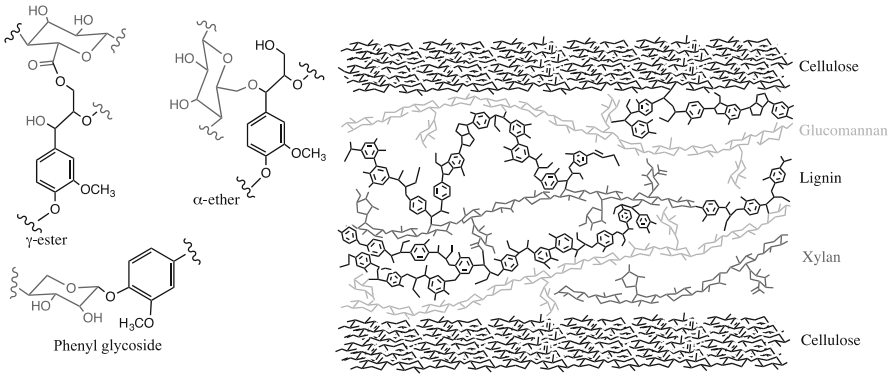

Fig. 2 Lignin carbohydrate complexes. The most common covalent bonds between lignin and polysaccharides (*left*) and a schematically lignin polysaccharide network in wood (*right*)

Moreover, phenyl glycoside bonds between lignin and polysaccharides have also been detected in wood, although the mechanism for formation is unknown (Fig. 2) (Balakshin et al. 2011). With a somewhat incongruous name, the covalent bonds between lignin and polysaccharides are called *lignin carbohydrate complexes (LCC)*.

In earlier studies, a method for the quantitative preparation of relatively intact molecules from wood carrying LCCs was developed, and the results indicated that lignin in wood cross-links different polysaccharides and that most lignin molecules in wood were covalently bound to polysaccharides (Lawoko et al. 2006). In unbleached kraft pulp and oxygen delignified kraft pulp, these bonds persisted—at least partly (Lawoko et al. 2005). Later studies suggested that individual hemicellulose molecules can carry more than one lignin functionality, which opens up the possibility that lignin in wood together with polysaccharides forms entire networks (Fig. 2) (Oinonen et al. 2015), that might be of central importance for the properties of wood, i.e., stiffness and resistance toward water adsorption.

Most LCC bonds seem to occur between lignin and hemicelluloses, although bonds to pectin and cellulose also occur (Jin et al. 2006; Cathala et al. 2001). Hemicellulose is a group of cell wall polysaccharides with some common properties, such as that the monosaccharide residues in the main chain are mainly connected by β, 1-4 glycosidic bonds, that the main chain carries monosaccharide side groups and that sugar residues are often acetylated. But there are also significant differences between different hemicelluloses; one of the most striking is that *arabinoxylan*, the dominating hemicellulose in hardwoods, but also common in softwoods, carries multiple carboxylic acids in the form of methyl glucuronic acid side groups, whereas *glucomannan*, the dominating hemicellulose in softwoods, and occurring in smaller amounts in hardwoods, is an uncharged polymer (Timell 1967). One unexpected observation was that there were differences between the structures of the softwood lignin, dependent on whether it was covalently bound to arabinoxylan or to glucomannan; the *glucomannan-bound lignin* seems to contain much more of condensed bonds and has a lower content of β–O-4 bonds than the

arabinoxylan-bound lignin (Lawoko et al. 2005). This is of scientific interest, since it suggests that hemicelluloses direct the lignin structure, and of technical interest, since hardwoods that are rich in xylan are faster to be delignified during chemical pulping; β–O-4 bonds are much easier broken than condensed bonds in chemical pulping (Gierer 1980). It should be noted, however, that there also are differences in the monolignol composition/structure between hardwoods and softwoods, and this might at least partly explain the differences between softwood and hardwood lignin (Boerjan et al. 2003).

