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A ^{13}C NMR Study of Milled Wood Lignins from Hybrid *Salix* Clones

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Phenylcoumaran
Salix
Willow

Summary

Acetylated milled wood lignins from four 1-year old and one 14-year old hybrid *Salix* (willow) clones were examined by qualitative and quantitative ^{13}C NMR spectroscopy. Intensity variations of given signals within a clone and between clones facilitated the characterization of minor functional entities. The lignins contained 7.1%-8.7% pinoresinol/syriligaresinol structures, 2%–4% phenylcoumaran structures, and 4.4%-6.5% p-hydroxybenzoate structures. Quantitation of these structures was based on prominent signals that were previously assigned. In addition, in the case of acetylated p-hydroxybenzoate structures, all the carbon signals were assigned by means of conventional proton decoupled spectra and DEPT spectra, and by comparison with model compounds. Examination of *Salix* milled wood lignins by ^{13}C NMK spectroscopy before and after mild hydrolysis indicated that the p-hydroxybenzoate units were attached to lignin via an ester linkage. No evidence for the existence of ether-linked benzoate subunits was found.

Introduction

Morphological variations in fast-growing hybrid species such as hybrid poplar (*Populus* spp.) have been known for some time (Anderson and Zsuffa 1975; Boyce and Kaeiser 1961). Differences in wood quality, such as specific gravity, were also reported in Salix (willow) clones (Scnnerby-Forsse *et al.* 1983). Variations in chemical composition and kraft pulping yield among clones of black cottonwood were reported, but whether they are caused by genetic factors is still unknown (Cole *et al.* 1984). The purpose of this study was to critically examine the chemical structure of milled-wood lignin (MWL) isolated from hybrid Salix clones by ^{13}C NMR spectroscopy. It was expected that the comparison of several very closely related lignins would reveal small differences in chemical constitution, which would facilitate assignment of signals from low abundance functional moieties.

Experimental

Materials

Stem tissue (free of bark) was taken from four 1-year-old and one 14-year-old hybrid *Salix* clones. The origins of the clones are indicated in Table 1.

Milled-wood lignin preparation

The MWLs (200 mg) were obtained from extracted woods of different clones following Björkman's procedure (Björkman 1956). The purified MWLs (yields of 8%-14%) were acetylated with acetic anhydride-pyridine (4 ml 1/1) at room temperature for 4 h. Toluene (10 ml) was then added and the resulting solution concentrated (40°C. 20 mm) to about 1/10th its volume. The toluene addition and concentration step was repeated several times to remove traces of pyridine; a mixture of toluene and acetone was then added and the procedure was repeated. Finally, pure acetone was added and the solution was evaporated under vacuunm. Traces of toluene were removed by spreading the concentrated acetylated lignin-acetone solutions on filter paper and air drying. The filter paper was then extracted with chloroform to recover the acetylated MWLs. An altermite acetylation product workup that proved to be more convenient is described in the next section.

Alkaline hydrolysis of Salix MWL

A solution of the MWL (250 mg) in 1 M NnOH (5 ml) was heated at 50°C in Teflon[5] Vessel for 5 h. The pH of the solution was then adjusted to about 5 with acetic acid and the resulting suspension was freeze-dried. Acetylation of thc residue with 1/1 acetic anhydride-pyridine for 7 h at room temperature followed by precipitation of the product in water, filtration on a Spectra/Por UF membrane (MW cutoff = 1,000,000), thorough washing with dilute hydrochloric acid and water, and freeze-drying resulted in the lignin product (234 mg).

NMR methods

The quantitative ^{13}C NMR spectra were obtained with a Bruker WM-250 spectrometer controlled by an Aspect-2000A minicompu-

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Table 1. Origin of hybrid *Salix* clones

Clone	Age (years)	Code	Common name	Origin
Salix alba, cl.187	1	SA11 (1)	White willow	Yugoslavia
Salix alba, cl.187	14	SA11 (14)	White willow	Yugoslavia
Salix alba 1/24	1	SA6	White willow	Yugoslavia
Salix dasyclados	1	SD1	–	Germany
Salix hastata L.	1	SH3	–	United Kingdom

ter as previously described (Landucci 1985). An 8% - 9% solution of the acetylated MWL in acetone-d_6 in a 10 mm-tube, with the probe tuned to 62.9 MHz, was used for all experiments. The DEPT spectra (Doddrell *et al.* 1982) were acquired with it standard Bruker microprogram.

