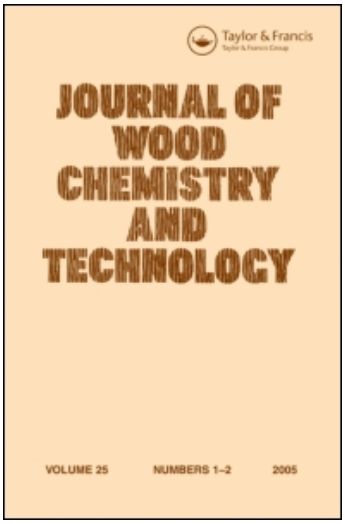

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NMR Studies on the Occurrence of Spirodienone Structures in Lignins

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NMR Studies on the Occurrence of Spirodienone Structures in Lignins

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Abstract: Spirodienone structures have been detected in spruce, birch, and kenaf lignin isolates. NMR signals corresponding to guaiacyl and syringyl spirodienones were fully identified and assigned based on ^{13}C, QUAT, HSQC, HSQC-TOCSY, and HMBC NMR data. Spruce lignin contains spirodienone structures of the guaiacyl type. Syringyl spirodienones dominate in kenaf and birch lignins. Each type of spirodienone was found to be present in two different stereoisomeric forms, with one of the isomers

This article is dedicated to the memory of Professor Josef S. Gratzl with gratitude and admiration for his ability to combine science and technology in a deeply humanistic manner.

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being more prevalent. Signal integrations indicate that about three spirodienones per 100 phenylpropanoid units are present in the spruce and birch lignins and about four in the kenaf lignin.

Keywords: Lignins, spruce, birch, kenaf, spirodienone structure, β–1-structure, NMR analysis

INTRODUCTION

The presence of β–1-structures **1** (1,2-diarylpropane-1,3-diols) in lignins was first noted by the isolation of dimeric and oligomeric lignin fragments containing these structures from spruce and beech wood after mild acidic hydrolysis in dioxane-water,[1,2] and by the isolation of degradation products that could be derived from them after acid hydrolysis.[3] A major fraction of the dimers resulting from the widely applied thioacidolysis/Raney-Ni degradative analysis were β–1-derived,[4] as were the dimers from the DFRC method.[5] However, results from some early NMR observations,[6–8] as well as permanganate oxidation[9,10] and ozonation[11] analyses have suggested that the β–1-structure, if present, might only be a minor component in lignin. To explain the discrepancies among the results from different studies, it has been suggested that a precursor of the β–1-unit **1**, a cyclohexadienone structure **2** might be present in native lignin.[3,12,13] On acid hydrolysis, the dienone structures would be converted to the conventional β–1-structures **1**, which would explain the observation that lignin fragments containing β–1-structures are obtained after acid hydrolysis of wood.

There have been several reports providing supporting evidence for the presence of a cyclohexadienone **2** precursor in lignin. Mild acid hydrolysis of spruce wood meal that was pre-methylated with diazomethane, resulted in the release of lignin β–1-dimers **1** predominantly carrying a free phenolic hydroxyl group on ring B.[14] This observation supported the idea that ring B did not have a phenolic-OH that could be methylated during treatment with diazomethane, that is, that it was originally present in a dienone structure. The NMR observation of an isochroman structure **3** and isolation of the derived dimer among lignin degradation products after DFRC treatment,[15] and the isolation of the sesquineolignan woorenol[16] **4** suggested that the β–1-unit may be present in the form of a spirodienone structure **6**. The possibility of spirodienone structures in lignin was further supported by the synthesis of a syringyl spirocyclohexadienone structure **5** by radical coupling of lignin-like model compounds.[17] NMR evidence for the presence of spirodienones in a ^{13}C-enriched poplar lignin was also tentatively observed and reported.[18]

In a previous communication,[19] spirodienone structures in spruce and aspen lignins were preliminarily revealed by compelling partial NMR evidence. It was found that the β–1-structure in the lignin polymer is

predominantly present in the form of the spirodienone structure **6** rather than the conventionally described 1,2-diarylpropane-1,3-diol ($\beta-1$) structure **1**. In this article, detailed NMR data are presented, clearly demonstrating the occurrence of guaiacyl spirodienone structures **6G** in spruce and, additionally, syringyl analogs **6S** in birch and kenaf lignins (Fig. 1).

