

Nondestructive Analysis of Lignin Structure by NMR Spectroscopy of Specifically ^{13}C -Enriched Lignins

Part 1. Solid State Study of Ginkgo Wood

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Summary

Guaiaacyl-type lignin is the major component of ginkgo lignin. Specific ^{13}C -enrichment of α , β and γ -carbons of the guaiaacylpropane side chains was achieved by administration of coniferin- $[\alpha\text{-}^{13}\text{C}]$, coniferin- $[\beta\text{-}^{13}\text{C}]$ and coniferin- $[\gamma\text{-}^{13}\text{C}]$, respectively, to growing stems of ginkgo trees. Unenriched coniferin was administered as a control. The xylem tissues containing specifically ^{13}C -enriched lignins or unenriched lignin were subjected to analysis by solid state NMR. Subtraction of the spectrum of tissue containing unenriched lignin from the spectra of tissues containing ^{13}C -enriched lignins gave difference spectra exclusively assigned to the enriched side chain carbons of intact protolignin in the cell walls. From the signal intensities determined under quantitative conditions and an estimate of corresponding standard uncertainties, the percentage ranges of the major inter-unit lignin bonds originating from the β -carbon of the coniferin precursor were estimated to be: $\beta\text{-O-4}/\alpha\text{-O-R}$ (R = H, polysaccharides or lignols) including $\beta\text{-OH}$, 53 % to 57 %; combined $\beta\text{-5}$, $\beta\text{-}\beta$ and $\beta\text{-1}$, 32 % to 36 %; coniferaldehyde end groups, 2 % to 4 %; and coniferyl alcohol end groups, 4 % to 6 %.

Introduction

Lignin as it exists in the cell wall is called protolignin to distinguish it from isolated lignin preparations, which are different from the protolignin in many respects. Protolignin is formed in polysaccharide gels by polymerization of monolignols in an irreversible manner, and the kinds of monolignol and polysaccharides vary with the age and type of the cell. Consequently, protolignin is physically and chemically bound to polysaccharides, and the structure and distribution of the protolignin macromolecule in the cell wall is not uniform. As a result, it is impossible to isolate protolignin quantitatively from the cell wall keeping its three dimensional heterogeneous structure.

Milled wood lignin (MWL) is frequently used for studies in lignin chemistry. However, a large part of MWL is presumed to be derived from secondary wall lignin (Whiting and Goring 1981; Terashima *et al.* 1992; Maurer and Fengel 1992; Kim and Koh 1997). Its maximum yield is usually less than half of the protolignin in the cell wall, and it contains a considerable amount of carbohydrates intimately bound to lignin.

The most commonly employed approaches to the elucidation of lignin structure have been various types of degradation analysis. At first, wood tissue or MWL is subjected to oxidation, reduction or solvolysis to produce monomeric, dimeric or oligomeric fragments, and then structure and

yield of the fragments are determined. Various structural schemes for lignin have been proposed based mainly on the information on these low-molecular-mass fragments. However, in the case of soft wood lignin, total fractional yield of low-molecular-mass fragments is less than 40 %; hence, more than half of the lignin macromolecule cannot be examined by the degradation methods.

These characteristic features of protolignin make study of its structure difficult. Tracer methods employing radio- and stable isotopes have been developed as promising approaches to circumvent these difficulties because they can provide information on the structure of protolignin without isolating it from the cell walls. By an improved radiotracer method using ^{14}C and ^3H , the heterogeneity in macromolecular structure and distribution of lignin in different morphological regions of cell walls have been studied (Terashima *et al.* 1979, 1986, 1988, 1993; Terashima and Fukushima 1988; Fukushima and Terashima 1991). However, the radiotracer method does not provide direct information on the chemical environment involving radio-labeled carbon or hydrogen. In contrast, the method employing specific ^{13}C -enrichment, combined with solid state NMR spectroscopy, can provide information on the chemical structure of protolignin without isolating it from the cell wall (Lewis *et al.* 1987, 1988, 1989). The selective ^{13}C -enrichment of a specific carbon of protolignin in various plants has been achieved by feeding a specifically ^{13}C -

