Dan Aoki, Kenta Nomura, Masashi Hashiura, Yoshinori Imamura, Sonoka Miyata, Noritsugu Terashima, Yasuyuki Matsushita*, Hiroshi Nishimura, Takashi Watanabe, Masato Katahira and Kazuhiko Fukushima

Evaluation of ring-5 structures of guaiacyl lignin in *Ginkgo biloba* L. using solid- and liquid-state ^{13}C NMR difference spectroscopy

<https://doi.org/10.1515/hf-2019-0011>

Received January 17, 2019; accepted June 13, 2019; previously published online July 13, 2019

Abstract: To discuss the macromolecular structure and properties of lignin, the complementary use of solid- and liquid-state nuclear magnetic resonance (NMR) combined with a specific ^{13}C -enrichment technique may provide useful information. The ^{13}C -enriched lignin was prepared by administering [guaiacyl ring-5(G5)- ^{13}C]-coniferin to a growing *Ginkgo biloba* L. shoot. The ^{13}C -enriched cellulosytic enzyme lignin (EL) and its acetate prepared from the ginkgo shoot were examined by solid- and liquid-state ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum derived only from the G5 carbon was obtained as a difference spectrum based on the spectra of the G5- ^{13}C enriched and un-enriched (UE) samples. The condensed structures, including the enriched G5-carbon, were evaluated using difference spectra. The chemical shifts of the enriched G5 carbon in both the solid- and liquid-state ^{13}C NMR spectra agreed with each other in principle. The quantitative ratio of the condensed and uncondensed structures at G5 was found to be larger by solid-state cross polarization/magic angle spinning (CP/MAS) NMR than by liquid-state NMR.

Keywords: ^{13}C -enriched lignin, difference spectrum, *Ginkgo biloba* L., solid-state ^{13}C CP/MAS NMR

Introduction

Native lignin in plant cell walls is a heterogeneous and complicated polymer formed by the combinatorial polymerization of several types of monolignol radicals and is related to polysaccharides (Terashima et al. 1993; Dimmel 2010). Various methods, primarily decomposition methods, have been developed to estimate the chemical structure of lignin. However, isolating lignin in its native state from plant cell walls and determining its chemical structure in detail using decomposition methods are difficult.

Most recent studies on the chemical structure of lignin were performed using liquid-state nuclear magnetic resonance (NMR) spectroscopy (Lundquist 1980; Lu and Ralph 2003; Capanema et al. 2004; Ralph and Landucci 2010). Various types of inter-unit linkages in lignin have been revealed, and the plant species and tissue of origin of the lignin structure have been discussed. However, due to the structural heterogeneity and isomeric complexity of lignin, the signals in ^1H and ^{13}C one-dimensional (1D) NMR spectra show extensive overlapping, making it difficult to assign and quantify the signals. Although ^1H - ^{13}C two-dimensional (2D) NMR techniques provide valuable information on lignin structures (Heikkinen et al. 2003; Ralph et al. 2006; Zhang and Gellerstedt 2007; Mansfield et al. 2012; Nishimura et al. 2018), lignin has several quaternary carbons in its polymeric structure. In particular, the guaiacyl ring-5 (G5) carbon can be the connection site of several types of inter-unit linkages, such as 5-5, β -5, 4-O-5 and dibenzodioxocine. NMR signals of quaternary carbons are quite weak, and a quantitative discussion of these linkages at the ring-5 position is still difficult.

A selective ^{13}C -enrichment process should be a promising approach to resolve these problems. Terashima et al. developed a method for the selective ^{13}C -enrichment of a specific lignin carbon by feeding the specifically ^{13}C -enriched coniferin, a precursor in lignin

*Corresponding author: Yasuyuki Matsushita, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan, e-mail: ysmatsu@agr.nagoya-u.ac.jp

Dan Aoki, Kenta Nomura, Masashi Hashiura, Yoshinori Imamura, Sonoka Miyata and Kazuhiko Fukushima: Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
Noritsugu Terashima: Nagoya University, 2-610 Uedayama, Tenpaku, Nagoya 468-0001, Japan

Hiroshi Nishimura and Takashi Watanabe: Research Institute for Sustainable Humanosphere, Kyoto University, Uji 611-0011, Japan

