

An Improved ^{13}C -Tracer Method for the Study of Lignin Structure and Reactions — Differential ^{13}C -NMR

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ABSTRACT

The technique of selective ^{13}C -enrichment of specific carbons in lignin combined with ^{13}C -NMR differential spectrometry between spectra of ^{13}C -enriched and unenriched lignins ($\Delta^{13}\text{C}$ -NMR) provides definitive information on the structure of the lignin macromolecule. Improvements were made on, (1) specific ^{13}C -enrichment of almost all carbons involved in inter-unit bonds and functional groups in lignin, (2) estimation of the frequencies of bonds and functional groups using $\Delta^{13}\text{C}$ -NMR under quantitative conditions, and (3) detection of specifically ^{13}C -labeled carbons by 2D-difference spectra. A softwood lignin model, guaiacyl-type ^{13}C -DHP, was prepared from specifically ^{13}C -enriched coniferin. Based on the $\Delta^{13}\text{C}$ -NMR, the structure of the DHP is proposed. Specifically ^{13}C -enriched MWLs were prepared from ginkgo wood fed with ^{13}C -enriched coniferins. Qualitative and quantitative differences in structure between MWL and DHP were shown by the comparison of $\Delta^{13}\text{C}$ -NMR determined under the same quantitative conditions. Despite a large difference in the structure of DHP from that of MWL, the ^{13}C -enriched DHP can be used as a model of lignin for a variety of studies on formation, structure and reactions of lignin in combination with the $\Delta^{13}\text{C}$ -NMR technique.

INTRODUCTION

Lignin is a structurally heterogeneous polymer formed from several kinds of monolignols by an irreversible, combinatorial radical polymerization. Therefore, it is impossible to degrade lignin quantitatively into monomeric or oligomeric units, and degradation loses information on its 3D macromolecular structure. Accordingly, destructive analyses can provide limited information on the chemical structure of protolignin in the cell wall. Among various non-destructive analytical methods, ^{13}C -NMR spectrometry is one of the most powerful techniques that provides direct information on the structure of lignin (1,2). In the ^{13}C -NMR spectrum, signals are distributed over a wide range of chemical shifts. However, considerable overlap of

the signals sometimes causes difficulty in assignments of weak signals and in quantitative determination of the signal intensities. Two-dimensional spectrometry provided a remarkable improvement in assignment and quantitative determination of the signal intensities (2). Nevertheless, there is still some overlapping of signals.

Selective ^{13}C -enrichment of a specific carbon in the lignin improved the signal intensity of the enriched carbon in its ^{13}C -NMR spectrum (3), and the difference spectrum between spectra of ^{13}C -enriched and unenriched lignins ($\Delta^{13}\text{C}$ -NMR) made further improvements in the assignment of the signal and quantitative determination of the intensity (4). Selective ^{13}C -enrichment is achieved by feeding ^{13}C -enriched lignin biosynthesis precursors to the growing stem of a tree (3,4) or inner cavity of a rice (5) or wheat plant stalk (6). The monolignol glucosides have been shown to be the best precursors (3,4,7).

The $\Delta^{13}\text{C}$ -NMR also gives information on the behavior of the bonds and functional groups during various types of reactions (8-10) that is difficult to obtain by any other conventional destructive analyses.

This paper deals with further improvements in the application of quantitative 1D and 2D $\Delta^{13}\text{C}$ -NMR for determination of the structure of DHP and lignin.

METHODS

Preparation of DHP- ^{13}C

Coniferins specifically ^{13}C -enriched at the side chain carbons, α , β , γ and at the aromatic ring carbons 1, 3, 4 and 5 were synthesized by the procedure of Terashima *et al.* (11-13), and the solution of the coniferins in phosphate buffer (pH:6.0) was treated with a mixture of three kinds of enzymes, β -glucosidase, glucose oxidase and peroxidase to produce guaiacyl (G) type DHP (14).

Preparation of ginkgo MWL- ^{13}C

Coniferins ^{13}C -enriched at the side chain carbons were fed to growing stems of ginkgo trees, and MWLs were prepared from the newly formed xylem.

RESULTS AND DISCUSSION

1. Differential NMR spectroscopy of ^{13}C -DHPs

Fig.1 shows the effective separation of overlapped signals from side chain carbons and guaiacyl ring carbons of unenriched G-DHP into component signals assigned to specific carbons shown in the spectra of $\Delta^{13}\text{C}_\gamma$ - $\Delta^{13}\text{C}_5$. The overlapping was greatly reduced in each $\Delta^{13}\text{C}$ spectrum, though some overlapping of signals still remains. The integration of signal intensity in the $\Delta^{13}\text{C}$ spectra determined under quantitative conditions gave information on the frequencies of major inter-unit bonds and functional groups involving the ^{13}C -enriched carbons.

