Identification of Side-Chain Structures in a Poplar Lignin Using Three-Dimensional HMQC−HOHAHA NMR Spectroscopy

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The structure of an acetylated ^{13}C-enriched poplar wood lignin preparation was studied using three-dimensional HMQC−HOHAHA NMR spectroscopy. This method takes advantage of the large dispersion of ^{13}C chemical shifts to resolve individual ^{1}H chemical shifts. The whole spin system of a ^{1}H−^{13}C correlation observed in an HMQC spectrum, even for minor components and unknown structural units, can be traced out. It is shown here that both trans- and cis-isomers of 6,7-dihydrodibenzo(*e*,*g*)(1,4)-dioxocin, a recently discovered prominent linkage in softwood lignins, occur also in hardwood lignin. Moreover, the poplar lignin preparation contains small amounts of noncyclic α-aryl ether linkages. Signals from a side chain of unknown structure are tentatively assigned to a spiro-cyclohexadienone.

Keywords: *NMR; 3D HMQC−HOHAHA; lignin*

INTRODUCTION

Lignin is an aromatic biopolymer, an essential component in the woody stems of arborescent gymnosperms and angiosperms. It is an integral cell wall constituent in all vascular plants including the herbaceous varieties (Sarkanen and Ludwig, 1971). Lignin is unique among biopolymers in that there is limited control over its biosynthesis, and the factors guiding this process are not yet fully understood. Lignin consists mainly of substituted phenylpropane units that are linked together to form a polymer lacking regularity, crystallinity, or optical activity. There is no known method for isolating unaltered lignin from plant cell walls, and chemical (or biochemical) degradation methods produce low molecular weight products in modest yields only. The most frequently used procedure to isolate lignin is milling wood in a ball mill and then extracting the lignin with aqueous dioxane (Björkman, 1956). This milled wood lignin (MWL) is regarded as fairly similar to the lignin in the wood (Adler, 1977; Lundquist, 1992), although there are unresolved problems regarding its morphological origin (Lai and Sarkanen, 1971; Whiting and Goring, 1981).

In structural studies on isolated lignins using NMR, the assignments of ^{1}H and ^{13}C NMR signals have mainly been based on comparison with data from synthesized model compounds (Lundquist, 1980; Lewis et al., 1988; Robert, 1992; Lapierre et al., 1984; Ede and Brunow, 1992; Ede et al., 1990). When one or more of the signals from a model compound have been found in a lignin sample, it has been assigned to a structural unit having the same structure as the model compound. This method of structural analysis has some weaknesses. Some signals overlap heavily and, in addition, the natural ^{1}H line didths are large (the half-width of Hα in **1** is ∼120 Hz at 11.7 T). This makes unambiguous assignment of proton spectra difficult. It, for instance, prevents carrying out decoupling experiments to check that the signals belong to the same structural unit.

The application of 2D methods is a solution in many cases. The large dispersion of ^{13}C chemical shifts makes ^{1}H−^{13}C and ^{13}C−^{13}C correlated methods attractive for structural analysis. The HMQC experiment has enabled structural investigations of lignin at natural abundance of ^{13}C (Ede and Brunow, 1992; Fukagawa et al., 1991). The ^{13}C−^{13}C correlated experiment, 2D INADEQUATE, has been applied to ^{13}C-enriched MWL (Guittet et al., 1985; Bardet et al., 1986), allowing the assignment of ^{13}C chemical shifts of predominant C−C bonds of lignin.

Most of the spin systems in lignins have been assigned reliably by applying ^{1}H-detected ^{1}H−^{13}C correlation (HMQC) and homonuclear Hartman−Hahn (HOHAHA) in concert (Ede and Brunow, 1992; Guittet et

