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SOLID STATE NMR SPECTROSCOPY OF SPECIFICALLY ^{13}C-ENRICHED LIGNIN IN WHEAT STRAW FROM CONIFERIN

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Key Word Index—*Triticum aestivum*; wheat; Gramineae; ^{13}C-enrichment; solid state ^{13}C NMR spectroscopy; coniferin; lignin; lignin structure; interunit linkages.

Abstract—Three coniferins, specifically ^{13}C-enriched at side chain α, β and γ carbons, and natural abundance (unenriched) coniferin were administered to internode cavities of lignifying culms of dwarf wheat. Difference ^{13}C CP/MAS spectra were obtained between the spectra of the coniferin-fed and the unfed wheat straws, or between the spectra of straws fed with enriched coniferin and unenriched coniferin. The difference spectra indicated that, although the feeding of coniferin increased the lignin content slightly, the normal lignification process was not affected seriously by feeding of the precursor. The lignin derived from the coniferin in the wheat stalk was specifically ^{13}C-enriched at the positions corresponding to the fed precursors. It is estimated that of the total lignin associated with the bottom and top sections of the wheat straws, respectively, 15.4 ± 2.0 and $9.5 \pm 2.5\%$ of the lignin originated from the labelled coniferin. The percentages of the major dimeric substructures of enriched lignin in the top and bottom of internodes, respectively, are: β-*O*-4 including β-*O*-4/α-*O*-R (R = carbohydrates and lignols), 74 ± 1.5 and $65 \pm 1.5\%$; combined β-5, β-β and β-1 structures, 18 ± 1.5 and $28 \pm 1.5\%$; and combined coniferyl alcohol and aldehyde end groups, 8 ± 1.5 and $6 \pm 1.5\%$. © 1997 Elsevier Science Ltd

INTRODUCTION

Lignin as it exists in the cell wall is called protolignin to distinguish it from isolated lignin preparations which are considered to be different from protolignin in many respects. The characteristic features of protolignin are summarized as follows: (1) protolignin is formed in polysaccharide gels by polymerization of monolignols in an irreversible manner [1–5]; and (2) the kinds of monolignol and polysaccharides vary with the age and type of the cell [4–8]; As a consequence, (3) protolignin is physically and chemically bound to polysaccharides; and (4) the structure and distribution of the protolignin macromolecule in the cell wall is heterogeneous [4, 5, 9–12]. As a result, it is impossible to isolate lignin quantitatively from the cell wall holding its heterogeneous nature and its three dimensional structure intact.

Milled wood lignin (MWL) is frequently used for studies in lignin chemistry. However, MWL is presumed to be derived mainly from secondary wall lignin [13–15], its maximum yield being less than half of protolignin in the cell wall, and it still contains a considerable amount of intimately bound carbohydrates.

These characteristic features of protolignin make the study of its structure and reactions very difficult. Methods employing radio [16] and stable isotopic [17–20] precursors have been developed as promising approaches to circumvent these difficulties, because they can provide information on the structure and reaction of protolignin without isolating it from cell wall.

By an improved radiotracer method using ^{14}C and ^{3}H, the structure and distribution of whole protolignin including their heterogeneous nature in the cell wall have been studied [4–8]. However, the radiotracer method does not provide information on the chemical environment associated with the labelled carbon or hydrogen. NMR spectroscopy, in contrast, provides some information about the structural moiety into which the label is incorporated. Thus the tracer method employing specific ^{13}C-enrichment technique combined with solid state NMR spectroscopy provides useful information on the chemistry of protolignin in the cell wall as pioneered by Lewis *et al.* [17–19]. The selective ^{13}C-enrichment of a specific car-

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bon of protolignin in various plants has been achieved by feeding a specifically ^{13}C-enriched ferulic acid [17–19], phenylalanine [20], monolignol glucosides [21–25] to seedlings, tissue cultured cells, or lignifying tissues of plants. The achievement of selective ^{13}C-enrichment at a specific carbon has been confirmed by solution state NMR of isolated MWLs [20–25] or dimethoxypropane lignin [23], or solid state NMR [17–20]. However, further improvements in the ^{13}C-enrichment technique and in the precise determination of NMR spectra were necessary to obtain reliable information on the structure of protolignin by a combination of these techniques.

This paper deals with the improvements of these techniques and its application to structural analysis of protolignin in wheat.