EXPERIMENTAL

Milled wood lignin (MWL) was prepared from pre-extracted wood meal from a freshly cut mature (50–60 year old) Norway spruce (*Picea abies*, lignin yield 15%) and from birch (*Betula pendula*, lignin yield 13%) according to a standard method.[20] Kenaf lignin was isolated from bast fibers from the core of Tainung kenaf stems.[21] Acetylation of lignin was carried out as described in Reference [22] but without performing the purification step. Acetylated lignin samples (~110 mg) were dissolved in acetone-d_6

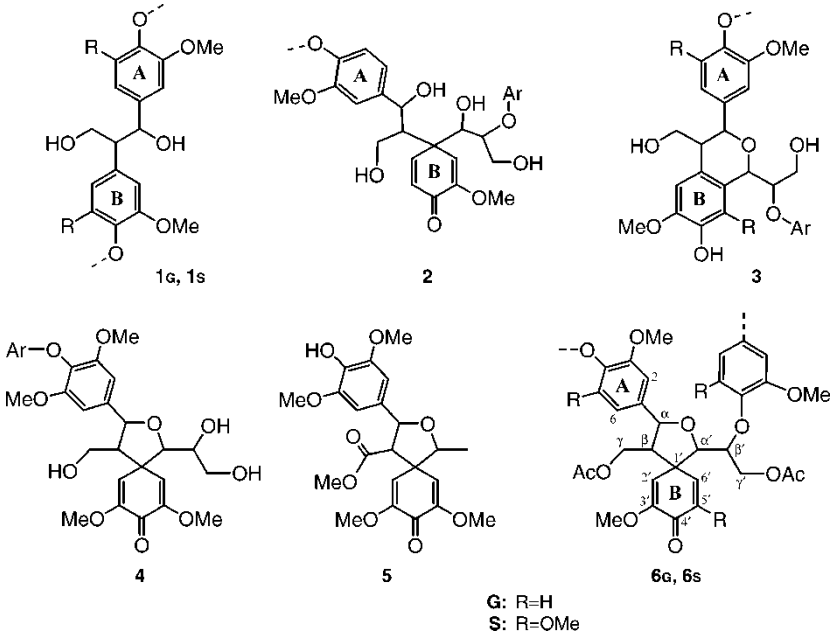


Figure 1. The conventional $\beta-1$-structures **1**, proposed dienone structures **2**, aryl isochroman structures **3** found in pine lignins, isolated natural sesquineolignan woorenol **4**, synthetic spirodienone **5**, and guaiacyl **6G** and syringyl **6S** spirodienone structures in lignins. **G** = guaiacyl, **S** = syringyl. Note: the parent compounds implicated in the lignins are shown; only 4–O-attachment is indicated but guaiacyl units may also be 5-linked.

(600 μL) and transferred into 5-mm NMR tubes. TMS was used as the reference for chemical shift values. NMR experiments were run on a Bruker Avance 400 MHz instrument or, when specified, an Avance 600 instrument fitted with a cryoprobe. Standard Bruker pulse programs were used in all experiments. The quantitative ^{13}C NMR experiments were performed with a 5-mm broadband probe and with an inverse-gated proton-decoupling sequence. A pulse angle of 90° and a delay time of 12 s between scans were applied during data acquisition.[23] QUAT (pulse program "quatd") NMR spectra that identify non-protonated carbons[24] were recorded with the same broadband probe. Both ^{13}C and QUAT NMR spectra were acquired with a spectral window of 250 ppm and an acquisition time of 0.65 s. 2D NMR experiments were performed on the 400 MHz instrument with a proton-carbon selective inverse probe equipped with a Z-gradient coil; on the 600 MHz instrument, a triple resonance inverse-detected cryoprobe with Z-gradients was used. Detailed explanation of the different 2D NMR techniques used in this work can be found in Reference [25]. HSQC (pulse program "invieagssi") spectra that correlated directly bonded proton and carbon signals were acquired with a spectral window of 12.8 ppm in F2 and 150 ppm in F1 with 2K × 1K increments, giving an acquisition time of 0.2 s in F2. The spectral center was set at 5.3 ppm in the ^{1}H dimension and at 91 ppm in the ^{13}C dimension. Typical parameters for HSQC data acquisition included: an acquisition time of 0.2 s, a relaxation delay of 0.7 s between scans, an average coupling constant of 150 Hz, an INEPT transfer delay time (1/4J) of 1.67 ms corresponding to a 150 Hz coupling constant, 32 scans per increment. HSQC-TOCSY (pulse program "invietgpml") spectra that correlate protons and carbons within a continuous chain of CH_n groups were acquired with a spectral window of 7.0 ppm in F2 and 80 ppm in F1 with 2K × 1K increments. The spectral center was set at 5.0 ppm in the ^{1}H dimension and at 65 ppm in the ^{13}C dimension. Typical parameters for HSQC-TOCSY data acquisition included: a relaxation delay of 0.5 s between scans, an average coupling constant of 150 Hz, a TOCSY mixing delay of 60 ms, 256 scans per increment. HMBC (pulse program "inv4gslplrnd") spectra that correlate protons and carbons two to three bonds away were acquired on the 600 cryoprobe instrument with a spectral window of 6.0 ppm in F2 and 150 ppm in F1 with 2K × 342 increments. The spectral center was set at 5.1 ppm in the ^{1}H dimension and at 115 ppm in the ^{13}C dimension. Typical parameters for HMBC data acquisition included: an acquisition time of 0.285 s, a relaxation delay of 1 s between scans, an average coupling constant of 145 Hz, an evolution delay of 80 ms for long range couplings, 104 scans per increment. HSQC and HSQC-TOCSY spectra were processed with 2K × 2K data points using the $\pi/2$ shifted sine bell window function in both dimensions. HMBC spectra were processed with 2K × 1K data points using matched Gaussian apodization in the F2 dimension and an unshifted squared-sine-bell window function in the F1 dimension.

