

2D-NMR (HSQC) difference spectra between specifically ^{13}C -enriched and unenriched protolignin of *Ginkgo biloba* obtained in the solution state of whole cell wall material

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Abstract

In the structural analysis of lignins by ^{13}C -NMR, signal overlap limits definitive assignment and accurate intensity measurement. Selective labeling by ^{13}C -enrichment of a specific carbon in lignin enhances its signal intensity in the spectrum. Further enhancement of the specifically labeled carbons can be realized via difference spectra created from the enriched and unenriched samples. Difference 2D ^{13}C - ^1H correlation (HSQC) NMR spectra, derived from the spectra of specifically ^{13}C -enriched lignin model polymers (so-called dehydrogenation polymers) and their unenriched counterparts, take advantage of the enhanced dispersion afforded by both ^{13}C and ^1H chemical shifts, diminishing the difficulties arising from the signal-overlap problem and aiding in definitive signal assignments. In this research, protolignin in xylem cell walls was specifically ^{13}C -enriched at all of the individual phenylpropanoid side-chain carbons by feeding ^{13}C -enriched coniferins to growing stems of *Ginkgo biloba*. The whole xylem fractions containing ^{13}C -enriched and unenriched protolignins were dissolved in a mixture of N-methylimidazole and DMSO, and then acetylated. Solution state 2D-NMR (HSQC) spectra of the acetylated whole cell wall were acquired. Difference spectra between the walls containing ^{13}C -enriched and unenriched

lignins afforded simplified 2D spectra in which well-separated signals were assigned exclusively to the specifically enriched carbons. This novel NMR technique provides a useful tool for elucidation of entire protolignin in the cell wall of ginkgo xylem.

Keywords: carbon-13 enrichment; carbon-13-NMR spectroscopy; dehydrogenation polymer (DHP); 2D-difference NMR spectroscopy; ginkgo; protolignin.

Introduction

In the growing stem of a tree, xylem cell walls are formed by deposition of lignin into the matrix of previously deposited polysaccharides, such as pectin, cellulose, and hemicelluloses. Physical, chemical, and biological properties of the lignified wall depend largely on the chemical structure and higher-order structure of those polymers, and their 3D assembly mode in the wall. Lignin, depositing last, is the key substance that fixes the architecture of the cell wall by formation of chemical and physical bonds between lignin and polysaccharides. This intact lignin in the cell wall is called protolignin to distinguish it from various isolated lignins that are considered to represent a part of protolignin. Protolignin is a structurally heterogeneous 3D polymer formed by an irreversible combinatorial polymerization of several types of monolignol radicals in the polysaccharide gel. It is technically possible to degrade protolignin into monomeric, dimeric, and oligomeric units, but all degradative methods lead to products with modified information on the macromolecular structure of the protolignin, i.e., destructive analyses provide only limited information on protolignin in the cell wall.

Among various non-destructive analytical methods, ^{13}C -NMR spectroscopy is one of the most powerful techniques that provides direct information on the structure of lignin (Lüdeman and Nimz 1973; Nimz et al. 1974; Robert 1992; Robert et al. 1998; Ralph et al. 1999). In a ^{13}C -NMR spectrum, signals are distributed over a wide range of chemical shifts. However, due to the structural heterogeneity and isomeric complexity of lignin, signals are broad and may extensively overlap. This causes difficulty in the assignment of weak signals and in quantitative determination of the signal intensities.

A variety of techniques (a–d) were described to circumvent difficulties arising from the signal-overlap problem:

- Selective ^{13}C -labeling (enrichment) of a specific lignin carbon by administration of specifically ^{13}C -enriched coniferin to a growing stem of pine, ginkgo, or euca-

lyptus: the signal intensity of the specific carbon was enhanced in 1D ^{13}C -NMR spectra of the derived milled wood lignin (MWL) (Terashima et al. 1991, 1999, 2003b; Xie and Terashima 1991; Xie et al. 1994; Robert et al. 1998; Evtuguin et al. 2003).