Results and Discussion

Main features of NMR spectra

Considerable information about a particular lignin can be obtained by quantifying major signals and signal groupings in a spectrum such as that illustrated in Figure 1. Shown in this quantitative partial spectrum of a typical acetylated *Salix* MWL are the signals due to methoxyl carbons (56 ppm), aliphatic carbons (51 - 101 ppm), aromatic carbons (101- 155 ppm), and acetoxy carbonyls (168–171 ppm). In addition, the presence of a signal at 101 ppm is indicative of a xylan impurity. Although an internal standard is normally used in quantitation, this is generally not practical for lignin analysis because of (1) the inconvenience of removing the standard substance, (2) the limited amount of signal-free area in a lignin spectrum, and (3) the lengthy relaxation times of a typically low molecular weight standard. Therefore, a part of the lignin spectrum must be used as an internal reference for the quantitation. With MWLs, a basic assumption is that the area of the aromatic signals is equal to 6.00, corresponding to the six carbon atoms in every lignin monomeric unit. This assumption is reasonable provided that there is no significant contribution from unsaturated (sp2) aliphatic carbons in this chemical shift range, such as those that may occur in the lignin side-chain. Generally, sp2 carbons in the lignin side-chain are protonated. Typically, one of these carbons gives a signal between 130 and 150 ppm (the unprotonated region), whereas the other carbon occurs among the signals from protonated aromatic sp2 carbons between 103 and 130 ppm. Therefore, the presence of a signal from a protonated carbon in the 130 to 150 ppm range is indicative of either an atypical aromatic carbon or an unsaturated aliphatic carbon. The latter case would invalidate the assumption of 6.00 carbons in the aromatic region and would require an appropriate correction.

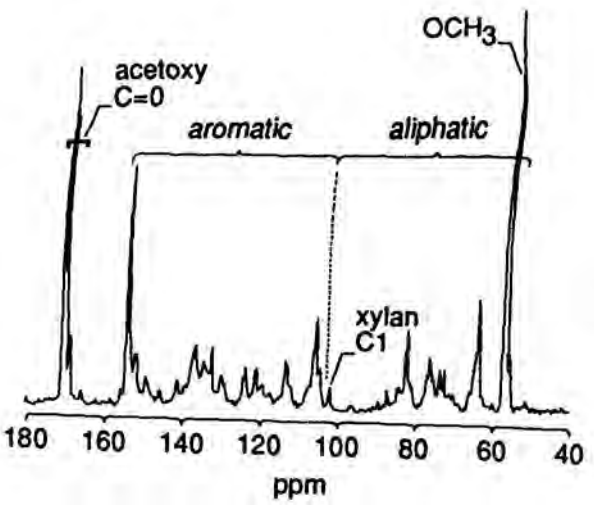

Fig. 1. ^{13}C NMR spectrum of acetylated *Salix* MWL (acetone-d_6).

Fortunately, the distinction between signals from protonated and unprotonated carbons is trivial with the use of one of the common spectral editing pulse sequences, such as DEPT, INEPT, APT, or JMOD, that have been applied to lignin characterization (Landucci 1991). These sequences give positive signals for protonated aromatic carbons and either do not detect unprotonated carbons (DEPT, INEPT) or give negative signals for such carbons (APT, JMOD). For example, Figure 2 shows the aromatic region of a DEPT spectrum of *Salix* MWL along with a conventional qualitative spectrum. Although the boundary between the protonated and unprotonated carbons is not sharp, it is generally at about 129 ppm. The relatively strong signal at 131.7 ppm, which is well into the unprotonated region, clearly results from a protonated carbon because it is present in the DEPT spectrum. However, this signal was assigned to the 2,6 carbons on p-acetoxybenzoate structures rather than an unsaturated aliphatic carbon (Landucci 1991). This assignment is consistent with the corresponding assignments (131.4 ppm in DMSO-d_6) of p-hydroxybenzoate structures in underivatized poplar MWL (Lapierre *et al.* 1984) and aspen MWL (Bardet *et al.* 1985).