RESULTS AND DISCUSSION

According to results from quantitative ^{13}C NMR analysis, the spruce lignin (Figure 2a) contained essentially only guaiacyl units, the birch lignin (Figure 2c) about 40% guaiacyl and 60% syringyl units whereas the aromatic units in kenaf lignin (Figure 2e) were found to be predominantly of the syringyl type (about 80%). Signals between 102–109 ppm were assigned to syringyl C-2,6 and those between 110–115 ppm were assigned to guaiacyl C-2. Spirodienone structures were found to be present in all three lignins. Their corresponding NMR signals were unambiguously assigned by a combination of NMR methods. In spruce lignin, the guaiacyl spirodienone **6G** structure was observed, whereas in kenaf lignin the syringyl spirodienone structure was predominant. In birch lignin, both the guaiacyl **6G** and syringyl **6S** spirodienones were observed, with the syringyl type being the more abundant.

In the ^{13}C NMR spectra of the acetylated birch (Figure 2c) and kenaf (Figure 2e) lignins, the two carbon signals appearing at 176.6 and 53.2 ppm are assigned to the carbonyl carbon (C-4′) and the quaternary carbon (C-1′) of the syringyl spirodienone structures (**6S** in Figure 1). These recorded chemical shift values are compatible with published NMR data of woorenol **4** (179.5 and 54.2 ppm, in CD_3OD)[16] and of a synthetic syringyl spirodienone structure **5** (176.0 and 52.1 ppm, in $CDCl_3$).[17] The identities of these two carbons as quaternary nuclei were confirmed by QUAT NMR spectra (e.g., Figure 2d for birch) in which protonated carbons are highly suppressed. In the ^{13}C NMR spectrum of a (unacetylated) spruce lignin (Figure 2a), a signal for the carbonyl carbon (C-4′) of the guaiacyl spirodienone structure **6G** can be observed at 182.5 ppm. After acetylation, this carbonyl carbon signal appears at 180.8 ppm (Figure 2b). A resonance from the quaternary C-1′ in the guaiacyl spirodienone structure could not be observed directly in the conventional ^{13}C NMR spectrum because of signal overlap by the intense methoxy peak (Figure 2a). It was, however, revealed in the QUAT NMR spectrum at 55.4 ppm (Figure 2b).

To confirm the aforementioned assignments and to further investigate the occurrence of the spirodienone structures in the lignins, 2D HSQC, HSQC-TOCSY and HMBC NMR spectra were recorded. In 2D HSQC spectra, signals for most of the CH_n groups from the syringyl and guaiacyl spirodienone structures in the lignin samples were observed and tentatively assigned. Signals for the γ-CH_2 groups could not be observed directly in the HSQC spectrum of spruce lignin (Figure 3a) because of signal overlap, but these signals can be clearly observed in the HSQC spectrum of kenaf lignin (Figure 3c) which is structurally simpler than the other two lignins because of its high syringyl content.[21] Both the guaiacyl and the syringyl spirodienone structures **6G** and **6S** seem to be a mixture of two different stereoisomers; most of the spirodienone CH_n signals appear in pairs in the HSQC NMR spectra, Figures 3 and 4. One of the isomers appears to be