Masato Katahira: Institute of Advanced Energy (IAE), Kyoto University, Uji 611-0011, Japan

biosynthesis (Whetten et al. 1998; Samuels et al. 2002; Aoki et al. 2016; Terashima et al. 2016), to a growing pine or ginkgo shoot. This method can enhance the peak intensity of the specific carbon in NMR spectra (Terashima et al. 1991; Xie and Terashima 1991). Moreover, by subtracting the spectrum of lignin-fed unenriched (UE) coniferin from the spectrum of lignin-fed specifically ^{13}C -enriched coniferin, a simple difference spectrum arising only from the ^{13}C -enriched carbon can be obtained (Terashima et al. 2002). Terashima et al. applied this method to acetylated ginkgo cell wall samples, which were individually and specifically ^{13}C -enriched at the C_α , C_β and C_γ side-chain carbons, and analyzed their difference spectra using 2D ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) NMR (Terashima et al. 2009).

When analyzing polymers, liquid-state NMR can reveal the details of the chemical structure of their soluble or swellable parts in the NMR solvents. Solid-state NMR may approximate the correlation between the chemical structure and physical properties of these compounds in a more natural (solid) state. To advance the discussion of lignin macromolecules from plant cell walls, the complementary use of solid- and liquid-state NMR will be most helpful. However, as reported previously (Maciel et al. 1981; Hatcher 1987; Mao et al. 2006; Bardet et al. 2007; Wheeler et al. 2015), the ^{13}C signals of lignin in solid-state NMR are generally broad, and it is difficult to specifically assign them to the lignin aromatic structures. Therefore, ^{13}C NMR difference spectrometry could be used to focus on specific carbon structures. Solid-state ^{13}C difference spectra of lignin samples specifically ^{13}C -enriched at C_α , C_β , and C_γ side chain carbons have been reported previously (Terashima et al. 1997, 2002).

The purpose of this study is the estimation of G5 condensed and uncondensed structures by solid- and liquid-state NMR combined with a specific ^{13}C -enrichment technique, which may be useful for understanding the macromolecular structure and properties of native lignin in the cell wall. In this study, specifically enriched [$\text{G5-}^{13}\text{C}$]-coniferin and UE-coniferin were administered to growing shoots of *Ginkgo biloba* L. Ball milling and enzymatic saccharification processes were employed to prepare the resultant enzyme lignin (EL) samples for solid- and liquid-state NMR analysis and compare the results. Lignin analyses using a specific ^{13}C -enrichment technique combined with ^{13}C NMR difference spectrometry between the enriched and UE samples were conducted to elucidate the structural variations with respect to the G5 carbon.

Materials and methods

Preparation of ginkgo lignin specifically ^{13}C -enriched at G5: The lignin in ginkgo xylem cell walls was specifically ^{13}C -enriched at the G5 position by using a previously described method (Terashima et al. 2002) with some modifications. UE-coniferin and [$\text{G5-}^{13}\text{C}$]-coniferin were synthesized according to a previously reported method (Terashima et al. 2003). Cut ginkgo shoots (3 years old, 20–25 cm in axial length) were placed into small vials containing an aqueous solution of either [$\text{G5-}^{13}\text{C}$]-coniferin or UE-coniferin. After feeding the solution (400 mg of coniferin in 100 ml of H_2O per shoot) in a growth chamber for 5 days under 14 h at 27°C :10 h at 20°C as the light:dark period, the shoots were grown for an additional 3 weeks in flasks containing tap water. Then, the bark was removed, and the newly formed xylem was collected and extracted with acetone for 4 h and hot water for 1 h, and the xylem sample was stored in a desiccator.

Structural unit composition and content of lignin: To estimate the structural unit composition of the ginkgo lignin, thioacidolysis (Nimz 1974; Lapierre et al. 1985) was performed according to the method reported by Roland et al. (1992). Approximately 3 mg of the extracted wood sample was placed in 5 ml of a dioxane/ethanethiol mixture (8.75:1, vol./vol.) containing 0.2 M (C_2H_5)₂OBF₃ in a test tube. As an internal standard for gas chromatography-mass spectrometry (GC-MS) measurements, 1.0 ml of 0.1 mg/ml docosane/dioxane solution was added to the tube. The thioacidolysis was conducted at 100°C for 4 h. Then, the reaction mixture was cooled on ice and 25 ml of 0.4 M NaHCO₃ aqueous solution (aq.) was added to stop the reaction. The pH of the mixture was adjusted to 2–3 with HCl aq. (1:3, vol./vol.), and the mixture was extracted using 3 ml of CH_2Cl_2 3 times. The thus obtained organic extract was dried over Na₂SO₄ and concentrated under reduced pressure at 40°C . The final solution was dissolved in 0.3 ml of CH_2Cl_2 and stored at 4°C until use. The silylated thioacidolysis product was analyzed by GC-MS (Trace 1300GC/ITQ 900, Thermo Fisher Scientific K.K., Tokyo, Japan) under the following conditions: capillary column, Rtx-1ms (30 m $\times$ 0.32 mm ID, 0.25- μm film thickness); temperature program, 180–230 $^\circ\text{C}$ at $2^\circ\text{C}/\text{min}$; carrier gas, He (1.5 ml/min). The monomeric products were assigned and evaluated by GC according to Lapierre et al. (1991).