RESULTS AND DISCUSSION

^{13}C-*Enrichment*

Three coniferins specifically ^{13}C-enriched at side chain α, β or γ carbons and unenriched coniferin (**2**, Fig. 1) were fed to internode cavities of lignifying culms of dwarf wheat. After feeding coniferin, the injected wheat plants grew to maturity in the same appearance as uninjected control plants. Figures 2(a–c) show the NMR spectra of the bottom part (b) of the wheat straw (WS) internodes fed, respectively, with coniferin enriched at the side chain γ-carbon (γWSb), β-carbon (βWSb) and α-carbon (αWSb). Included for reference is the spectrum [Fig. 2(d)] of the unfed control wheat straw internode (cWSb). The signal intensities in the spectra of the labelled internodes increase relative to the intensities of the cWSb spectrum, at the chemical shifts corresponding to the enriched side chain carbons. To demonstrate this increase in signal intensity, a difference spectrum was obtained by subtraction of a spectrum of straw fed with unenriched precursor from the spectrum of straw fed with an enriched precursor as shown in Fig. 3. In generating the difference spectrum, the normalization of each spectra was chosen to minimize the polysaccharide-carbon resonance contributions. Thus those claims we make, which are solely based on the difference spectra, are predicted on the assumption that coniferin does not affect the polysaccharide synthesis.

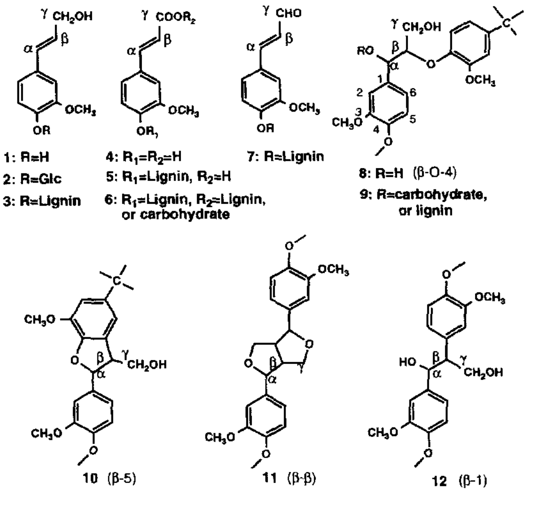

Fig. 1. Lignin precursors, coniferyl alcohol (**1**), coniferin (**2**), ferulic acid (**4**); various end groups with unsaturated side chain in lignin, coniferyl alcohol- (**3**), ferulic acid- (**5**), and ferulic acid ester- (**6**), coniferaldehyde- (**7**) end groups; and lignin substructures with various inter-unit linkages, β-O-4 (**8**), β-O-4/α-O-R (**9**), β-5 (**10**), β-β (**11**) and β-1 (**12**).

Figure 4(a) shows the spectra of the bottom and top parts of the internodes of wheat straws fed with unenriched coniferin (uWSb and uWSt). The signal intensities are slightly different between bottom and top parts as shown in the difference spectrum [uWSb-uWSt: Fig. 4(b)], which spectrum shows intensity in regions characteristic of lignin. This indicates that the lignin content of the bottom part of internode fed with unenriched coniferin is slightly higher than that of the top part. The bottom part was more immature than the top part at the time of feeding with lignin precursor, thus the bottom part is characterized by more active growth over the period of exposure to the precursor solution. Figure 4(c) also shows the difference spectrum between uWSb and cWSb. This spectrum too shows characteristic features of a lignin spectrum except for the slightly lower content of acetyl groups in uWSb near 22 ppm ($\underline{C}H_3$-) and 174 ppm ($\underline{C}O$). Both of the difference spectra of Fig. 4 should, in large measure, reflect changes in lignin quantity or composition associated with the added coniferin. Although the signal to noise ratios are poor in both difference spectra, the similarity in aromatic resonance shapes (110–160 ppm) to that of lignin indicates two things. First, the feeding of coniferin increases lignin content depending on the maturity of the tissue. Second, the coniferin-induced lignin seems to be not radically different chemically from normal wheat lignin. The participation of monolignol glucosides in biogenesis of lignin proposal by Freudenberg [1] has been debated for many years [2]. Microautoradiograms of wood xylem tissues administered with radio-labelled monolignol glucosides showed that a specific type of monolignol was incorporated into lignin in a specific region of cell wall depending on the age and type of cell [4–8]. A part of the label on p-glucocoumaryl alcohol and coniferin were incorporated into guaiacyl- and/or syringyl lignin [4–8], probably by biochemical modification of monomeric units before polymerization. High resolution NMR spectroscopy of MWLs prepared from shoots of pine, ginkgo, oleander and rice straw administered with ^{13}C-enriched coniferin showed that feeding of the precursor did not significantly disturb the normal formation of lignin [21–25]. It has been shown that the cell-wall bound β-glucosidase in spruce seedlings has different substrate specificity towards three kinds of monolignol glucosides [26] and β-glucosidase specific for coniferin has been found in lignifying xylem of