It is difficult to find any obvious explanation for the differences in structure between the glucomannan- and arabinoxylan-bound lignins. The two hemicelluloses appear to be closely associated in the cell wall, and there is no reason to believe that the two lignins are not synthesized simultaneously; on the contrary, it has been demonstrated that a single lignin molecule can have covalent bonds to both glucomannan and arabinoxylan (Lawoko et al. 2006). Furthermore, there are suggestions in the literature that there is a kind of "template effect" in the lignification, but this statement lacks convincing experimental support (Holmgren et al. 2008), and it is not easy to understand how such templates should explain these differences, although it has been suggested as an explanation (Chen and Sarkanen 2010). However, there might be a more direct effect of the hemicelluloses on the lignin structure; the carboxylic acid groups on the xylan might create a local lowering of the pH around the polymer, due to electrostatic repletion of hydroxyl ions and attraction of hydrogen ions (Fig. 3), which do not exist close to the uncharged glucomannan polymer. If a lower pH gives a more linear structure rich in β–O-4 bonds, this might be a plausible explanation of the differences in lignin structure related to type of hemicellulose.

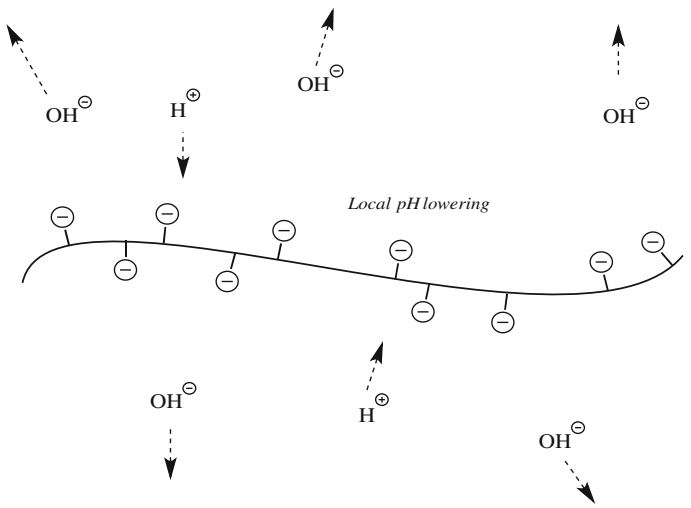

Fig. 3 A negatively charged polymer, as arabinoxylan, is expected to generate a locally lower pH around the polymer, as carboxylic acid with negative charges repels hydroxyl ions and attracts hydrogen ions

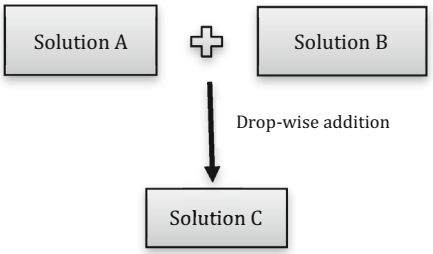

Fig. 4 Dropwise addition of solution A and B to solution C

In this work, structures of synthetic lignins synthesized at different pH are discussed to investigate whether micro-pH gradients, such as described above, might be an explanation of the differences in lignin structure depending on which hemicellulose it is bound to.

Materials and methods

Chemicals

Chemicals including coniferyl aldehyde (4-hydroxy-3-methoxycinnamaldehyde, Sigma-Aldrich), horseradish peroxidase (HRP, 250–330 units/mg, Sigma-Aldrich), hydrogen peroxide solution (30% wt in H_2O, Sigma-Aldrich), sodium borohydride (Sigma-Aldrich) and ethyl acetate (>99.5%, Sigma-Aldrich) with analytical grades were used.

Manufacture of coniferyl alcohol

A sample (4 g, 22.49 mmol) of coniferyl aldehyde was dissolved in 250 ml ethyl acetate (>99.5%). Precisely, 1.65 g (43.61 mmol) of sodium borohydride was added to the solution as a reductive agent and the solution was stirred over night at room temperature. The reaction was quenched by water after one overnight run and the organic phase was separated and the aqueous phase was extracted with excessive amount of ethyl acetate (2 × 50 ml) to improve efficient extraction. The extracted product was dried by anhydrous $MgSO_4$ and solvent evaporated under reduced pressure at 40 °C. The oily product was recrystallized from dichloromethane/petroleum ether to result in pale yellow powder. H NMR result verified the complete reduction in initial sample (Ludley and Ralph 1996).