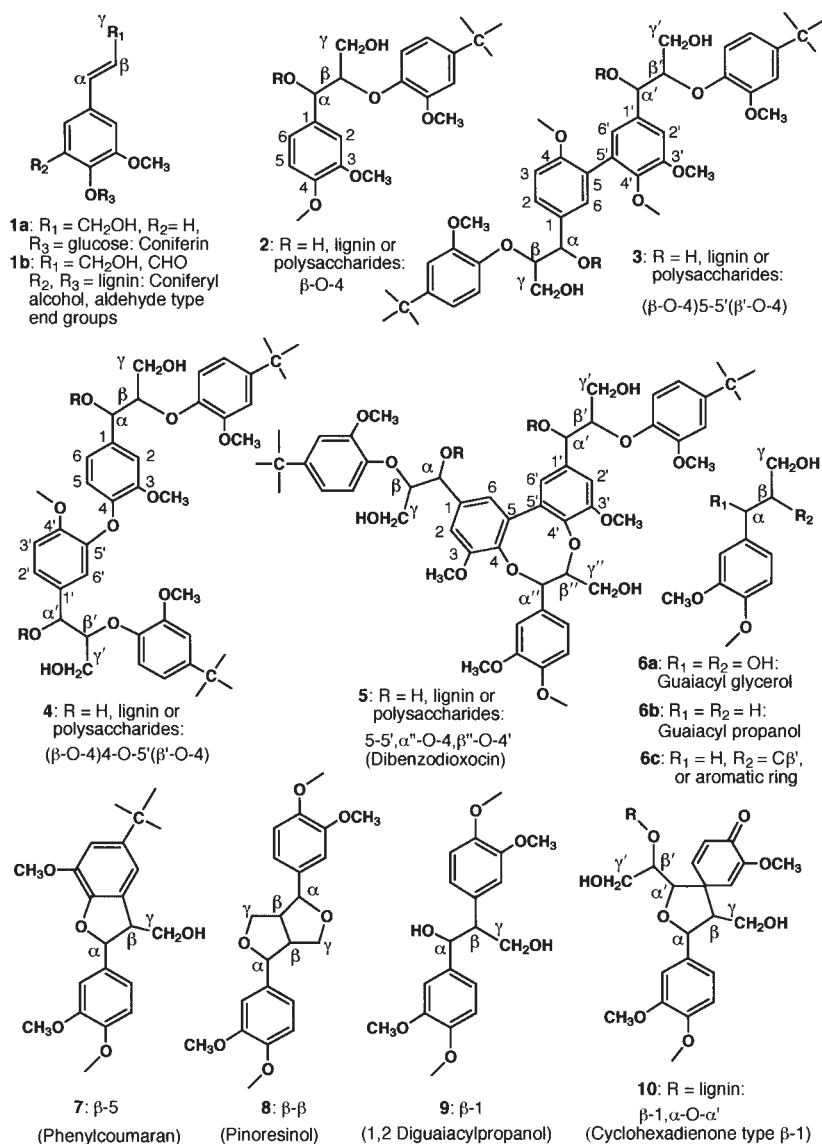


Fig. 1. Substructures of guaiacyl lignin.

enriched ferulic acid to plant seedlings (Lewis *et al.* 1987, 1988, 1989), ^{13}C -enriched phenylalanine to cultured cells (Eberhardt *et al.* 1993) and ^{13}C -enriched monolignol glucosides to lignifying tissues of plants (Terashima *et al.* 1991, 1997; Xie and Terashima 1991, 1993; Xie *et al.* 1994a, b). The achievement of selective ^{13}C -enrichment at a specific carbon has been confirmed by solution state NMR of isolated MWLs (Terashima *et al.* 1991; Xie and Terashima 1991; 1993; Eberhardt *et al.* 1993; Xie *et al.* 1994a, b) or dimethoxypropane lignin (Xie and Terashima 1993), or by solid state NMR (Lewis *et al.* 1987, 1988, 1989; Eberhardt *et al.* 1993; Terashima *et al.* 1997). However, further improvements in the ^{13}C -enrichment technique and in the determination of NMR spectra were necessary to obtain more useful information for the study of structure and reaction of protolignin in the wood cell walls by a combination of these techniques.

This paper deals with nondestructive analysis of protolignin structure in ginkgo wood by careful application of

the technique of specific ^{13}C -enrichment combined with solid state NMR spectroscopy.