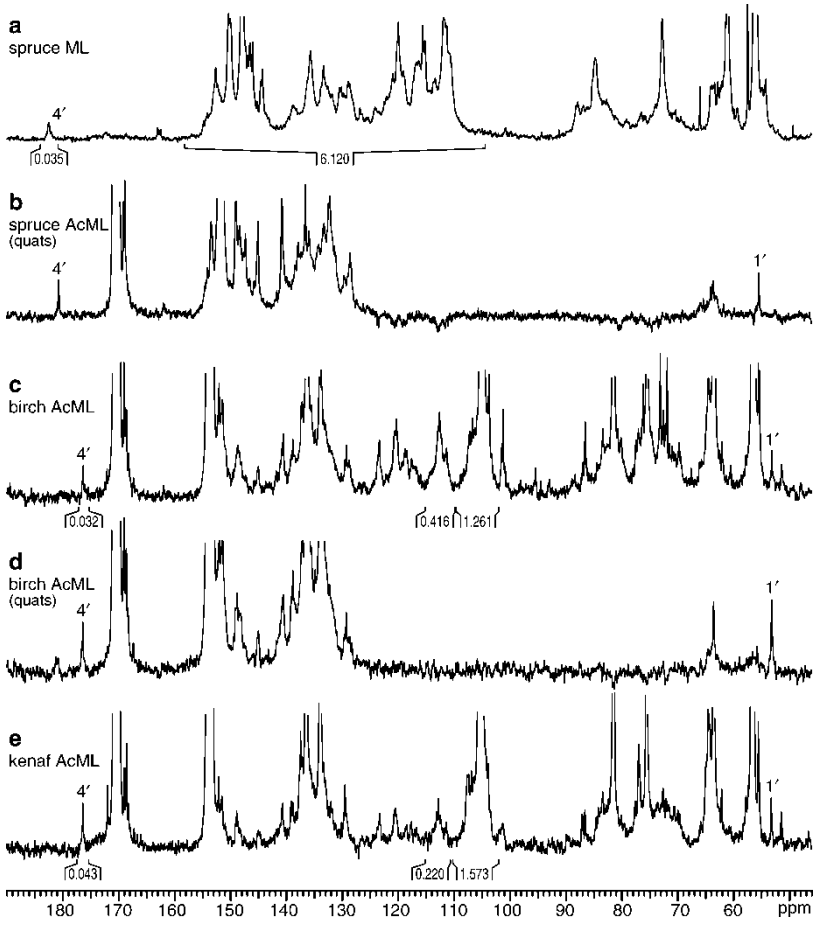


Figure 2. Observation of C4′ and C1′ carbons from the guaiacyl spirodienone structures **6G** in: (a) the quantitative ^{13}C NMR spectrum of spruce lignin, (b) the QUAT NMR spectrum of acetylated spruce lignin; observation of C4′ and C1′ from the syringyl spirodienone structures **6S** in: (c) the quantitative ^{13}C NMR spectrum of acetylated birch lignin, (d) the QUAT NMR spectrum of acetylated birch lignin, and (e) in the quantitative ^{13}C NMR spectrum of acetylated kenaf lignin.

more abundant than the other one, particularly in the syringyl case. The major differences between the NMR signals from the guaiacyl **6G** and the syringyl **6S** dienone structures were observed in the double bond signal area in the HSQC spectra, Figure 4. Signals 2′,5′, and 6′ in Figure 4a are assigned to the guaiacyl spirodienone structures **6G**, whereas signals 2′ and 6′ in Figure 4c are assigned to the syringyl analogs **6S**. Both guaiacyl and syringyl spirodienone structures are present in birch lignin (Figure 4b), with the syringyl ones being more abundant.

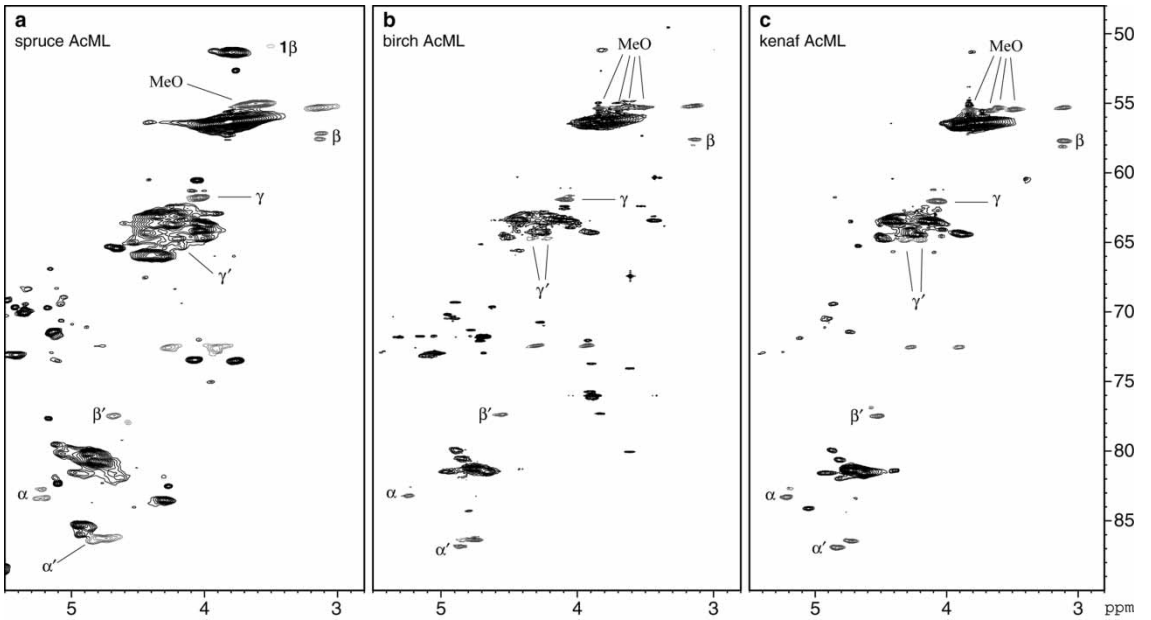


Figure 3. Partial aliphatic (side-chain) regions of 2D HSQC NMR spectra of acetylated lignins from: (a) spruce, (b) birch, and (c) kenaf. Signals from **6G** and **6S** are marked and identified. A signal from **1G** can also be seen in spectrum (a). (Spectra for the web version are colored to make identification easier, **6** in red, **1** in cyan and, for reference, resinol structures in purple).