- b) CP/MAS difference spectra of whole wood containing ^{13}C -enriched and unenriched lignin (solid-state CP/MAS- $\Delta^{13}\text{C}$ -NMR): whole ginkgo wood difference spectra provided improved assignment and intensity determination of the specific ^{13}C -signals. Relative frequencies of linkages between side-chain carbons of C_6 - C_3 units and those between lignin and polysaccharides have been estimated from the difference spectra (Terashima et al. 2002). However, the poor resolution of signals in solid state CP/MAS- $\Delta^{13}\text{C}$ -NMR proved to be unsatisfactory for detailed qualitative and quantitative analyses of the linkages.
- c) Difference spectra between solution state NMR of ^{13}C -enriched and unenriched MWL ($\Delta^{13}\text{C}$ -NMR of MWL): the low resolution problem of solid state CP/MAS- $\Delta^{13}\text{C}$ -NMR has been circumvented by using high-resolution solution state $\Delta^{13}\text{C}$ -NMR between the spectra of specifically ^{13}C -enriched and unenriched MWLs of ginkgo (Terashima et al. 1999, 2003b) and eucalyptus (Evtuguin et al. 2003). However, this NMR analysis of MWL does not provide information on the linkages between protolignin and polysaccharides in the cell wall.
- d) 2D ^{13}C - ^1H correlation (HSQC) NMR spectroscopy: 2D-NMR spectroscopy provides a substantial improvement in dispersion/resolution and allows more detailed assignment of signals (Robert et al. 1998; Ralph et al. 1999; Lu and Ralph 2003; Evtuguin et al. 2003, 2005; Capanema et al. 2004; Holtman et al. 2007). Nevertheless, the signal-overlap problem cannot be completely solved due to the structural complexity of lignin. In solution state 2D spectra of whole wood, lignin exhibits a low signal intensity compared to the polysaccharide signals. Polysaccharides can be partially removed (by treatment with crude cellulases) to provide spectra suitable for lignin structural analysis (Holtman et al. 2007). However, it can be anticipated that solution state 2D-NMR difference spectra from whole cell wall material would yield definitive information without requiring the removal of polysaccharides, even if the lignin content in such samples is very low.

Ginkgo (*Ginkgo biloba*) is called a living fossil tree. It retains primitive characteristic features of gymnosperms that appeared in the early stages of tree evolution on this planet. Ginkgo tracheid walls are quite morphologically similar to those of representative conifers (Timell 1986). Ginkgo lignin is composed of more than 95% guaiacyl-propanoid units and its chemical structure closely resembles that of pine and spruce (Terashima 2007). Ginkgo is one of the most suitable tree species for selective ^{13}C -enrichment of protolignin, because a cut shoot of ginkgo put in a dilute aqueous solution of ^{13}C -coniferin can grow normally for more than 1 month and forms new xylem containing ^{13}C -enriched protolignin (Xie and Terashima 1991; Xie et al. 1994; Terashima et al. 2002; Terashima 2007).

This paper focuses on a new 2D-NMR method for structural analysis of ginkgo protolignin in which the above listed techniques (a–d) are combined. For development and confirmation of usefulness of this method, specifically ^{13}C -enriched dehydrogenation polymers (DHPs) were prepared from the ^{13}C -enriched coniferins, and 2D- $\Delta^{13}\text{C}/^1\text{H}$ -NMR of those DHPs were determined.

Materials and methods

Preparation of specifically ^{13}C -enriched synthetic guaiacyl lignins

Coniferins individually and specifically ^{13}C -enriched at C_α , C_β , C_γ side-chain carbons, and unenriched coniferin were synthesized by the procedure described in a previous paper (Terashima et al. 1996). To produce guaiacyl type DHPs, solutions of coniferin in phosphate buffer (pH 6.0) were treated with a mixture of three types of enzymes: β -glucosidase (to remove the phenolic glucoside), glucose oxidase (to produce in situ H_2O_2 from the released glucose, necessary to re-oxidize the active center in peroxidase), and peroxidase (to generate the monolignol and oligolignol radicals for the radical coupling reactions) (Terashima et al. 1995; Parkäs et al. 2004).