Synthetic lignin (dehydrogenized polymer, DHP)

Manufacture of synthetic lignin (dehydrogenized polymer, DHP) was carried out at three different pHs: 3.5, 4.5, and 6.5. For this reason, 0.1 M sodium citrate buffers were prepared for pH 3.5 and pH 4.5, and 0.1 M sodium phosphate buffer for pH 6.5 (Ludley and Ralph 1996). Samples (synthesized coniferyl alcohol) of 400 mg were

dissolved in 10 ml acetone and mixed with 200 ml of 5 mM of the corresponding buffer to obtain solutions with a certain pH (including pH 3.5, pH 4.5, and pH 6.5) making *solution A*. Approximately 1.5 ml 30% hydrogen peroxide (48.9 mmol) was mixed with 200 ml of the corresponding buffer (*solution B*), and 5 mg of the enzyme horseradish peroxidase (1250 – 1650 units) was dissolved in 200 ml of the same buffer (*solution C)* (Fig. 4). Three batches of DHPs were synthesized at pH 3.5, 4.5, and 6.5, respectively. The DHPs were made according to "endwise polymerization" (Terashima et al. 1996) in which solutions A and B were dropped in solution C at the controlled speed of 10 ml/min. The reaction was run for 1 day at room temperature. Thereafter additions of solid enzyme (2 mg, 500–660 units) and 0.5 ml of 30% wt hydrogen peroxide were done, and the mixture was incubated for 2 days during magnetic stirring at room temperature. The turbidly light brownish solutions were centrifuged and the products were obtained as a pellet. Successive clean water washing was applied to remove salt. The yields of the products were 40–50%.

Size exclusion chromatography (SEC/THF)

DHP samples of 5 mg were acetylated with equal amount of pyridine and acidic acid anhydride (1/1 V/V) overnight to enhance the solubility. Excess amount of pyridine and acidic anhydrous was removed by gradually adding methanol/toluene and roto-evaporation at 50 °C (Gellerstedt 1992). The residue was dissolved in 1 ml of HPLC-grade tetrahydrofuran (THF) and the resulting solution filtered through a 5 μm PTFE syringe filter. Size exclusion chromatography (SEC) analysis was performed using a Waters instrument system (Waters Sverige AB, Sollentuna, Sweden) consisting of a 515 HPLC pump, 2707 autosampler and 2998 photodiode array detector (operated at 254 and 280 nm). HPLC-grade tetrahydrofuran, filtered through a 2 μm PTFE membrane filter and degassed, was used as a mobile phase using a flow of 0.3 ml/min. Separation was achieved on Waters Ultrastyragel HR4, HR2 and HR0.5 4.6 × 300 mm solvent efficient columns connected in series and operated at 35 °C. For analysis, a sample volume of 20 μl was injected using the partial loop needle overfill injection technique. Data were collected at both 254 and 280 nm to ensure a minimal peak drift. Calibration was performed at 254 nm using polystyrene standards with nominal molecular weights ranging from 480 to 176,000 Da. Quantification was performed using the Waters Empower 3 build 3471 software.