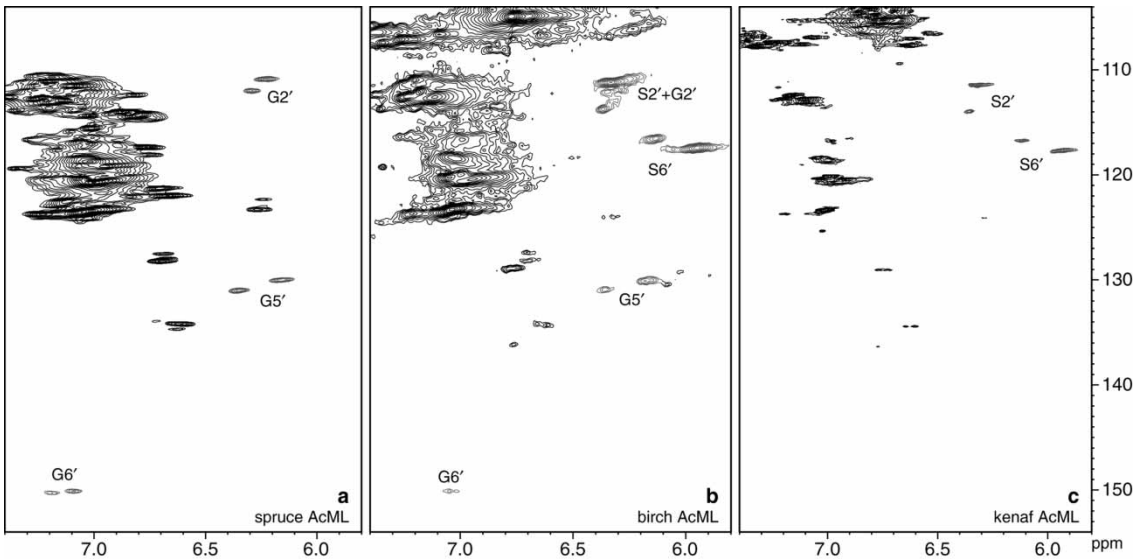


Figure 4. Partial aromatic/ethylenic regions of 2D HSQC NMR spectra of acetylated lignins from: (a) spruce, (b) birch, and (c) kenaf. Signals from **6G** and **6S** are marked and identified. Note: contour levels have been chosen to readily visualize the peaks of interest; the birch lignin is shown nearer the baseplane level to reveal the **6G** components. (Colorized in the web version).

Correlations between neighboring CH_n groups in the same coupling network were identified in the elegant 2D HSQC-TOCSY NMR spectrum (Figure 5). Usefully redundant correlations between a sidechain carbon and not only its attached proton but all of the protons in the same sidechain (or alternatively viewed, between a sidechain proton, its attached carbon, and all carbons in its sidechain) allow the carbon and proton networks for each of the sidechains to be clearly delineated. Long-range connections over 2–3 bonds between the two side chains and the dienone ring were established by 2D HMBC NMR spectra. As shown in Figure 6, a signal for C2′ at 111.4 ppm can be observed to have long range correlations with H6′, Hα' and, diagnostically, with Hβ from the other unit. Many other long-range correlations can also be observed in the HMBC NMR spectrum that support the occurrence of the spirodienone structures, the most diagnostic being the correlations of quaternary carbon C1′ with Hγ and Hβ, and Cα' with Hα and H6′.

^{13}C chemical shift values for C3′ and C5′ as well as ^{13}C and ^{1}H chemical shift values for 3′,5′-methoxy groups were also identified by combining signals observed in the HSQC and HMBC spectra (Figure 7). C5′ is expected to show long range correlations with H6′ and the 5′-OMe proton signals, whereas C3′ should show correlations with H2′ and 3′-OMe protons in the HMBC spectrum. In Figure 7, it is the NMR signals from the more abundant spirodienone isomeric structures that are marked and assigned.