Preparation of acetylated whole cell walls in which lignin is specifically ^{13}C -enriched

The protolignin in ginkgo xylem cell walls was specifically ^{13}C -enriched by feeding a dilute aqueous solution of ^{13}C -enriched coniferin according to the procedure described in a previous paper (Terashima et al. 2002). The xylem was finely milled, and 100 mg of the milled material was dissolved in DMSO/*N*-methylimidazole (2 ml/1 ml), a solvent shown to dissolve the whole cell wall fraction of ball-milled woods and other plants (Lu and Ralph 2003). Dissolution of the wood meals was complete. Addition of acetic anhydride (0.5 ml, 1.5 h), followed by precipitation into water, and filtration through 0.2 μm nylon filters, gave acetylated samples (123% yield by weight for control: 122% for α -labeled sample, 123% yield for β -labeled sample and 123% yield for γ -labeled sample). The following NMR measurements were performed as previously described (Lu and Ralph 2003).

Determination of 2D- $\Delta^{13}\text{C}/^1\text{H}$ -NMR

The NMR spectra presented here were acquired on a Bruker Biospin (Rheinstetten, Germany) DMX-500 instrument fitted with a sensitive cryogenically-cooled 5-mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ gradient probe with inverse geometry (proton coils closest to the sample). Acetylated lignin or cell wall preparations (60 mg) were dissolved in 0.5 ml CDCl_3 ; the central chloroform solvent peak was used as internal reference (δ_{C} 77.0, δ_{H} 7.26 ppm). HSQC experimental conditions were as described previously (Wagner et al. 2007). Volume integration of contours in HSQC plots was accomplished by the Bruker TopSpin 2.0 software as described recently (Ralph et al. 2006). Difference spectra (2D- $\Delta^{13}\text{C}/^1\text{H}$ -NMR spectra) were created in TopSpin 2.0 by subtracting the unlabeled control 2D spectrum from that from the labeled sample, nulling the methoxy contours as effectively as possible; some subtraction artifacts remain in the whole cell wall spectra, particularly with the intense polysaccharide contours.

Results and discussion

Validity of the ^{13}C -enrichment technique

The present 2D- $\Delta^{13}\text{C}/^1\text{H}$ -NMR method is based on the assumption that the structure of ^{13}C -enriched ginkgo protolignin formed by feeding ^{13}C -enriched coniferin to growing ginkgo stems is similar to that of native protolignin in the cell wall. The following experimental observations support this assumption.

- 1) Coniferin and coniferin-specific β -glucosidase have been found in the cambial sap of representative gymnospermous trees when lignification is in progress (Freudenberg and Harkin 1963; Dharmawardhana et al. 1995; Dharmawardhana and Ellis 1998).
- 2) It was shown by microautoradiography that radio-labeled coniferin is effectively incorporated into protolignin in the proper location of differentiating ginkgo cell walls (Fukushima and Terashima 1991). Location analysis of the ^{13}C in newly formed ginkgo xylem after 4 weeks feeding of ^{13}C -coniferin indicated that incorporation occurred in the mature cell wall with the same pattern as that observed by microautoradiography (Xie and Terashima 1991).
- 3) Coniferyl alcohol is biosynthesized inside the plasma membrane in cytosol and may be converted to its glucoside coniferin for transportation through plasma membrane to the site of lignification in the differentiating cell wall (Kawai et al. 2006). Coniferin is therefore considered to be a proven natural precursor for the biosynthesis of guaiacyl lignin.
- 4) Before incorporation into protolignin, exogenously fed ^{13}C -coniferin must be hydrolyzed to ^{13}C -coniferyl alcohol and glucose by naturally occurring β -glucosidase. The enzyme-controlled incorporation into ginkgo lignin resulted in a maximal ^{13}C -enrichment being approximately 3.5 times higher than the natural abundance (Xie and Terashima 1991). This means that approximately 2.5% of exogenous ^{13}C -coniferyl alcohol and 97.5% of naturally occurring coniferyl alcohol (including a few percent of *p*-coumaryl alcohol) were polymerized into lignifying cell walls under the control of the native β -glucosidase. Therefore, it can be reasonably assumed that slow feeding of dilute aqueous solutions (0.1–0.2%) of ^{13}C -coniferin will not significantly disturb the original biosynthetic processes for producing protolignin in the cell wall. No remarkable differences were observed between NMR spectra of unfed ginkgo lignin with those fed with unenriched coniferin (Terashima et al. 2002). No enrichment in either *p*-hydroxyphenyl and syringyl units occurred; their content in ginkgo lignins totals less than 5% (Fukushima and Terashima 1991).