The lignin content was estimated by the acetyl bromide method (Iiyama and Wallis 1990). The ball-milled ginkgo wood (see following text) was added into a mixture of 2.5 ml of 25% acetyl bromide/acetic acid solution. After the addition of 0.1 ml of 60% perchloric acid, the solution was stirred at 70°C for 30 min. Then, the mixed solution was cooled with ice water. The solution was poured into a mixture of 5 ml of 2 M NaOH aqueous solution and 13 ml of acetic acid. The whole solution was diluted to 50 ml using a volumetric flask. The absorbance at 280 nm was measured using a ultraviolet-visible (UV-Vis) spectrophotometer (V-530, JASCO, Tokyo, Japan), and the lignin content in the solution was evaluated. The resulting lignin content was 34.8 wt% for the $\text{G5-}^{13}\text{C}$ -enriched sample.

Determination of the ^{13}C abundance in the newly formed xylem: The ^{13}C incorporation in the ball-milled ginkgo wood containing $\text{G5-}^{13}\text{C}$ -enriched lignin was evaluated using isotope ratio mass spectrometry (IR-MS; Delta Plus, ThermoFinnigan, San Jose, CA, USA)

connected to an elemental analyzer (NC2500, ThermoFinnigan, San Jose, CA, USA). $^{13}\text{C}/^{12}\text{C}$ at the G5 position, $(^{13}\text{C}/^{12}\text{C})_{\text{G5}}$, was calculated using the following equation:

$$(^{13}\text{C}/^{12}\text{C})_{\text{sample}} = 0.348 \times 1/10 \times (^{13}\text{C}/^{12}\text{C})_{\text{G5}} + (1 - 0.348 \times 1/10) \times (^{13}\text{C}/^{12}\text{C})_{\text{control}}$$

where 0.348 is the resultant lignin content evaluated by the acetyl bromide method as described earlier, 1/10 is the specific carbon ratio at the position concerning the guaiacyl unit having a C_{10} structure, $(^{13}\text{C}/^{12}\text{C})_{\text{sample}}$ is the obtained value for the G5- ^{13}C sample, and $(^{13}\text{C}/^{12}\text{C})_{\text{control}}$ is for the UE sample. The experiments were performed in triplicate, and the average value was used to estimate the ^{13}C ratio (%).

Sample preparation for NMR measurements: The ^{13}C -enriched and UE ginkgo xylem samples were ground in a Wiley mill and passed through a 24-mesh screen and then sequentially extracted with acetone for 4 h and hot water for 1 h. Wood meal (1 g) and zirconia balls (5-mm diameter, 100 g) were placed into a zirconia jar. The wood meal was finely ball-milled using a Fritsch Pulverisette 6 planetary ball-mill (FRITSCH, Idar-Oberstein, Germany) at 24.7 g for 6 h (grinding for 2 min, waiting for 2 min, 15 cycles $\times$ 12 sets with 20-min intervals).

The EL samples were prepared as follows: Finely milled wood (1 g) was immersed in 5 ml of 7 wt% LiCl/*N,N*-dimethylacetamide and allowed to stand at 40°C for 24 h, and then 25 ml of pH 4.8 acetate buffer and 50 mg of meicelase (cellulase originating from *Trichoderma viride*, Meiji Seika Pharma Co. Ltd., Tokyo, Japan) were added to the resultant swollen mixture. The mixture was shaken at 40°C for 72 h. During the shaking period, additional 50-mg portions of meicelase were added at 24 h and 48 h after the start of the reaction. The resultant EL samples were washed with H_2O by centrifugation and freeze-dried.

