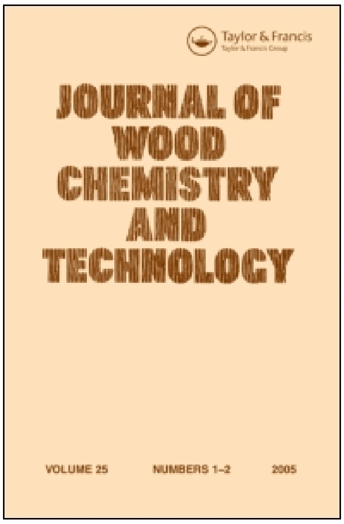

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A TWO-DIMENSIONAL NMR STUDY OF BIRCH MILLED WOOD LIGNIN

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ABSTRACT

Chemical structures of underivatized and acetylated birch Milled Wood Lignins were investigated in solution by various two-dimensional NMR experiments such as COSY, HOHAHA, HMQC, and HMBC.

INTRODUCTION

Nuclear Magnetic Resonance Spectroscopy (NMR) is one of the most powerful tools for research into chemical structures. In lignin studies, NMR has provided a large quantity of information concerning structures. However, for ^{1}H NMR[1-4] and also ^{13}C NMR[5-9], severe peak overlap has prevented wood chemists from making detailed and well-defined assignments for chemical structures. Therefore, chemical shifts of model compounds have been invaluable for the determination of chemical structures of lignin.

In this decade, various pulse sequences have been developed and have applied in structural studies of natural products and biopolymers. Unambiguous assignments of overlapping peaks have become possible using these pulse sequences. Although lignin has very large transverse relaxation rates, utilization of these pulse sequences on lignin has become possible as a result of progress in NMR equipment. Recently, Guittet *et al* [10] applied the INADEQUATE experiment to ^{13}C-enriched poplar lignin, and identified pinoresinol structures and confirmed many assignments. In the case of MWL with natural ^{13}C abundance, INADEQUATE, and also a normal ^{13}C-^{1}H shift correlation experiments cannot provide useful information because of low sensitivity. Ede *et al*[11] applied the COSY experiment to acetylated MWL and DHP, and discussed the proportion of each side chain structure.

Recently, the HOHAHA (Homonuclear Hartmann-Hahn) experiment has been used for the identification of ^{1}H-^{1}H coupling networks.[12] Whereas the COSY experiment detects the scalar coupling between vicinal protons, the HOHAHA experiments can detect the whole scalar couplings within the network, with a sufficiently long mixing time. In addition, detection of ^{13}C-^{1}H shift correlation has been possible in low concentration under natural ^{13}C abundance by the HMQC (^{1}H-Detected Multiple Quantum Coherence) and the HMBC (^{1}H-Detected Multiple-bond Heteronuclear Multiple Quantum Coherence) experiments.[13,14] The HMQC detects directly-bonded ^{13}C-^{1}H systems via short-range couplings and the HMBC picks up vicinal and more remote ^{13}C-^{1}H patterns via long-range couplings.

We have applied these experiments to acetylated and underivatized MWLs. The acetylated form has been used frequently in the conventional study of MWL by NMR, because it has an advantage of the higher solubility in non-viscous solvents; such solvents are suitable for high-resolution experiments.[15] However, peaks in the aliphatic and carbonyl regions in MWL spectra are overlapped by acetyl group resonances. Therefore, both acetylated and underivatized MWLs were examined in this report.

RESULTS AND DISCUSSION

General Discussion of Pulse Sequences

COSY Experiments

COSY spectra under the conditions used in this work showed sufficient sensitivity to provide significant details. However, since there are many peaks from 3 to 4 ppm in the ^1H spectrum of underivatized MWL, the COSY cross peaks in this region overlap, even under the resolution-enhanced conditions. Therefore, COSY experiments are less useful for underivatized MWL than other 2D-NMR experiments. This restriction was less severe for acetylated MWL.

HOHAHA Experiments

HOHAHA spectra had the same tendency to overlap as COSY spectra. However, some series of cross peaks could be observed in uncrowded regions. Coherence transfer from C_5 to C_3 in acetylated anhydropyranose residues was observed in the experiment with a mixing time of 30 ms, and that from C_5 to C_1 was observed in the experiment with a mixing time of 60 ms.

HMQC Experiments

HMQC experiments were found to be useful for structural studies of lignin. Although in the case of underivatized MWL, peaks in the region (^1H, 3.3-4.1 ppm; ^{13}C, 59-64 ppm) overlapped, the other peaks were resolved (Figure 1-b). However, the peaks derived from the two diastereomers, *erythro* and *threo* β-O-4 structures, were not discernible. The sensitivity under the conditions used here was high enough for definition of the main side-chain structures. Since a large number of scans was accumulated, the spectrum was nearly free from t_1 noise, which is a problem in HMQC experiments.

HMBC Experiments

The HMBC experiment can supply very useful information on carbon skeletons. However, only a little information was obtained under the conditions used in this work because of low S/N ratio and t_1 noise overlapping with important peaks.

Chemical Structures of Underivatized MWL

β-O-4 Ether Structures

This type of structure showed intense peaks in the HMQC spectrum (Figure 1-b). That is, α;peak 1, β;peak2 and γ;peak3. The two non-equivalent methylene protons at the γ position were not well resolved in the f_2 dimension. The high intensities of these peaks indicated that this structure is the predominant structure in hardwood MWLs, in good agreement with the previous investigation.[16]

Two series of cross peaks ascribable to β-O-4 structures were observed in the HOHAHA spectrum (Figure 2-a, 1/2 and 1/3).

Phenylcoumaran Structures

Peak 4 in the HMQC spectrum (Figure 1-b) was assigned to the α position of phenylcoumaran structures. The intensity of this peak was much weaker than that of β-O-4 structures (peak 1) and that of pinoresinol structures (peak 7). Moreover, the peaks of the β position (5, Table 1) and the γ position (6, Table 1) were obscure because of their low intensities and overlap with other peaks.

Pinoresinol Structures

The relative abundance of pinoresinol structures in hardwood MWL has been thought to be no higher than that of phenylcoumaran species.[16] In the HMQC