Differential NMR spectroscopy of ^{13}C -DHPs, and of ginkgo MWL

It was shown by Nimz et al. (1974) that guaiacyl type DHPs produce high resolution ^{13}C -NMR spectra that are useful for the assignment of signals in conifer lignin spectra. For the detailed assignment of weak signals and quantitative estimation of signal intensities, the resolution

of signals is not satisfactory, in part due to the broadness of each signal and due to the close neighborhood of different signals. This broadness and extensive signal overlap is derived partly from the fact that even a simple lignin model DHP contains a huge number of isomeric structures (Ralph et al. 2004a). In a previous paper by Parkås et al. (2004), it has been demonstrated that multiple signal clusters of side-chain carbons in the 1D ^{13}C -NMR spectrum of unenriched DHP were resolved into a set of three simple difference spectra for ^{13}C - α -enriched DHP ($\Delta^{13}\text{C}$ - α -spectrum), ^{13}C - β -enriched DHP ($\Delta^{13}\text{C}$ - β -spectrum), and ^{13}C - γ -enriched DHP ($\Delta^{13}\text{C}$ - γ -spectrum). The resolved signals of specific side-chain carbons can be used for definitive assignment of the signals and quantitative estimation of frequencies of a variety of linkages at a specific carbon (Parkås et al. 2004).

The overlapped signals from side-chain carbons observed in the spectrum of unenriched isolated lignins (MWL) were also reasonably well resolved in a set of difference spectra, namely in $\Delta^{13}\text{C}$ - α -, $\Delta^{13}\text{C}$ - β -, and $\Delta^{13}\text{C}$ - γ -spectra, although signal overlap still remained in the MWLs due to the greater complexity of a real lignin structure than that of DHP (Terashima et al. 2003b).

Figure 1 shows the 2D ^{13}C - ^1H correlation (HSQC) NMR spectrum of an unlabeled (unenriched) DHP (a), 2D $\Delta^{13}\text{C}/^1\text{H}$ -NMR spectra of the DHPs labeled (^{13}C -enriched) at C- α (b), C- β (c), and C- γ (d). The $^{13}\text{C}/^1\text{H}$ side-chain contours in the unlabeled G-DHP (a) are effectively separated into correlations (b–d) that are assigned exclusively to the specific C/H environments. The overlap observed in the spectrum of unlabeled DHP (a) was more clearly resolved in the set of 2D- $\Delta^{13}\text{C}/^1\text{H}$ spectra (b–d) than the set of 1D- $\Delta^{13}\text{C}$ spectra. Assignments are described more fully below.

In the 2D-NMR (HSQC) spectrum from the unfractionated whole cell wall (e), signals arising from polysaccharides and lignin are extensively overlapped. The lignin side-chain signals are, however, effectively resolved into a set of 2D- $\Delta^{13}\text{C}/^1\text{H}$ spectra (f–h). There are still some weak signals partly due to imperfect normalization and subtraction.