2. Differential 2D spectra between ^{13}C -DHPs and unenriched DHP

The technique of $\Delta^{13}\text{C}$ -NMR can be applied to 2D

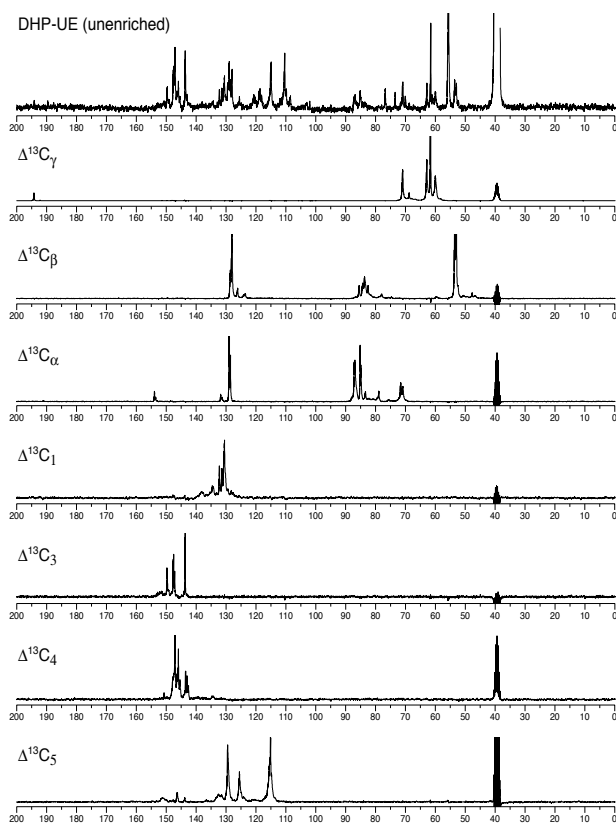


Fig. 1. Quantitative ^{13}C -NMR spectra of G-DHPs.

The NMR data were obtained with a Bruker DPX-250 spectrometer (62.9 MHz carbon) fitted with a 5 mm quadrupole probe. The sample concentration was 40 mg DHP in 0.4 ml of $\text{DMSO}-d_6$. Quantitative ^{13}C NMR spectra were obtained with a pulse width of 6.5 μs corresponding to a tip angle of 90° . The relaxation delay was 15 s and sample temperature was 300 K, and 3,800 transients (16K data points) were collected over 16 hours.

DHP-UE: unenriched DHP. $\Delta^{13}\text{C}_\gamma$ - $\Delta^{13}\text{C}_5$: Difference spectra obtained by subtraction of the spectrum of unenriched DHP from the spectra of DHPs specifically ^{13}C -enriched at side-chain carbon C_γ , C_β , C_α and guaiacyl ring carbon at C_1 , C_3 , C_4 and C_5 .

NMR spectroscopy. Fig. 4 shows effective separation of overlapped signals of the side chain carbons in the 2D spectrum of unenriched DHP into 3 component spectra. Coniferin unfortunately appears to be incorporated in an unknown way in these DHPs. Evidence for the excellent subtraction is the disappearance of the strong methoxyl group in the 2D difference spectra for C_α - C_γ . The 2D spectra clearly delineate the ^{13}C and ^1H assignments arising from specific labeled carbons.

3. Structure of G-DHP

Based on the above information, a set of structures for this DHP is tentatively proposed, shown in part in Fig.3. Information on the formation of a tetralignol, G-