The EL samples were acetylated as follows (Lu and Ralph 2003): 100 mg of EL was added to a mixture of 2 ml of dimethylsulfoxide (DMSO) and 1 ml of *N*-methylimidazole and stirred at 25°C for 3 h under an Ar atmosphere. Then, 0.6 ml of acetic anhydride was added into the solution, and the mixture was stirred at 25°C for 24 h. The resultant homogeneous solution was poured into excess ice-cold H_2O . The acetylated EL (ELAc) samples were washed with H_2O repeatedly and dried *in vacuo* at 40°C for 48 h.

Hereafter, the samples containing ^{13}C -enriched lignin are described as G5-EL or G5-ELAc, and the samples containing UE lignin are described as UE-EL or UE-ELAc.

Solid-state ^{13}C CP/MAS NMR: Solid-state ^{13}C cross polarization/magic angle spinning (CP/MAS) NMR measurements were conducted using a JEOL ECA-700 spectrometer (JEOL, Ltd., Tokyo, Japan). Dried samples were filled into 4-mm zirconia sample tubes and measured under the following conditions: ^{13}C resonance frequency, 175 MHz; magic angle spinning rate, 10.0 kHz; scan number, 17 000; acquisition time, 14.54 ms; repetition time, 5.0 s; contact time, 3, 4, 5, 6 or 7 ms. The obtained free induction decays (FIDs) were multiplied with an exponential window function using a line broadening of 200 Hz prior to Fourier transform. The chemical shifts were calibrated using the methoxy carbon as an internal standard (56.0 ppm). Phase and baseline corrections were

performed using the Delta software (v5.0.5.1, JEOL). The room temperature was 293 K, and the actual sample temperature in the 10.0 kHz spinning sample tube was estimated to be 317.5 K (r.t. + 24.5 K) using samarium acetate (Campbell et al. 1986).

Liquid-state quantitative ^{13}C NMR: Liquid-state quantitative ^{13}C NMR measurements were carried out on a Bruker Avance 600 spectrometer equipped with a cryo probe (Bruker BioSpin GmbH, Rheinstetten, Germany) using an inverse-gated decoupling mode. The acetylated samples were soluble in CDCl_3 . The measurement conditions were as follows: ^{13}C resonance frequency, 150 MHz; sample concentration, 100 mg/ml; solvent, CDCl_3 containing 0.01 M Cr(III) acetylacetonate as a relaxation reagent (Gansow et al. 1972; Rokhin et al. 1994; Xia et al. 2001); scan number, 43 000; acquisition time, 1.4 s; pulse delay, 1.7 s; temperature, 295 K.

NMR data processing: For solid-state NMR, five spectra were obtained for each EL sample with contact times of 3, 4, 5, 6 and 7 ms. The spectrum of the UE sample was subtracted from the spectrum of the G5-enriched sample after normalization using the methoxy carbon signal. Gaussian peak fitting by the least squares method was carried out using the solver function in Excel 2010 software (Microsoft, Redmond, WA, USA). The resultant peak area variation was plotted vs. the contact time, and linear approximation was calculated using Excel 2010 software.

For liquid-state NMR, the obtained quantitative ^{13}C NMR spectra were exported to ASCII data using the Bruker Topspin software (ver. 4.0, Bruker BioSpin GmbH, Rheinstetten, Germany). The spectrum of UE-ELAc was subtracted from the spectrum of G5-ELAc after normalization using the methoxy carbon signal. The peak areas were integrated using Excel 2010 software.

Results and discussion

Preparation of specifically ^{13}C -enriched ginkgo lignin

The preparation and analyses of the specifically ^{13}C -enriched ginkgo lignin are schematically illustrated in Figure 1. The ^{13}C abundance was estimated by IR-MS, and the resultant ^{13}C -enrichment ratio at G5 was 2.72%, which is approximately 2.5 times the natural abundance.

Figure 1: Schematic illustration of the preparation and analyses of specifically ^{13}C -enriched ginkgo lignin.

Solid-state NMR measurements and signal assignments of the lignin G5 structures

The solid-state ^{13}C CP/MAS NMR spectra of UE-wood meal, G5-EL and UE-EL as well as a difference spectrum (between G5-EL and UE-EL) are shown in Figure 2. The aromatic carbon signals in the spectrum before enzymatic saccharification (Figure 2a) were relatively weak. After the enzymatic saccharification (Figure 2b), these signals were enhanced. The signals in the range from 50 to 110 ppm

derived from residual polysaccharides were still detected, probably because of the imperfect removal by the present saccharification procedures.