Nuclear magnetic resonance spectroscopy (2D-HSQC and C13 NMR)

All NMR measurements were acquired at 25 °C on a Bruker Avance III HD 400 MHz instrument. The probe head used was a 5-mm BBFO broadband probe equipped with a Z-gradient coil. The instrument had an automatic tuning and matching unit as well as an automatic shimming unit. The NMR sample was prepared by dissolving the samples (100 mg) in 1.0 ml of Dimethyl sulfoxide (DMSO) and shaken vigorously to obtain clear solution. The 2D HSQC (heteronuclear single quantum coherence) NMR spectrum was acquired by using

the Bruker pulse program "hsqcetgpsi," acquisition time 0.12 s, relaxation delay 0.82 s, a coupling constant of 145 Hz, an INEPT transfer delay time of 1.72 ms ($d4 = 1/4$ J), a spectral window of 10.5 ppm in $F2$ and 166 ppm in $F1$ with 1024 × 512 increments, 240 scans per increment, a spectral center set at 90.0 ppm in $F1$ and 5.3 ppm in $F2$. The 2D HSQC NMR data set was processed with 2 K × 1 K data points using a π/2 shifted sine bell window function in both dimensions. Chromium acetylacetonate (1.5 mg) was added to the NMR samples before carrying out the ^{13}C NMR experiment. The ^{13}C NMR spectrum was acquired by using an inverse gated proton decoupling program, a 90-degree pulse angle, a spectral window of 239 ppm, 64 K data points in the time domain, an acquisition time of 1.36 s, a relaxation delay time of 2 s between each scan, and a total number of 20 K scans. Data from 2D-HSQC in combination with ^{13}C were used to quantify the amount of the different structures.

Results and discussion

Synthetic lignin, or dehydrogenation polymer (DHP), was made by peroxidase polymerization at three different pH: 3.5, 4.5, and 6.5. The experiment was repeated, and thus, two sets of DHPs were reported. The molecular weights of synthesized polymers were analyzed by size exclusion chromatography (SEC/THF). The main purpose of SEC is to understand the size of samples produced at different pH which resulted in the size range between 2000 and 3000 Da. As shown in Fig. 5, the trend in batch 1 is that lower pH gives somewhat higher molecular weight, whereas the molecular weights for the DHPs in batch 2 are rather similar. The molecular weights are normal for these types of DHPs, and the structural information can therefore be compared with the material.

As mentioned earlier, the DHPs were analyzed with HSQC and ^{13}C NMR. With these methods several important intermonolignol bonds can be quantified (Zhang and Gellerstedt 2007). Figure 6 shows one example of the 2D HSQC and ^{13}C NMR to represent how the synthesized DHP peaks look like. In order to quantify the different interunit linkages, aromatic carbon (C2) in phenolic unit was used as an internal standard and the rest of the peaks were assigned based on the amount of aromatic carbon (C2). The results of two different series of DHPs are shown in Table 1. Although there are differences in the absolute values in the two different batches, the result indicates that their relative proportion of β-O-4 bonds toward the condensed bonds (β-β and β-5), which could be measured with this method, is significantly higher at pH 3.5 than at the higher pH values. Furthermore, the amounts of free phenols are higher at higher pH. Both these observations are in line with the idea that the lowest pH during lignin polymerization gives a lignin richer in β-O-4 bonds and poorer in condensed bonds, as the latter type will leave more free phenols in the polymer. The molecular weight data of batch 1 (Fig. 5) are also in line with this.

Thus, lower pH leads to lignin getting a higher relative content of β–O-4 bonds, and this therefore supports the idea that xylan can affect the lignin structure by creating a locally lower pH, thereby giving a plausible explanation of the