An alternate method for signal quantitation, which would not be affected by unsaturated aliphatics, would be to set the total area of the aromatic and aliphatic signals (minus the methoxyl signal) to 9.00 for a C_9 phenylpropane unit. However, the presence

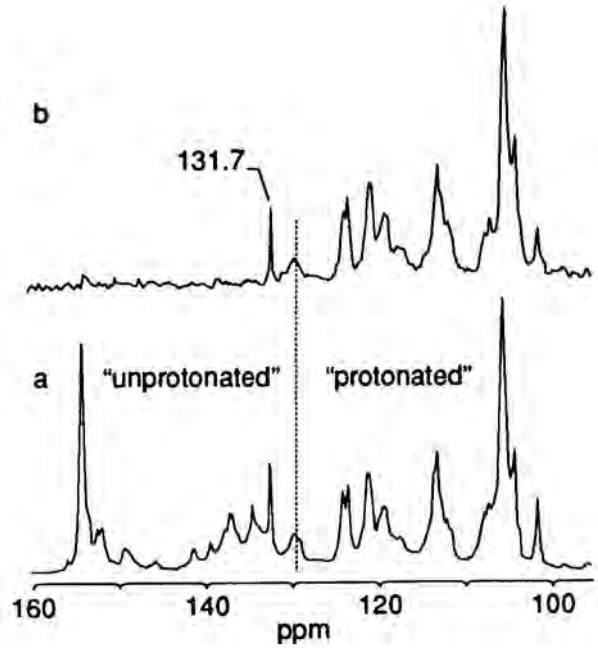

Fig. 2. Qualitative ^{13}C NMR spectrum and DEPT spectrum of aromatic region of acetylated *Salix* MWL (acetone-d_6).

of the signal at 101 ppm resulting from the C1 of xylan units would render this method invalid because the other xylan signals are in the aliphatic region along with the lignin aliphatics.

Setting the area of the aromatic signals to 6.00 and integrating selected signals and signal clusters in the quantitative spectrum (Fig. 1) resulted in the methoxyl, xylan C1, and acetoxy carbonyl content values shown in Table 2. The carbonyl signals can be further resolved into those associated with primary, secondary, or phenolic hydroxyl groups. The greater syringyl contents in the MWLs of the SA 11 clones are indicated by the higher methoxyl values. The values for the aliphatic acetoxy carbonyls must be treated with caution in a lignin that contains carbohydrate material because the acetoxy carbonyls associated with the carbohydrates overlap with corresponding lignin signals. With hardwood MWLs, the major carbohydrate impurity is xylan, which contains only secondary hydroxyl groups. Therefore, the signals in the 169.7–170.0 ppm region are due to secondary acetoxy carbonyl groups in both lignin and xylan. For example, as illustrated in Table 2, the higher xylan content in the MWLs of clones SA11 (1), SA6, and SH3 is reflected in correspondingly high values for secondary acetoxy carbonyls.

To correct these erroneously high values, the relationship of the xylan C1/C9 value (obtained from the area of the C1 signal at 101 ppm) and total secondary hydroxyl/C_9 can be extrapolated to zero xylan content, as illustrated in Figure 3. With this method, a value of 0.47 is obtained for the secondary acetoxy carbonyls on lignin units. The difference between the uncorrected values for the secondary carbonyls (Table 2) and 0.47 gives the contribution of xylan secondary acetoxy groups. When these values are divided by the corresponding values for the xylan C1 carbon, the number of xylan acetoxy carbonyl groups per xylose unit is obtained. For example, for the SD1 lignin: (0.56–0.47)/0.045 = 2.0. Values of 1.8–2.2 were obtained among the clones. This indicates two acetoxy groups per xylan unit, which is consistent with the postulated structure of a fully acetylated 4-0-methylglucuronoxylan structure (Fengel and Wegener 1983).