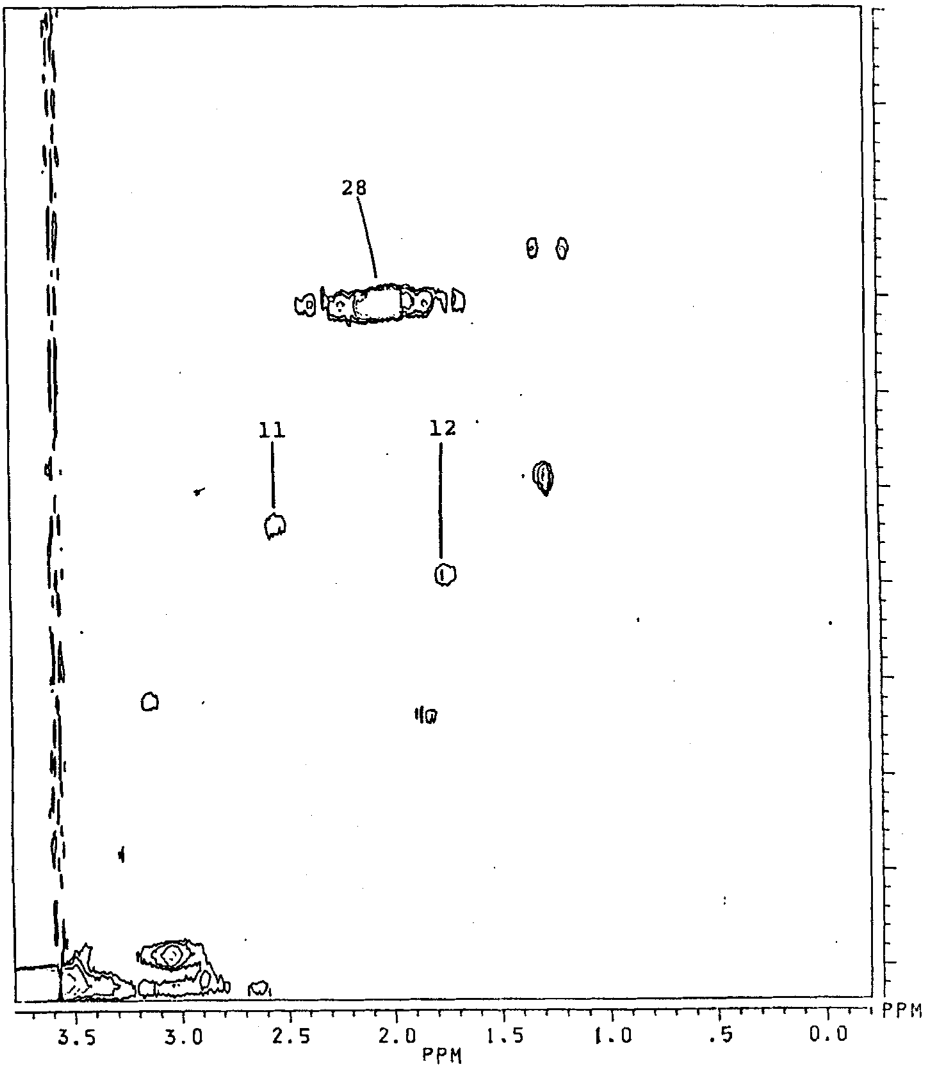

FIGURE 1-a. The high-field region of the HMQC spectrum of underivatized birch MWL. The peak numbers correspond to those in Table 1.

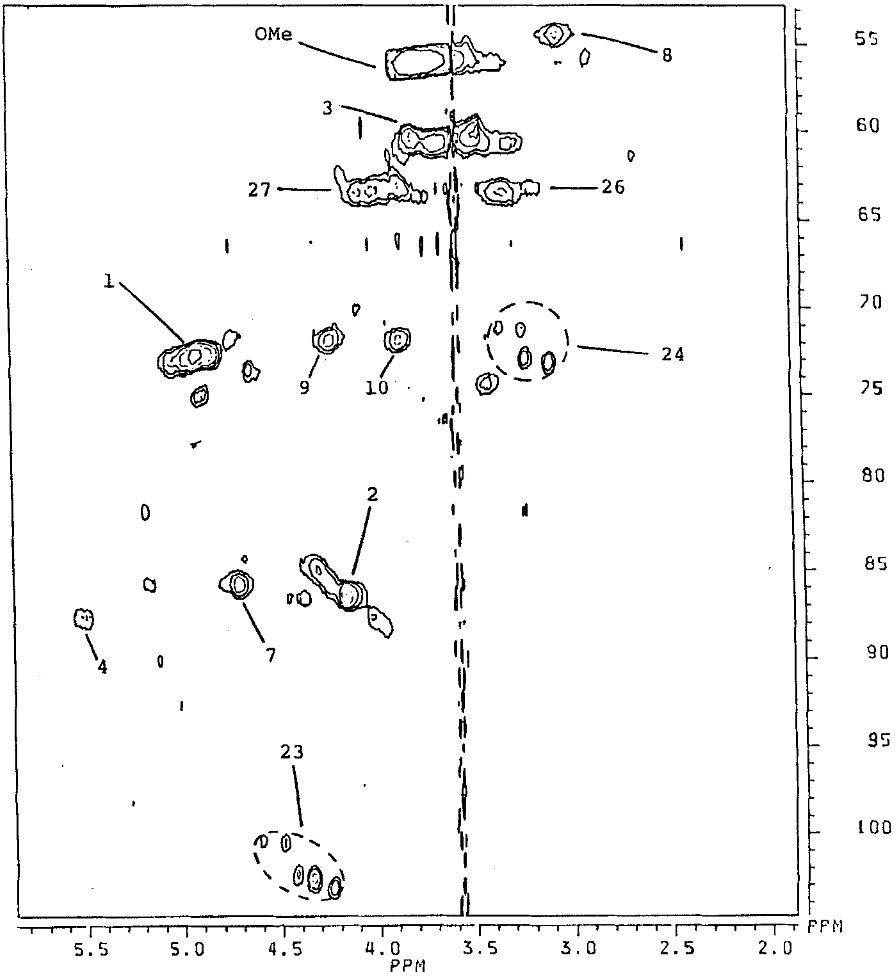

FIGURE 1-b. The aliphatic region of the HMQC spectrum of underivatized birch MWL. The peak numbers correspond to those in Table 1.