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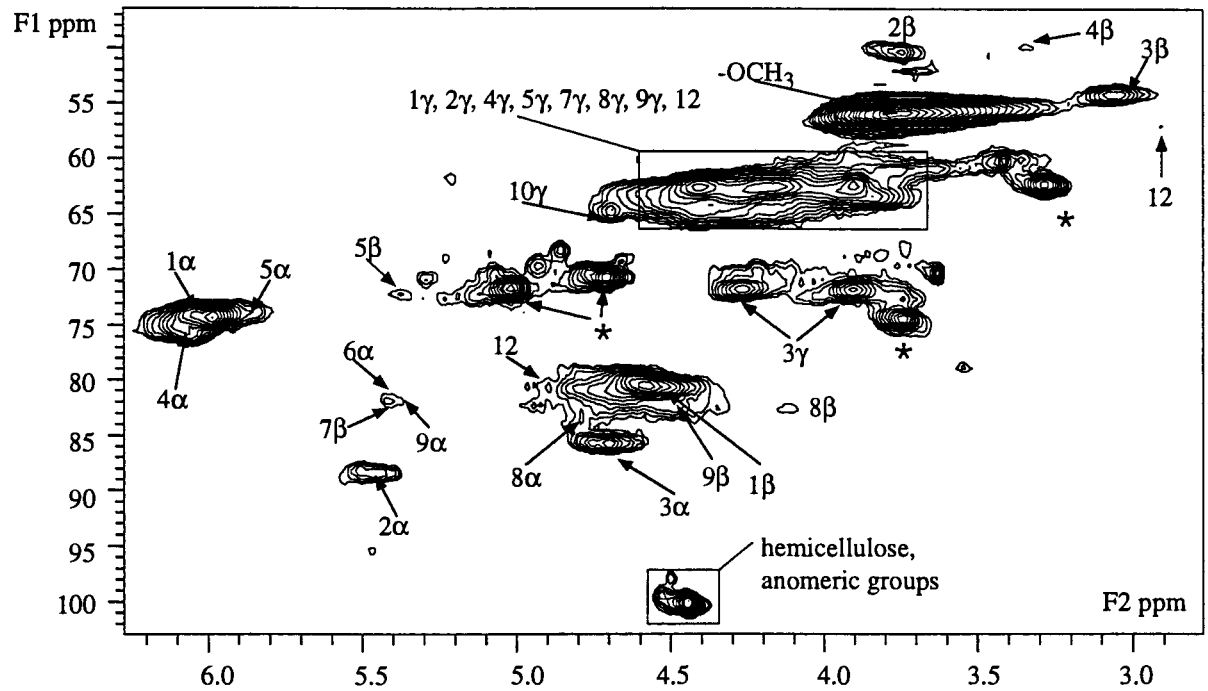

Figure 1. Expansion of the 2D HMQC spectrum of ^{13}C-enriched acetylated poplar MWL. The correlations are labeled as follows: the number refers to the structure and the letter to the proton, for example, for structure **1** 1α = Hα/Cα, 1β = Hβ/Cβ, 1γ = Hγ/Cγ, Hγ'/Cγ'. The correlations of xylan are marked with asterisks.

al., 1985; Kilpeläinen et al., 1994a; Kilpeläinen, 1994). There is also a method that employs a combination of HOHAHA and HMQC data, providing HMQC correlations together with HOHAHA in the same 2D plot (Ralph et al., 1997). This method (2D HMQC−HOHAHA) is very useful when preliminary results are wanted quickly, especially if one already has some supporting data or one is looking for some specific correlations. In all of these methods some overlap of resonances remains and the assignments are still guesswork.

We have found that a three-dimensional HMQC−HOHAHA method makes it possible to remove most of the remaining doubts in assigning NMR signals to structural units in a lignin sample. In a three-dimensional HMQC−HOHAHA spectrum the HOHAHA correlations are scattered according to their carbon chemical shifts, not only in one plane, as in 2D application, but in space (Brunow et al., 1998b). There are two advantages to this method: first, there is less overlap in the spectrum, because both carbon and proton chemical shifts in two spin systems very seldom coincide, and, second, one can always cross-check any ambiguous assignments from different planes of the 3D spectrum. This means that one can approach the data in the plane in which proton−proton correlations are extended according to their carbon chemical shifts (F2F3, i.e., HOHAHA) or in the plane in which proton−carbon correlations are spread according to proton chemical shifts (F1F2, i.e., HMQC). The main drawback of the 3D method is that ^{13}C enrichment is essential for obtaining reasonable measuring times. Three-dimensional heteronuclear NMR methods have been widely used for structural analysis of proteins, DNA, and RNA, but applications to other biopolymers are rare. In this paper we report ^{1}H and ^{13}C assignment of side-chain structures found in an acetylated ^{13}C-enriched hardwood (poplar) MWL preparation.