The 2D- $\Delta^{13}\text{C}/^1\text{H}$ spectra obtained here reaffirm the reliability of the assignments of well-known major structural units of lignin in ^{13}C - ^1H correlation (HSQC) spectra: β -aryl ether (β -O-4, structure **A**), phenylcoumaran (β -5, **B**), resinol (β - β , **C**), and dibenzodioxocin (5-5/4-O- β , **D**) structural units which have been assigned in detailed studies since the 1970s, based on the NMR chemical shifts of numerous lignin model compounds (Lundquist 1979; Hauteville et al. 1986; Karhunen et al. 1995; Li et al. 1997; Ralph et al. 2004b). Different NMR techniques, such as HSQC-DEPT (which helps distinguish CH, CH_2 , and CH_3 carbons in the HSQC format), long-range ^{13}C - ^1H correlation (HMBC, which correlates protons and carbons within 3-bonds of each other), and particularly useful experiments, such as HSQC-TOCSY (in which a side-chain carbon correlates with all protons in that structure's side-chain), have also been applied to lignin studies. The results reinforced the assignments and novel structures

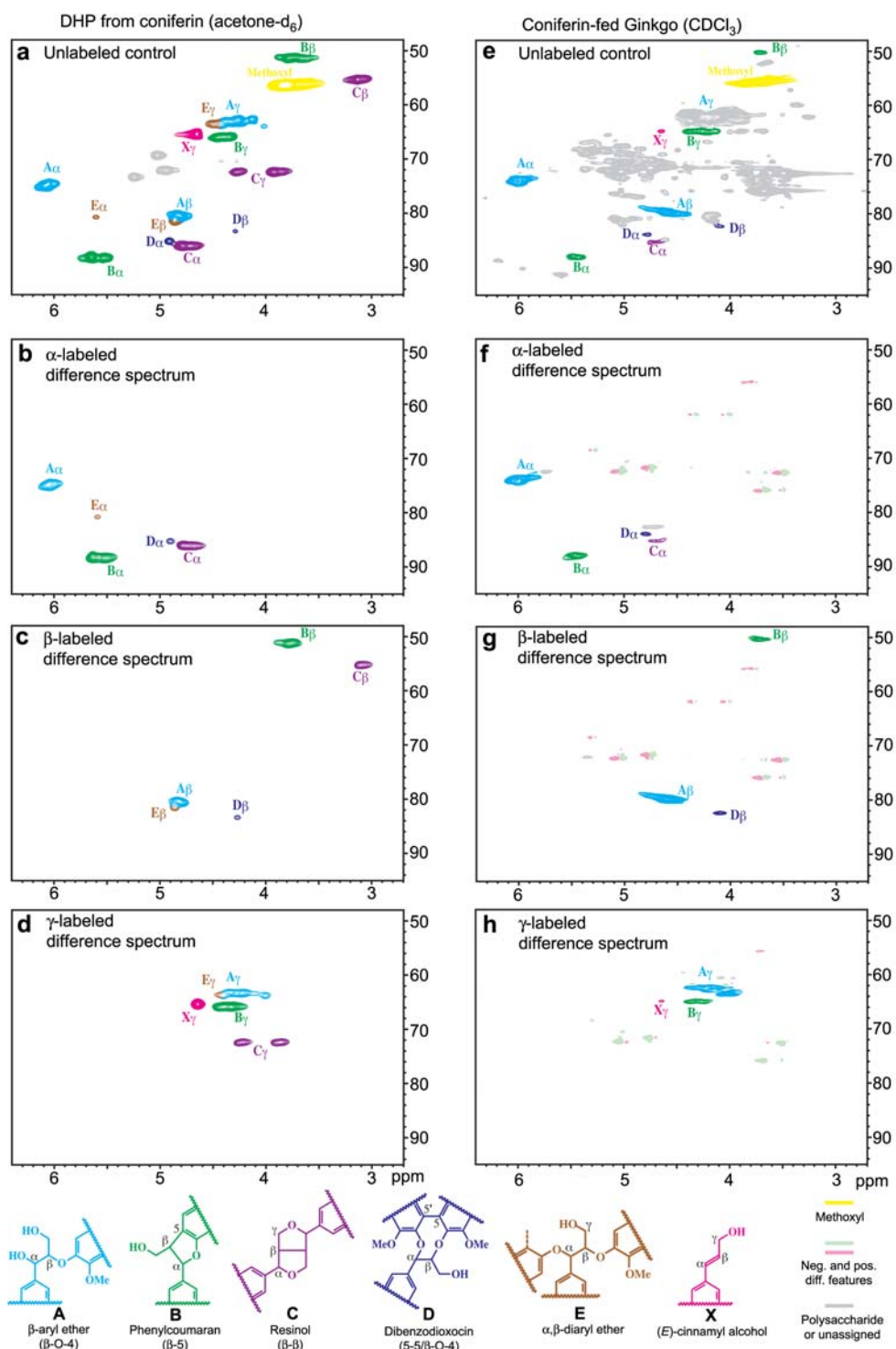


Figure 1 2D-HSQC spectra of unenriched (acetylated) DHP (a) and acetylated whole ginkgo wood (e), difference spectra between DHPs ^{13}C -enriched at side-chain carbons α , β , γ , and unenriched DHP (left column b–d), and difference spectra between ^{13}C -enriched and unenriched ginkgo protolignin acetates (right column f–h) via solution state whole cell wall NMR.

were found by providing information additional to that obtained by an HSQC spectrum (Marita et al. 1999; Kim et al. 2003; Zhang et al. 2003).

Signals in $2\text{D}-\Delta^{13}\text{C}/^1\text{H}$ spectra indicate the chemical shifts of the side-chain carbons and their directly attached protons in lignin, whereas HSQC-TOCSY and HMBC techniques provide bonding information among the signals. As shown in the $2\text{D}-\Delta^{13}\text{C}/^1\text{H}-\alpha$ spectrum (f), only correlations for the α -position (A α , B α ,