Details of NMR spectra

Precise quantitation of individual signals in crowded regions of a lignin spectrum is not feasible, even when spectra of rather high signal/noise (SIN) ratios can be obtained. However, if the overall SIN ratio is sufficient, even signals from low abundance structural groups can frequently be estimated to within ± 20%

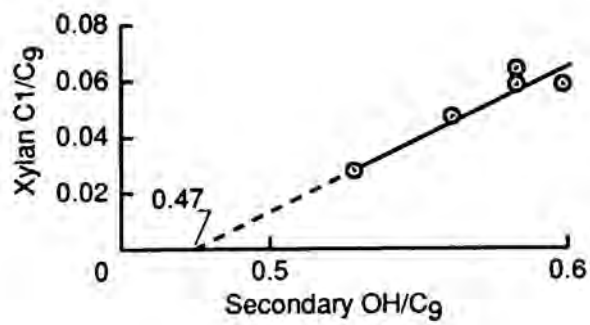

Fig 3. Relationship of xylan C1/C_9 and secondary hydroxyl/C_9 and extrapolation to zero xylan content.

Table 2. Contents of selected functional groupls/C_9 unit

Clone	Methoxyl	Xylan C1	Acetate C=O	Hydroxyl group		
				Primary	Secondary	Phenolic
SA11 (1)	1.44	0.060	1.57	0.69	0.60	0.28
SA11 (14)	1.45	0.028	1.45	0.70	0.53	0.22
SA6	1.33	0.062	1.48	0.68	0.58	0.22
SD1	1.36	0.045	1.47	0.68	0.56	0.23
SH3	1.39	0.058	1.51	0.67	0.58	0.26

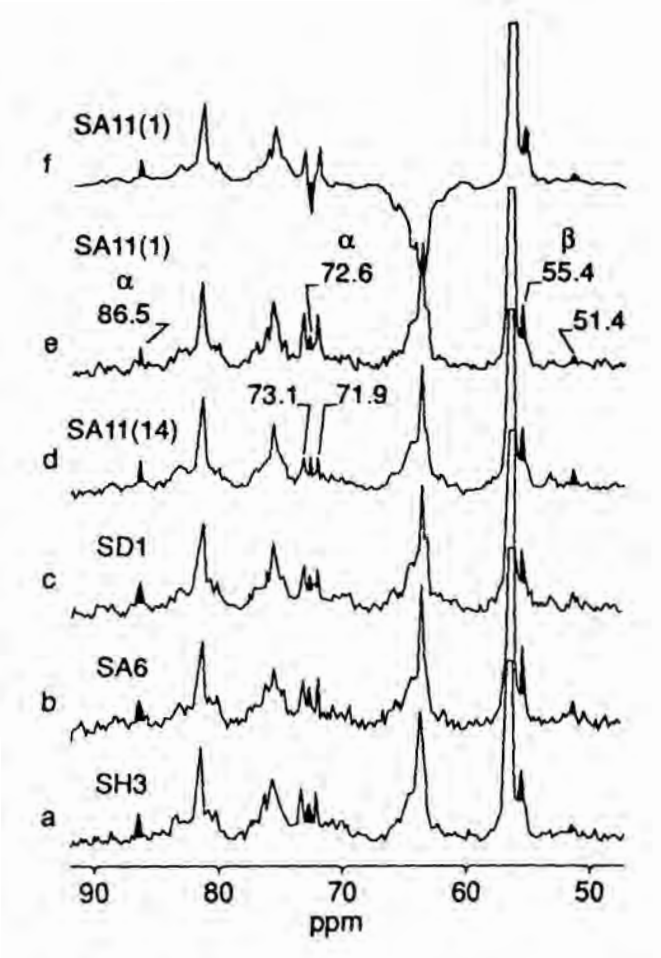

Fig. 4. Partial quantitative ^{13}C NMK spectra (a-c) and DEPT spectrum (f) of aliphatic region of acetylated *Salix* MWLs (acetone-d_6).

if the signals do not coincide with others. For example, in Figure 4a-e, the partial quantitative spectra of the aliphatic region of all the acetylated *Salix* MWLs are shown. Figure 4f is the corresponding DEPTspectrum of the MWL from the SA11 (1) clone. The shaded signals at 55.4, 72.6. and 86.5 ppm are those arising from the beta, gamma, and alpha carbons, respectively, from pinoresinol units (Nimz and Lüdemann 1976). These three signals can also be assigned to the corresponding signals in syringaresinol units because it was found that the syringaresinol side-chain signals in a syringyl synthetic lignin (DHP), prepared from synapyl alcohol had essentially the same chemical shifts as the pinoresinol side-chain signals in a guaiacyl DHP, prepared from coniferyl alcohol (Landucci and Hammel, unpublished results). Note that the gamma carbon at 72.6 ppm is inverted in the DEPT spectrum, confirming its identity as a methylene carbon. By determining an average area of the three signals along with the initial assumption of 6.00 units in the aromatic region, a range of 0.071 to 0.087 resinol units/C_9 was calculated among the clones. The two signals on either side of the gamma carbon at 71.9 and 73.1 ppm are presumably due to C3 and C4 of xylan units (Sorvari *et al.* 1986). The large difference in the intensity of these two signals in the young and old SA11 MWLs (Fig. 4d, e) is consistent with the corresponding difference in the xylan content for these two lignins given in Table 2.