As we have reported in a previous paper (Kilpeläinen et al., 1994b), we have been able to find unambiguous evidence in a ^{13}C-enriched poplar MWL sample for the presence of a small amount of noncyclic α-aryl ether structures, **6**, for which there have been conflicting reports from previous studies. We can also show that both *trans*- and *cis*-dibenzodioxocin structures, **8** and **9**, which are recently discovered prominent structural units in softwood lignins (Karhunen et al., 1995), occur in poplar lignin. We have not been able to detect these structures at natural abundance of ^{13}C in hardwood lignin samples. In addition, a new yet unidentified side-chain structure is presented.

EXPERIMENTAL METHODS

All NMR spectra were obtained with a Varian Unity 500 spectrometer (11.7 T), with 5 mm inverse detection probe, and referenced to internal tetramethylsilane (0.0 ppm). The inverse detected ^{1}H−^{13}C correlation spectra, HMQC (Summers et al., 1986), and the homonuclear Hartman−Hahn spectra, HOHAHA (Griesinger et al., 1988), were measured as described earlier (Ede and Brunow, 1992; Ede et al., 1990).

^{13}C-enriched poplar wood MWL sample (16 mg) was acetylated and dissolved in $CDCl_3$ to increase the solubility and chemical shift dispersion of the side-chain units. The amount of ^{13}C in the sample was ∼6% (Lapierre et al., 1983).

Three-dimensional, ^{1}H-detected ^{1}H−^{13}C correlation−homonuclear Hartman−Hahn spectra, HMQC−HOHAHA, were acquired as described by Wijmenga et al. (1989). The spectral width was set to 6 kHz in F2 and F3 and to 25 kHz in F1; 32 transients in 70 time increments in F1 and F2 were collected using the hypercomplex method. The delay for polarization transfer between ^{1}H and ^{13}C was set to 3.6 ms ($\Delta = {}^{1}/_{2}$ J), with a relaxation delay of 0.5 s, and spin-lock lengths of 30, 60, and 100 ms were used to obtain the spectra. The time domain data of F2 was extended to 128 points using forward linear prediction, and the spectra were processed using Π/2 shifted squared sinebell functions in all dimensions prior to Fourier transformation.

RESULTS AND DISCUSSION

The spin systems were traced out as described previously (Kilpeläinen et al., 1994b). First, a correlation in