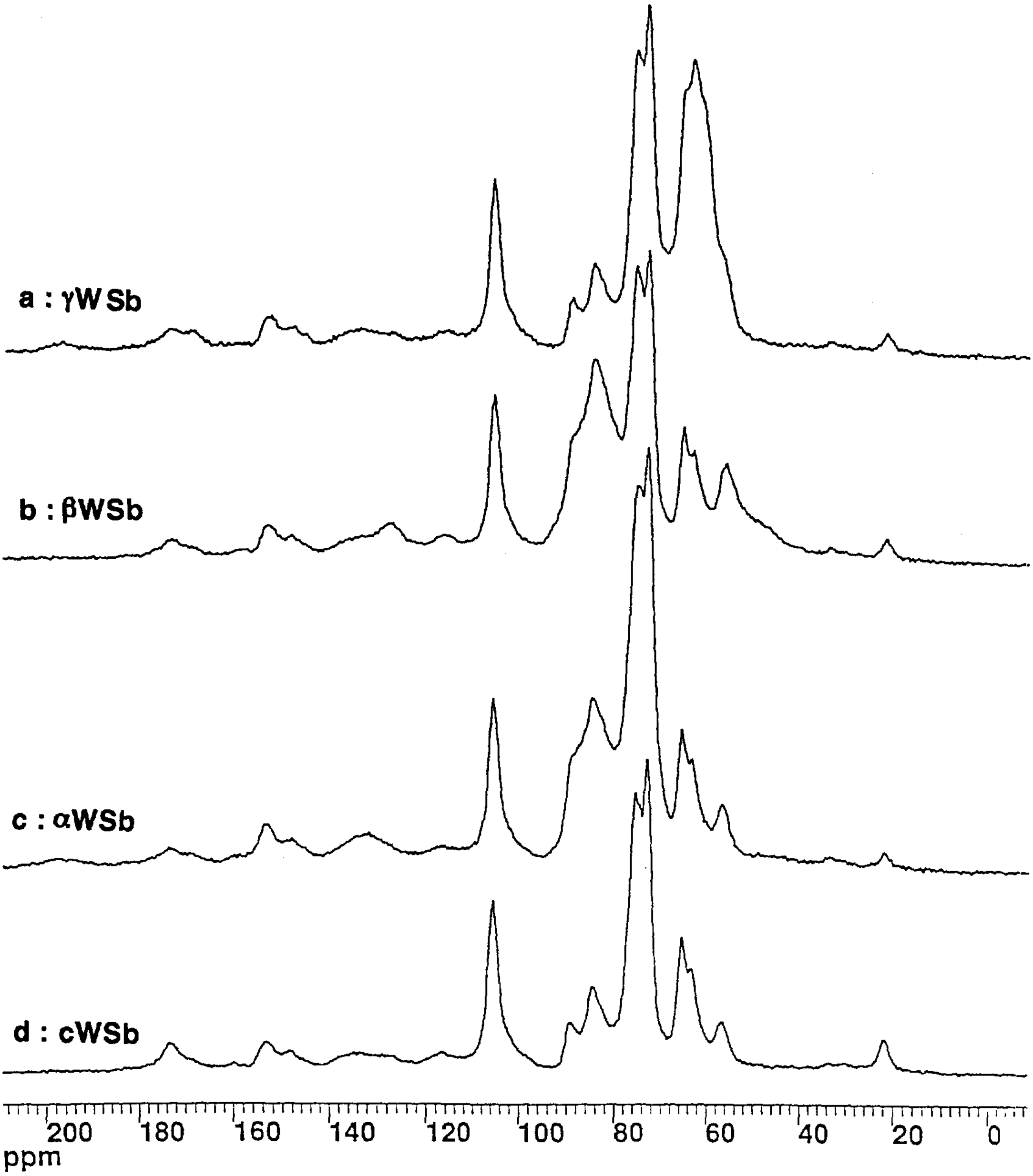

Fig. 2. Solid state NMR spectra of bottom parts of wheat straw internodes fed with coniferin ^{13}C-enriched at the side chain γ-carbon (a:γWSb), β-carbon (b:βWSb), α-carbon (c:αWSb) and unfed control wheat straw (cWSb). Chemical shifts are defined relative to liquid tetramethyl silane at 0.0 ppm.

lodge-pole pine [27]. These facts suggest that monolignols administered as their glucosides are incorporated into protolignin in a biologically regulated manner.

