Application of Two-Dimensional Homo- and Heteronuclear Correlation NMR Spectroscopy to Wood Lignin Structure Determination

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A released suspension culture lignin (RSCL) from Norway spruce (*Picea abies*) was subjected to a detailed two-dimensional NMR study. It was found that the most informative techniques were homonuclear Hartmann–Hahn (HOHAHA) spectroscopy and ^{13}C-decoupled ^{1}H-detected multiple quantum ^{1}H–^{13}C-correlation (HMQC) spectroscopy. While correlations from the side chains of most of the commonly proposed lignin structural units were observed, no resonances assignable to the noncyclic benzyl aryl ether or diarylpropane-1,3-diol substructures, which have previously been claimed to account for up to 20% of the interunit linkages in lignins, could be observed, suggesting that the relative importance of these units to the structure of lignin is somewhat questionable. The rotating-frame NOE experiment (ROESY) showed correlations attributable to intraunit magnetization transfer. From the ROESY data, and by consideration of molecular modeling results, it was concluded that in the predominant β-aryl ether interunit linkage most of the side chains were in the gauche conformation, with some in an anti conformation.

Introduction

Lignin is the material which encrusts the cellulose fibers in woody plants, and it is lignin which is removed from wood in most pulping processes. Lignin, however, is unlike any other natural polymer in that there appears to be limited enzymatic control over its biosynthesis.[1] Although lignification in plants is initiated enzymatically to give monolignol radicals, the coupling of the radicals gives a random, racemic polymer with no easily identifiable repeating units. Investigations into lignin structure have also been hampered by the difficulty in isolating lignin in an unadulterated form.

Detailed chemical studies[1] on wood lignins have led to the proposal of a range of bonding schemes.[1,2] More recently, solution- and solid-state 1D ^{1}H and ^{13}C NMR techniques have been used to assist in lignin structure elucidation.[3-7] However, all NMR spectrum interpretation has been based on comparison of the chemical shifts of the lignin resonances with those of model compounds. Owing to the number of interunit linkages, polydispersity (2.4–2.6) and high molecular weight ($Z_{\text{ave}} = 2\text{–}3 \times 10^4$),[8] and the concommittantly large NMR line widths, there are many overlapping resonances. Furthermore, the ^{1}H line widths exceed the size of the scalar couplings, hindering interpretation of the proton spectrum. Thus, there is often only one δ value of the model compound side chain that can be compared with the appropriate lignin resonance, making the interpretation of the spectral data somewhat open to error.

The more sophisticated and informative 2D NMR techniques, which have been applied so successfully to the characterization of other important biomacromolecules, have had a rather limited application to lignin characterization.[9-12] Presumably, a reason for this is that the random nature of the lignin polymer and the types of interunit linkages which apparently exist make lignin an even more formidable challenge to the NMR spectroscopist than a large protein, where at least all the molecules in the NMR tube have the same chemical structure, configuration, and conformation.

Recently, we reported the application of 2D COSY and J-resolved NMR techniques to soluble lignin samples[12] and showed that although we could observe correlations due to all side-chain protons in the majority of the substructures in Figure 1, we could find no evidence of structures **2** and **3** (the appropriate correlations were also missing from spectra in work published simultaneously[11]). However, owing to instrumental limitations on both hardware and software, we were limited in the NMR pulse sequences we could apply. Also problematic were the intrinsically short $T_{1\rho}$'s and T_2's of the protons (ca. 50–100 ms), which caused severe cross-peak intensity loss in the COSY experiments.