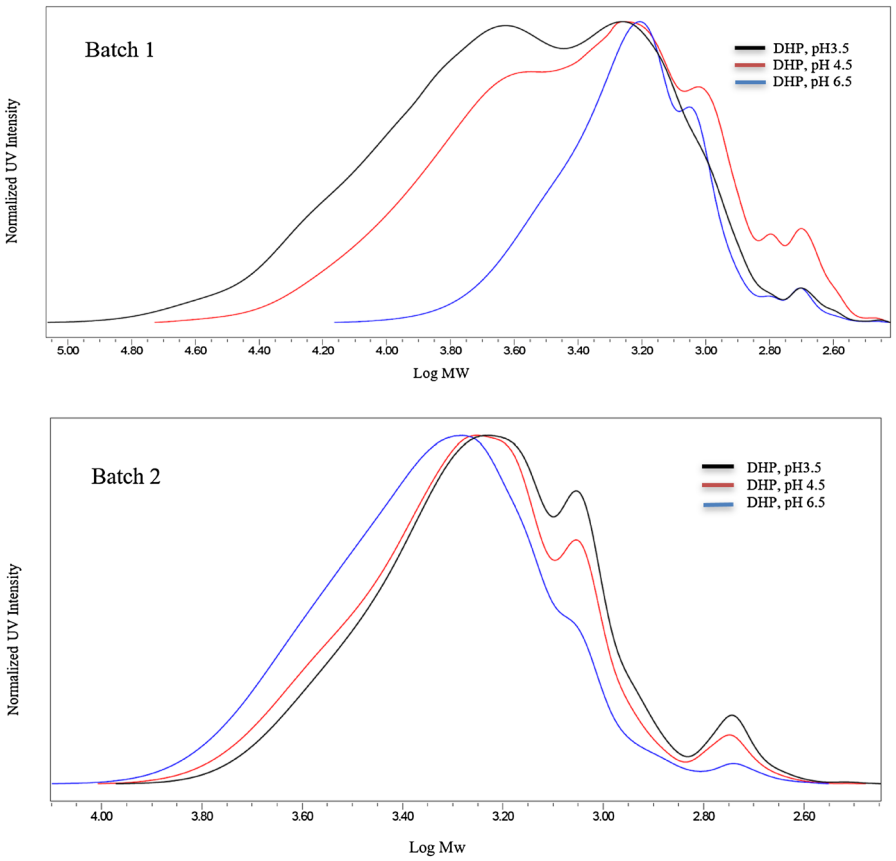

Fig. 5 Size exclusion chromatography of DHPs made at the different pH

hemicellulose-dependent inhomogeneity of lignin structure in the cell wall. However, this raises the question what the exact mechanism of the pH effect is. Since the experiments in this work were done in vitro and thus were well defined with horseradish peroxidase as a sole biomolecule, any "template effect" (Sarkanen 1997) appears to be an unlikely explanation. In Fig. 7, a possible suggestion is given that deprotonations might stabilize coupling products in the pathway leading to condensed bonds and that protonation might have similar effect on the pathway leading to β-O-4 bonds. The current model might be controversial as the radical coupling is suggested to be reversible and further studies are needed for unveiling the mechanistic details of lignin polymerization. However, reversible radical couplings are known from polymer chemistry (Kulis et al. 2009).

Biological significance

Is this hypothetical effect by xylan to make lignin more linear and rich in β–O-4 bonds just an unimportant curiosity, or does it perform an important biological