Materials and Methods

Syntheses of specifically ^{13}C -enriched coniferins and unenriched coniferin

Coniferins (**1a** in Fig. 1, $\text{R}_1 = \text{CH}_2\text{OH}$, $\text{R}_2 = \text{H}$, $\text{R}_3 = \text{Glucose}$), both ^{13}C -enriched at specific side chain carbons and unenriched (natural abundance), were synthesized by the procedure of Terashima *et al.* (1996). For synthesis of coniferin- $[\alpha\text{-}^{13}\text{C}]$, vanillin- $[\text{carbonyl-}^{13}\text{C}]$ was prepared from sodium acetate- $[\text{1-}^{13}\text{C}]$ (99 atom %, Aldrich Chem., Milwaukee, WI, USA¹) as an intermediate compound by the procedure of Terashima *et al.* (1991). For syntheses of coniferin- $[\beta\text{-}^{13}\text{C}]$, coniferin- $[\gamma\text{-}^{13}\text{C}]$ and unenriched coniferin,

¹) Certain commercial companies are named in order to specify adequately the experimental procedure. This in no way implies endorsement or recommendation by NIST.

malonic acid-[2-¹³C] (99 atom %, Aldrich Chem.), malonic acid-[1,3-¹³C] and monoethyl malonate respectively were employed.

Administration of coniferins to ginkgo shoots

Ginkgo trees (5-year-old) were grown in pots. Cut shoots (15 cm to 20 cm) were put in small vials containing an aqueous solution of either coniferin-[α -¹³C], coniferin-[β -¹³C], coniferin-[γ -¹³C] or unenriched coniferin (as unfed control). After feeding the solution (300 mg coniferin/300 ml water) for about one week under natural conditions (sunlight in daytime and dark at night), the shoots were allowed to grow further for one month in flasks containing water. The bark was removed, and newly formed xylem was collected and extracted with ether and hot water, and dried in a vacuum desiccator.

Solid state NMR

Solid state ¹³C-NMR spectra were obtained using a non-commercial spectrometer operating at a ¹³C frequency of 25.19 MHz, using cross polarization (CP) and magic angle spinning in the usual way (Schaefer *et al.* 1975; Terashima *et al.* 1997). This spinning frequency was fixed at 4 kHz so that there would be no overlap between center bands and side bands. By fixing the spinning frequency, side band intensities would not contribute to the differences in center band intensities, which, in turn, formed the basis for our interpretations. The proton and ¹³C rf levels used in CP and decoupling corresponded to nutation frequencies of 66 kHz and 70 kHz, respectively. The CP time was 1.0 ms and the time between scans was 4 s. Typically 15 000 to 20 000 transients were averaged and chemical shifts are referenced, by substitution, to the methine resonance of adamantane at 29.50 ppm.

Measurement uncertainties

Uncertainties in measurements will be expressed in the form ($a \pm b$) where, unless otherwise indicated, 'b' is the estimated standard uncertainty.

Results and Discussion

Monolignol glucoside, coniferin, as an effective precursor for ¹³C-enrichment

Ginkgo is one of the oldest surviving trees from the viewpoint of plant evolution. The anatomy and chemical composition of ginkgo wood are close to that of conifer wood, though the difference between compression wood and normal wood is less in ginkgo than in conifers. The structure of ginkgo lignin is also close to that of conifer lignin. Alkaline nitrobenzene oxidation of ginkgo wood gave combined yields (as mass fractions) of aldehydes and acids as follows; *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid derived from *p*-hydroxyphenyl lignin: (3.5 ± 0.2) %; vanillin and vanillic acid derived from guaiacyl lignin: (95.3 ± 0.2) %; syringaldehyde and syringic acid derived from syringyl lignin: (1.3 ± 0.2) % based on the total aromatic aldehydes and acids (Fukushima and Terashima 1991). Pyrolysis-gas chromatography of ginkgo wood also showed that the content of syringyl units in ginkgo lignin is very low (Obst and Landucci 1986). These results indicate that the major part of protolignin in ginkgo wood consists of guaiacyl lignin containing a small amount of *p*-hydroxyphenyl lignin in the middle lamella region, and a very small amount of syringyl lignin in the secondary wall (Fukushima and Terashima 1991).