It is expected, however, that use of the spin-locked 2D isotropic mixing experiments[13] would provide improvements in sensitivity owing to the effects of the spin-lock on the rapidly relaxing transverse magnetization. Also, useful new structural information should be obtainable from heteronuclear chemical shift correlation experiments and conformational information from nuclear Overhauser experiments. This paper reports here the successful application of the 2D homonuclear Hartmann–Hahn experiment,[13] the ^{1}H-detected one-bond ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC),[14,15] and rotating-frame Overhauser effect (ROESY)[16] experiments to the

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Table I. Comparison of Lignin Model Compound Chemical Shifts,[a] with Connectivities Observed from 2D HOHAHA Spectra of Acetylated RSCL

structure	Hα (model)	α–β (Figure 2)	Hβ (model)	β–γ (Figure 2)	Hγ (model)[b]	α–γ (Figure 2)[c]	α–γ (Figure 3)[d]
1[e]	6.02–6.12	6.07–4.65	4.63–4.70		4.01′, 4.46″		6.00–4.00, 4.20, 4.36
2	5.45		4.73–4.50		4.73–4.50		
3	6.05		3.35		4.11′, 4.28″		
4	5.51	5.46–3.68	3.76	3.68–4.33, 4.52	4.29′, 4.45″		5.46–4.3′, 4.4″
5	4.80	4.78–3.07	3.10	3.07–3.95′, 4.26″	3.94′, 4.28″		4.78–3.95′, 4.26″
6	6.65	6.57–7.41	7.41	7.41–9.65	9.64	9.65–6.57	9.65–6.57
7	6.55	6.57–6.15	6.16	6.15–4.67	4.70	4.67–6.57	4.67–6.57
8	2.67	2.61–1.97	1.96	1.97–4.08	4.11	4.08–2.61	4.08–2.61

[a] Model compound data from refs 4, 5, 11, and 24. [b] Primes and double primes denote upfield and downfield signals, respectively, of diastereotopic protons. [c] Four-bond correlation observed from experiment carried out with 25-ms spin-lock. [d] Four-bond correlations observed from experiment carried out with 150-ms spin-lock. [e] Range of shifts dependent on side-chain stereochemistry and on whether the aromatic ring is etherified to other lignin units or phenolic.

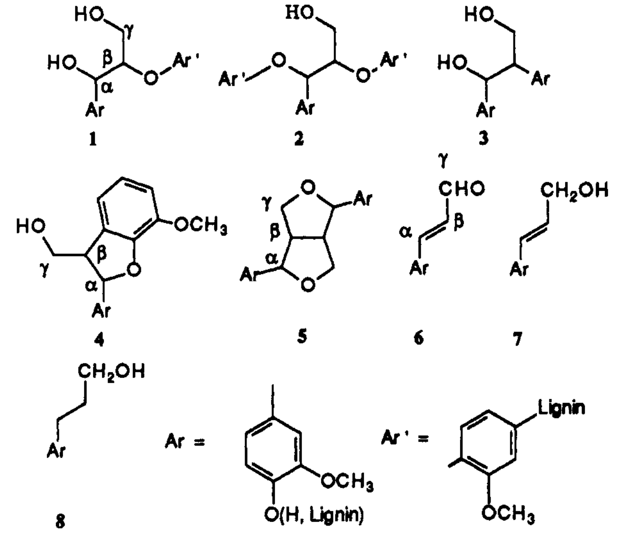

Figure 1. Typical side-chain structures proposed for softwood lignins.

structural analysis of a lignin released into a Norway spruce (*Picea abies*) suspension culture. This lignin has been shown[17] to be quite similar to lignin extracted from spruce wood and has the advantage that it has not undergone any chemical or mechanical degradation process which could lead to structural changes.

Results and Discussion

HOHAHA Spectra. The HOHAHA spectra (Figures 2 and 3) which were recorded with spin-lock periods of 25 and 150 ms, respectively, show a range of correlations from a variety of side-chain structures present in the RSCL. In the spectrum in Figure 3, the longer spin-lock period has allowed observation of both direct scalar coupling and relayed magnetization transfer between the α, β, and γ protons in structures 1 and 4–7 (the coupling networks are mapped out in the Figures). Comparison of the chemical shifts of the cross-peaks with data from appropriate model compounds shows extremely close correspondence, and the assignment of the cross-peaks is given in Table I.