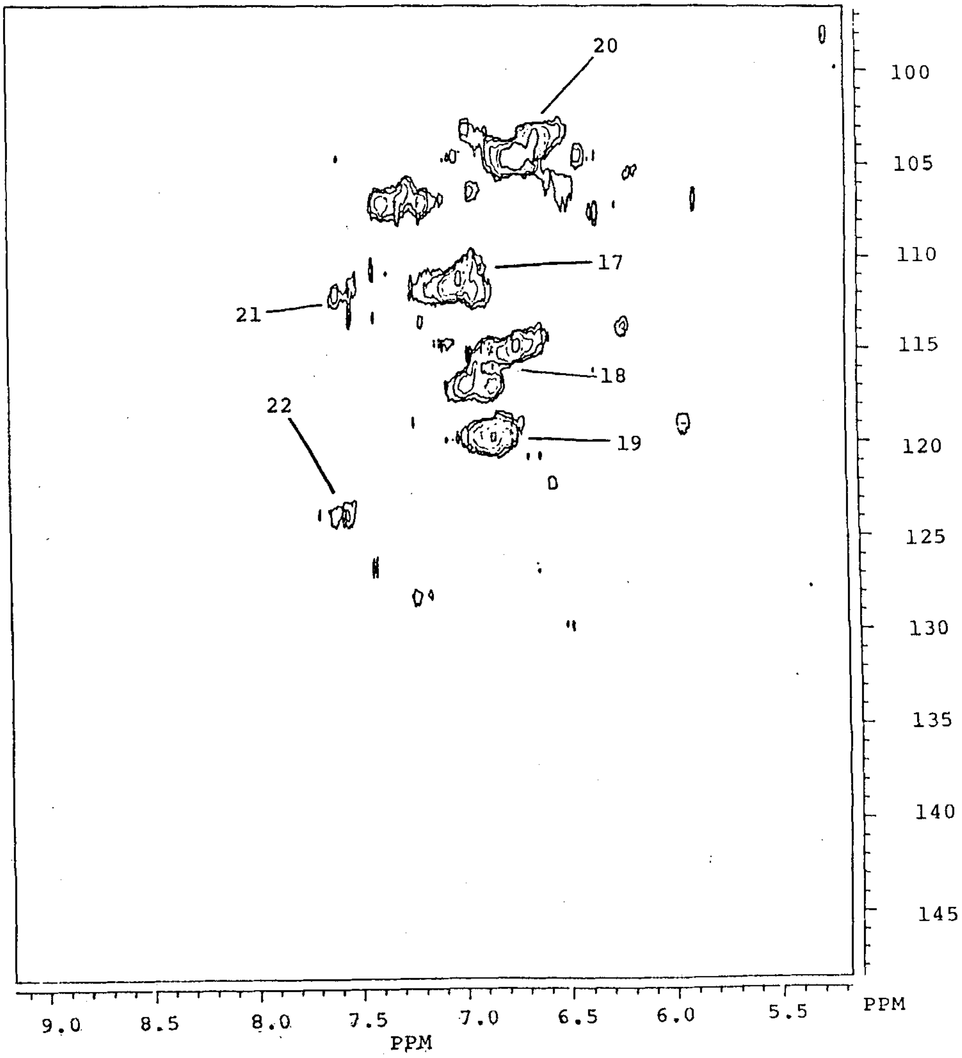

FIGURE 1-c. The aromatic region of the HMQC spectrum of underivatized birch MWL. The peak numbers correspond to those in Table 1.

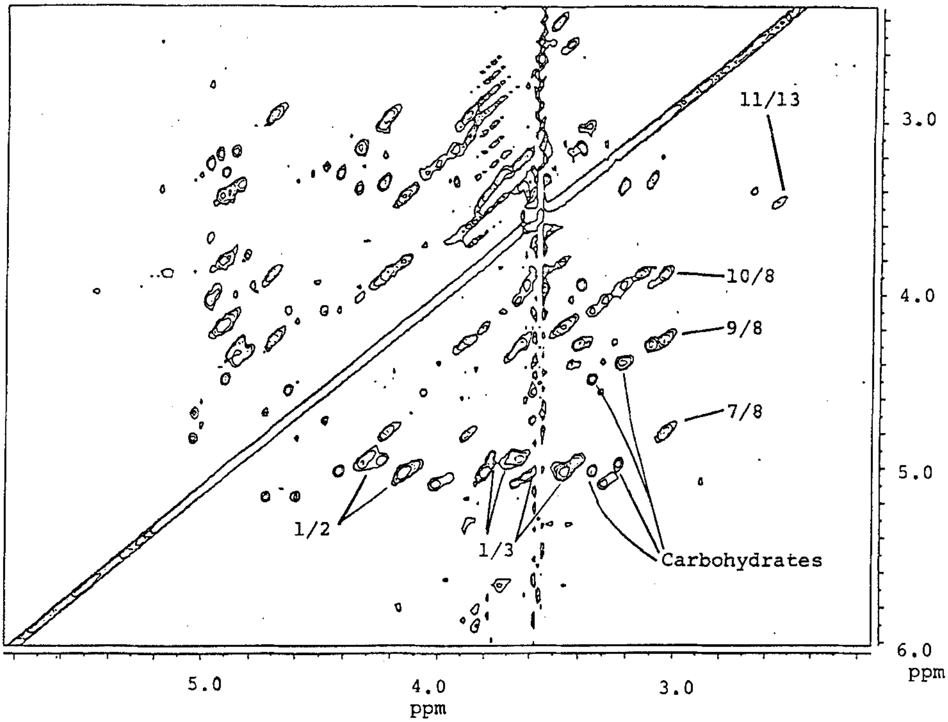

FIGURE 2-a. The high-field region of the HOHAHA spectrum with a mixing time of 35 ms of underivatized birch MWL. The peak numbers correspond to those in Table 1.

spectrum (Figure 1-b), two peaks from the non-equivalent methylene protons at the γ position, at 3.9 ppm (axial) and 4.2 ppm (equatorial) with the same ^{13}C chemical shift of 72 ppm were observed. The other peaks of α (peak 7) and β (peak 8) were also observed. Although the intensities of these peaks were much smaller than those of β-O-4 structures, they were larger than those of the phenylcoumaran structures. Since $^{13}C-^{1}H$ coupling constants and relaxation rates

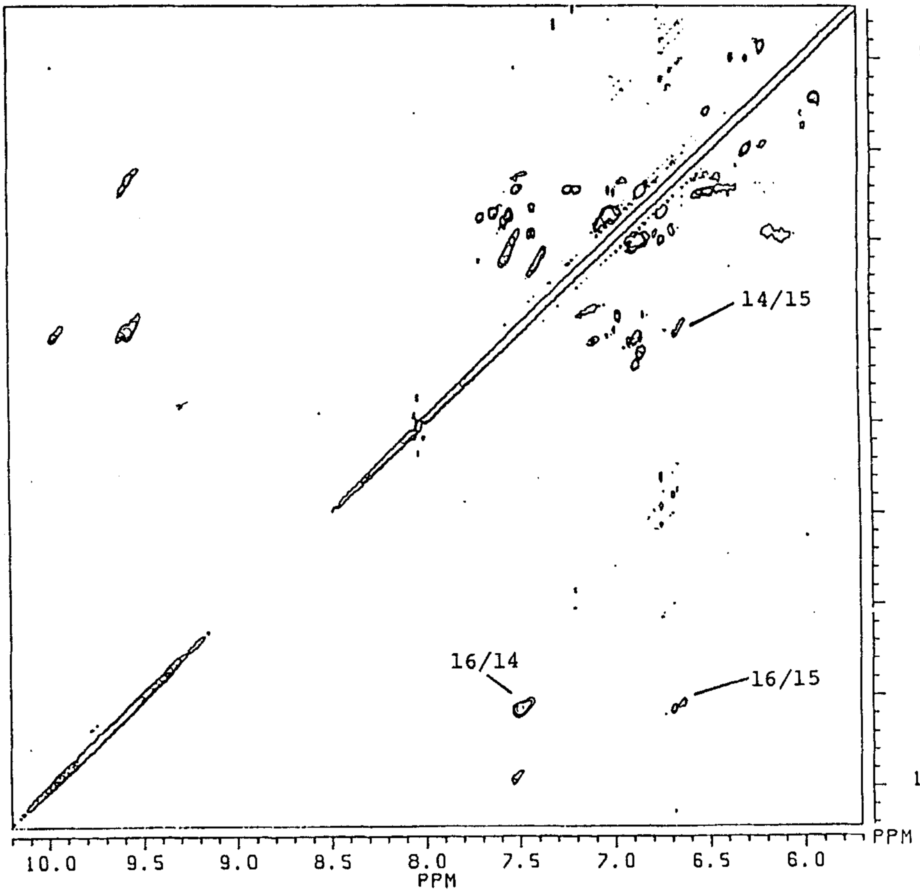

FIGURE 2-b. The low-field region of the HOHAHA spectrum with a mixing time of 60 ms of underivatized birch MWL. The peak numbers correspond to those in Table 1.

of transverse magnetization vary in these structures, quantitative discussion is not always possible on the basis of their peak intensities in this spectrum. However, indications are that pinoresinol structures must be next in importance to β-O-4 type structures among the side-chain structures of hardwood MWL. In

TABLE 1.