It is worthwhile to discuss the suitability of using coniferin as a precursor for the specific ¹³C-enrichment of ginkgo lignin. There are many facts and observations which support the hypothesis that labeled monolignols, administered as their glucosides, are effectively incorporated into cell wall lignin in a biologically controlled manner. We mention the following:

1. Coniferin is present in lignifying tissue of conifers (Freudenberg 1968).

2. β -Glucosidase is present in the lignifying cell wall of conifers (Freudenberg 1968; Marcinowski and Griesebach 1978) and coniferin-specific β -Glucosidase has been found in differentiating xylem of pine (Dharmawardhana *et al.* 1995).

3. When radio-labeled coniferin was administered to lignifying spruce, the radioactive aglycon, coniferyl alcohol, was incorporated irreversibly into lignin (Freudenberg *et al.* 1955; Kratzl *et al.* 1957; Kratzl and Hofbauer 1958; Freudenberg 1968).

4. By microautoradiography, it was shown that different kinds of radio-labeled monolignols, administered as their glucosides, were incorporated preferentially into different morphological regions of cell walls (Terashima and Fukushima 1989; Fukushima and Terashima 1991; Terashima *et al.* 1993). These results coincide with those obtained by UV microspectroscopy and SEM-EDXA analysis (Fergus and Goring 1970; Saka and Goring 1985) as well as degradation analyses of isolated cell wall layers (Hardell *et al.* 1980a, b).

5. During the incorporation of monolignols administered as their glucosides, some progressive biochemical modification of the aromatic ring occurs, passing from *p*-hydroxyphenyl to guaiacyl units and from guaiacyl to syringyl units (Terashima *et al.* 1986; Terashima and Fukushima 1988; Fukushima and Terashima 1991; Matsui *et al.* 1994). This fact indicates that the administered precursor was incorporated into lignin through the normal metabolic pathway.

6. Radio-labeled monolignol glucosides were administered to ginkgo and magnolia woods; then MWL and LCC were prepared. The distribution of label in the MWL, LCC and residual wood indicated that the labeled lignin was intimately bound to polysaccharides in the cell wall (Terashima *et al.* 1992).

7. Solution NMR spectra of MWLs prepared from pine or ginkgo xylem, to which coniferin or ¹³C-enriched coniferin were administered, showed no difference compared with that of MWL prepared from unfed control xylem, except for an increased signal intensity assigned to the ¹³C-enriched carbon (Terashima *et al.* 1991; Xie and Terashima 1991; Xie *et al.* 1994a).

8. Solid state NMR spectra of suspension-cultured pine cells to which ¹³C-phenylalanine had been applied (Eberhardt *et al.* 1993) were similar to the spectra of ¹³C-coniferin-administered wheat tissue, except that a part of the phenylalanine was incorporated into the protein of the suspension-cultured cells (Terashima *et al.* 1997).

From above facts, it is reasonable to expect that the ¹³C-enriched coniferyl alcohol derived from coniferin would be

incorporated effectively into guaiacyl lignin, the major part of protolignin in ginkgo wood.

Solid state NMR of specifically ^{13}C -enriched guaiacyl lignin in ginkgo tissue

Figure 2 shows the solid state NMR spectra of ginkgo xylem tissues to which coniferin- $[\alpha\text{-}^{13}\text{C}]$, coniferin- $[\beta\text{-}^{13}\text{C}]$, coniferin- $[\gamma\text{-}^{13}\text{C}]$ or unenriched coniferin were administered. At the chemical shifts corresponding to the enriched side chain carbons, the signal intensities in the spectra of labeled xylem tissues (Fig. 2A, 2B and 2C) increased relative to the intensities of the unenriched-control spectrum (Fig. 2D). To demonstrate these increases in signal intensity, difference spectra (Fig. 3A, 3B and 3C) were obtained by subtraction of 2D from 2A, 2B or 2C. In generating the difference spectra, the scaling of spectrum 2D had to be established. In choosing this scaling, we applied two principles. First, chemistry and formation of lignin in the presence of the enriched or unenriched coniferin is assumed to be the same. Hence, in generating the difference spectra, it is appropriate to null certain resonance regions (e.g. portions of the aromatic region between 110 ppm and 160 ppm) associated exclusively with lignin carbons whose resonances do not overlap with those carbons associated with the enriched sites. In this manner, the amount of lignin represented is the same in both components of each difference spectrum. Second, we did not want the saccharide (mainly cellulosic) contributions to contribute to the difference spectrum. These latter contributions have their special signatures (including the narrower spectral features of crystalline cellulose) in the 60 ppm to 107 ppm region. Hence, we nulled the saccharide intensities in each difference spec-