Of particular interest in these spectra is the absence of correlations in the spectrum in Figure 2 at δ 5.4–4.7 (Hα–Hβ in 2) and 6.1–3.4 (Hα–Hβ in 3) and δ 5.4–4.7 (Hα–Hβ–Hγ–Hγ″ in 2) and δ 6.1–3.4–4.1–4.3 (Hα–Hβ–Hγ–Hγ″ in 3) in Figure 3. These chemical shifts should be representative of side-chain correlations in structures 2 and 3, respectively. Although the absence of correlations is not proof in itself of a low or nonexistent amount of these structural units in lignin, some doubt must be cast on their presence. It is also significant that both of these structural types have been the subject of a continuing debate. Estimates have varied between <3% to >8% of the interunit linkages for structure 2[1,12,18] and 1–7% for structure 3.[1,19] Degradation products from structure 3 have been observed in some experiments,[20,21] but it is possible that these structures may be generated during the degradation process from a precursor.[22] The chemical evidence for

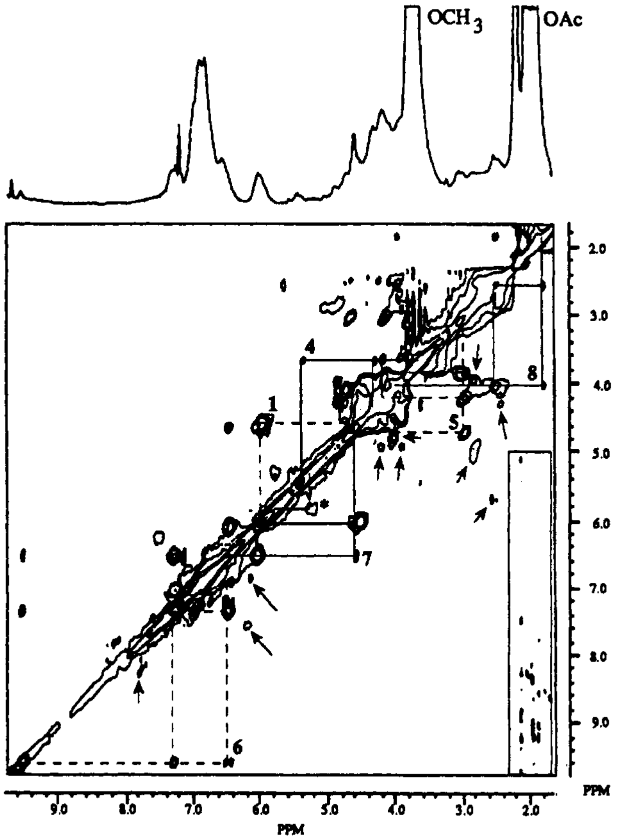

Figure 2. Phase-sensitive 2D HOHAHA spectrum of acetylated RSCL, acquired with a 25-ms spin-lock. The *J*-connectivity patterns for structures 1 and 4–8 are mapped out. Cross-peaks marked with arrows are from as yet undetermined structures, and the boxed area in the bottom right is from F_1 noise from the acetate methyls associated with a slightly twisted F_1 base line (also observed in Figure 3). The cross-peak marked with an asterisk may be due to Hα–Hβ coupling in aryl glycerol units.

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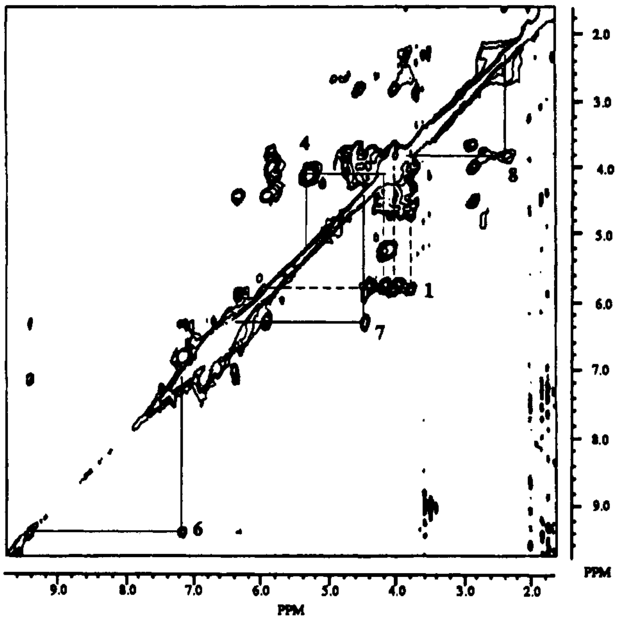