Assignments in NMR Spectra of Underivatized MWL

No.	Chemical Shift (ppm) ^1H	^{13}C	Assignment
1.	4.8-5.0	72-74	β-O-4-α
2.	4.1-4.4	86-87	β-O-4-β
3.	3.4-3.8	60-61	β-O-4-γ
4.	5.5	88	Phenylcoumaran-α
5.	3.5	--	Phenylcoumaran-β
6.	4.1	--	Phenylcoumaran-γ
7.	4.7	86	Pinoresinol-α
8.	3.0-3.1	54	Pinoresinol-β
9.	4.2	72	Pinoresinol-γ (equatorial)
10.	3.9	72	Pinoresinol-γ (axial)
11.	2.6	32	3-Aryl-1-propanol-α
12.	1.7	35	3-Aryl-1-propanol-β
13.	3.5	--	3-Aryl-1-propanol-γ
14.	7.5	--	Coniferyl aldehyde-α
15.	6.7	--	Coniferyl aldehyde-β
16.	9.6	--	Coniferyl aldehyde-γ
17.	6.9-7.2	110-112	Guaiacyl-2
18.	6.6-7.0	114-118	Guaiacyl-5
19.	6.8-7.0	119-121	Guaiacyl-6
20.	6.5-6.9	104-106	Syringyl-2,6
21.	7.5	112	Biphenyl-2
22.	7.6	124	Biphenyl-6
23.	4.2-4.6	100-104	Carbohydrates-1
24.	3.1-3.4	71-74	Carbohydrates-2
25.	--	74-76	Carbohydrates-3
26	3.3-3.5	63-64	Carbohydrates-5 (axial)
27.	3.8-4.1	63-64	Carbohydrates-5 (equatorial)
28.	1.8-2.3	19-22	Me in acetyl groups
29.	--	168-174	Carbonyl in acetyl groups

the HOHAHA spectrum (Figure 2-a), the coupling network was identified.

Other Side-Chain Structures

In model compound experiments, the β position of α-carbonyl β-O-4 structures to which photo-discoloration of lignin had been ascribed showed chemical shifts of 5.2-5.3 ppm in ^{1}H NMR and 84-86 ppm in ^{13}C NMR.[17] The peak (5.2 ppm, 86 ppm) in the HMQC spectrum (Figure 1-b) could arise from this position. However, the peak expected from long-range coupling between the α-carbonyl ^{13}C and β ^{1}H was not observed in the HMBC spectrum (Figure 3-a).

Two peaks (11 and 12) corresponding to 3-aryl-1-propanol structures were detected in the HMQC spectrum (Figure 1-a).[18]

The cross peaks (6.7 ppm/7.5 ppm) and (9.6 ppm/6.7 ppm) were observed in the COSY spectrum (not shown). In addition, another cross peak (7.5 ppm/9.6 ppm) was observed in the HOHAHA spectrum (Figure 2-b). This series of peaks was assigned to coniferyl aldehyde structures α (7.5 ppm), β (6.7 ppm) and γ (9.6ppm). A weak cross peak (10.0 ppm/7.5 ppm) was also observed in the HOHAHA spectrum (Figure 2-b), but no cross peak was observed at 10.0 ppm in the COSY spectrum. Therefore this cross peak is thought to be an artifact in the HOHAHA experiment.

Aromatic Structures

In the HMQC spectrum (Figure 1-c), five major peaks in the aromatic region were observed. Four of these peaks were assigned to syringyl 2 and 6 positions (peak 20), guaiacyl 2 position (peak 17), guaiacyl 6 position (peak 19), and guaiacyl 5 position (peak 18), respectively. However, the peak (7.1-7.4 ppm, 106-108 ppm) could not be assigned on the basis of model compound experiments. Two weak peaks at (7.5

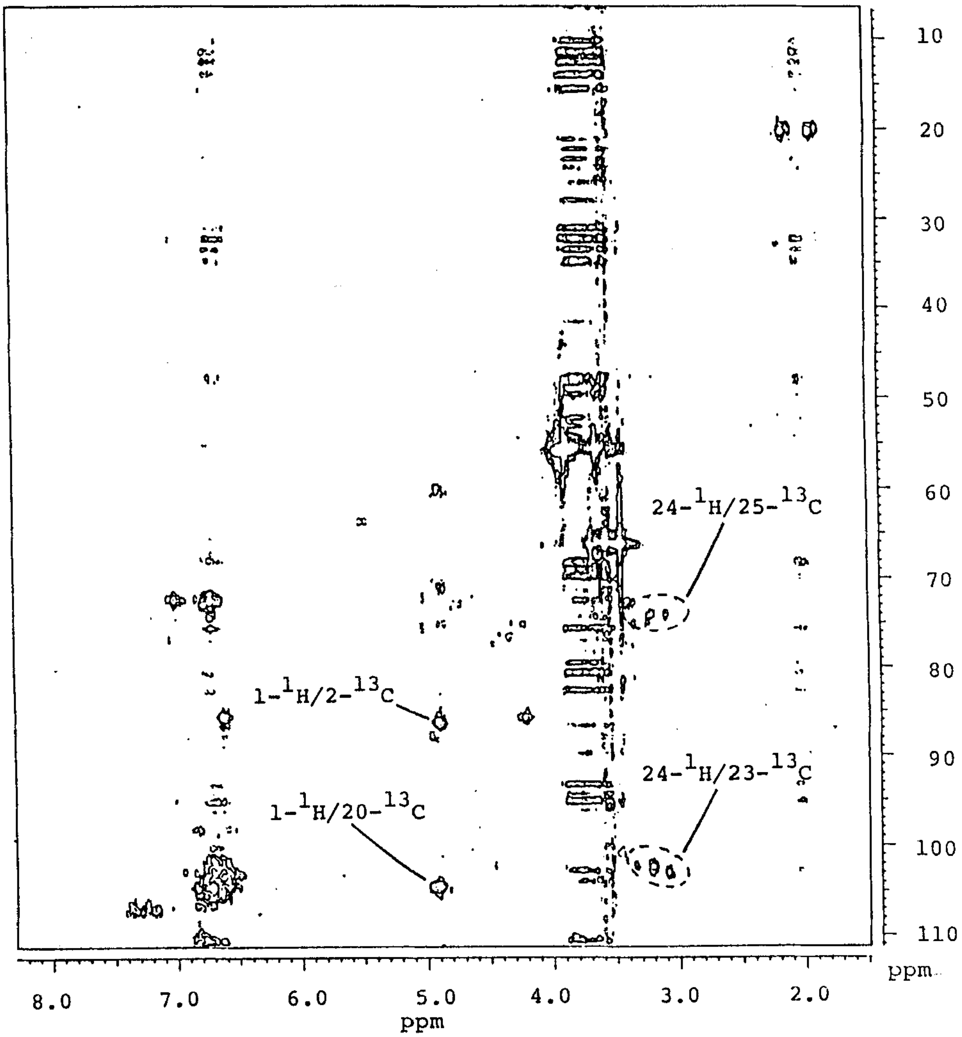

FIGURE 3-a. The high-field region of the HMBC spectrum of underivatized birch MWL. The peak numbers correspond to those in Table 1.