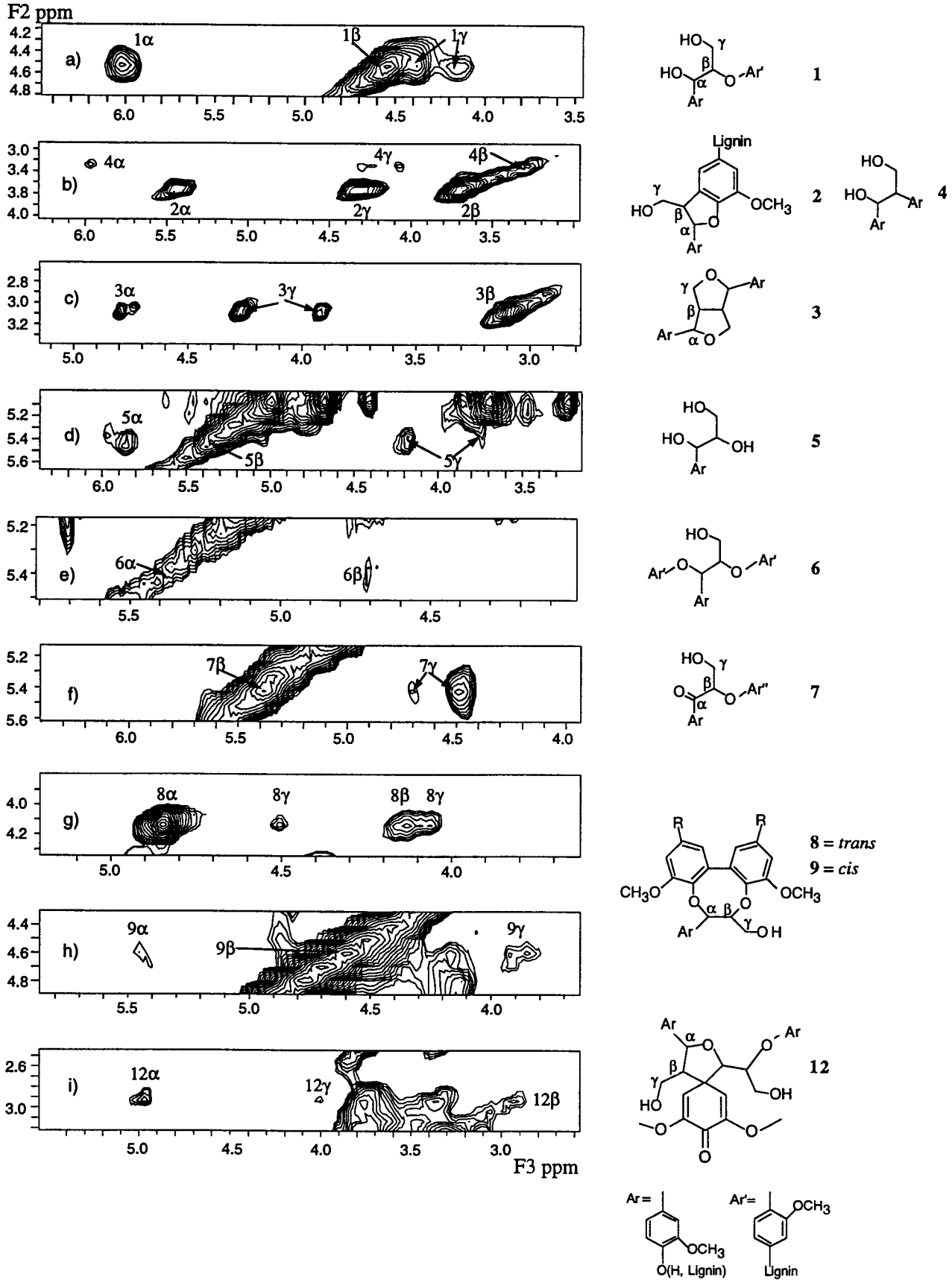

Figure 2. Selected HOHAHA (F2F3) slices of 3D HMQC−HOHAHA spectrum of ^{13}C-enriched acetylated poplar MWL. Spectrum is recorded with 30 ms spin-lock period. Slices are selected to show usually Hβ/Cβ correlation at diagonal, and on the horizontal line are shown the other protons of the same spin system. (a) Hβ/Cβ (4.60/80.6 ppm) of structure **1**; (b) Hβ/Cβ (3.80/50.4 ppm) of structure **2** and Hβ/Cβ (3.35/50.4 ppm) of structure **4**; (c) Hβ/Cβ (3.10/54.4 ppm) of structure **3**; (d) Hβ/Cβ (5.41/72.9 ppm) of structure **5**; (e) Hα/Cα (5.44/81 ppm) of structure **6**; (f) Hβ/Cβ (5.45/82.3 ppm) of structure **7**; (g) Hβ/Cβ (4.14/82.5 ppm) of structure **8**; (h) Hβ/Cβ (4.54/83.0 ppm) of structure **9**; (i) Hβ/Cβ (2.94/57.4 ppm) of unknown structure **12**.

Table 1. ^{1}H and ^{13}C Assignments of ^{13}C-Enriched Acetylated Poplar MWL Side-Chain Structures on the Basis of 3D HMQC−HOHAHA Spectra (Chemical Shift Dispersion in Parentheses)[a]

	^{1}H assignments			^{13}C assignments		
structure	Hα	Hβ	Hγ	Cα	Cβ	Cγ
1	6.02 (5.88−6.18)	4.60	4.20, 4.43 (4.00−4.48)	74.5 (73.0−76.5)	80.6	63.4 (60.9−67.3)
2	5.51 (5.44−5.56)	3.80 (3.73−3.93)	4.33, 4.46 (4.12−4.66)	88.3 (87.0−90.0)	50.4	65.3 (60.9−67.3)
3	4.72	3.10	3.93, 4.30	85.8	54.4	71.9
4	6.03 (5.88−6.18)	3.35	4.14 (4.08−4.29)	74.7 (66.1−79.7)	50.4	63.4 (60.2−66.8)
5	5.90 (5.81−6.10)	5.41	3.81, 4.24	74.7 (73.4−74.4)	72.9	62.5
6	5.44	4.75	*	81.9	*	*
7		5.45 (5.35−5.56)	4.56, 4.77		82.3	65.3
8	4.85	4.14	4.50, 4.05	84.4	82.5	63.8
9	5.33	4.54	3.79	81.6	83.0	63.3
10	6.57	6.19	4.75	134.0	123.3	65.2
11	6.58	7.38	9.85	127.6	153.1	191.1
12	4.96	2.94	4.01	82.3	57.4	61.8