trum as well. If the lignin to cellulose ratio is constant, then one choice of scaling produces nulls for both the unenriched lignin and the saccharide carbons. In Figure 3, spectrum 3A satisfied this expectation. However, for spectra 3B and 3C, generation of the lignin null left a small, but not negligible, saccharide contribution because the cellulose to lignin ratio was not exactly the same in the control as in the enriched samples. In the difference spectra, the identifiable signature of the cellulose component is the suite of spectrally sharper resonances, near 66, 73, 76, 90 and 105 ppm, corresponding to the crystalline carbons. In order to get rid of this small, residual saccharide contribution, we subtracted an additional, small amount of intensity using the spectrum (not shown) of a Kraft pulp. This spectrum was chosen from our extensive library of cellulose spectra on the basis of having the best lineshape match to the saccharide contribution in the ginkgo spectrum.

In Figure 3 it is noteworthy that characteristic signals assigned to $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$ or $^{13}\text{C}\gamma$ appear in the specific region, and no remarkable spectral overlap is observed. From this we conclude that very little, if any, random enrichment of side-chain carbons occurs during the incorporation of the precursor coniferin into the macromolecular protolignin of the cell wall. Thus, we will assume that there is no scrambling as we discuss the spectra of Figure 3 in terms of the major substructures of protolignin (Fig. 1). In the following discussion, chemical shifts are assigned to structures based on assignments from solution-state, high resolution NMR (Lüdemann and Nimz 1974; Robert 1992; Ralph *et al.* 1999). Table 1 summarizes the assignments and the relative intensities (with standard error estimates) corresponding to the distinct resonance regions for each difference spectrum of Figure 3.

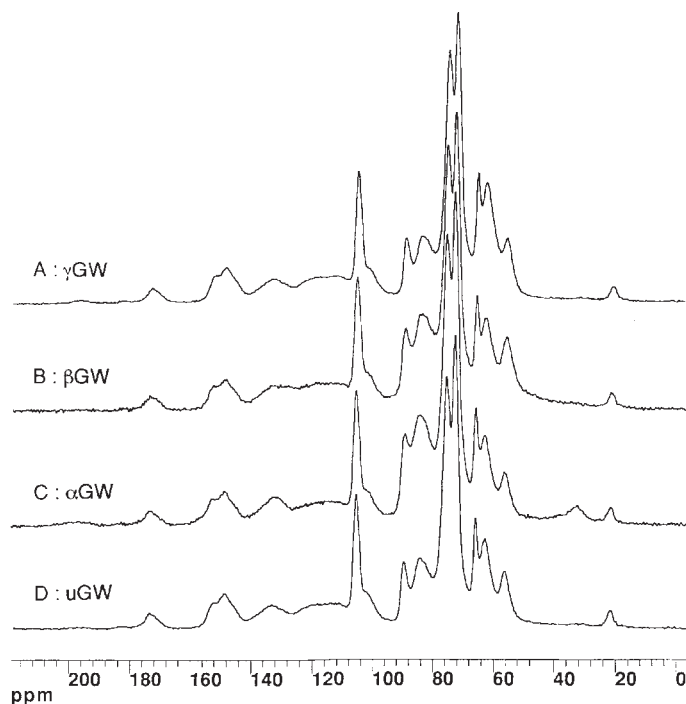


Fig. 2. Solid state NMR spectra of ginkgo wood fed with coniferins ^{13}C -enriched at side chain $\text{C}\gamma$ (A: γGW), $\text{C}\beta$ (B: βGW), $\text{C}\alpha$ (C: αGW) and unenriched coniferin (D: uGW).

