

# Application of Two-Dimensional NMR Spectroscopy to Wood Lignin Structure Determination and Identification of Some Minor Structural Units of Hard- and Softwood Lignins

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Two-dimensional NMR techniques, homonuclear Hartmann-Hahn (HOHAHA), and  $^1\text{H}$ -detected multiple quantum  $^1\text{H}$ - $^{13}\text{C}$  correlation spectroscopy were applied to hard- and softwood milled wood lignin (MWL) samples. The structural units of MWLs were found to be very similar to those of released suspension culture lignin, but the MWL samples contained diarylpropane-1,3-diol units, which have now been identified unambiguously for the first time in lignin samples. Correlations which may arise from arylpropane-1,3-diol 2-aryl ether structures etherified in the 3-position were also observed. Furthermore, some not yet identified structures have been detected, as well as novel structural differences between hard- and softwood lignins.

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**Keywords:** *Lignin structure; 2D NMR; MWL; HOHAHA; HMQC*

## INTRODUCTION

Lignin is an amorphous, aromatic biopolymer which forms an essential part of plant cell walls. By binding the individual plant cells together to form a compact tissue, it gives the plant rigidity to resist external forces, such as wind or gravity. In the utilization of wood lignin plays an essential role; for instance, lignin is removed during most pulping processes. Lignin is composed of phenylpropane units that are linked together with various types of bonds to form a polymer network lacking crystallinity or optical activity.

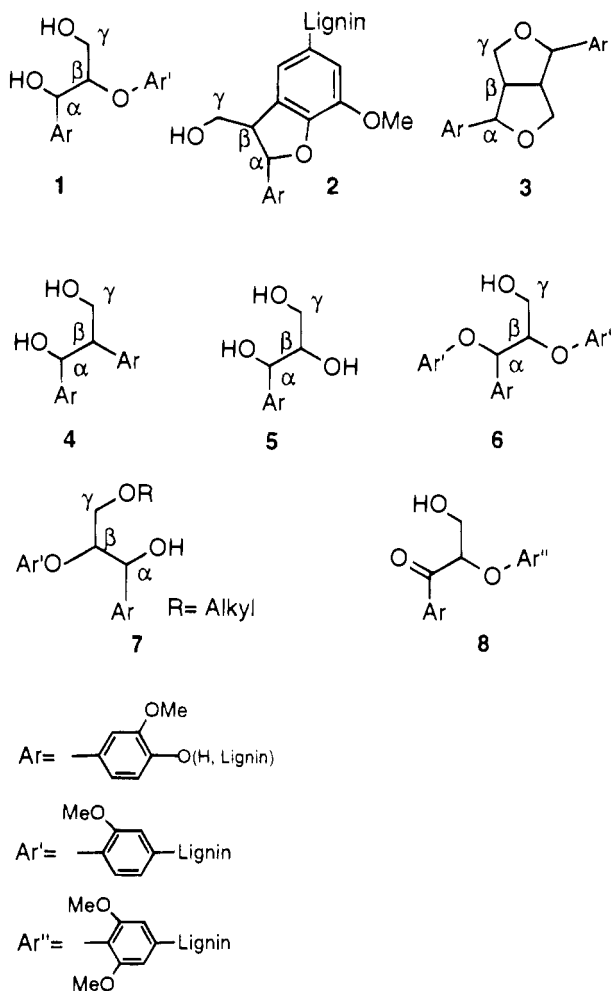
The application of one-dimensional  $^1\text{H}$  and  $^{13}\text{C}$  NMR to wood and lignin preparations in solid and solution state has been of great help in the elucidation of lignin structure (Lüdemann and Nimz, 1974; Lundquist, 1980; Lewis et al., 1988; Robert, 1992; Lapierre et al., 1984). However, these techniques suffer from signal overlap, and only tentative assignments can be done by using reference data from model compounds. Moreover, the  $^1\text{H}$  line widths are broader than the scalar coupling constants, therefore hindering the use of coupling information. The use of two-dimensional NMR techniques has improved confidence in the conclusions regarding the presence or absence of lignin structural units.