On the other hand, when ferulic acid-[β-^{13}C] (4) was administered to wheat seedlings, solid state NMR spectra of the root tissues were different from the spectra expected for native lignin from model studies [17–19]. When ^{13}C-enriched phenylalanine was fed to cultured pine cells, a part of the precursor was incorporated into protein, as revealed by its solid state NMR [20]. Because phenylalanine is known to be a common precursor of phenylpropanoid compounds including lignin, it may be a suitable precursor for specific ^{13}C-enrichment of side chain carbons in lignin, if the plant tissue does not contain appreciable amounts of protein and phenylpropanoids other than lignin. In general, an intermediate compound nearest to protolignin in its biosynthetic pathway may be a suitable precursor as regards to the selectivity of labelling. All of these observations indicate that monolignol glucosides are suitable precursors for specific ^{13}C enrichment as well as specific radio-labelling of protolignin [1, 4].

It should be noted that wheat lignin is composed of *p*-hydroxyphenyl, guaiacyl- and syringyl-propane

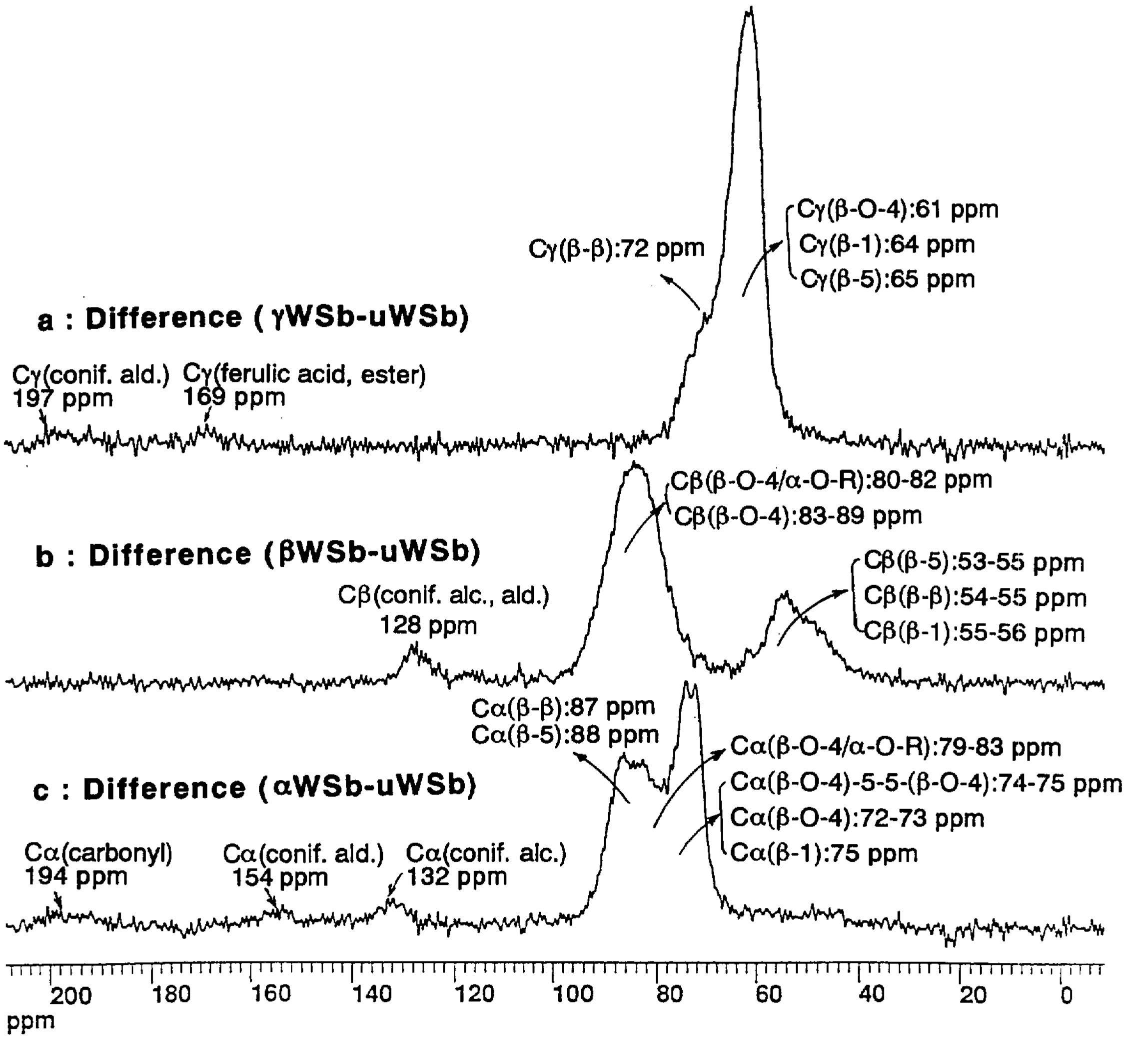

Fig. 3. Difference spectra obtained by subtracting the spectrum of straw fed with natural abundance coniferin from the spectra of straws fed with Cγ-enriched coniferin (a:γWSb-uWSb), Cβ-enriched coniferin (b:βWSb-uWSb), Cα-enriched coniferin (c:αWSb-uWSb). The criterion for generating those difference spectra is the nulling of the cellulose resonances.