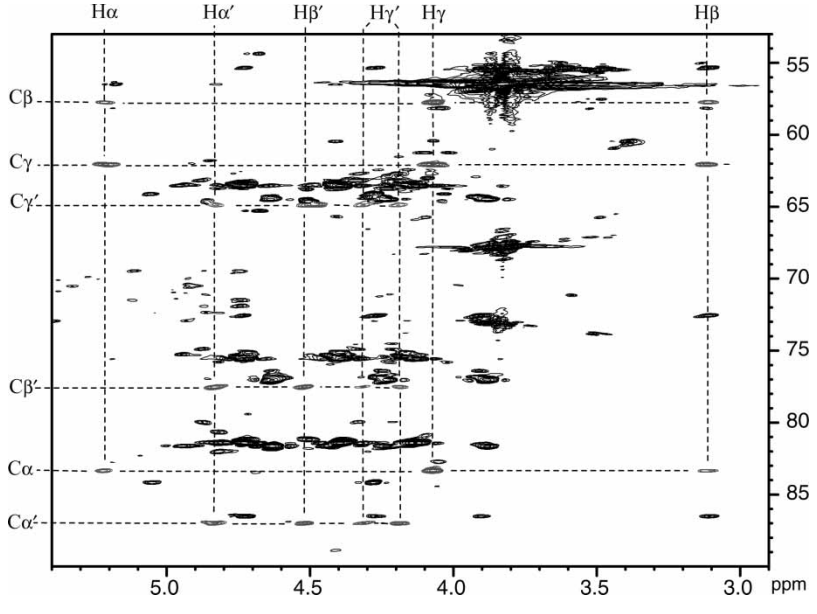


Figure 5. Partial HSQC-TOCSY spectrum of acetylated kenaf lignin, revealing the two independent side chain CH_n coupling networks of the syringyl spirodienone structures **6S**. (Colorized in the web version).

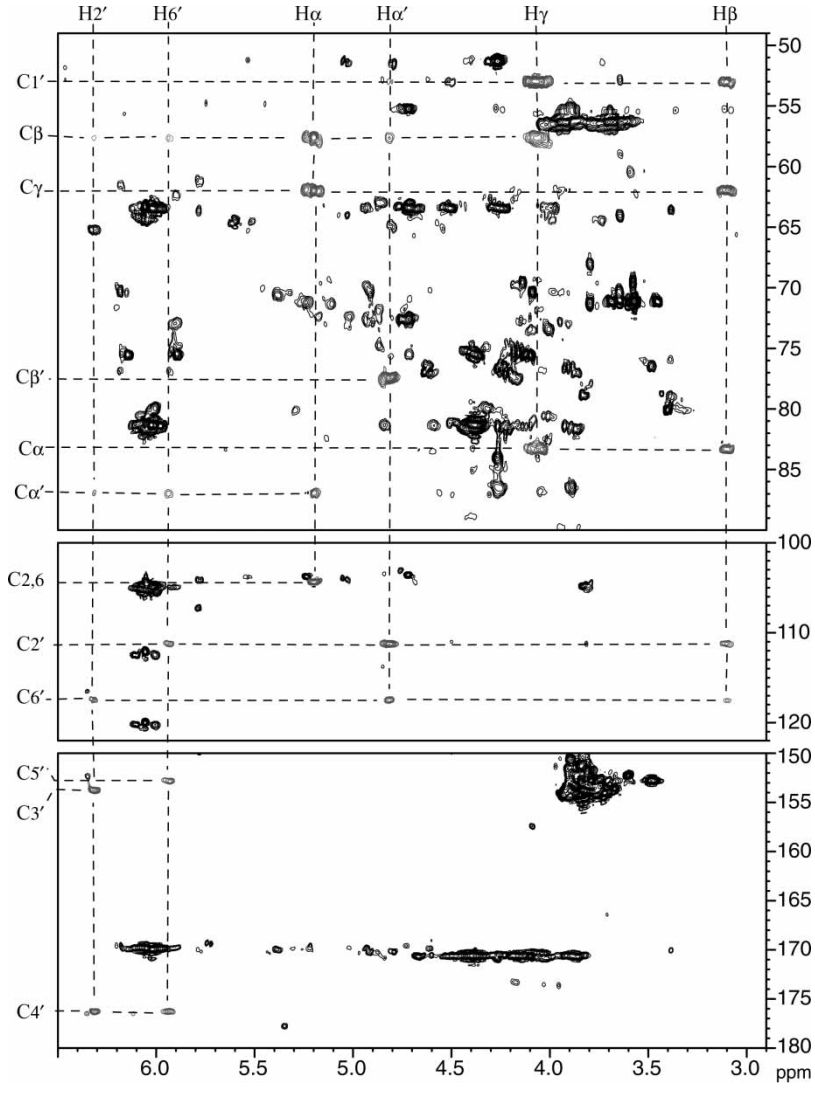


Figure 6. Partial HMBC spectra of acetylated kenaf lignin, establishing the long-range correlations between the two side chains and the spirodienone ring of **6S**. (Colorized in the web version for increased clarity).

The NMR signals from the less abundant isomer can also be clearly observed in the spectra and their assignments are listed in Table 1.