$^1\text{H}$ - $^1\text{H}$  correlated methods provide valuable information about the spin systems of the compounds studied. By the application of  $^1\text{H}$ - $^1\text{H}$  correlated methods (2D COSY and *J*-resolved spectroscopy), additional structural information has been obtained (Ede et al., 1990; Lundquist and Stern, 1989). With large molecules,

where the  $^1\text{H}$  natural line width exceeds coupling constants, the HOHAHA (or TOCSY) experiment provides better sensitivity than COSY (Briand and Ernst, 1993). The gain in sensitivity in HOHAHA is due to both the in-phase character of the multiplet structure and the beneficial effects of the spin-lock on the  $T_{1\rho}$  (Briand and Ernst, 1993; Bax and Davis, 1985).

The large dispersion of the  $^{13}\text{C}$  NMR signals makes  $^1\text{H}$ - $^{13}\text{C}$  and  $^{13}\text{C}$ - $^{13}\text{C}$  correlated methods powerful tools for structural analysis.  $^{13}\text{C}$ - $^{13}\text{C}$  correlated spectroscopy (2 D INADEQUATE) has been applied to  $^{13}\text{C}$  enriched aspen wood MWL (Guittet et al. 1985; Bardet et al., 1986). The sensitivity of these techniques is, however, quite poor and they have had limited application to lignin characterization. The HMQC experiment is much more sensitive than traditional HETCOR (Summers et al., 1986), therefore enabling the use of  $^1\text{H}$ - $^{13}\text{C}$  correlated spectroscopy for lignin structure elucidation at natural abundance of  $^{13}\text{C}$ .

The combined use of HOHAHA and HMQC techniques allows not only the assignment of the protons bound to a particular carbon atom but also the assignment of other protons and carbons belonging to the same spin system. This provides a much better basis for structural elucidation than the use of single signals obtained from model compounds. Fukagawa et al. (1991) have applied both HOHAHA and HMQC techniques to birch wood MWL and were able to identify its main structural units (Fukagawa et al., 1991). Recently, some of us have reported the application of these



**Figure 1.** Proposed bonding schemes in wood lignin (Adler, 1977; Sakakibara, 1980; Leary, 1980). (In this study the lignin preparations were acetylated to increase solubility and spectral dispersion of aliphatic side-chain signals.)

techniques to released cell suspension culture lignin (RSCL) (Ede and Brunow, 1992).

It is known that there are structural differences in lignins from different origins (Nimz et al., 1981). Therefore, we subjected acetylated MWL samples from softwood (Scotch pine, *Pinus sylvestris*) and hardwood (birch, *Betula verrucosa*) to a closer examination using HMQC and HOHAHA techniques. Detailed chemical analyses of lignin have led to the proposal of various bonding schemes in lignin (Adler, 1977; Sakakibara, 1980; Leary, 1980) (Figure 1). In the present study we report, for the first time, unambiguous proof for the existence of diarylpropane-1,3-diol structures (4) as well as arylglycerol (5) and  $\alpha$ -carbonyl structures (8). Signals that may be attributed to arylpropane-1,3-diol 2-aryl ether structures that are etherified in the 3-position have also been detected. Such structures may represent important cross-linking in the lignin.

The main structural units (1–3) in these MWLs were found to be similar to those of RSCL (Ede and Brunow, 1992) and will not be discussed here.

#### EXPERIMENTAL METHODS

The MWL samples were prepared using the method of Björkman et al. (1956) and acetylated with 1:1 acetic anhydride–pyridine. Sample sizes varied from 30 to 60 mg.

Spectra were obtained with a Varian Unity 500 spectrometer (11.7 T). The spectra were measured at 27 °C, nonspinning, in CDCl<sub>3</sub> and referenced to internal TMS.