units, and it contains a considerable amount of esterified and etherified *p*-coumaric and ferulic acids [28, 29]. When radio-labelled coniferin was administered to rice, 90% of incorporated label was on guaiacyl lignin, and 10% was on syringyl lignin [30]. In this experiment, ^{13}C-enrichment of wheat straw lignin may be achieved mainly on guaiacyl lignin and partly on syringyl lignin; and *p*-hydroxyphenyl lignin and phenolic acid moieties may be not enriched. For enrichment of these units, *p*-glycocoumaryl alcohol, syringin or glucosides of *p*-coumaric and ferulic acids may be used as suitable precursor as shown in ^{13}C-enrichment of syringyl lignin in oleander [25] and ferulic acid in rice [23].

Difference spectra

Figures 3(a–c) show the difference spectra, obtained by substraction of the spectrum of straw fed with unenriched coniferin [uWSb, Fig. 4(a)] from those of αWSb, βWSb, γWSb, respectively. All signals are exclusively assigned to the α-, β- or γ-side chain carbons in each spectra as shown in Fig. 3. Their chemical shifts coincide well with those expected, given the ^{13}C-enriched positions in coniferin and the chemical shifts observed by solution state NMR spectroscopy of isolated rice straw lignin [23], and model compounds [31, 32] and given the variations, usually limited at ±3 ppm, arising from solid-state effects [33].

It is notable that these difference spectra obtained with wheat straw lignin share similar resonance positions with the difference solid state NMR spectra obtained with specifically ^{13}C-enriched dehydrogenation polymers (DHPs) derived from coniferyl alcohol-[γ-^{13}C], [β-^{13}C], [α-^{13}C] (**1**) and unenriched coniferyl alcohol [18, 19]. However there are significant differences in relative signal intensities between the spectra of DHPs and wheat straw lignin. In the difference spectra of DHPs, the intensities of signals assigned to Cα and Cβ of coniferyl alcohol end