Figure 3. Phase-sensitive 2D HOHAHA spectrum of acetylated RSCL acquired under the same conditions as in Figure 1, but with a spin-lock period of 150 ms. The connectivity patterns due to relayed magnetization transfer are shown mapped out.

structure 2 is more tenuous (and is based on the formation of phenolic groups after mild acid hydrolysis of the lignin),[1,23] and although this structure has been commonly postulated as an important type of labile interunit linkage that is cleaved during many pulping processes, there is now strong biosynthetic evidence from model compound and other studies[12,23,24] that this linkage may well be an insignificant contributor to the structure of lignin.

Of further interest is the presence of the correlations marked with arrows in the HOHAHA spectrum in Figure 2. These correlations are not assignable to any of the currently proposed lignin side-chain structures, and we are actively investigating possible candidates for new types of interunit linkage.

HMQC Spectrum. To further clarify the situation, it was decided to obtain a ^{1}H–^{13}C heteronuclear shift correlation spectrum, since structures 2 and 3 would both be expected to show well-resolved correlations. Owing to the limited sample available, the most suitable technique was judged to be the inverse-mode HMQC experiment. An expanded section of the spectrum is shown in Figure 4, showing the region where the side-chain C–H correlations are observed. The assignments of the correlations for this region are given in Table II. In this case also, the expected unique correlations for structure 2 at δ 80.5–5.5 (Cα–Hα) and 82.0–4.75 (Cβ–Hβ) and for structure 3 at δ 75.9–6.0 (Cα–Hα) and δ 49.5–3.35 (Cβ–Hβ) are not present in the spectrum. The Cγ–Hγ'/Cγ–Hγ'' correlations would be expected to appear in the region of the cross-peaks from the Cγ–Hγ'/Cγ–Hγ'' of the other structures.

There are a number of as yet unassigned resonances observed in the HMQC spectrum, and, in combination with the information obtained from the HOHAHA spectra and chemical shift information from model compounds, other candidate structures which would account for the presence of these correlations are under investigation.

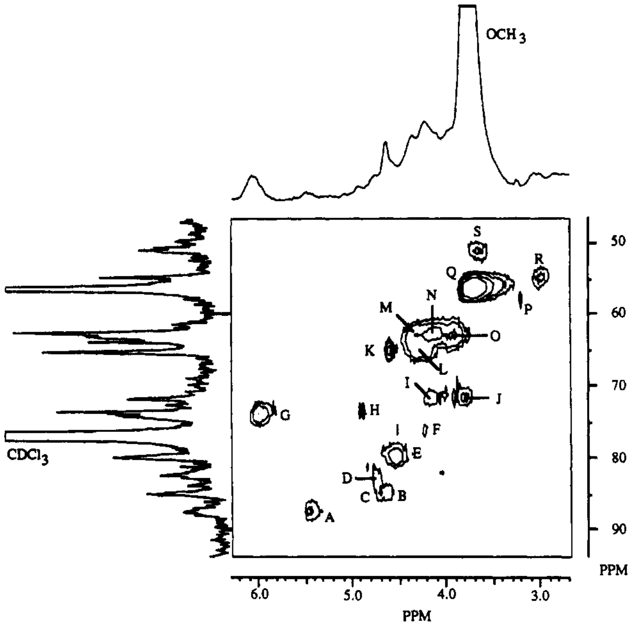

Figure 4. Aliphatic side-chain region of a phase-sensitive ^{13}C-decoupled ^{1}H-detected ^{1}H–^{13}C HMQC chemical shift correlation spectrum of acetylated RSCL.