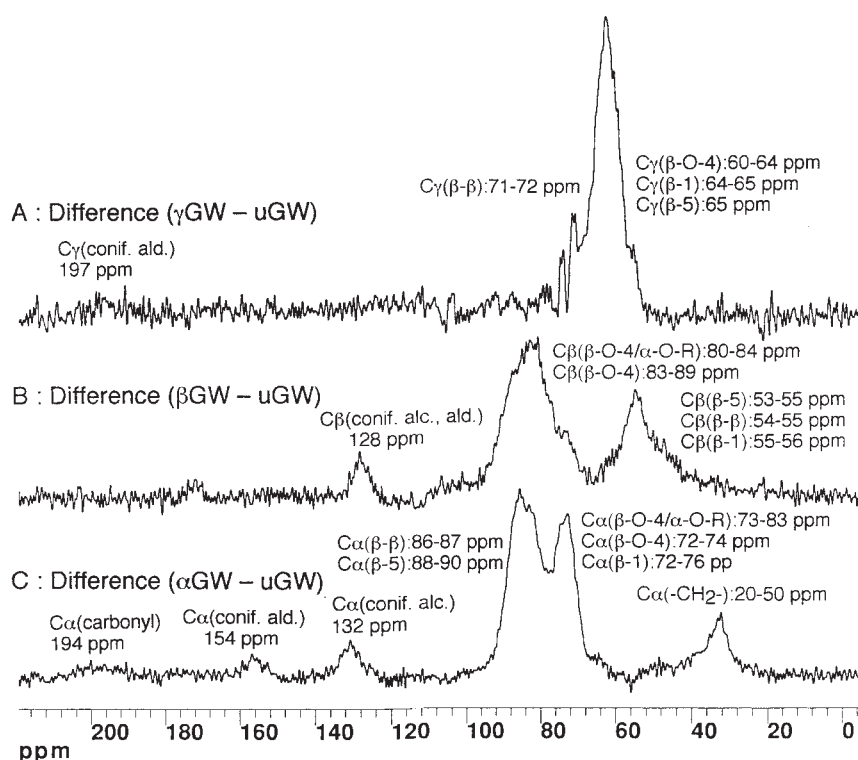


Fig. 3. Difference spectra obtained by subtracting the spectrum of ginkgo wood fed with unenriched coniferin from the spectrum of ginkgo wood fed with either $C\gamma$ -enriched coniferin, A: Difference (γ GW-uGW), $C\beta$ -enriched coniferin, B: Difference (β GW-uGW) or $C\alpha$ -enriched coniferin, C: Difference (α GW-uGW). Spectra B and C also involved subtraction of a very small amount of intensity using a spectrum of a Kraft pulp (not shown). The latter, predominantly-cellulose spectrum, was used to make minor corrections for differences in lignin-to-cellulose ratios (see text).

Deductions about lignin structure from the difference spectra

The signals exclusively assigned to $C\gamma$ (Fig. 3A) mainly appear in a narrow range, and resolution is not high enough to estimate chemical structures related to the γ -carbon. The rapidly oscillating features in the 70 ppm to 74 ppm range are not true peaks but are artifacts which arise from the subtraction of slightly shifted, intense cellulose peaks with limited digital resolution. In this latter region, $C\gamma$ is contributing a shoulder whose origin is probably the (β - β) pinosresinol-type (**8**) substructure. There is also a weak intensity near 197 ppm associated with coniferyl aldehyde. From the signal intensity, the mass fraction of $C\gamma$ in the form of coniferaldehyde end group (**1b**) is estimated to be $3 \pm 1.5\%$.

The signal near 128 ppm in Figure 3B, representing $6.5 \pm 1.5\%$ of the total spectrum intensity, is assigned to β -carbons ($C\beta$) in coniferaldehyde and coniferyl alcohol end groups (**1b**). The signals observed in the 64 ppm to 100 ppm region in Figure 3B are assigned to several substructures: $C\beta$ in β -O-4 (**2**), in β -O-4/ α -O-polysaccharides in ester or ether form (**2**), in (β -O-4)5-5'(β' -O-4) (**3**) and $C\beta''$ in dibenzodioxocin structure (**5**) (Karhunen *et al.* 1995; Brunow *et al.* 1998). The signals assigned to $C\beta$ in (β -O-4)4-O-5'(β' -O-4) (**4**) and to the $C\beta$ of guaiacylglycerol (**6a**) also appear in this region. From the integrated sig-