The aforementioned NMR observations unambiguously demonstrate the occurrence of spirodienone structures in spruce, birch, and kenaf isolated lignins. The ^{13}C and ^{1}H chemical shift values for both guaiacyl and syringyl spirodienone structures **6G** and **6S** are fully identified and

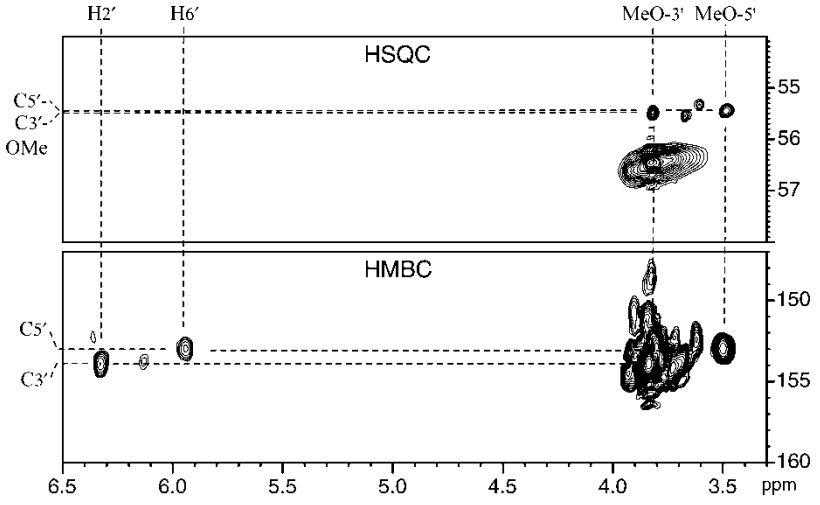


Figure 7. Assignment of NMR signals for C3′, C5′, and MeO groups from **6S** by combining HSQC and HMBC NMR spectra of kenaf lignin.

assigned. The observed NMR data are summarized in Table 1. The proportions of the dienone structures in the lignins were determined by signal integration of the carbonyl signal (C4′, 180.8 ppm for guaiacyl spirodienones and 176.4 ppm for syringyl spirodienones) in the quantitative ^{13}C NMR spectra (Figure 2). Signal integrations for the whole aromatic and ethylenic

Table 1. ^{1}H and ^{13}C NMR data for **II** and **III** in acetone-d_6

Guaiacyl spiro-dienone (**II**)		Syringyl spiro-dienone (**III**)	
Position	δ_H/δ_C (ppm)	Position	δ_H/δ_C (ppm)
α	5.22/82.7, 5.19/83.3	α	5.21/83.3, 5.19/82.7
β	3.12/57.2, 3.13/57.6	β	3.10/57.8, 3.11/58.2
γ	4.03/61.8	γ	4.07/62.1
α'	4.84/86.5	α'	4.81/87.1, 4.85/87.0
β'	4.68/77.5, 4.57/78.0	β'	4.53/77.6, 4.59/77.0
γ'	4.19, 4.31/65.1	γ'	4.21, 4.32/64.9
1′	55.4	1′	53.2
2′	6.23/110.9, 6.29/112.0	2′	6.36/113.9, 6.33/111.4
3′	153.5, 154.1	3′	154.0, 152.3
4′	180.8	4′	176.4
5′	6.15/130.0, 6.35/131.0	5′	153.0, 153.9
6′	7.09/150.1, 7.18/150.3	6′	5.94/117.7, 6.12/116.7
MeO	3.57/55.0, 3.70/55.2	3′-MeO	3.82/55.5, 3.60/55.4
		5′-MeO	3.50/55.5, 3.67/55.6

carbon region between 102 to 158 ppm were set to 6.12 carbons for spruce lignin, 6.06 carbons for birch, and 6.04 for kenaf lignin. These values were used as the internal references for calculation of relative signal integrals.[23] The contribution of ethylenic carbons in these lignins were estimated by measuring the signals for coniferaldehyde and coniferyl alcohol structures. Coniferaldehyde contents in the spruce, birch, and kenaf lignins were found to be 3.5, 1.5, and 1.0 units per 100 phenylpropanoid (C9) units respectively, according to both the proton signal at 9.67 ppm and the ^{13}C signal at 194.1 ppm. It is quite difficult to estimate the content of coniferyl alcohol structures in lignin polymer by conventional NMR analysis. By applying a newly developed quantitative 2D HSQC analysis,[26] it could be estimated that the contents of coniferyl alcohol in the spruce, birch, and kenaf lignins were less than 2.2, 1.4, and 1.0 units per 100 phenylpropanoid (C9) units, respectively. Therefore the content of guaiacyl and syringyl spirodienones can be determined and it was found that the spruce and the birch lignins contained about 3 spirodienone units per 100 phenylpropanoid (C9) units and the kenaf lignin about 4. To our knowledge, compounds containing the guaiacyl spirodienone structure have so far been neither synthesized nor isolated from plants. Attempts to synthesize appropriate model compounds bearing all the important functionalities in **6** are currently proving elusive.