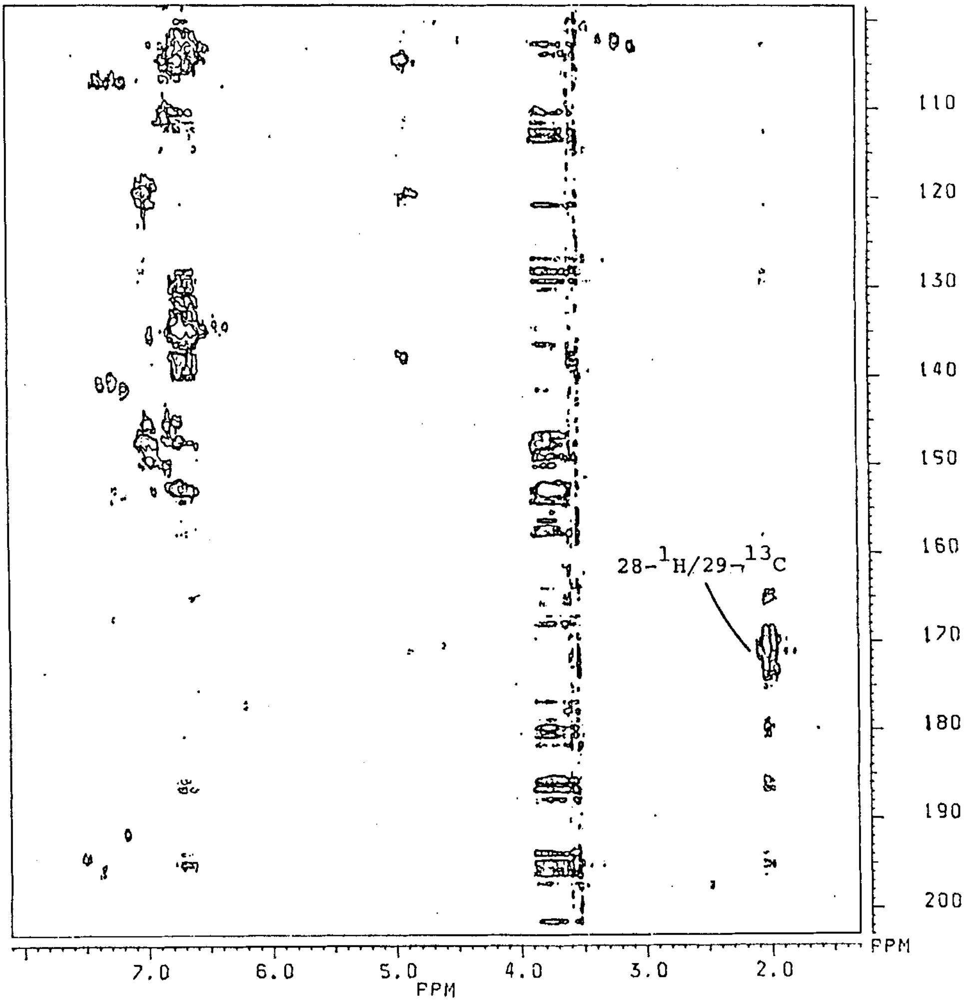

FIGURE 3-b. The low-field region of the HMBC spectrum of underivatized birch MWL. The peak numbers correspond to those in Table 1.

ppm, 112 ppm) and (7.6 ppm, 124 ppm) probably arise from biphenyl 2 and 6 positions, respectively.

Carbohydrates

It has been pointed out that hemicellulose remains in MWL and is not easily removed without chemical modification of the lignin. Five peaks ascribed to anomeric positions (peak 23) were observed in the HMQC spectrum (Figure 1-b). This indicates that at least five kinds of anomeric carbons are involved in the hemicellulose fraction of this MWL. Two intense peaks which probably arose from C_5 of anhydropentopyranoside residues (axial; peak 26 and equatorial; peak 27) were observed in the HMQC spectrum(Figure 1-b).[19] In the HMBC spectrum (Figure 3-a), peaks derived from long-range coupling were observed in the region of 3.1-3.4 ppm and 74-76 ppm (the coupling between ^1H-C_2 and ^{13}C-C_3), and in the region of 3.1-3.4 ppm and 102-104 ppm (the coupling between ^1H-C_2 and ^{13}C-C_1). Two series of cross peaks derived from these carbohydrate skeletons were observed in the HOHAHA spectrum (Figure 2-a). In the HMQC spectrum (Figure 1-a), the peak derived from a methyl group (1.8-2.3 ppm, 19-21 ppm) was observed. Moreover, in the HMBC spectrum (Figure 3-b), a long-range coupling between this proton and the carbonyl carbon of an ester group (1.9-2.2 ppm, 168-174 ppm) was observed. Therefore, this methyl group and ester carbonyl group were attributed to acetyl groups which are probably substituents on xylan.

Chemical Structures of Acetylated MWL

β-O-4 Ether Structures

Three peaks ascribable to these structures, α (peak 1, Table 2 and Figure 4), β (peak 2), γ (peak 3) were observed in the HMQC spectrum, and the couplings among these protons were detected in both the COSY (Figure 5) and the HOHAHA spectra (Figure 6-a).