For phenylcoumaran structures, the only signal that was unobscured by others was that from the beta carbon at 51.4 ppm (Nimz and Lüdemann 1976). However, this signal is very weak in all the clones and was estimated to correspond to 0.02 to 0.04 units/C_9.

Of particular interest in this MWL series was the relatively high concentration of *p*-acetoxybenzoate structures. Quantitation based on the prominent signal at 131.7 ppm (Fig. 2) gave values of 0.044 to 0.063/C_9. A series of closely related lignins, such as those examined in this study, are particularly valuable to the extent that fine detail in the spectra can be compared to confirm chemical shift assignments or to make additional assignments based on intensity changes in corresponding signals. For this type of comparison, it is best to use the conventional inverse-gated (quantitative) spectra because the intensities of signals from both protonated and unprotonated carbons can be directly compared. This comparison technique along with model studies has allowed the complete assignments of the carbons in the relatively minor *p*-acetoxybenzoate units, which are derived from the corresponding *p*-hydroxybenzoate units in the unde-

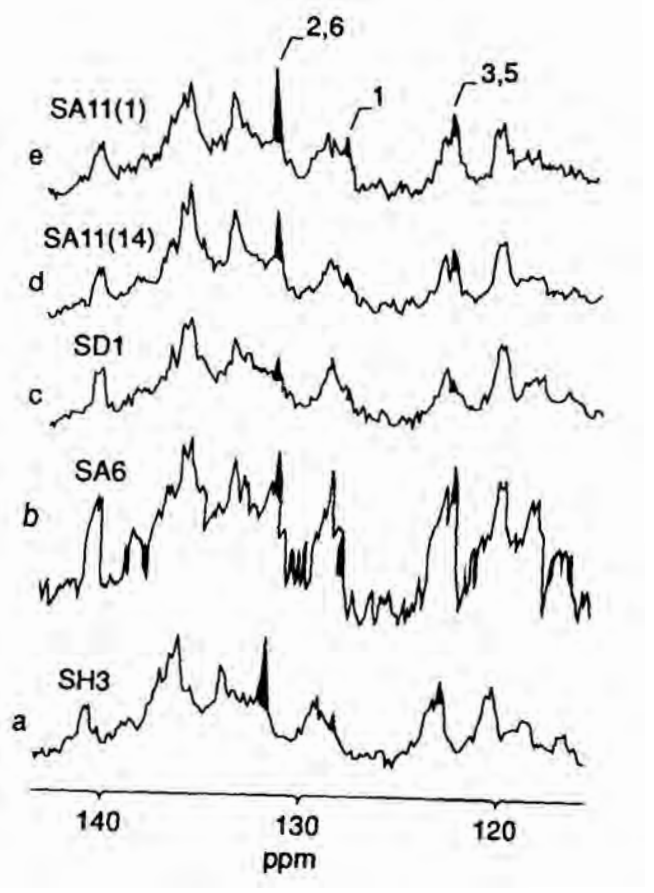

Fig. 5. Partial quantitative ^{13}C NMR spectra of aromatic region of acetylatcd *Salix* MWLs (acetone-d_6).

rivatized MWL. This subunit has recently been implicated as the cause of unusually high concentrations of phenol produced during hydrothermolysis and alkaline hydrolysis of hybrid poplar lignins (Agblevor and Boocock 1989a) and phenol found in mill effluents during sulfur-free semichemical pulping of hardwood (Shariff *et al.* 1989).