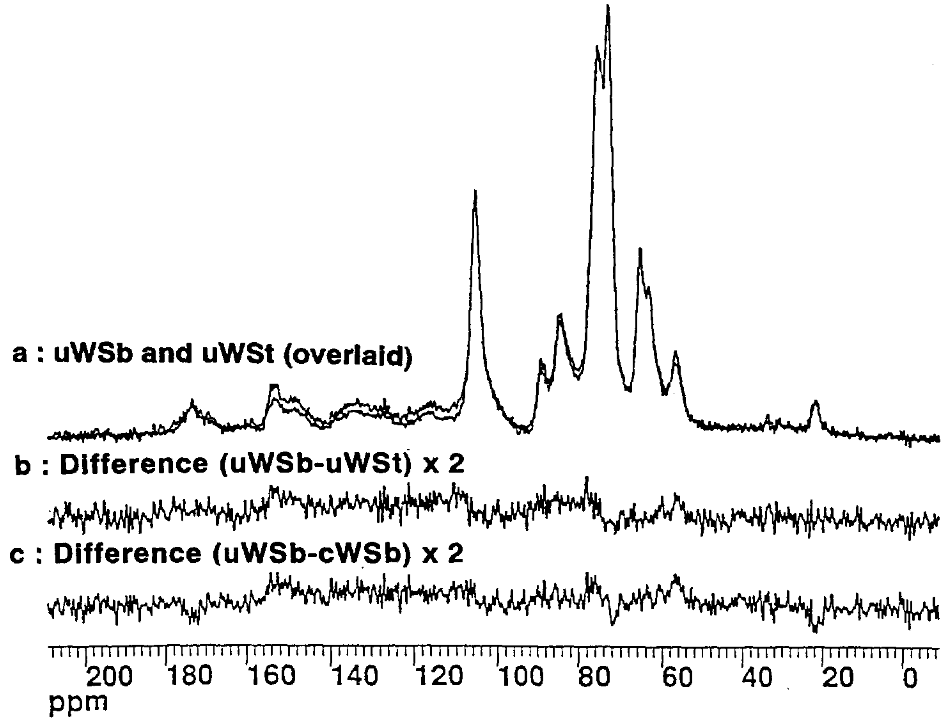

Fig. 4. Comparison of spectra between bottom and top parts of the straw fed with natural abundance coniferin (a:uWSb and uWSt, overlaid), their difference spectrum (b:uWSb-uWSt) and a difference spectrum between unfed control (c:uWSb-cWSb) corresponding to the bottom parts of wheat straws, unfed vs fed with natural abundance coniferin. The difference spectra, even though somewhat noisy, are dominated by lignin resonances. The lignin, relative to the cellulose, is more plentiful in the uWSb sample relative to either the uWSt or the cWSb sample. Scaling of the difference spectra is chosen for minimizing polysaccharide-carbon contributions from the cellulose region (60–110 ppm).

groups (**3**) at 132 ppm and 128 ppm are relatively strong. Those same signals are weak in wheat straw lignin as shown in Figs 3(b and c). The signal intensity assigned to Cβ(β-*O*-4) (**8**) at 83.8 ppm in the spectrum of the DHP derived from coniferyl alcohol-[β-^{13}C] is lower than the combined intensities assigned to Cβ(β-5) (**10**), Cβ(β-β) (**11**) and Cβ(β-1) (**12**) [18, 19], while in the spectra of β-^{13}C-enriched wheat straw lignin [Fig. 3(b)], the intensity of the former was far higher than that of the latter, as discussed in detail later. DHPs derived from coniferyl alcohol (**1**) under conventional preparative conditions contain a larger amount of coniferyl alcohol end groups (**3**) and a smaller amount of β-*O*-4 (**8**) than MWL does [34, 35]. All these features of ^{13}C-enriched wheat straw lignin are those expected from the known characteristic features of MWL. It has been shown by microautoradiography that coniferin is incorporated effectively into cell wall lignin. These facts suggest that the structure of ^{13}C-enriched wheat straw lignin are close to protolignin in the cell wall.

Long term feeding of coniferin (**2**) may cause biochemical transformation of guaiacyl units to syringyl to some extent as shown in case of rice plant [30]. This may also contribute to broadening of the signals. However, the three side chain carbons are incorporated into protolignin holding the original position in the precursor as indicated by Fig. 3.

We estimated the lignin mass fraction of the uWSb and the uWSt samples to be 0.23 and 0.19, respectively, based on (a) the intrinsic carbon intensities for different kinds of carbons discussed in the Experimental section, (b) the experimental fraction of total integrated intensity from 110–190 ppm in the spectra of the uWSb and uWSt samples (this spectral region is assigned to aromatic and carbonyl carbons which, in turn, are assigned to lignin, except for the acetate carbonyl contribution, which acetates would more likely be associated with the polysaccharides; adjustments for the acetate contribution were based on the acetate methyl intensity at 22 ppm), (c) the assumption that about 60% of the carbons in lignin are aromatic or carbonyl as it is true for coniferyl alcohol, the lignin precursor obtained from coniferin, and (d) the assumption, based on typical structures, that the carbon density per unit weight for lignin is about 1.5 times that of polysaccharide.

We also estimated the percentage of the lignin which derived from the labelled coniferin in the ^{13}C-enriched samples. The exercise of computing the weight fraction of lignin involved estimating the fractions of the total integrated intensity of the uWSb and uWSt spectra attributed to lignin. With this knowledge, the total integrals of Fig. 3 could be interpreted in terms of enrichment levels per single carbon site in a precursor. Assuming that an average lignin precursor has 10 carbons, and making corrections for variations in carbon intensities as given in the Experimental section, we found the following: the percentage of lignin precursors originating from the labelled coniferin was 9.5±2.5% for the wheat straw tops and 15.4±2.0% for the wheat straw bottoms where the uncertainties represent the extremes of the ranges. Given that the ^{13}C isotope has a natural abundance of 1.1%, this means that, on average, the tops and bottoms of the wheat straws achieved enrichment levels of 8.6 and 14.0 times natural abundance levels associated with a single carbon site in a precursor molecule. These values are also in good agreement with published results involving mass spectral analysis on lignin from rice straws where, similar to the present study, internodes of the rice plants were fed with ^{13}C-enriched coniferin; in that case the labelled coniferin gave enrichment levels of about 10 times natural abundance [23] and no distinction was made between the tops and bottoms of the straws.