It was also remarkably easy to identify correlations in spectra from acetylated and solubilized ball-milled entire cell wall material,[27] that is, without requiring lignin isolation. This was particularly trivial in kenaf where the levels are quite striking. Figure 8 shows partial HSQC spectra from a small region around the methoxyl groups of a kenaf isolated lignin and the solubilized whole cell wall sample. Although the resolution and signal-to-noise of the spectrum are necessarily lower in the more complex whole cell wall sample due to the presence of the overwhelming polysaccharides in the NMR tube, resinol and spirodienone β-C/H correlations are outstandingly well delineated from other signals.

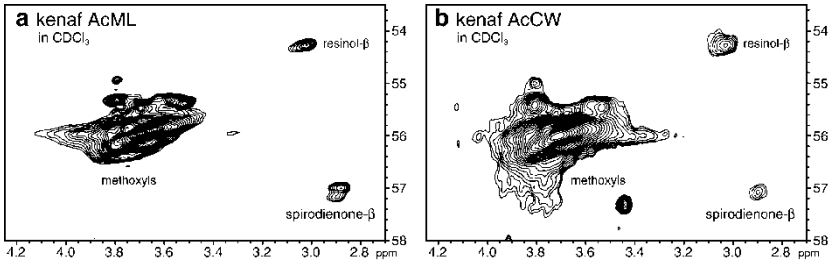
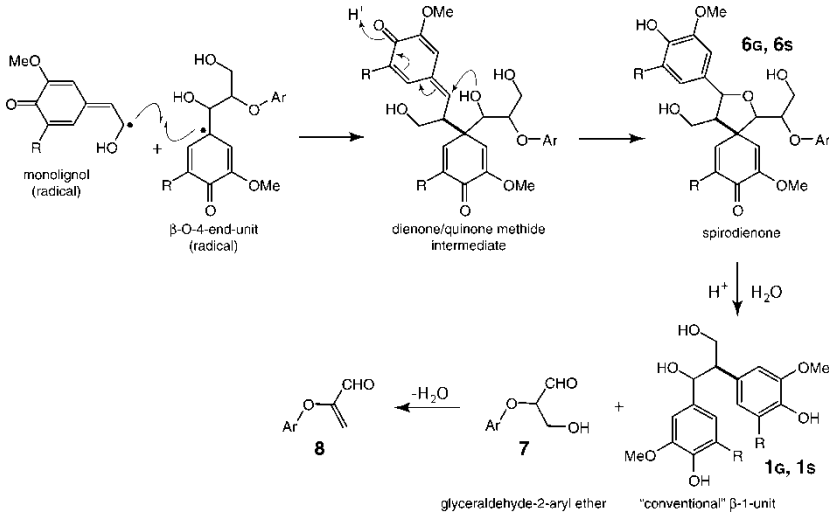


Figure 8. Partial HSQC spectra illustrating how readily spirodienones can be evidenced even in entire cell wall samples; (a) isolated kenaf acetylated lignin, (b) kenaf acetylated cell walls. These spectra are run in $CDCl_3$, a solvent in which acetylated ball-milled cell walls are more soluble; note that spirodienone resonances are markedly solvent-dependent, as can be seen in comparisons with Figure 3c.



Scheme 1. Suggested biosynthetic pathway for formation of spirodienone structures **6** during lignification, and the likely degradation of the spirodienone **6** to the conventional β–1-dimer **1** during mild acid hydrolysis. Note that incipient lignification may alkylate available phenols.

The suggested biosynthetic pathway leading to the formation of spirodienone structures in lignins is shown in Scheme 1. The syringyl spirodienone structure **6S** may be more stable than the guaiacyl counterpart **6G**. Low levels of the 1,2-diarylpropane-1,3-diol structure **1G** were observed in the spruce guaiacyl lignin, Figure 3a, but **1S** can scarcely be observed in the kenaf or birch samples. Structures **1** might be formed in the spruce lignin as a result of degradation of spirodienones **6** during aging or ball-milling.[19] During mild hydrolysis (pH 3–4, 100°C) of spruce wood in aqueous media, part of the guaiacyl spirodienones **6G** has also been found to be converted into the β–1-signatures **1G** and **8**.[14,28] Structure **8** was presumably formed via structure **7** by loss of water.

CONCLUSIONS

Spirodienone structures are present in spruce, birch, and kenaf isolated lignins and presumably in all lignins. Spruce lignin contains spirodienones of the guaiacyl type. In birch and kenaf lignins, syringyl spirodienones dominate; small amounts of guaiacyl spirodienones are detected in birch lignin. The spirodienone structures are present in the lignin polymer in two different stereoisomeric forms, with one of the isomers more abundant than the other. Assigning these isomers awaits the synthesis of appropriate model

compounds. The spruce and birch lignins contained about 3 spirodienone structures per 100 phenylpropanoid units, and the kenaf lignin contained about 4.

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