Figure 5a-e illustrates the 115–145 ppm region of the acetylated *Salix* MWL spectra. The key signal at 131.7 ppm was easily assigned to C2,6 of *p*-acetoxybenzoate units on the basis of the DEPT spectrum (Fig. 2b) and previous assignments. Unfortunately, some confusion and questionable assignments exist in the literature because of the lack of discrimmination between *p*-hydroxyphenyl and *p*-hydroxybenzoate. The introduction of the alpha carbonyl causes a downfield shift (greater ppm) of 3–4 ppm. The NMR data from numerous model compounds in both acetone and DMSO indicated that the chemical shift of the 2,6 carbons is < 130 ppm (127.2–129.1 ppm) in the presence of an alpha hydroxyl or alkoxyl, and > 130 ppm (130.6–132.5 ppm) in the presence of an alpha carbonyl.

A *p*-hydroxybenzoate entity could conceivably be attached to lignin by an ester linkage, an ether linkage, or both, as illustrated in Figure 6. Also illustrated in this figure are model compounds I–III,which represent these structure types. Compound IV models the acetylated version of structure type I, which is pertinent when dealing with acetylated MWLs. Table 3 lists the chemical shifts of C2,6 of models I–IV,a *Salix* MWL (clone SA11), and the corresponding acetylated MWL.

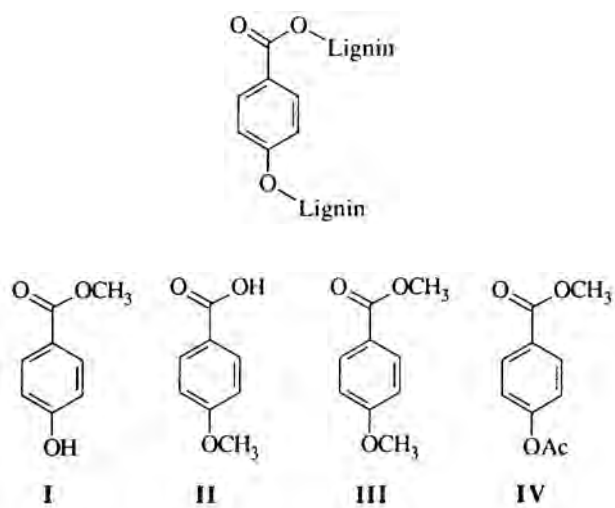

Fig. 6. Possible lignin linkages to *p*-hydroxybenzoate structures and corresponding model compounds.

With DMSO as the solvent, the value for the MWL is 131.4 ppm, which matches precisely the corresponding value for the benzoate ester. Similarly, the value of 130.6 ppm for the acetylated MWL is consistent with that of the acetylated benzoate ester. Also, in both acetone and chloroform, the chemical shifts of the acetylated MWL are consistent with those of the acetylated benzoate ester. Signals corresponding to the etherified acid or etherified ester model compounds could not be found in the lignin spectra. However, further confirmation was desired with the other signals in the *p*-acetoxybenzoate unit. Unfortunately, these signals overlapped with many others and could not be reliably assigned on the basis of any one sample. Upon careful study of intensities relative to the C2,6 signal among the quantitative spectra illustrated in Figure 5 and of the values obtained from the model compounds, all the carbons of the p-acetoxybenzoate subunit in the acetylated *Salix* MWL could be assigned. The complete assignments with both acetylated and underivatized MWLs along with the corresponding chemical shifts of the model compounds I–IV are given in Table 4.

As illustrated in Table 4, there is excellent agreement between the chemical shifts of model compound I (benzoate ester) with corresponding signals in the underivatized MWL and those of model compound IV (acetylated benzoate ester) with corresponding signals in the acetylated MWL. The two weak signals at 169.0 and 169.4 ppm in the underivatized MWL are most likely due to 0-acetyl groups on the xylan backbone of some residual hemicellulose, not to carbonyls of *p*-hydroxybenzoate structures as reported previously in poplar lignins (Agblevor and Boocock 1989b). Examination of several benzoate model compounds was inconsistent with this previous assignment.