Deductions about lignin structure from difference spectra

The signals at 197 ppm and 169 ppm in Fig. 3(a) are assigned to Cγ(coniferaldehyde end group) (**7**) and Cγ(ferulic acid/ester end group) (**5**, **6**), respectively. The signal at 128 ppm in Fig. 3(b) is assigned to Cβ(coniferaldehyde and coniferyl alcohol end group) (**7**, **3**). In Fig. 3(c), the broad weak signals at 194–200 ppm, 154 ppm and 132 ppm are assigned to Cα(carbonyl), Cα(coniferaldehyde end group) (**7**) and Cα(coniferyl alcohol end group) (**3**), respectively. Thus small portions of the α- and γ-carbons in coniferin (**2**) are oxidized to carbonyl or carboxylic carbons during incorporation into protolignin.

Based on the signal intensities in the solid state NMR spectra, the combined content of β carbons in coniferaldehyde and coniferyl alcohol end groups in this enriched wheat lignin can be estimated tentatively to be 6±1.5% (bottom part) and 8±1.5% (top part, spectra is not shown), respectively.

The major signals of Cβ [Fig. 3(b)] are observed in two regions, 75–94 ppm and 45–60 ppm. The former signals are assigned to Cβ(β-O-4) (**8**), Cβ(β-O-4/α-O-4) (**9**), and Cβ(β-O-4/α-O-carbohydrate in ester or ether form) (**9**), while the latter signals are assigned to Cβ(β-5) (**10**), Cβ(β-β) (**11**) and Cβ(β-1) (**12**), respectively. From the signal intensity, the relative content of β-carbons in combined β-O-4 type units (**8** and **9**) can be estimated to be 65±1.5% (bottom part) and 74±1.5% (top part). The β carbons in β-5 (**10**), β-β (**11**) and β-1 (**12**) substructures are 28±1.5% (bottom part) and 18±1.5% (top part, spectra is not shown).

The difference in the content of combined β-5, β-β and β-1 type substructures between top and bottom parts of the internode is shown in Fig. 5. Figures 5(a–b) are the difference spectra for bottom and top parts, βSWb-uSWb, and βSWt-uSWt, respectively. Figure 5(c) is the difference obtained by subtracting 5(a) from 5(b). The higher content of combined β-5, β-β and β-1 substructures (signals around 55 ppm) in the bottom part lignin indicates that the polylignol formed in more immature tissue is more bulk type, and the polylignol formed in more mature tissue is more endwise. It has been shown by ozonolysis that the ratio of β-5/β-O-4 is higher in more immature wheat tissues than that in mature tissues [36]. Structural difference between lignins in upper and lower parts of an internode of wheat straw has also been reported [37].

The estimated amounts of 18–28% for combined β-5, β-β and β-1 substructures and 65–74% for β-O-4 substructures seem to be reasonable because the latter substructure includes tetrameric structures in which two β-O-4 dimers are connected by 5-5, 4-O-5 or other type of linkages. By destructive analysis of MWLs, the amount of combined β-5, β-β and β-1 substructures has been estimated to be 18–21% for spruce [38] and 23% for beech [39].

EXPERIMENTAL

Specific ^{13}C-enrichment of wheat lignin. Four coniferins, ^{13}C-enriched (estimated ^{13}C content = 99.2%) at side chain α, β or γ carbons and unenriched coniferin (^{13}C content = 1.1%) were synthesized by the method of Terashima *et al.* [40]. Dwarf wheat (*Triticum aestivum* L. cv. Marshall, MN.) was grown in a pot and, just after the head appeared, an aliquot (*ca* 0.1–0.2 ml) of the aq. soln of coniferin (10 mg ml^{-1}) was injected using a fine glass capillary in a culm to fill up the inner cavity of the second internode from the top of the plant stem. After growing for one further month, the mature wheat was harvested and dried. The straw internodes fed with coniferin were carefully cut out from the wheat plants, and the top and bottom directions of the internode were noted. The straws were extracted thoroughly with ether and hot H_2O (70°) successively and dried in a vacuum desiccator.