C α , and D α) were expectedly but successfully derived from ^{13}C - α -enriched coniferin fed ginkgo, i.e., without correlations from the β - and γ -positions. In the same manner, the $2\text{D}-\Delta^{13}\text{C}/^1\text{H}-\beta$ spectrum (g) and the $2\text{D}-\Delta^{13}\text{C}/^1\text{H}-\gamma$ spectrum (h) exclusively showed correlations for the β - and γ -positions, respectively. Thus, the reliability of the past assignment of NMR signals from structures A, B, C, and D was reinforced by resolving the spectra into three groups: α -, β -, and γ -position-specific 2D spectra.

Note, in addition, that the α,β -diaryl ether structures **E** observed in the lignin model polymer DHPs ("synthetic lignin") are not present in the actual lignins, as noted previously (Ralph et al. 1999).

Additional advantages of 2D- $\Delta^{13}\text{C}/^1\text{H}$ spectra lie in the potential to find novel chemical structures in lignin without the influence of the otherwise overwhelming carbohydrates. As shown in the 2D- $\Delta^{13}\text{C}/^1\text{H}$ - α spectrum (**f**), additional correlations at 4.71/82.7 and 5.74/72.5 ppm ($^1\text{H}/^{13}\text{C}$ ppm) were observed as well as the known correlations for **A α** , **B α** , **C α** , and **D α** . The correlations can be assigned to phenylpropanoid units rather than carbohydrates, as the 2D- $\Delta^{13}\text{C}/^1\text{H}$ - α spectrum enhanced the signals from the α -carbon originating from ^{13}C - α -enriched coniferin involved in lignification and diminished the signals from carbohydrates. Further predictions will be possible. The origin of the additional correlation can be narrowed down to hydroxyl or etherified benzyl carbons from examination of the chemical shift values.