[a] The asterisk indicates that these structures are of very low abundance and still overlapping with other correlations so that exact chemical shifts are indefinable. Structures **1**−**9** and **12** are shown in Figure 2, **10** is coniferyl alcohol, and **11** is coniferyl aldehyde.

the 2D HMQC spectrum (Figure 1) was chosen, and the corresponding F2F3 plane at a given ^{13}C chemical shift (Figure 2) was viewed. The ^{1}H chemical shifts of each known spin system can then be seen on HOHAHA slices, examples of which are shown in Figure 2. The carbon of each proton was then traced out from the F1F3 planes [not shown here; for more details in the interpretation of 3D spectra, see Brunow et al. (1998b)]. Three 3D spectra were recorded with various HOHAHA mixing times to allow the development of short- and long-range couplings.

Most MWL preparations contain variable amounts of carbohydrates. The correlations of anomeric protons (at ∼4.5/100 ppm, Figure 1) are strongly overlapping with each other in the spectrum of acetylated sample, and thus we did not assign them except for the correlations of xylan (marked with asterisks in Figure 1; Bardet et al., 1986).

Comments on the Assignments of Side-Chain Structures in the MWL. The results of the interpretation of the 3D NMR spectra are shown in Table 1. The HMQC spectrum (Figure 1) offers a starting point for the discussion of the assignments and the improvement in reliability obtained by the 3D method.

Carbon Signals at 80−89 ppm. The strongest signal in this region is at 88.4 ppm. The signal from the proton bound to this carbon is at 5.51 ppm. In the HOHAHA dimension this proton signal correlates with protons at 3.80 and at 4.33 and 4.46 ppm. These protons in turn are bound to carbons that give signals at 50.4 and 65.3 ppm, respectively. The correlation at 3.80/50.4 ppm is clearly visible in the HMQC spectrum, but the other signals are obscured by overlap from other signals. The existence of these correlations can be definitely proved only by doing a 3D experiment. This pattern corresponds to the α, *β*, and *γ* carbons of the trans-isomer of a phenylcoumaran, or *β*-5, structural unit in the lignin (structure **2**, for comparison with model compound data; Kilpeläinen et al., 1994a). In a similar manner the correlation centered at 4.72/85.8 ppm can be assigned to the α position in the resinol structure **3**. The signals caused by the side chain of the resinol structure are all clearly visible.

A small but distinct signal can be seen at 4.14/82.5 ppm, which can be traced and shown to belong to the side-chain spin system of the trans-isomer of the dibenzodioxocin structure (**8**).

Around 5.4/82 ppm there is a small group of signals, the strongest of which can be assigned to be H*β* of the α-carbonyl side chain **7**; this structure seems typical for hardwood lignins and may be a consequence of the lower oxidation potential of syringyl units as compared to guaiacyl units (Brunow et al., 1998a). In the same group there is also an Hα signal of *cis*-dibenzodioxocin structure **9**. In a 2D proton spectrum both structures, **7** and **9**, would have a correlation between protons 5.4 and 4.5 ppm, which are also very close to Hα and H*γ* correlations of phenylcoumaran (**2**). The difference between structures **7** and **9** can be done on the basis of H*γ* of **9** (3.8 ppm), but again structure **2** causes a proton signal exactly at the same position. In a 2D HMQC−HOHAHA spectra structure **2** can be distinguished reliably from **7** and **9**, but distinguishing **7** from **9** is more difficult as all of the correlations lie between 81 and 83 ppm and 63−65 ppm. However, using 3D methods, the correlations can be assigned reliably and it is clearly shown that both structures are present.

The third correlation in a small group visible in this region is the Hα positions of **6**. In Figure 2e a weak correlation can be seen to a proton at 4.75 ppm. The other signals of the noncyclic benzyl aryl ether **6** are below the threshold for reliable assignments. Further signals in this region are the strong signal from the *β* position in the predominant *β*-O-4 structural unit and a weaker signal that belongs to an unassigned spin system (**12**, Table 1).

Carbon Signals in the Range 66−75 ppm. The most prominent signals are those from the benzyl alcohol groups (as acetates) of structures **1** and, obscured by this strong signal, the corresponding signals from aryl glycerol and *β*-1 structures. The strong signals from xylans that occur in this region are marked with asterisks. The *γ*-positions in the resinol units (**3**) are also clearly visible.