Solid state NMR. The wheat straw, whose overall length varied from about 50–80 mm, were cut into 2–3 mm lengths starting with the bottom end of each internode and moving up about 35 mm. Approximately 40 straws were utilized to make up a sample weighing about 220 mg; these are the 'wheat-straw-bottoms' samples. The remaining top portions of the straws were used to generate 'wheat-straw-tops' samples. Samples were equilibrated at ambient humidity prior to loading into the sample rotors. For packing the rotors, the cut straws were pressed, under a force of about 1.5 kg mm^{-2}, into an overall cylindrical shape, about 6 mm OD and 10 mm high. Solid state ^{13}C NMR spectra were obtained on a non-commercial spectrometer operating at a ^{13}C frequency of 25.19 MHz, using cross polarization (CP) and magic angle spinning in the usual way [41]. This spinning frequency was fixed at 4 kHz so that there would be no overlap between centrebands and sidebands. By fixing the spinning frequency, sideband intensities would not contribute to the differences in centreband

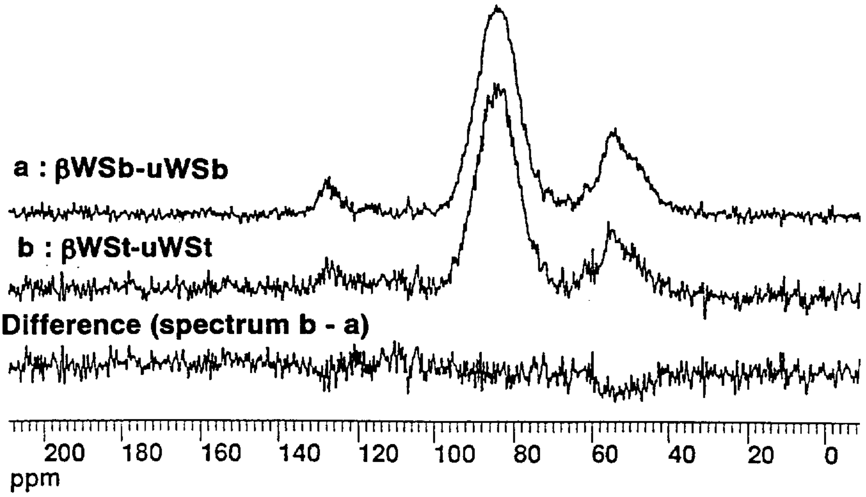

Fig. 5. Comparison of difference spectra showing Cβ-enriched lignin in bottom (a:βWSb-uWSb) and top parts (b:βWSt-uWSt) of internode, and their difference (spectrum b-a). The criterion for generating the difference spectrum is the nulling of the peak at 83 ppm. Relative intensity differences point to changes in lignin chemistry as a function of stem maturity.

intensities which, in turn, formed the basis for our interpretations. The proton and ^{13}C rf levels used in CP and decoupling corresponded to nutation frequencies of 66 and 70 kHz, respectively. The CP time was 1.0 ms and the time between scans was 4 s. A careful monitoring of the rf levels, so as to optimize CP, was maintained because the intensities of unprotonated carbons can be sensitive to relative changes in rf levels. Since we evaluate spectral intensities quantitatively in this paper, we assign to each carbon type our best estimate of its relative intensity per carbon in the spectra we will be discussing. These relative centreband intensities are based on the combined effects of (a) the loss of intensity into spinning sidebands in model compounds, (b) incomplete CP over 1 ms based on different CP efficiencies for different kinds of carbons in model compounds and (c) the difference in proton rotating frame relaxation time, $T^{H}_{1\rho}$, between the lignin-rich and cellulose-rich phases. The latter correction is based on wheat straw measurements where the average $T^{H}_{1\rho}$ of the cellulose is about 15 ms and that of the lignin-rich phase is about 10 ms. Thus, after 1 ms of CP, the intensity per carbon for carbons in the lignin-rich phase is less than for carbons in the celulose-rich phase. The relative centreband intensities per carbon are referenced to cellulose carbons in the cellulose-rich phase having a value of 1.00. Then for other carbons we estimate the following values. For carbons in the lignin-rich phase: 0.95 for aliphatic carbons, 0.87 for protonated aromatic carbons, 0.80 for non-protonated aromatic carbons and 0.81 for carbonyl carbons; in the cellulose-rich phase, 0.85 for any carbonyls. The foregoing numbers allow us to make a more qunatitative assessment of the relative numbers of the various kinds of carbon atoms present.

As to the estimates of the relative intensities of the lines in the spectra of the β-enriched precursors, a conservative estimate is ±1.5%, including all sources of error associated with the method and the uncertainties in the integration of signals.

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