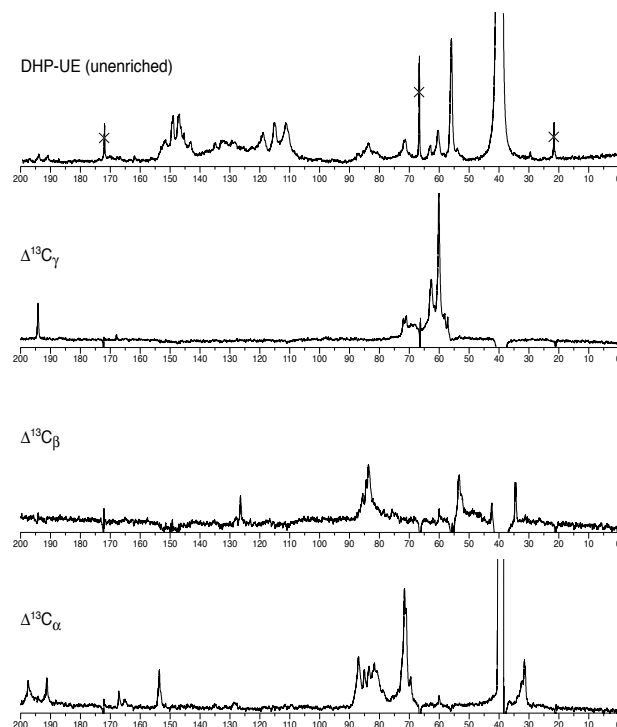


Fig. 2. Quantitative ^{13}C -NMR spectra of ginkgo MWLs determined under the same conditions as for DHPs.

MWL-UE: unenriched ginkgo MWL. $\Delta^{13}\text{C}_\gamma$ - $\Delta^{13}\text{C}_\alpha$: Difference spectra obtained by subtraction of the spectrum of unenriched MWL from the spectra of MWLs specifically ^{13}C -enriched at side chain carbon, C_γ , C_β , and C_α . X = solvent impurities.

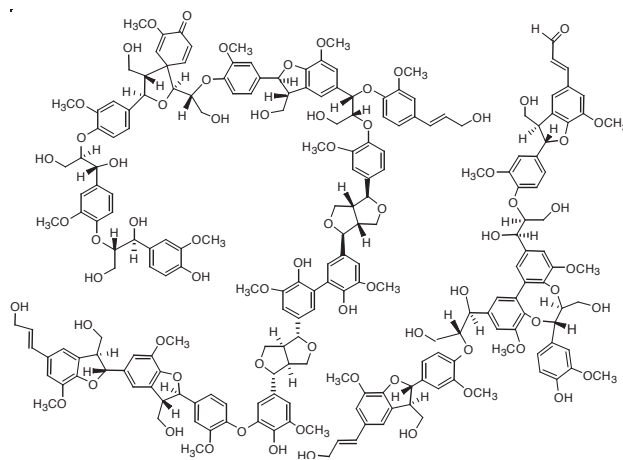
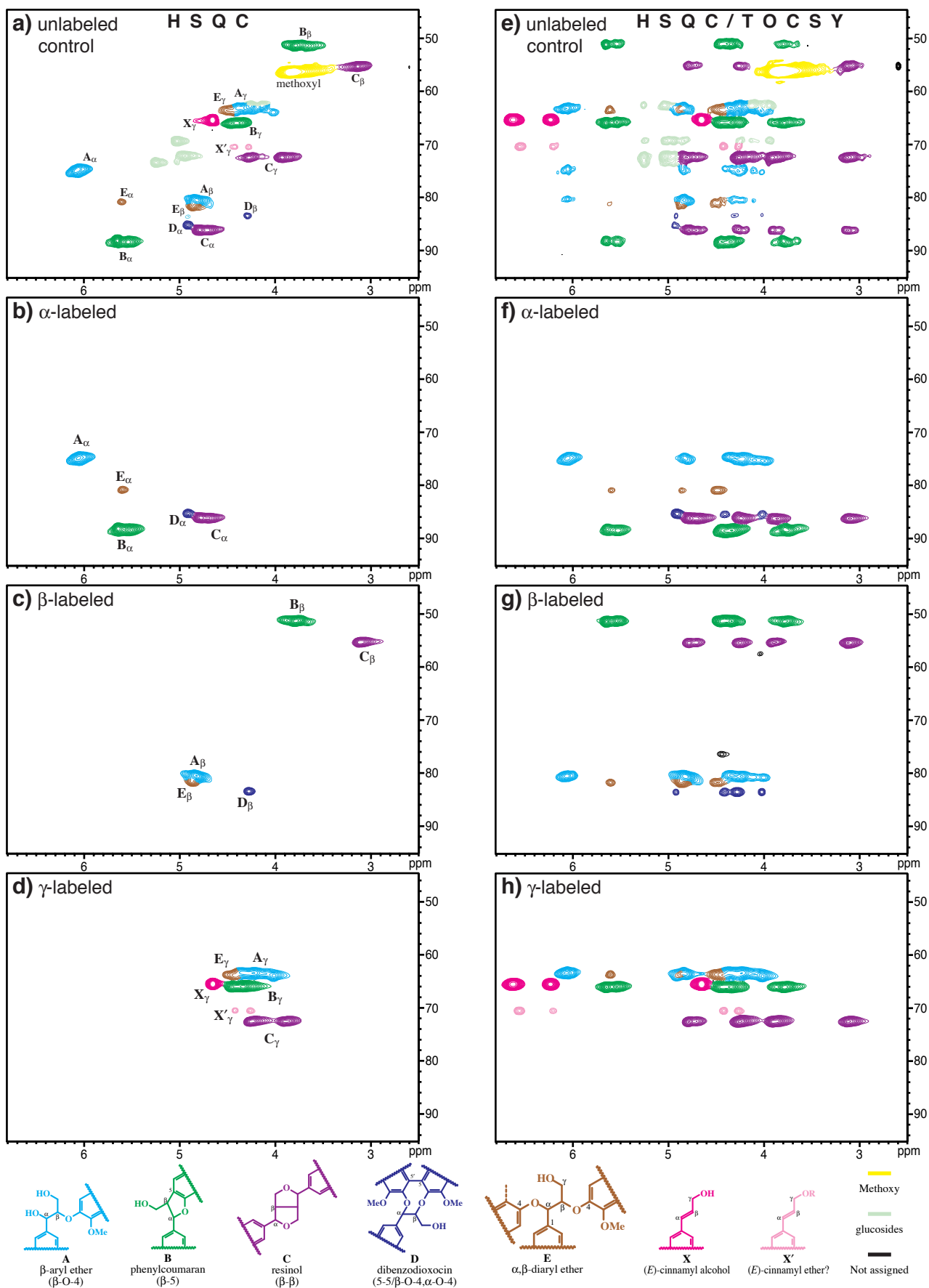