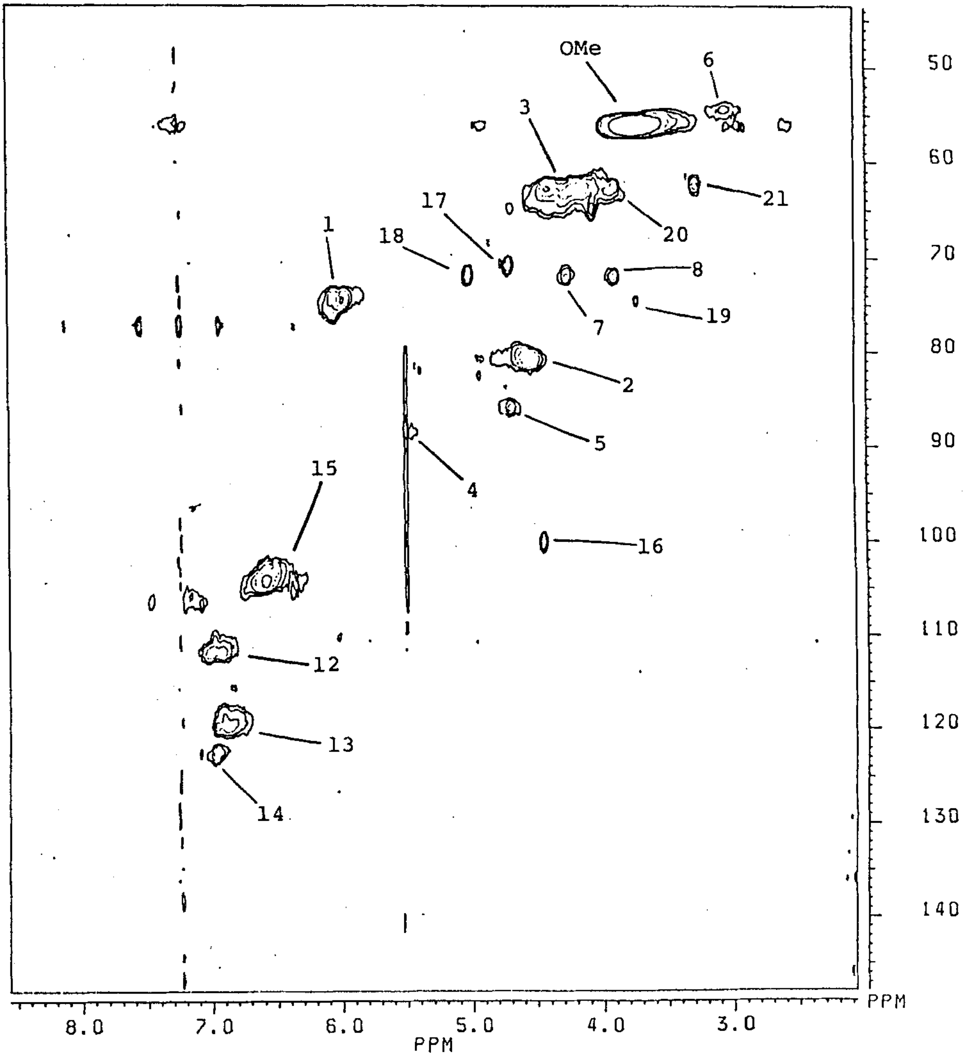

FIGURE 4. The HMQC spectrum of acetylated birch MWL. The peak numbers correspond to those in Table 2.

TABLE 2.

Assignments in NMR Spectra of Acetylated MWL

No.	Chemical Shift (ppm) ^1H	^{13}C	Assignment
1.	5.8-6.1	73-76	β-O-4-α
2.	4.4-4.7	79-82	β-O-4-β
3.	3.9-4.4	62-65	β-O-4-γ
4.	5.4	88	Phenylcoumaran-α
5.	4.7	86	Pinoresinol-α
6.	3.1	54	Pinoresinol-β
7.	4.3	72	Pinoresinol-γ (equatorial)
8.	3.9	72	Pinoresinol-γ (axial)
9.	7.4	--	Coniferyl aldehyde-α
10.	6.6	--	Coniferyl aldehyde-β
11.	9.7	--	Coniferyl aldehyde-γ
12.	6.8-7.1	109-112	Guaiacyl-2
13.	6.8-7.0	117-121	Guaiacyl-5,6(=OAc) & Guaiacyl-5(= not OAc)
14.	6.9-7.0	122-124	Guaiacyl-6(=not OAc)
15.	6.3-6.7	102-105	Syringyl-2,6
16.	4.4	100	Anhydroxylose residue-1
17.	4.7	71	Anhydroxylose residue-2
18.	5.0	72	Anhydroxylose residue-3
19.	3.7	74	Anhydroxylose residue-4
20.	3.9	62	Anhydroxylose residue-5 (equatorial)
21.	3.3	62	Anhydroxylose residue-5 (axial)
22.	0.1	--	Contaminants and artifacts
	0.9	--	
	1.3	--	
	1.6	--	
	1.8	--	
	2.6	--	
	2.8	--	
	4.2	--	
	5.3	--	
	7.4	--	

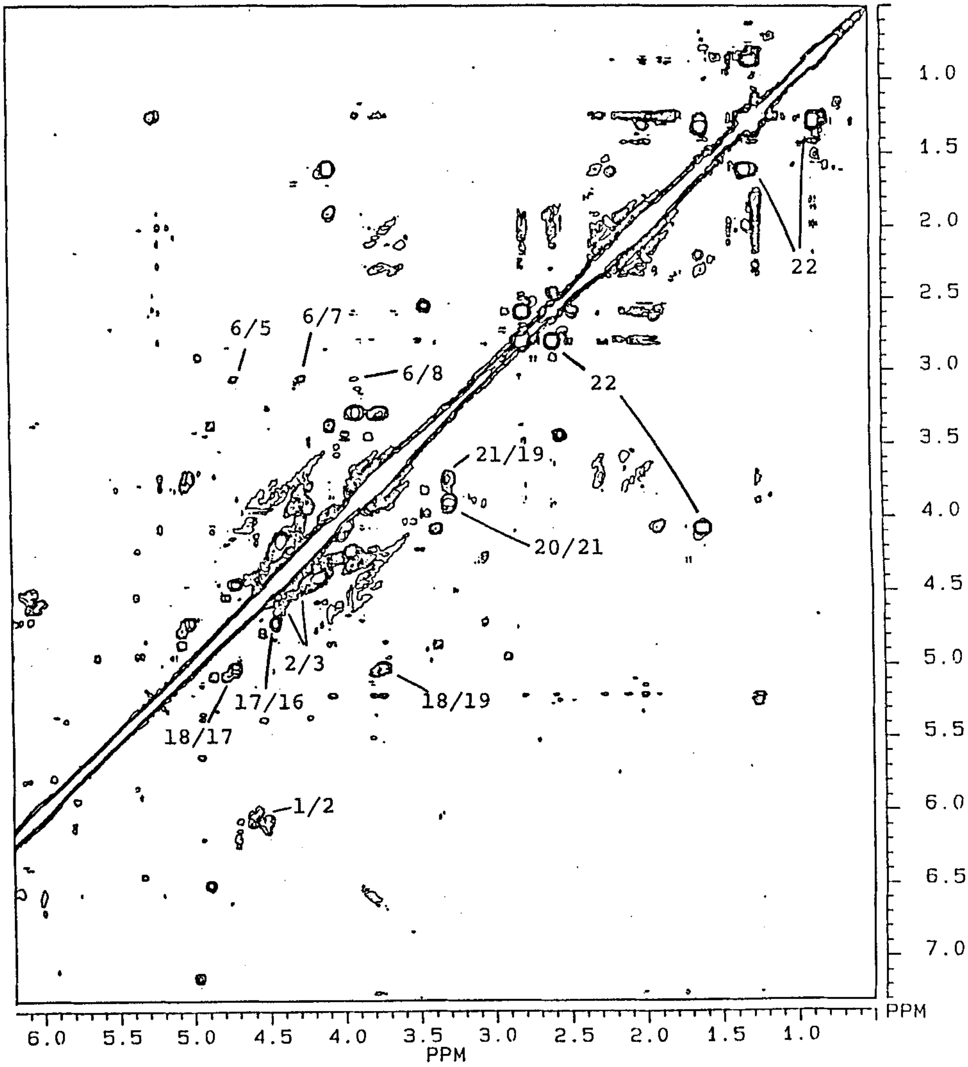

FIGURE 5. The high-field region of the COSY spectrum of acetylated birch MWL. The peak numbers correspond to those in Table 2.