The inverse detected <sup>1</sup>H–<sup>13</sup>C correlation spectra, HMQC, were measured according to the method of Summers et al. (1986), but the BIRD inversion of <sup>13</sup>C-bound protons was omitted (Bax and Subramanian, 1986). The delay for polarization transfer between <sup>13</sup>C and <sup>1</sup>H was set for an assumed  $J = 140$  Hz, and a relaxation delay of 0.8 s was used between scans. The spectral width in F2 was set to 6 kHz and in F1 to 25 kHz. GARP-1 decoupling was used in the <sup>13</sup>C channel during acquisition. One hundred ninety-two time increments, 256 scans per increment, were collected by the hypercomplex method. The spectra were processed using  $\pi/2$  shifted squared sinebell functions in both domains prior to Fourier transformation.

The total correlation spectra, HOHAHA, were recorded using the method of Griesinger et al. (1988). The spectral width was 6 kHz (F1 = F2). A relaxation delay of 0.8 s was used between scans, and spin-lock periods of 20–150 ms (MLEV-17) were used to deliver the magnetization. Two hundred forty time increments, 128 scans per increment, were collected by the hypercomplex method. The spectra were processed using  $\pi/2$  shifted squared sinebell functions in both domains prior to Fourier transformation.

#### RESULTS AND DISCUSSION

The observed connectivities in the side-chain region of HMQC and HOHAHA spectra were compared with chemical shift data obtained from relevant model compounds (Table 1).

**Diarylpropane-1,3-diols ( $\beta$ -1).** The  $\beta$ -1 structures, 4, were first discovered by the isolation of dimeric degradation products of beech wood (Nimz, 1965) and spruce MWL (Lundquist and Miksche, 1965). Since then, the estimates of their occurrence have varied in different lignin preparations (Lundquist, 1987; Habu et al., 1990; Lapierre et al., 1991; Gellerstedt and Zhang, 1991), but no irrefutable evidence for their occurrence in native or isolated lignins has been obtained so far.

In the HMQC spectrum of pine MWL there is a small correlation at 3.35/50.5 ppm (T in Figure 2a) which can be assigned to be the H $\beta$ /C $\beta$  correlation of diarylpropane-1,3-diol ( $\beta$ -1), 4, structures (Lundquist, 1979; Mörk and Kringstad, 1985). In the HOHAHA spectra of pine MWL the proton at 3.35 ppm gives correlations to 6.05 (III in Figure 3a) and to 4.28 and 4.10 ppm (XIV and XV in Figure 3c). These correlations match very well with expected H $\alpha$ /H $\beta$  (III) and H $\gamma$ /H $\beta$  (XIV and XV) correlations of a model compound of structure 4 (Lundquist, 1979), and thus provide strong evidence for the presence of structure 4. Structures of type 4 were also detectable in birch MWL (III in Figure 3b), although in the birch MWL HMQC spectrum in Figure 2b the H $\beta$ /C $\beta$  correlation of structure 4 at 3.35/50.5 ppm is of low intensity and not visible.

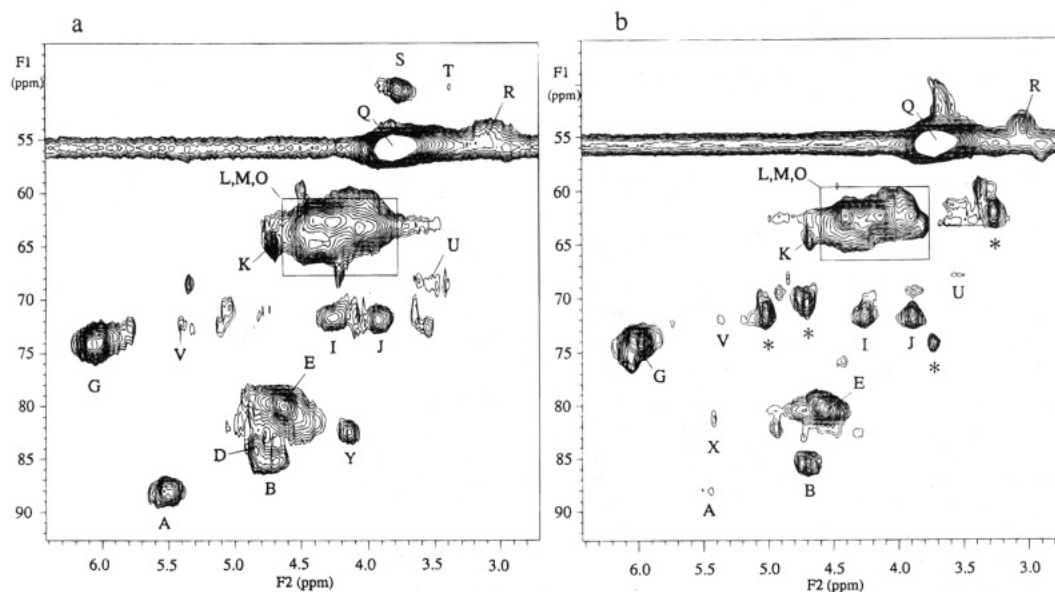
In the HMQC spectra of MWLs, the H $\alpha$ /C $\alpha$  correlation of 4 will be hidden under the H $\alpha$ /C $\alpha$  correlation (G in Figure 2) of structure 1 and the H $\gamma$ /C $\gamma$  correlation under intense H $\gamma$ /C $\gamma$  correlations (L, M, and O in Figure 2a) of structures 1 and 2, which are the predominant structures in lignins (Ede et al., 1990; Ede and Brunow, 1992). In the HOHAHA spectra, the H $\alpha$ /H $\gamma$  correlation will be hidden under the strong H $\alpha$ /H $\gamma$  correlation of structure 1 (II in Figure 3).