Although the spectra of UE-EL (Figure 2b) and G5-EL (Figure 2c) are similar, the difference spectrum (Figure 2d) indicates that there are several enhanced signals assignable exclusively to the G5 carbon between 110 and 160 ppm. The gray line is the ± 0 line, and the arrows indicate the methoxy signals. The downfield signals (>160 ppm) may be spinning side bands derived from the aromatic carbons. In this study, the magic angle spinning rate was at 10 kHz, and the spinning side band positions should be ± 57.1 ppm. At this point, the upfield signals (<50 ppm) showed a nearly flat baseline in the difference spectrum, and we believe that the spinning side bands of the residual polysaccharides are negligible in the aromatic region, although the spinning side bands of the aromatic carbons should overlap in the polysaccharide region. The possible structures assigned exclusively to the G5- ^{13}C signals in the 110–160 ppm region are uncondensed guaiacyl rings with β -O-4, α -O-4, β - β and β -1 linkages, and condensed structures with β -5, 5-5 and 4-O-5 linkages (Figure 3).

These signals were separated into 6 G5- ^{13}C signals (signals **a**, **b**, **c**, **d**, **e** and **f**) and a polysaccharide signal (signal **s**, corresponding mainly to the polysaccharide C1) by Gaussian peak fitting (Figure 3b) (Atalla et al. 1980). The difference spectrum between the G5- ^{13}C -enriched and UE dehydrogenated polymers showed no signals in this region (near **s**) (Parkäs et al. 2004). The plotted curve with circles is the fitting total. For these signals, the signals were assigned according to previous reports using solid- and liquid-state NMR measurements (Nimz et al. 1981; Haw and Schultz 1985; Hatcher 1987; Hatfield et al. 1987; Drumond et al. 1989; Xie and Terashima 1991; Hawkes et al. 1993; Alves et al. 2000; Parkäs et al. 2004; Ralph and Ralph 2009, 2010; Li et al. 2016; Yue et al. 2016).

Signals **a** and **b** were assigned to the uncondensed G5 carbons (C4-OH or C4-OR). Signal **c** was determined to be an overlapping signal of the G5 carbons in β -5 [(**c**)-1] and 5-5 linkages with a neighboring C4-OH [5-5 (OH), (**c**)-2] moiety. Signal **d** was assigned to the G5 carbons involved in 5-5 linkages with a neighboring C4-OR [5-5 (OR), including 5-5 in the dibenzodioxocine unit]. Signals **e** and **f** were assigned to the G5 carbons of the 4-O-5 linkages having a neighboring C4-OH [4-O-5 (OH)] or C4-OR [4-O-5 (OR)] unit.

The thioacidolysis and GC-MS measurements showed that the obtained sample did not produce thioethylated H (*p*-hydroxyphenyl)-unit monomers (data not shown); however, previous studies suggested the presence of condensed H-units in the compound middle

Figure 2: Solid-state ^{13}C CP/MAS NMR spectra of (a) UE-wood meal, (b) UE-EL, (c) G5-EL, and (d) the difference between (b) and (c). The arrows indicate the methoxy carbon at 56 ppm. The gray line is the ± 0 line resulting from spectral subtraction. Contact time = 5 ms.

Figure 3: (a) Solid-state ^{13}C CP/MAS NMR difference spectrum between UE-EL and G5-EL, (b) the result of Gaussian peak fitting of (a), and (c) the solid-state ^{13}C CP/MAS NMR difference spectrum between UE-ELAc and G5-ELAc.

The arrows in (b) and (c) suggest the peak maxima in (b). The dashed arrow in (c) indicates the missing carbon. Contact time = 5 ms.

lamella and cell corner regions (Terashima and Fukushima 1988; Saito and Fukushima 2005) in gymnosperm lignin. Thus, although the neighboring unit in Figure 3 might be a guaiacyl and/or *p*-hydroxyphenyl moiety in the gymnosperm lignin, the difference should not affect these signal assignments.