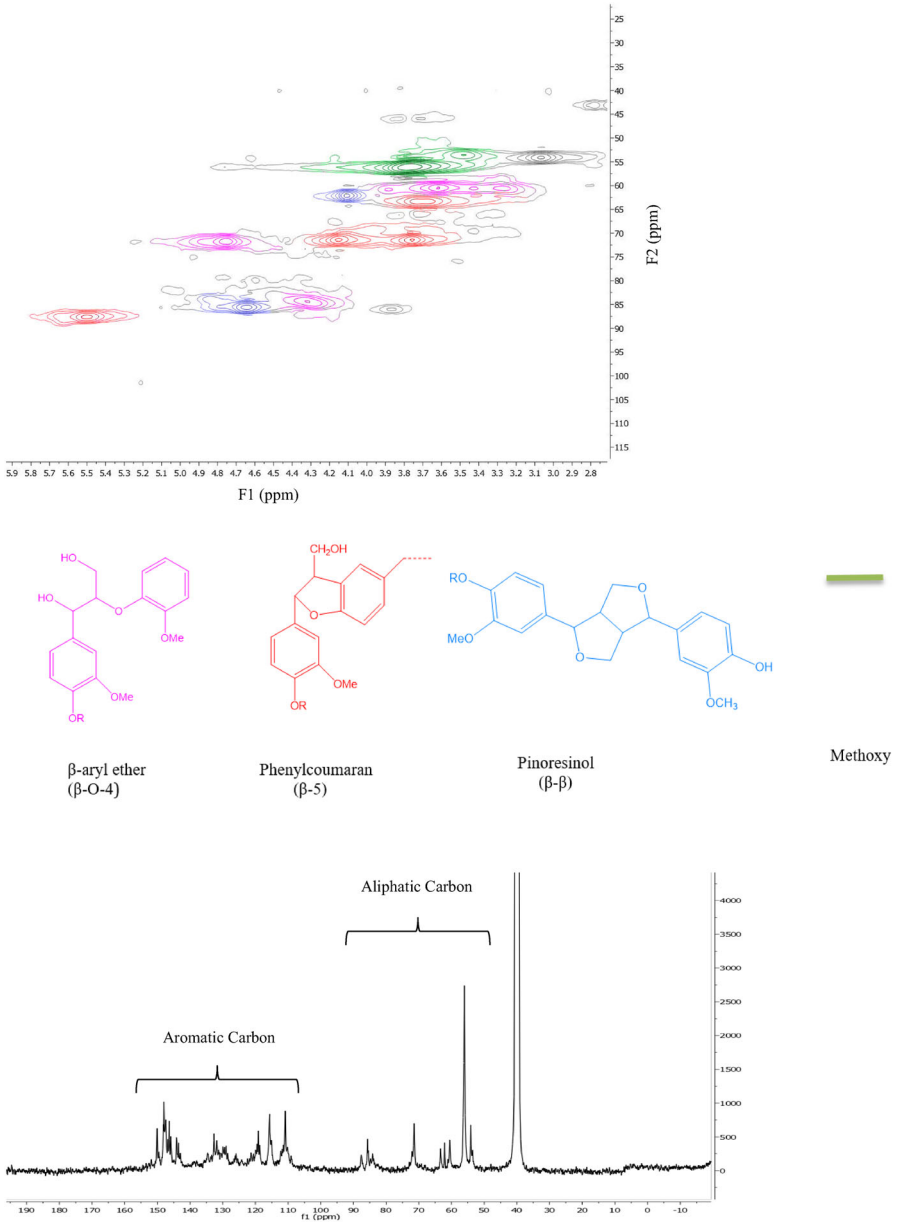

Fig. 6 Example of results of 2D NMR and 13C, respectively

function? High content of xylan in a cell wall should give lignin a more linear structure, and this is actually in accordance with wood chemical observations; hardwood has higher content of arabinoxylan than softwood and also lignin with higher content of β–O-4 bonds. In primary cell wall and middle lamella, the content

Table 1 Results of 2D-NMR quantification. The ratio β-O-4/(β-5 + β-β) is a measurement of the relative content of linear lignin related to condensed bonds

Batch	pH, during synthesis	β-5	β-β	β-O-4	Free phenol	β-O-4/(β-5 + β-β)
1	3.5	0.17	0.27	0.36	0.10	0.818
1	4.5	0.24	0.30	0.28	0.14	0.518
1	6.5	0.26	0.27	0.27	0.36	0.509
2	3.5	0.15	0.18	0.28	0.19	0.848
2	4.5	0.22	0.23	0.22	0.24	0.488
2	6.5	0.23	0.20	0.29	0.25	0.674