Although it is not possible to accurately estimate the amounts of different structural units from this kind of 2D NMR spectra, it is evident that structure 4 exists in lignin, but is a minor constituent in lignin structure, at least in MWL lignin preparations. On the other hand, hydrolysis experiments have indicated that  $\beta$ -1 structures are not uniformly distributed in lignins but seem to accumulate in low molecular weight fractions

**Table 1. Comparison of Lignin Model Compound Chemical Shifts with Observed Connectivities in the Side-Chain Region of HMQC and HOHAHA Spectra of Softwood and Hardwood MWL**

structure <sup>e</sup>	<sup>1</sup> H/ <sup>13</sup> C chemical shifts in model compounds (ppm)			observed correlations in					
	$\alpha$	$\beta$	$\gamma$	HMQC			HOHAHA		
				$\alpha$	$\beta$	$\gamma$	$\alpha/\beta$	$\alpha/\gamma$	$\beta/\gamma$
1 <sup>a</sup>	6.02–6.12/ 73.9–74.5	4.63–4.65/ 80.2	4.01, 4.46/ 63.1–62.6	G 6.01/73.9	E 4.58/79.5	L, M, O <sup>g</sup> 3.80–4.55/ 60.6–66.6	I 6.07/4.65	II 6.00/ 4.00–4.46	– <sup>h</sup>
2 <sup>b</sup>	5.51/87.6	3.76/50.7	4.29, 4.45/ 65.5	A 5.50/ 87.9	S 3.66/ 50.5	L, M, O <sup>g</sup> 3.80–4.55/ 60.6–66.6	IX 5.46/3.68	VII 5.46/4.3–4.4	– <sup>h</sup>
3 <sup>a</sup>	4.80/85.5	3.10/54.4	3.94, 4.28/ 72.0	B 4.68/ 85.5	R 3.06/ 54.5	I, J 3.85, 4.20/ 71.8	X 4.78/3.07	– <sup>h</sup>	– <sup>h</sup>
4 <sup>c</sup>	6.05/75.2	3.35/50.4	4.11, 4.28/ 63.9	– <sup>i</sup>	T 3.35/ 50.5	L, M, O <sup>g</sup> 3.80–4.55/ 60.6–66.6	III 6.05/3.35	– <sup>j</sup>	XIV, XV 4.28, 4.10/ 3.35
5 <sup>a</sup>	5.92–5.94/ 72.8–73.5	5.41–5.44/ 72.3–72.5	3.81, 4.24/ 61.7–62.1	– <sup>i</sup>	V 5.41/ 72.7	L, M, O <sup>g</sup> 3.80–4.55/ 60.6–66.6	IV 5.92/5.41	V, VI 5.92/ 4.24, 3.80	VIII 5.41/4.24 <sup>k</sup>
6 <sup>d</sup>	5.45/ 80.2–80.7	4.69–4.75/ 80.7–82.0	4.13–4.70/ 63.4–66.2	–	–	–	–	–	–
7 <sup>e</sup>	5.92–6.14/ 74.8–75.5	4.50–4.64/ 80.5–81.0	3.50–3.75/ 67.6–69.2	– <sup>j</sup>	– <sup>l</sup>	U 3.53–3.66/ 68.7	– <sup>m</sup>	XIII 5.96–6.06/ 3.45–3.68	XI 4.58–4.67/ 3.49–3.73
8 <sup>f</sup>	–/194.4	5.45/81.7	4.56/64.6	–	X 5.42/ 81.5	L, M, O <sup>g</sup> 3.80–4.55/ 60.6–66.6	–	–	XII 5.42/4.55