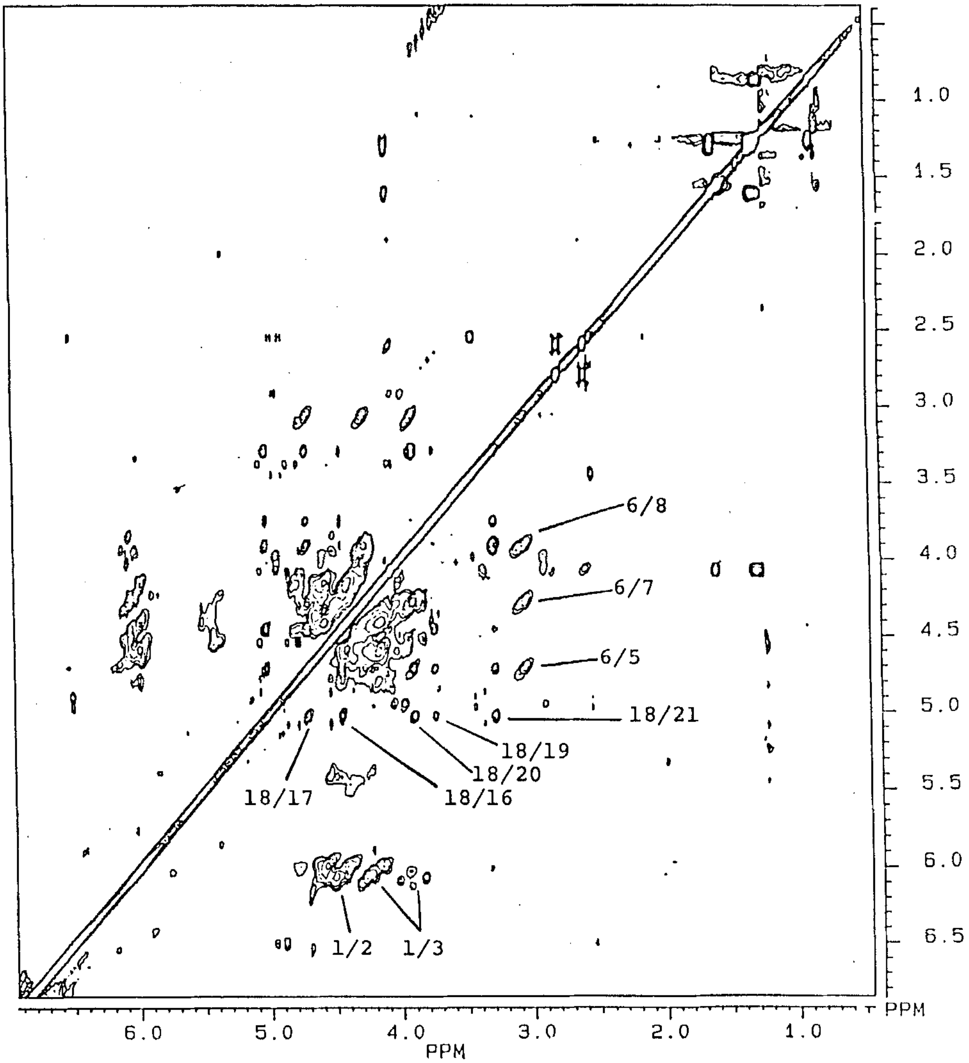

FIGURE 6-a. The high-field region of the HOHAHA spectrum with a mixing time of 60 ms of acetylated birch MWL. The peak numbers correspond to those in Table 2.

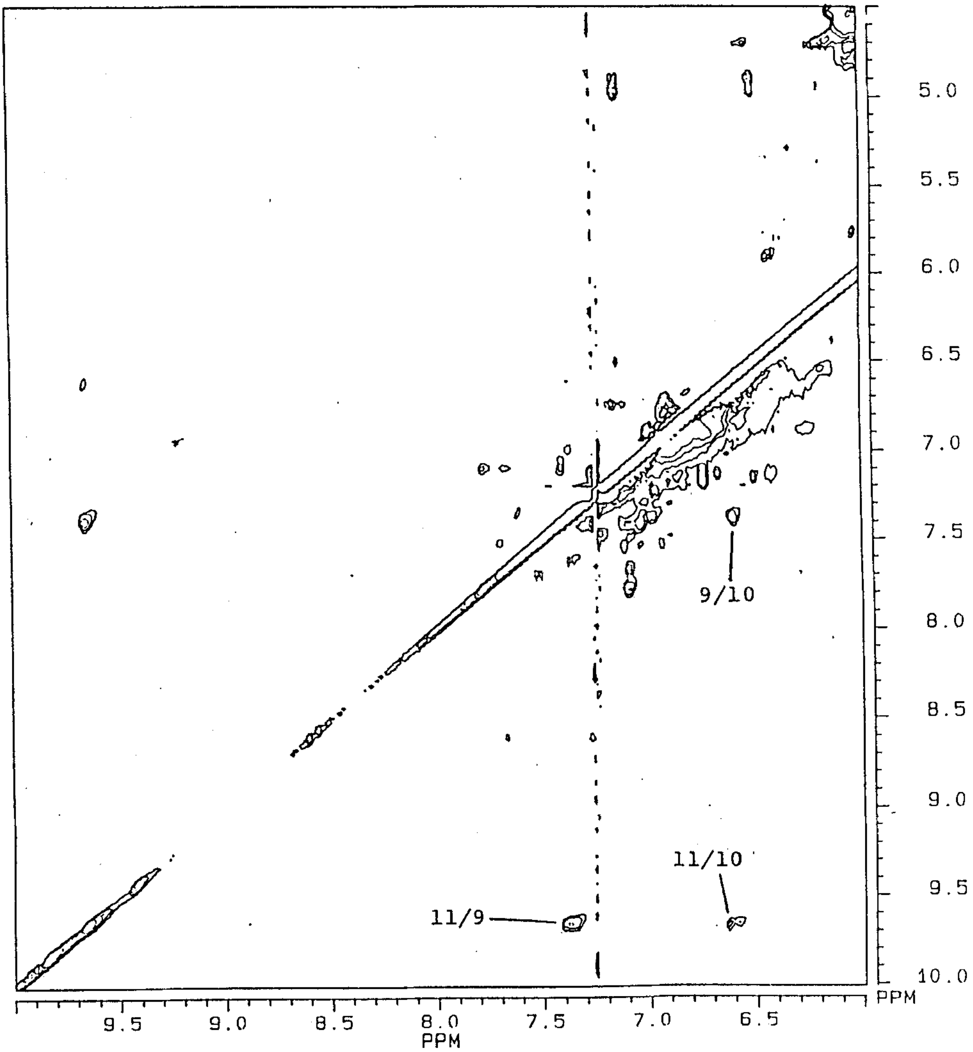

FIGURE 6-b. The low-field region of the HOHAHA spectrum with a mixing time of 60 ms of acetylated birch MWL. The peak numbers correspond to those in Table 2.