Carbon Signals in the Range 50−66 ppm. The primary alcohol groups of the lignin side chains all give overlapping signals in this region; there is also strong broadening of the signals caused by geminal couplings of the different diastereomers present in the lignin. Another strong signal that interferes with assignments

is the methoxyl at 56 ppm. With the aid of 3D correlations, however, the spin systems and chemical shifts can be traced. The spin system of structure **4** can be traced out, although the signals are very weak. Moreover, two signals from an unknown structure **12** are visible in this region. Although definite identification of the structure **12** cannot be made at present, we suggest a tentative structure for this unit in Figure 2. Details for this are discussed below.

Conclusions. The 3D technique makes it possible not only to detect minor components but also to chart spin systems of unknown structures. For instance, the spin system **12** has also been detected in spectra of RSCL and DHP preparations (Brunow et al., 1998b). The recent publication of the structure of a neolignan with a spiro skeleton (Yoshikawa et al., 1998) makes it possible to somewhat speculatively assign the three side-chain carbons in structure **12** to a part of a similar spiro structure. The rest of the spiro structure side chain has not been found (or assigned). It may even overlap with signals from structure **8**. The exact nature of the structure must await confirmation by synthesis of a satisfactory model compound.

In conclusion, it can be safely assumed that all major, and most of the minor, structural units of a lignin sample can be charted by using a combination of 2D and 3D techniques. The use of 3D enhances the power of HMQC and HOHAHA as a tool for structural analysis. It does give the advantage of clear assignments in complex cases when broad signals and strong overlap obscure assignments by other means. Because of the requirement for ^{13}C enrichment, 3D NMR in this form cannot yet be a routine procedure. It will be of great value, however, for determining structures in cases when 2D spectra at natural abundance of ^{13}C are not sufficient.

LITERATURE CITED

Adler, E. Lignin chemistry—past, present and future. *Wood Sci. Technol.* **1977**, *11*, 169−218.

Bardet, M.; Gagnaire, D.; Nardin, R.; Robert, D.; Vincendon, M. Use of ^{13}C Enriched Wood for Structural NMR Investigation of Wood and Wood components, Cellulose and Lignin, in Solid and in Solution. *Holzforschung* **1986**, *40* (Suppl.), 17−24.

Björkman, A. Studies on finely divided wood. Part I. Extraction of lignin with neutral solvents. *Svensk Papperstidn.* **1956**, *59*, 477.

Brunow, G.; Kilpeläinen, I.; Sipilä, J.; Syrjänen, K.; Karhunen, P.; Setalä, H.; Rummakko, P. Oxidative Coupling of Phenols and the Biosynthesis of Lignin. In *Lignin and Lignan Biosynthesis*; Lewis, N. G., Sarkanen, S., Eds.; ACS Symposium Series 697; American Chemical Society: Washington, DC, 1998a; pp 131−147.

Brunow, G.; Ämmälahti, E.; Niemi, T.; Sipilä, J.; Simola, L. K.; Kilpeläinen, I. Labeling of a lignin from suspension cultures of *Picea abies*. *Phytochemistry* **1998b**, *47*, 1495−1500.

Ede, R. M.; Brunow, G. Application of Two-Dimensional Homo- and Heteronuclear NMR Spectroscopy to Wood Lignin Structure Determination. *J. Org. Chem.* **1992**, *57*, 1477−1480.

Ede, R. M.; Brunow, G.; Simola, L. K.; Lemmetyinen, J. Two-Dimensional ^{1}H-^{1}H Chemical Shift Correlation and J-Resolved NMR Studies on Isolated and Synthetic Lignins. *Holzforschung* **1990**, *44*, 95−101.

Fukagawa, N.; Meshitsuka, G.; Ishizu, A. A Two-Dimensional NMR Study of Birch Milled Wood Lignin. *J. Wood Chem. Technol.* **1991**, *11*, 373−396.

Griesinger, C.; Otting, G.; Wüthrich, K.; Ernst, R. R. Clean TOCSY for ^{1}H Spin System Identification in Macromolecules. *J. Am. Chem. Soc.* **1988**, *110*, 7870−7872.

Guittet, E.; Lallemand, J. Y.; Lapierre, C.; Monties, B. Applicability of the ^{13}C NMR "Inadequate" experiment to lignin, a natural polymer. *Tetrahedron Lett.* **1985**, *26*, 2671−2674.