unit = number per C9 units

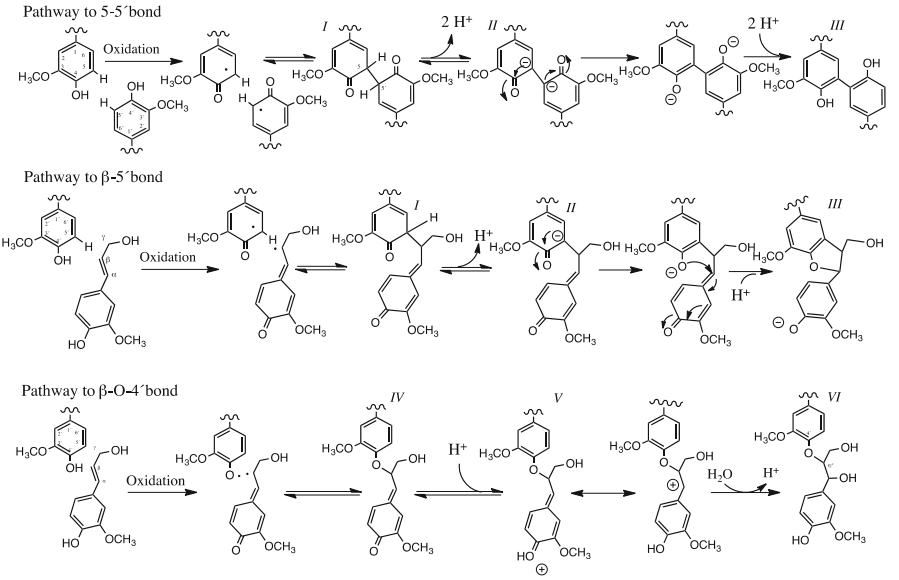

Fig. 7 Model explaining why non-condensed bonds are favored at lower pH. For the creation of 5-5 and β-5 bonds, it is suggested that the immediate products (I) are instable and that the radical couplings are reversible. To get "irreversible products," the structure needs to be deprotonized (II) which leads to stable end products (III). Thus, this reaction is stimulated by higher pH, as the reaction I to II produces protons. The formation of β-O-4 bond on the other side does have an immediate reaction product (IV) that might be more difficult to cleave up to radicals, and it is converted to a stable end product (VI) with the help of a protonation (V). Therefore, this pathway is rather favored by lower pH. Thus, lower pH is expected to favor a higher content of β-O-4 bonds over condensed bonds

of xylan is lower, and the lignin has also a higher content of condensed bonds than in the secondary cell wall (Terashima and Fukushima 1998). However, in the primary cell wall/middle lamella another polysaccharide that carries negative charge is located—pectin (Sjöström 1993)—which does not necessarily mean that the overall pH is low during the lignification of this location, as the pectin carboxylic acids can have been titrated with other substances, and the pH of the

middle lamella/primary cell wall region can thus be relatively high in spite of the presence of pectin. The effects of a pH gradient caused by charged polysaccharides such as xylans are probably more important on a "nanolevel" (Fig. 3).

Under all circumstances, arabinoxylan content in a location could therefore be a way for the plant to affect the lignin structure, but what should the plant gain by having lignin with varying content of β–O-4 bonds? It is difficult to answer this question, but a high content of β–O-4 bonds could lead to higher content of LCC (two of the most important types of LCC, α-ethers and γ–esters, require formation of β–O-4 bonds) and therefore a stiffer wood structure. Could the higher content of xylan in hardwood make the LCC content higher and therefore the wood harder? On the other hand, it might be possible that the biodegradability of the lignin might be higher.

It should be underlined that the structure-directing effect of arabinoxylan as described in this work is working on a statistical level, i.e., a lowering of the pH affects only the proportions of different intermonolignol bond types; according to the above assumptions, lignin should still have a mainly random distribution of different bond types, although with different proportions.

Conclusion

- Synthetic lignin made at pH 3.5 has a higher content of β–O-4 bonds related to condensed bonds, than lignin made at higher pH.
- Since xylan may produce a lower local pH around the polymer under the influence of its carboxylic acids, this can explain why arabinoxylan-bound lignin has high content of β–O-4 bonds and low content of condensed bonds.
- The explanation for this might be that deprotonation stabilizes intermediates in pathways leading to condensed bonds, during lignin polymerization and that protonation might stimulate pathways leading to β–O-4 bonds (Fig. 7).
- High content of xylan in wood may lead to a more linear lignin with higher content of covalent bonds to polysaccharides, which may lead to a harder and stiffer wood.

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