nal area, the percentage of β -O-4 type $C\beta$ including substructures **2**, **3**, **4**, **5** and **6a** is estimated to be $55 \pm 2\%$. A part of the β -1 substructure (**9**) may be present in wood as its precursor form, tetrahydrofuran-3-spiro-4'-cyclohexadienone structure (**10**) (Brunow and Lundquist 1991; Brunow *et al.* 1998) or its hydrolyzed form (**10**, α -OH, α' -OH) (Zhang and Gellerstedt 1999). Cleavage of the bond between $C\alpha'$ and the cyclohexanone ring of the latter forms β -1 (**9**) and glyceraldehyde- $C\beta'$ -OR. The signal of this $C\beta'$, located on the side chain of **10** and its hydrolyzed form, as well as $C\beta'$ located on the glyceraldehyde-O-R ether will also appear in this region. The signals in the 30 ppm to 64 ppm range (Fig. 3B) are assigned to $C\beta$ in β -5 (**7**), β - β (**8**) and β -1 (**9**) substructures. The combined percentage of $C\beta$ in these types of substructures is determined to be $35 \pm 2\%$. Sakakibara (1980) proposed 38% for combined $C\beta$ in β -5, β - β and β -1 substructures in his structural model for softwood lignin. We speculate that signals from the $C\beta$ associated with the α - CH_2 sites (discussed below) will also be found in this region.

The signals in Figure 3C were assigned exclusively to $C\alpha$. The broad signal at 185 ppm to 208 ppm was assigned to various types of carbonyl groups at the α position, and the combined percentage was determined to be $4 \pm 1\%$. Signals from $C\alpha$ in coniferaldehyde and coniferyl alcohol appear at different chemical shifts, around 154 and 132 ppm, respectively. From the signal intensity, the percentages of

Table 1. Measured relative intensities and assignments for each distinct region in the difference spectra of Figure 3. Standard uncertainties are given in parentheses

Difference spectrum	Region (ppm)	Relative intensity	Region assignments
C- α	20 to 50	0.14 (.02)	α -CH ₂
C- α	65 to 95	0.74 (.02)	β -O-4, β -O-4/ α -O-R, β -1, β -5, β - β ,
C- α	65 to 77	0.28 (.05)	β -O-4, β -O-4/ α -O-R, β -1
C- α	77 to 95	0.46 (.05)	β -O-4/ α -O-R, β -5, β - β
C- α	122 to 140	0.05 (.01)	Conif. alcohol
C- α	148 to 164	0.03 (.01)	Conif. aldehyde
C- α	188 to 210	0.04 (.02)	C- α carbonyl
C- β	30 to 65	0.35 (.02)	β -1, β -5, β - β , and β carbon adjacent to α -CH ₂
C- β	65 to 100	0.57 (.02)	β -O-4, (β -O-4/ α -O-R)
C- β	120 to 135	0.065 (.015)	Conif. alcohol, conif. aldehyde.
C- β	166 to 178	0.015 (.01)	?
C- γ	50 to 76	0.97 (+0.15, -0.05)	Everything except conif. aldehyde
C- γ	180 to 206	0.03 (.015)	Conif. aldehyde

coniferinaldehyde and coniferyl alcohol end groups were estimated to be $3 \pm 1\%$ and $5 \pm 1\%$, respectively. These latter values are consistent with the integrals involving these endgroups in the C β and C γ difference spectra. In contrast, by $^1\text{H-NMR}$ spectroscopy of spruce MWL, the content of cinnamaldehyde and cinnamyl alcohol end groups has been estimated to be 4% and 1% (Lundquist and Olsson 1977; Brunow and Lundquist 1980). Analysis of cinnamaldehyde end groups by reduction difference spectroscopy gave also similar results (Adler and Marton 1959). While the reported sum of these two components is similar to our results, we differ from these reported results in that we see a dominance of the cinnamyl alcohol over the cinnamyl aldehyde. On the other hand, solid state $^{13}\text{C-NMR}$ of a man-made dehydrogenation polymer of coniferyl alcohol (DHP) (Lewin *et al.* 1989; Hafrén *et al.* 1999; Parkås *et al.* 1999) indicates that the DHP contains a large number of these end groups.