<sup>a</sup> <sup>1</sup>H, Lundquist (1979); <sup>13</sup>C, Mörck and Kringstad (1985). <sup>b</sup> <sup>1</sup>H, Lundquist (1979); <sup>13</sup>C, the shifts of the model compound (Brunow and Lundquist, 1984) have not been published previously. <sup>c</sup> <sup>1</sup>H, Lundquist, (1979); <sup>13</sup>C, Ahvonen et al. (1983). <sup>d</sup> <sup>1</sup>H and <sup>13</sup>C, Sipilä (1990). <sup>e</sup> <sup>1</sup>H, Sipil and Brunow (1993); <sup>13</sup>C, Sipilä and Kilpeläinen, unpublished data. <sup>f</sup> <sup>1</sup>H, Lundquist (1992); <sup>13</sup>C, the shifts of the model compound (Lundquist, 1992) have not been published previously. <sup>g</sup> The H $\gamma$ /C $\gamma$  correlations of structures 1, 2, 4, and 5 are overlapping in HMQC spectra. <sup>h</sup> These correlations are of high intensity and are not resolved or visible in Figure 3 (see ref 12). <sup>i</sup> The correlation is obscured by the strong H $\alpha$ /C $\alpha$  correlation of structure 1. <sup>j</sup> The correlation is obscured by the strong H $\alpha$ /H $\gamma$  correlation of structure 1. <sup>k</sup> The correlation to the  $\gamma$ -proton at higher field is obscured by the H $\alpha$ /H $\beta$  correlation of structure 2. <sup>l</sup> The correlation is obscured by the strong H $\beta$ /C $\beta$  correlation of structure 1. <sup>m</sup> The correlation is obscured by the strong H $\alpha$ /H $\beta$  correlation of structure 1.



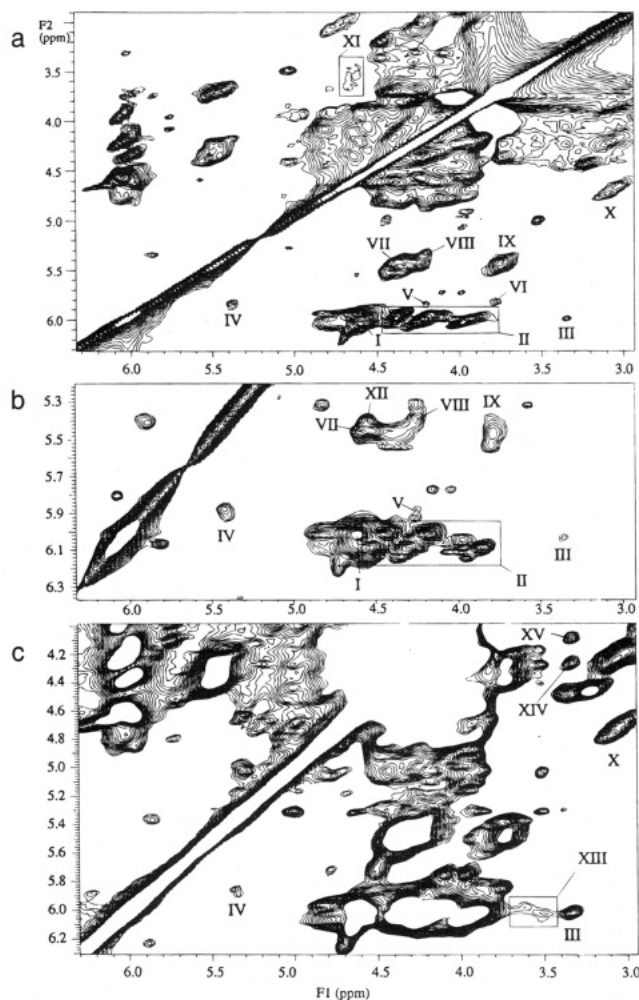
**Figure 2.** Expansion of aliphatic side-chain region of HMQC spectra of pine (a) and birch (b) MWL. The correlations are labeled analogously to our previous publication (Ede and Brunow, 1992). The assignments of correlations S and R have been corrected (see Table 1). The asterisks (\*) denote correlations that originate from hemicellulose (xylan) (Fukagawa et al., 1991).