Phenylcoumaran Structures

In the HMQC spectrum (Figure 4), only the weak peak for the α position (peak 4) was observed.

Pinoresinol Structures

In the HMQC spectrum (Figure 4), four peaks (α ;peak 5, β;peak 6, equatorial-γ;peak 7 and axial-γ ;peak 8) ascribable to these structures were observed. These chemical shifts were nearly the same as those in the underivatized MWL. Moreover, the cross peaks in the HOHAHA spectrum (Figure 6-a) clearly showed the coupling network of this structure.

Other Side-Chain Structures

The peaks ascribable to other structures could not be observed in the HMQC spectrum (Figure 4), because of low sample concentration and the relatively low abundance of these structures. However, in the HOHAHA spectrum (Figure 6-b), the cross peaks of the coniferyl aldehyde structure were observed at α;peak 9, β;peak 10 and γ;peak 11.

Aromatic Structures

In the HMQC spectrum (Figure 4), four main peaks (12-15) were observed and assigned as shown in Table 2 according to previous studies.[20]

Carbohydrates

Five peaks (16-21) in the HMQC spectrum (Figure 4) were assigned to acetylated xylan: C_1;peak 16, C_2;peak 17, C_3;peak 18, C_4;peak 19, and C_5-axial;peak 21. The peak ascribable to the C_5-equatorial was overlapped by the intense peak from the γ position in β-O-4, phenylcoumaran and other structures. However, a cross peak in the COSY spectrum (Figure 5) and one in the HOHAHA spectrum (Figure 6-a) indicates the existence of this structure. Whereas five peaks were observed in the anomeric region of the HMQC spectrum of underivatized MWL (Figure 1-b), only one peak was observed in that of acetylated MWL (Figure 4). Therefore significant loss of carbohydrates is thought to occur during acetylation and/or subsequent extraction.

EXPERIMENTAL

Sample

Birch (*Betula Maximowicziana Regel*) MWL was prepared by the method of Lee *et al.*[21]

Acetylation of MWL

To 50 mg of MWL, 1 ml of pyridine and 1 ml of acetic anhydride were added at room temperature, and the mixture was kept at this temperature. After 48 hr, the reaction mixture was poured into ice-cooled deionized water and extracted twice with 30 ml of CH_2Cl_2. The CH_2Cl_2 layer was washed twice with 30 ml of 10% $NaHCO_3$, twice with 2*N* HCl, once with water, and dried over anhydrous Na_2SO_4. The solvent was removed at 40 °C *in vacuo*, and the residue was dried at 45 °C under vacuum. Ten milligrams of acetylated MWL was obtained. Some very sharp peaks at 0.1, 0.9, 2.6, 2.8, 4.2, 5.3, 7.1 and 7.4 ppm were observed in 1D ^1H spectrum. The underivatized MWL did not show these peaks in its 1D ^1H spectrum. They are thought to be due to low molecular weight contaminants introduced during acetylation treatment.[22] However, these peaks did not overlap the cross peaks derived from acetylated MWL in any of the 2D spectra.

General

All NMR spectra were recorded on a Bruker AM600 spectrometer.

NMR Experiments on Underivatized Milled Wood Lignin

Five milligrams of MWL in 0.8 ml of a mixture of 98% d_8-dioxane and 99% D_2O (v/v=9/1) was used for the absolute-mode COSY experiment and the phase-sensitive

HOHAHA experiment. In these experiments, data were recorded with a 5 mm selective probe for ^1H at 45 °C. The peak corresponding to HDO, which was at first smaller than that of dioxane, increased to be larger than that of dioxane because of D/H exchange between D_2O and hydroxyl groups in lignin. Therefore, whereas the dioxane peak was presaturated in the COSY experiment, the HDO peak was presaturated in the HOHAHA experiment.

The absolute-mode COSY spectrum was measured at 600 MHz, by acquiring 512 2k spectra and transforming to a 2k x 1k final matrix. A non-phase-shifted sinebell squared window was applied to both dimensions. In each t_1 value, 128 scans were accumulated. The data were symmetrized.

The phase-sensitive HOHAHA spectra were recorded at 600 MHz, by acquiring 512 2k spectra and transforming to a 2k x 1k final matrix. The minimum t_1 was 3 μs and the t_1 increment was 56 μs. In the experiment with a mixing time of 35 ms, 96 scans were accumulated at each t_1 value, and a $\pi/4$ phase-shifted sinebell squared window was applied to both dimensions. In the experiment with a mixing time of 60 ms, 128 scans were accumulated at each t_1 value, and a $\pi/8$ phase-shifted sinebell squared window was applied to both dimensions.

Phase-sensitive HMQC data were recorded on 50 mg of MWL dissolved in 0.7 ml of a mixture of 98% d_8-dioxane and 99% D_2O (v/v=9/1) at 600 MHz with a 5 mm broad-band probe at 45 °C by acquiring 400 2k spectra and transforming to a 2k x 1k final matrix. The HDO peak was presaturated. The minimum t_1 was 3 μs and the t_1 increment was 7.9 μs. For each t_1 value, 256 scans were accumulated, and a $\pi/4$ phase-shifted sinebell squared window was applied to both dimensions.

Absolute-mode HMBC data were recorded on 50 mg of MWL dissolved in 0.7 ml of a mixture of 98% d_8-dioxane and 99% D_2O (v/v=9/1) at 600 MHz with a 5 mm broad-band probe at 45 °C by acquiring 320 2k spectra and transforming to a 2k x 1k final matrix. The HDO peak was presaturated. The minimum t_1 was 3 μs and the t_1 increment was 5.8 μs. For each t_1 value, 320 scans were accumulated. A non-phase-shifted sinebell window was applied to both dimensions.

NMR Experiments on Acetylated MWL

Ten milligrams of acetylated MWL in 1 ml of 99.5% $CDCL_3$ was used for the following three experiments. The measuring temperature was 27 °C. For both the COSY and the HOHAHA experiments a 5 mm selective probe for ^1H was used, and a 5 mm broad-band probe was used for the HMQC experiment.

The absolute-mode COSY spectrum was measured using the same acquiring and processing conditions as for the underivatized sample.

The phase-sensitive HOHAHA spectra were recorded using the same acquiring and processing conditions as for the underivatized sample, except that the t_1 increment was 35 μs.

The phase-sensitive HMQC data were recorded by acquiring 300 2k spectra and transforming to 2k x 1k final matrix. The minimum t_1 was 3 μs and the t_1 increment was 35 μs. For each t_1 value, 256 scans were accumulated. A $\pi/2$ phase-shifted sinebell squared window was applied to both dimensions.

CONCLUSIONS

1) HMQC spectra of MWL showed peaks sufficiently resolved to provide well-defined detail of the structures in MWL.

2) By using HOHAHA experiments, $^1H-^1H$ coupling networks in side chain structures of MWL were identified.

3) Pinoresinol structures were found to be next in importance to β-O-4 structures in hardwood MWL.

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