To clarify the signal assignments, the difference spectrum between the acetylated G5-EL (G5-ELAc) and acetylated UE-EL (UE-ELAc) samples is shown in Figure 3c. Comparing these difference spectra before (Figure 3b) and after (Figure 3c) acetylation, we found that signal **b** at 116 ppm disappeared in Figure 3c. From this result, we determined that signal **b** was derived from the uncondensed G5 carbon having a neighboring C4-OH group [uncondensed (OH)]. Similarly, the peak shape of signal **c** changed slightly after acetylation, as indicated by the arrow. Here, we found that the peak shape of signal **d** was seldom changed by acetylation, indicating that this signal corresponds to 5-5 linkages (OR). From these results, we concluded that the overlapping signal in **c** was 5-5 (OH).

Additionally, we surmised that signal **e** should originate from 4-O-5 (OH) because of the change in the peak shape upon acetylation.

As a consequence of the aforementioned discussion, the G5 carbon signals in the solid-state ^{13}C NMR spectrum were assigned, and these assignments along with previously reported assignments are listed in Table 1 (Nimz et al. 1981; Haw and Schultz 1985; Hatcher 1987; Hatfield et al. 1987; Drumond et al. 1989; Xie and Terashima 1991; Hawkes et al. 1993; Alves et al. 2000; Parkås et al. 2004; Ralph and Ralph 2009, 2010; Li et al. 2016; Yue et al. 2016, see Supplementary Table S1 for details). Although the sample state was different, these signal assignments agreed in principle with those of the liquid-state ^{13}C NMR spectrum (see following text). After acetylation, the signals were shifted, overlapped, broadened and more complex. Some signals were not determined after acetylation. Therefore, in this study, we focused on the results obtained from the G5-EL and UE-EL samples.

Table 1: Chemical shifts (ppm) of the G5 carbons in solid- and liquid-state ^{13}C NMR.

G5-structures	Solid-state		Liquid-state	Previous reports	
	Before Ac	After Ac	After Ac	Solid-state	Liquid-state
Uncondensed (OR)	111.7	112.5	118.3	111–115	112–120
Uncondensed (OH)	116.1	–	–	112–116	113–119
Uncondensed (OAc)	–	n.d.	122.8	123–125	123–125
β -5	124.2	n.d.	127.2	129–130	127–134
5-5 (OH)	–	–	–	123	123–127
5-5 (OAc)	–	n.d.	131.2	–	130–137
5-5 (OR)	132.6	132.1	132.5	–	130–134
4-O-5 (OH)	147.7	–	–	(147–148) ^a	143–147
4-O-5 (OAc)	–	146.5	147.9	–	150–152
4-O-5 (OR)	151.8	151.5	150.5	(153) ^a	151–156

Ac, acetylation; n.d., not determined; NMR, nuclear magnetic resonance. ^aReferred to syringyl C3/C5 signals.

Evaluation of the G5 carbons by solid-state NMR

In the solid-state ^{13}C CP/MAS NMR measurements, the signal intensity is not proportional to the molar abundance. For the quantitative evaluation by solid-state ^{13}C NMR, the direct polarization method can be used if the signal intensity is sufficiently strong. However, in the cases of lignin, the signals were usually too weak to conduct an adequate number of scans within practical measurement times. To quantitatively evaluate the lignin G5 carbon signals using solid-state NMR, in this study, we used the contact time extrapolation method (Ottenbours et al. 1998). The following section will briefly explain this method.

In solid-state NMR, the ^{13}C signal sensitivity was compensated by the CP/MAS method. In the CP/MAS sequence, the ^1H magnetization was transferred to ^{13}C , and the transfer efficiency strongly depends on the environments of the proton and the carbon. Therefore, the obtained signals are not quantitative. The simplified evolution of carbon magnetization as a function of the contact time can be expressed as eq. (1).

$$M = M_0 [1 - \exp(-T_{\text{CT}}/T_{\text{CH}})] \exp(-T_{\text{CT}}/T_{\text{ip}}^{\text{H}}) \quad (1)$$

where M is the experimentally obtained magnetization value (peak area), M_0 is the theoretical maximum magnetization value, T_{CT} is the contact time, T_{CH} is the cross-polarization time constant and T_{ip}^{H} is the proton relaxation time constant in the rotating frame. When $T_{\text{CH}} \ll T_{\text{CT}}$, the term $[1 - \exp(-T_{\text{CT}}/T_{\text{CH}})]$ can be approximated to 1, and eq. (1) can be simplified to eq. (2).

$$M = M_0 \cdot \exp(-T_{\text{CT}}/T_{\text{ip}}^{\text{H}}) \quad (2)$$

Here, T_{ip}^{H} is the intrinsic constant of the structure, and we can draw a line in proportion to T_{CT} . As a result, the extrapolated intercept ($T_{\text{CT}}=0$) is the theoretical maximum magnetization value, and we can evaluate the signal quantitatively.