after acid hydrolysis (Lapierre et al., 1991; Gellerstedt and Zhang, 1991).

**Arylglycerols.** The occurrence of arylglycerol structures has been suggested on the basis of hydrolysis experiments; low yields of formaldehyde formed on periodate oxidation imply that only small amounts of such groupings can be present in lignins (Nimz, 1967; Lundquist and Lundgren, 1972).

In the HMQC spectra of pine and birch MWL, the correlation at 5.41/72.7 ppm (V in Figure 2) could be

assigned to the H $\beta$ /C $\beta$  correlation of arylglycerol, 5, structures (Lundquist, 1979; Mörck and Kringstad, 1985). In the HOHAHA spectra this proton at 5.41 ppm has correlations to 5.92 and 4.24 ppm (IV and VIII in Figure 3), and the proton at 5.92 ppm has correlations to 4.24 and 3.81 ppm (V and VI in Figure 3a,b). The correlation VI is of low-intensity birch MWL and not visible in Figure 3b. These correlations match very well with values reported (H $\alpha$  5.92, H $\beta$  5.44, and H $\gamma$  4.24, 3.81) (Lundquist, 1979) for model compounds and thus



**Figure 3.** Expansions of pine (a, c) and birch (b) MWL HOHAHA spectra recorded with 60 (a, b) and 120 ms (c) spin-lock periods.

provide strong evidence for the presence of structure **5**. Previously, the arylglycerol structures have been tentatively assigned on the basis of  $H\alpha/H\beta$  correlation in COSY and HOHAHA spectra (Ede et al., 1990; Ede and Brunow, 1992). Now we have confirmed these assignments by observation of the HOHAHA correlations of  $H\alpha/H\gamma$  and the HMQC correlation of  $H\beta/C\beta$ .

**Other Structures.** The occurrence of noncyclic benzyl aryl ether structures, **6**, is still an open question in lignin structure (Sipilä, 1990; Freudenberg and Friedman, 1960; Nimz, 1981; Leary, 1982). Even though studies on synthetic lignins (DHPs) suggest that such structures can be formed under certain conditions during lignin biosynthesis (Adler, 1977; Sipilä et al., 1993), their existence in lignins has not been confirmed by direct spectroscopic or other methods. In a previous study (Ede et al., 1990), a weak correlation peak observed in a COSY spectrum was tentatively assigned to structures of type **6**, but the authors indicated that this may have been an artifact.

In the HMQC spectrum of birch MWL there is a correlation at 5.42/81.5 ppm (X in Figure 2b), which could be assigned to the  $H\alpha/C\alpha$  correlation of such structures. The correlation was not observed in pine MWL spectrum. The proton at 5.42 ppm in birch MWL is linked to a proton at 4.55 ppm. The correlation at 5.42/4.55 ppm (XII in Figure 3b) in the 2D HOHAHA spectrum of birch MWL is partially overlapped by the  $H\alpha/H\gamma$  correlation (VII in Figure 3c) of structure **2**, but