The signals of C α involved in the major inter-unit bonds in substructures, β -O-4, β -5, β - β and β -1 were not resolved enough to estimate the percentage of C α in each of these substructures. However, there is a partially resolved splitting over this 65 ppm to 95 ppm resonance range with an intensity ratio of the downfield to the upfield components of approximately 5:3. In Table 1, the total as well as the component intensities are listed along with the corresponding assignments (Fig. 3C).

The signal of C α in the methylene form has a maximum near 34 ppm and the corresponding intensity in the 20 ppm to 50 ppm range is $14 \pm 2\%$. A type of C α on a side chain, i.e. a guaiacyl propanol type end group (**6b**), is consistent with this chemical shift and has been proposed on the basis of degradation analyses (Sakakibara 1980). However, if structure (**6b**) were to account for the resonance near 34 ppm in spectrum 3C, then we would also expect to see

the methylene carbon in the β position of this structure near 32 ppm in spectrum 3B. But such a resonance is very weak in spectrum 3B. It is interesting that high resolution NMR spectra (to be discussed in a forthcoming paper) of MWL's derived from these C α - and C β -enriched samples both showed resonances in the vicinity of 35 ppm, but with relative intensities in the 5%, rather than the 14% range. This difference between protolignin and MWL suggests the possibility of either a chemical bias (a preferential reactivity) or a chemical modification associated with the production of the MWL. The resonance around 34 ppm in spectrum 3C, we believe, should be assigned mainly to methylene C α in substructures (**6c**) in which C β is bonded to C β' such as larciresinol type substructure, or C β is bonded to an aromatic ring of another guaiacylpropane unit. Sakakibara (1980) has proposed the presence of these types of substructures in softwood lignin. The latter suggested structures have C α carbons that fall into the 30 ppm to 65 ppm range in Table 1 and the listed assignment reflects that.

It is noted that the above information on the type and frequency of inter-unit bonds pertains to *intact* protolignin in the cell wall. The intensity information summarized in Table 1 is not sufficient for determining the probabilities for all type of bonds. Only the coniferyl alcohol, the coniferyl aldehyde and the C α -CH₂ carbons have direct estimates. The remaining categories of carbons considered in Table 1 cannot be uniquely determined. We simply note that, considering the intensities and uncertainties of Table 1, the β -O-4 substructures including β -O-4/ α -O-R are the main structures in intact lignin.

By utilizing this technique, it will be possible to trace the structural change of lignin under various reaction conditions, such as pulping, bleaching or biodegradation, if the tissues containing ^{13}C -enriched lignin and unenriched lignin are treated under identical conditions to obtain an adequate difference spectrum. The application of difference solid state NMR spectroscopy in a yellowing study of a specifically ^{13}C -enriched DHP-cell wall complex has also been shown useful by Parkås *et al.* (1999).

The probabilities of β -5, β - β , β -1 and other substructures can be estimated under higher resolution conditions by difference NMR in the solution state. The major question remains whether MWL truly represents the intact lignin. Results obtained in this paper point to significant possible discrepancies between intact lignin and MWL, but more work needs to be done. In particular, the MWL derived from the same samples used for solid state NMR analysis, will be helpful in sorting out this question. Presentation and analysis of the difference NMR spectra of specifically ^{13}C -enriched MWLs in solution will be offered in later papers.

Conclusion

Specific ^{13}C -enrichment of side chains α , β , and γ -carbon of guaiacyl lignin in ginkgo wood was achieved by administration of coniferin- $[\alpha\text{-}^{13}\text{C}]$, coniferin- $[\beta\text{-}^{13}\text{C}]$, and coniferin- $[\gamma\text{-}^{13}\text{C}]$ to growing stems of ginkgo trees. Difference NMR spectra between wood tissues containing specifically ^{13}C -enriched protolignin and unenriched protolignin provided

information on the frequencies of major linkages with which a specific side chain carbon is involved. The solid state NMR spectroscopy combined with the technique of specific ¹³C-enrichment can be employed as one of the general nondestructive approaches for structural analysis of lignin in the cell wall.

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