Further confirmation of the signals corresponding to *p*-hydroxybenzoate units was obtained by mild alkaline hydrolysis (5 h at 50°C) of the sensitive ester

Table 3. Chemical shifts of C2,6 of *p*-hydroxybenzoate structures of lignin and lignin model compounds

Solvent	MWL	Benzoate ester (I)	Etherified ester (III)	Etherified acid (II)	Acetylated MWL	Acetylated benzoate ester (IV)
DMSO	131.4	131.4	131.2	131.3	130.6	130.7
Acetone	–	132.4	132.1	132.5	131.7	131.6
Chloroform	–	132.0	131.6	132.4	131.1	131.1

Table 4. Chemical shifts of *p*-hydroxybenzoate and *p*-acetoxybenzoate model compounds and corresponding assignments in underivatized and acetylated *Salix* MWLs in different solvents

	DMSO-d_6				Acetone-d_6			
		Model				Model		
Carbon	MWL	I	II	III	Acetylated MWL	II	III	IV
C1	nr[a]	120.2	123.0	121.9	127.9	123.7	123.4	128.5
C2,6	131.4	131.4	131.3	131.2	131.7	132.5	132.1	131.6
C3,5	115.2	115.3	113.7	114.0	122.8	114.5	114.8	122.8
C4	161.9	161.9	162.8	163.1	155.7	164.4	164.4	155.6
Benzoate C=O	165.3	166.1	167.0	165.9	166.5	167.5	166.8	166.5
Acetate C=O	169.0,4	–	–	–	169.2	–	–	169.2

[a] Not resolved.

linkage in the MWL. Figure 7a shows a qualitative spectrum of acetylate d Salix MWL; the shaded signals originated from the *p*-acetoxybenzoate structures. The only carbon of this submit not in this range is the acetoxy methyl carbon, which is masked by the other acetoxy methyl signals at about 21 ppm. After hydrolysis and reacetylation, another spectrum was obtained (Fig. 7b). Clearly, these signals are no longer present in the spectrum of the lignin polymer. The lack of any significant signal at 131.6 ppm (2.6 carbons) in the DEPTspectrum (Fig. 7c) of the hydrolyzed product con firm s the efficiency of the hydrolysis. Although the signal at 122.9 ppm, assigned to the 3,5 carbons, is apparently still present (but much less intense), this residual signal must he due to a different structure that coincidentally has t he same chemical shift.

Based on these results, we established that the *p*-hydroxybenzoate structures in *Salix* MWL are present primarily as free phenolic benzoate esters. If ethers or either-esters are present, they are below the range of detection by NMR technique.

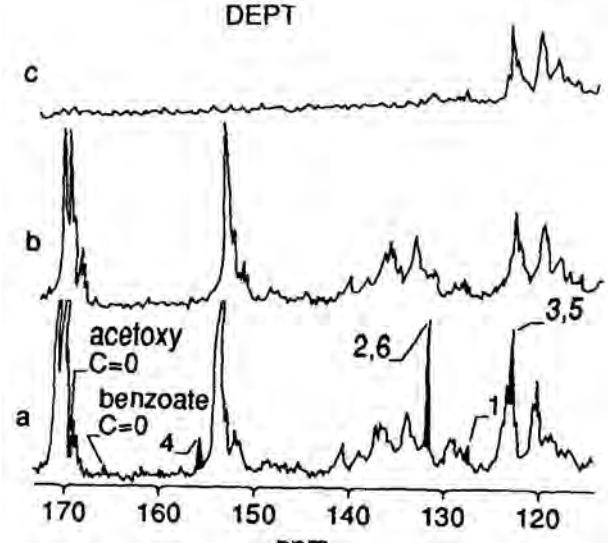

Fig 7. Partical ^{13}C NMR spectra of acetylated *Salix* MWL (a) before mild alkaline hydrolysis and (b) after hydrolysis and reacetylation; (c) DEPT spectrum corresponding to (b).

Conclusions

Comparison of quantitative ^{13}C NMR spectra of acetylated MWLs from five *Salix* clones indicated small differences in the intensities of some signals. Most of these differences were associated with carbohydrate material, variations in free phenolic content, and minor structure types such as *p*-acetoxybenzoate structures. By noting the changes in signal intensities among clones. along with lignin model studies, assignments were made of the signals of the p-acetoxybenzoate structures in the lignin spectra. In addition, mild alkaline hydrolysis of the MWL resulted in the disappearance of these signals in the lignin spectrum. which is consistent with an ester linkage. No evidence for corresponding ether-linked structures was found.

Acknowledgement

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