the correlation of 5.42/81.5 to 4.55 ppm has been confirmed by a three-dimensional HMQC–TOCSY experiment of  $^{13}\text{C}$ -enriched aspen wood MWL (the results of this 3D study will be published separately). We have synthesized a number of  $\alpha,\beta$ -diaryl ether lignin model compounds with guaiacyl and syringyl structures (to be published separately). The chemical shifts of  $\beta$ -protons in all of these (acetylated) model compounds are in the range 4.7–4.9 ppm. Therefore, we conclude that the correlation at 5.42/81.5 ppm is not originating from a noncyclic benzyl aryl ether structure. Instead, these correlations can be attributed to structure **8**, a syringyl  $\beta$ -ether with an  $\alpha$ -carbonyl group. In this structure the  $H\beta/C\beta$  correlation occurs at 5.43/81.7 ppm and the  $H\beta/H\gamma$  at 5.43/4.56 ppm (Lundquist, 1992). In earlier work (Lundquist, 1992) it has been difficult to find clear-cut evidence for the presence of such carbonyl groups using NMR techniques.

**Arylpropane-1,3-diol 2-Aryl Ether Structures Etherified in the 3-Position (7).** The correlation at 3.53–3.66/68.7 ppm in the HMQC spectra (U in Figure 2) of pine and birch MWL matches well with the  $H\gamma/C\gamma$  correlation of a model compound where the 3-hydroxy of arylpropane-1,3-diol 2-aryl ether, **1**, is etherified with the 1-hydroxy group of another arylpropane-1,3-diol 2-aryl ether unit (Sipilä et al., 1991). In the HOHAHA spectrum of pine MWL recorded with a 60 ms spin-lock period, there is a correlation at 4.58–4.67/3.49–3.73 ppm (XI in Figure 3a), which matches with model compound data for  $H\beta/H\gamma$  chemical shifts. The correlation at 5.96–6.06/3.45–3.68 ppm (XIII in Figure 3c), which becomes visible by a somewhat longer spin-lock period (120 ms), matches well with the expected  $H\alpha/H\gamma$  correlations of the model compound. We were, however, not able to find correlations to the other part of the  $\gamma$ -ether bond (in two-dimensional ROESY, NOESY, or HMBC experiments). Therefore this assignment remains somewhat uncertain because these correlations can also originate from other kinds of  $\gamma$ -alkyl ether structures. Moreover, the correlations for  $H\alpha'/C\alpha'$  and  $H\beta'/C\beta'$  of the second arylpropane unit (the 1-etherified) of the model compound are very close to those of  $H\beta/C\beta$  of structure **1**. Therefore, the HOHAHA and HMQC correlations of the second arylpropane unit would be obscured by the strong correlations of structure **1**.

It is of particular interest to compare NMR data from hardwood and softwood lignins. Differences in interunit structures between hardwood and softwood lignins may partly explain mechanical and chemical differences between wood species. Of the structures discussed in this paper, **4** and **5** exist in both hard- and softwood MWLs. The HMQC spectra in Figure 2 reveal some differences; one is the correlation at 5.42/81.5 ppm (X in Figure 2b, structure **8**) that is missing in pine MWL. Other noticeable differences are, for instance, the correlations D and Y which have been found in spruce RSCl and were found in pine MWL but are missing in birch MWL. We have not been able to assign these correlations to any of the currently proposed lignin side-chain structures; they have to be attributed to new types of interunit linkages.

In conclusion, the use of HMQC and HOHAHA spectroscopy offers a powerful tool for the structural analysis of lignins. By using these techniques in concert, we have unambiguously shown that  $\beta$ -1, **4**, arylglycerol, **5**, and probably  $\alpha$ -carbonyl, **8**, structures are present in milled wood lignins. The sensitivity of these techniques is good, and lignin can be analyzed at natural abun-

dance of  $^{13}\text{C}$ . However, some of the resonances overlap heavily in two-dimensional spectroscopy, and thus three-dimensional techniques, such as HMQC-TOCSY and HMQC-NOESY experiments, are needed to resolve them. The major drawback of these 3D techniques is poor sensitivity at natural abundance of  $^{13}\text{C}$ . This can be circumvented by using  $^{13}\text{C}$  labeled samples.

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