Karhunen, P.; Rummakko, P.; Sipilä, J.; Brunow, G.; Kilpeläinen, I. Dibenzodioxocins; A Novel Type Of Linkage In Softwood Lignins. *Tetrahedron Lett.* **1995**, *36*, 169−170.

Kilpeläinen, I. Synthesis of lignin model compounds and analysis of lignin structure by NMR spectroscopy. *Ann. Acad. Sci., Ser. A II* **1994**, 1−373.

Kilpeläinen, I.; Sipilä, J.; Brunow, G.; Lundquist, K. Application of Two-Dimensional NMR Spectroscopy to Wood Lignin Structure Determination and Identification of Some Minor Structural Units of Hard- and Softwood Lignins. *J. Agric. Food Chem.* **1994a**, *42*, 2790−2794.

Kilpeläinen, I.; Ämmälahti, E.; Brunow, G.; Robert, D. Application of Three-Dimensional HMQC−HOHAHA NMR Spectroscopy to Wood Lignin, a Natural Polymer. *Tetrahedron Lett.* **1994b**, *35*, 9267−9270.

Lai, Y. Z.; Sarkanen, K. V. Isolation and structural studies. In *Lignins, Occurrence, Formation, Structure and Reactions*; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley-Interscience: New York, 1971; pp 214−215.

Lapierre, C.; Gaudillere, J. P.; Monties, B.; Guittet, E.; Rolando, C.; Lallemand, J. Y. Enrichissement photosynthetique en carbone-13 de lignines de peuplier: caractérisation préliminaire par acidolyse et RMN ^{13}C. *Holzforschung* **1983**, *37*, 217−224.

Lapierre, C.; Monties, B.; Guittet, E.; Lallemand, J. Y. Photosynthetically Carbon-13 Labeled Poplar Lignin: Carbon-13 Experiments. *Holzforschung* **1984**, *38*, 333−342.

Lewis, N. G.; Razal, R. A.; Dhara, K. P.; Yamamoto, E.; Bokelman, G. H.; Wooten, J. B. Incorporation of [2-^{13}C]-Ferulic Acid, a Lignin Precursor, into *Leucaena leucocephala* and its Analysis by Solid state ^{13}C NMR Spectroscopy. *J. Chem. Soc., Chem. Commun.* **1988**, 1626−1628.

Lundquist, K. NMR Studies of Lignin 4. Investigation of Spruce Lignin by ^{1}H NMR Spectroscopy. *Acta Chem. Scand.* **1980**, *B34*, 21−26.

Lundquist, K. ^{1}H NMR spectral studies of lignins. Results regarding the occurrence of β-5 structures, β−β structures, non-cyclic benzyl aryl ethers, carbonyl groups and phenolic groups. *Nord. Pulp Pap. Res. J.* **1992**, *7*, 4−8.

Ralph, J.; Mackay, J. J.; Hatfield, R. D.; O'Malley, D. M.; Whetten, R. W.; Sederoff, R. R. Abnormal Lignin in a Loblolly Pine Mutant. *Science* **1997**, *277*, 235−239.

Robert, D. Carbon-13 Nuclear magnetic resonance Spectroscopy. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Heidelberg, 1992; pp 250−265.

Sarkanen, K. V.; Ludwig, C. H. *Lignins, Occurrence, Formation, Structure and Reactions*; Wiley-Interscience: New York, 1971.

Summers, M. F.; Marzilli, L. G.; Bax, A. Complete ^{1}H and ^{13}C Assignments of Coenzyme B_{12} through the Use of New Two-Dimensional NMR Experiments. *J. Am. Chem. Soc.* **1986**, *108*, 4285−4294.

Whiting, P.; Goring, D. A. I. The morphological origin of milled wood lignin. *Svensk Papperstidn.* **1981**, *84*, R120−R122.

Wijmenga, S. S.; Hallenga, K.; Hilbers, C. W. A Three-Dimensional Heteronuclear Multiple-Quantum Coherence Homonuclear Hartmann−Hahn Experiment. *J. Magn. Reson.* **1989**, *84*, 634−642.

Yoshikawa, K.; Kinoshita, H.; Arihara, S. Woorenol, a Novel Sesquineolignan with a Unique Spiro Skeleton, from the Rhizomes of *Coptis japonica* var. *dissecta*. *J. Nat. Prod.* **1997**, *60*, 511−513.

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