Table II. Assignment of Cross-Peaks from Aliphatic Side-Chain Region of 2D HMQC Spectrum of Acetylated RSCL

cross-peak	δ ^{13}C	δ ^{1}H	assignment
A	87.9	5.50	Cα/Hα, 4
B	85.5	4.68	Cα/Hα, 5
C	85.0	4.75	?[a]
D	83.4	4.80	?
E	79.5	4.58	Cβ/Hβ, 1
F	76.2	4.27	?
G	73.9	6.01	Cα/Hα, 1
H	73.5	4.85	?
I	71.8	4.20	Cγ/Hγ'', 5
J	71.8	3.85	Cγ/Hγ', 5
K	65.0	4.65	Cγ/Hγ, 7
L	65.0	4.30	?
M	62.6	4.32	Hγ, 1, 4
N	62.6	4.16	Hγ, 1, 4
O	62.6	4.00	Hγ, 1, 4
P	57.5	3.27	?
Q	56.0	3.75	OCH_3
R	54.5	3.06	Cβ/Hβ, 4
S	50.5	3.66	Cα/Hα, 7

[a] ? denotes unassigned correlation.

ROESY Spectrum. Although lignin is commonly assumed to be random in structure, it has been postulated that there is a degree of regularity in its structure. It has been suggested that the aromatic rings in lignin take up a parallel orientation,[25] and other evidence based on size exclusion chromatography[26] suggests some form of order in the structure of lignin. If the lignin does in fact possess some form of ordered structure, it may be possible to observe through-space interactions using NOE techniques.

A 2D rotating-frame Overhauser experiment (ROESY) was carried out, and a region of this spectrum is shown in Figure 5. Intraunit magnetization transfer correlations were observed between the following: methoxyl groups and H2 on the aromatic rings (cross peak A); Hγ in structures such as 1 and 4 and H2 and H6 on the aromatic rings (cross peak B); Hβ in structure 1 and H2/H6 (cross-peak C); Hα

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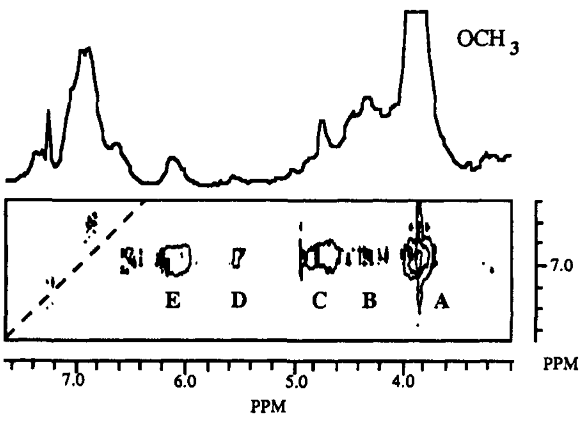
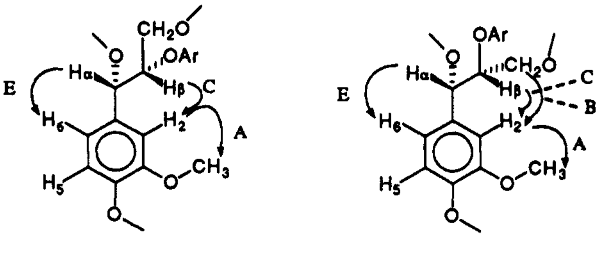


Figure 5. Part of the 2D phase-sensitive ROESY spectrum of acetylated RSCL, showing cross-peaks assigned to intraunit NOE. Contours negative with respect to the diagonal are plotted. The dotted line represents the position of the diagonal.



(a) (b)

Figure 6. (a) NOE interactions observed from interunit linkages of type 1 in a gauche conformation. (b) NOE interactions observed from interunit linkages of type 1 in an anti conformation.

in structure 4 and H2/H6 (cross peak D); and Hα in structure 1 and 7 and H2/H6 (cross-peak E).

Apart from the presence of cross-peak B, these data are consistent with a solution conformation of the lignin side-chain structures as shown in Figure 6a. This is in accord with conformations observed in NOE[27,28] and crystallographic[29] studies on certain lignin model compounds, where the two aromatic rings are gauche with respect to each other. The presence of the weaker cross-peak B suggests that there is an interaction between the methylene protons (Hγ) and the aromatic ring protons in structure 1. For such a cross-peak to occur, the aromatic rings must be anti with respect to each other (see Figure 6b). Both the gauche and anti conformations are potential energy minima as evidenced by molecular mechanics modeling, and it appears that since cross-peak C is so much stronger than cross-peak B, more of the structure 1 side chains are in a gauche conformation than are in an anti conformation.