Fig. 3. A part of proposed structure for G-DHP.

Fig. 4. (Next page) Two dimensional HMQC and HMQC-TOCSY spectra of acetates of ^{13}C -enriched DHPs.

a): unenriched control. b)-d): Difference spectra obtained by 2D subtraction of the spectrum of the unenriched control from the spectra of DHPs specifically ^{13}C -enriched at side-chain carbons, C_γ , C_β , C_α showing clean labeling.



β - β' -G-5-5'-G- β - β' -G, and a trilignol, G-5- β' -G-5- β' -G by Freudenberg (15), and the results of thioacidolysis on a DHP from coniferin (16) are incorporated in the proposed structure. The *erythro/threo* ratio of the β -O-4 units was estimated to be slightly higher than 1 from $\Delta^{13}\text{C}\alpha$ and $\Delta^{13}\text{C}\beta$ spectra. It was shown by GPC analysis that this DHP consists of polymers higher than heptalignol (81%), hexa- to tetralignols (17%) and trilignols + dilignols (2 %). Because the frequency of terminal functional groups (and certain kinds of inter-unit bonds) depends largely on the molecular weight, the wide molecular weight distribution of this DHP caused difficulty in bringing all of the NMR data into a single structural model. Fractionation into DHPs with narrow MW distribution should reduce signal overlapping in $\Delta^{13}\text{C}$.

4. Comparison of structure of DHP and MWL by $\Delta^{13}\text{C}$

Figure 2 shows $\Delta^{13}\text{C}$ -NMR spectra of ginkgo MWLs. Comparison of Fig. 1 with Fig. 2 indicates that the DHP contains most of the major inter-unit bonds and functional groups found in MWL, though their frequencies are greatly different. There are also differences in minor substructures and functional groups such as dihydrocinnamyl side chains and carbonyls which may be caused by a difference in the formation mechanism between DHP and protolignin in the cell walls. Despite the considerable differences in structure, ^{13}C -DHPs are good models for studying the structure and reactions of native lignin using $\Delta^{13}\text{C}$ -NMR, because this technique enables us to make non-destructive quantitative evaluation of the structural difference between DHP and MWL, and this will contribute to further improvements in the preparative methods for a better DHP model.

ACKNOWLEDGEMENTS

Financial support from the research foundation Stiftelsen Nils och Dorthi Troëdssons Forskningsfond is gratefully acknowledged. We are grateful to Mr. K. Yamauchi, Nagoya University, for his help in preparing MWLs.

Note: The pdf on the ISWPC CD for this manuscript has Fig. 4 in color, considerably clarifying the interpretation of the various units.

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12th ISWPC

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