For the future, it would be worth investigating the bonding connections between the unknown α -correlations and the other unassigned correlations at 5.35/72.1 ppm in the 2D- $\Delta^{13}\text{C}/^1\text{H}$ - β spectrum (**g**) and 4.44/59.6, 3.96/60.6, and 4.08/60.6 ppm in the 2D- $\Delta^{13}\text{C}/^1\text{H}$ - γ spectrum (**h**), which can possibly be investigated by HSQC-TOCSY and/or HMBC spectra. Thus, the specific 2D spectra for α -, β -, and/or γ -positions have the potential for detection of new structural units in lignin.

Quantitative determination of the signal intensities for estimating the frequencies of major inter-unit bonds and functional groups from these 2D- $\Delta^{13}\text{C}/^1\text{H}$ spectra needs further development. Nevertheless, it is clearly shown that the content of the resinol (β - β unit, structure **C**) in the protolignin is very low, as can be seen in $\Delta^{13}\text{C}$ - $\alpha/^1\text{H}$ (**f**), or too low to be seen at the contour levels used in the $\Delta^{13}\text{C}$ - $\beta/^1\text{H}$ (**g**) and $\Delta^{13}\text{C}$ - $\gamma/^1\text{H}$ (**h**) spectra, in contrast with the high content of resinol structure **C** evident in the corresponding DHPs (**b-d**). The ratio of β -O-4 (structure **A**) to β -5 units (structure **B**) is obviously higher in protolignin than that in the DHP. Tentative estimation of major bond frequencies based on the signal intensity on the 2D- $\Delta^{13}\text{C}/^1\text{H}$ - α spectrum (**f**) showed that the ratio of **A α** :**B α** :**C α** :**D α** was 70:17:6:5. This ratio for the major structural units in ginkgo protolignin is quite reasonable compared to the integrated estimation by tracer methods and thioacidolysis (Terashima 2001). All these observations coincide well with the previously reported comparison of structural differences between DHP and lignin (Terashima et al. 1995; Terashima 2001).

Conclusion and future prospects

Difficulties associated with NMR analysis of cell wall protolignin, arising from signal-overlap and low-resolution, can be considerably diminished by the described solution state high-resolution 2D- $\Delta^{13}\text{C}/^1\text{H}$ -NMR method. This analytical method consists of a series of technically sophisticated steps. Nevertheless, it appears to allow definitive and reliable information about a specific lignin carbon/proton in the cell wall that cannot be obtained by any other method.

The special features of this method are:

1. it is a relatively non-destructive method, aside from the required fine milling of the wood sample;
2. it does not require lignin isolation (and therefore avoids the inherent potential fractionation possibilities);
3. it provides lignin-specific information by subtraction of interfering information from carbohydrates, etc.; and
4. it will allow tracing of the behavior of a specific carbon/proton connection during any type of reaction resulting in structural changes of lignin, such as in pulping, bleaching, biodegradation, as all signals are exclusively assigned to the enriched carbon in the difference spectra.

Parkås et al. (2004) applied 1D- $\Delta^{13}\text{C}$ -NMR spectroscopy to the study of light-induced yellowing of a lignin model DHP. This traceability of a specific lignin carbon/proton will also be useful for variation of lignin reaction theories which are based on simple experiments previously employing low-molecular lignin model compounds.

Further improvements are necessary in order to draw maximal advantage from this method in the following experimental processes: (1) more perfect subtraction of the spectrum of the unenriched sample from the ^{13}C -enriched sample and (2) establishment of quantitative analysis methods for signal intensities or contour volumes in 2D spectra. This will provide more reliable information on increasingly low-abundance structural moieties.

In addition, specific ^{13}C -enrichment of all of the carbons, including aromatic ring carbons (Terashima et al. 2003a; Terashima 2007), in *p*-hydroxyphenyl-, guaiacyl- and syringyl-propanoid units in various plants will extend the usefulness of this method. A trial in this direction has been reported for *Eucalyptus* lignin by Evtuguin et al. (2003, 2005).

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