The ROESY data presented above represent the first reported information on the solution-state conformation of lignin, albeit in an organic solvent. The discussion of the ROESY data above assumes that the cross-peaks observed arise from intraunit magnetization transfer; the possibility does exist, however, that owing to the broadness of the resonances, cross-peaks arising from interunit magnetization transfer could be hidden under those rising from intraunit magnetization transfer.

In summary, the interpretation of the ^{1}H and ^{13}C NMR spectra of lignin has been facilitated by application of the HOHAHA, HMQC, and ROESY techniques. The presence of most major structural units in acetylated RSCL has been confirmed, and the lack of correlations from structures 2 and 3 in all three techniques suggests that the importance of these structures should be reevaluated. Further investigations, on this, and other types of lignin, aimed at determining connectivity between different types of interunit linkage involving selective excitation and long-range C–H correlations are underway. The application of these techniques to the detection of covalently bonded lignin–carbohydrate moieties in lignocellulosics is also an area of active investigation.

Experimental Section

The RSCL sample was obtained and acetylated as described previously.[17] The lignin was acetylated before analysis to improve its solubility in CDCl$_3$ and to improve spectral dispersion of side-chain protons. A 30-mg sample in 0.4 mL of CDCl$_3$ (Aldrich) was used.

All spectra were obtained at 7 T and 300 K on a Bruker AC300 spectrometer, operating at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C, using a dual ^{1}H/^{13}C probe with conventional geometry. Chemical shifts were measured relative to hypothetial internal TMS. The 2D spectra were obtained using standard Bruker software (DISNMR89).

Phase-sensitive HOHAHA spectra were acquired over a 3333 Hz window, using a 1K × 512 data matrix, and 256 increments were obtained , with 16 scans per t_1 increment. The MLEV-17 spin-lock period of 25 ms (Figure 2) and 150 ms (Figure 3) was preceded and followed by 2.5-ms trim pulses. The data was processed with a sine-bell squared function shifted by $\pi/2$ and was not symmetrized.

The phase-sensitive ^{1}H-detected HMQC spectrum (incorporating the bilinear rotation sequence[15]) was acquired over an F_2 spectral window of 9056 Hz, and an F_1 window of 6060 Hz, with a 2K × 128 matrix (zero-filled to 256 in F_1), and 128 scans per increment. Sine-bell window functions shifted by $\pi/2$ were applied in both domains. The delay for polarization transfer was set for an assumed $^{1}J_{C-H}$ of 140 Hz, and a delay between the BIRD portion and the HMQC portion of the pulse sequence was set to 400 ms. The ^{13}C GARP-1 decoupling during acquisition of the HMQC spectra was delivered by a Bruker BFX-5 amplifier. Total measuring time was 6 h.

The phase-sensitive ROESY spectrum was acquired over a 3246-Hz spectral window, with a 2K × 256 matrix (zero-filled to 512 in F_1), with 16 scans per increment. Sine bell window functions shifted by $\pi/2$ were applied in both dimensions. The CW spin-lock was applied for 200 ms at a power level of ca. 0.5 W, resulting in a spin-lock field of 2 kHz.

To offset problems with base-line distortion in all spectra (a particular problem with the large dynamic ranges required in lignin spectra), the calculated preacquisition delay (DE) was reduced by ca. 25%, the filter width (FW) was increased by 100%, and the spectra were acquired in simultaneous acquisition mode.[30]

Molecular modeling was performed using ChemMod II software from Advanced Processor Research Ltd, distributed by Fraser Williams (Scientific Systems) Ltd (U.K.). Calculations were performed on an Apple Macintosh IIfx. Structures were minimized using the Newton–Raphson minimizer and further structural refinements were obtained by selecting minima in the potential energy vs torsion angle plots.

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Registry No. Lignin, 9005-53-2.

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