The T_{CH} of the aromatic signals of gymnosperm lignin were previously reported as $T_{\text{CH}} \leq 0.46$ ms (Hatfield et al. 1987). If $T_{\text{CT}} > 5T_{\text{CH}}$, the term $[1 - \exp(-T_{\text{CT}}/T_{\text{CH}})]$ is larger than 0.99 and can be approximated to 1. Considering these points, we used the measurement parameters of contact times of 3, 4, 5, 6 and 7 ms in solid-state ^{13}C CP/MAS NMR measurements. The obtained ^{13}C NMR spectra were processed in the same way as described earlier. For example, the spectrum of UE-EL with $T_{\text{CT}}=5$ was subtracted from the spectrum of G5-EL with the same T_{CT} ($=5$), and the peak areas were evaluated by Gaussian fittings as displayed in Figure 3b. We obtained five data sets measured using different T_{CT} values; the obtained values were plotted vs. contact time, and the approximated results are shown in Figure 4.

The slope of the lines obtained for the six signals varied from -0.0143 to -0.0573 . The values correlated with the relaxation rate of the G5 carbons, allowing us to estimate the mobility in the structures. The order of the absolute values was as follows: $b \leq e \leq a \leq c \approx f \leq d$, i.e. uncondensed (OH) $\leq$ 4-O-5 (OH) $\leq$ uncondensed (OR) $\leq$ β -5 and 5-5 (OH) $\approx$ 4-O-5 (OR) $\leq$ 5-5 (OR). Although some coefficients of correlation (R^2) were relatively low, probably because of the signal overlapping and the procedure accuracy of the difference spectrum calculation, the absolute values tend to increase when the G5 carbon gains a new connection in the structural unit. To discuss the structural mobility of lignin in more detail, further experiments should be conducted; nevertheless, we have attempted a quantitative discussion of lignin G5 structures based on these data.

The peak area ratios to the total aromatic area in $T_{CT}=0$ and 5 ms are summarized in Table 2. $T_{CT}=5$ ms is one of the usual measurement conditions in solid-state CP/MAS NMR, and the values in $T_{CT}=0$ ms are the result of contact time extrapolation. As a result of the extrapolation ($T_{CT}=0$), the ratio of uncondensed G5 carbons was $13+15=28\%$, G5 carbons having a carbon-carbon bond (β -5 and 5-5) was $26+27=53\%$, and G5 carbons having an

ether linkage (4-O-5) was $5+14=19\%$. These values are slightly different from the values obtained in $T_{CT}=5$ ms. The G5 structures resulting in a large slope, such as 5-5 (OR), might be underestimated in the usual measurement conditions for solid-state NMR. These results will be discussed further with the results of the quantitative liquid-state ^{13}C NMR measurements.

Quantitative evaluation of the G5 carbons by liquid-state NMR

The liquid-state quantitative ^{13}C NMR spectra of G5-ELAc and UE-ELAc and their difference spectrum in the aromatic region are shown in Figure 5. The ^{13}C signals in Figure 5c were sharp and better resolved in comparison with the solid-state NMR signals. The signals were assigned to the G5 carbon structures according to previous

Figure 4: Peak area variations in G5 carbon signals depending on the contact time by solid-state ^{13}C CP/MAS NMR measurements. The plots (a, b, c, d, e and f) correspond to the signals shown in Figure 3b.

Table 2: G5 carbon structural ratios as determined by solid- and liquid-state ^{13}C NMR.

G5-structures	Solid-state		Liquid-state	Previous reports ^a
	$T_{CT}=5$	$T_{CT}=0$	After Ac	Liquid-state
Uncondensed (OH)	15%	15%	—	—
Uncondensed (OAc)	—	—	23%	—
Uncondensed (OR)	12%	13%	17%	—
(Total) Uncondensed	28%	28%	40%	48–54%
β -5	31%	26%	18%	8–12%
5-5 (OH)	—	—	—	—
5-5 (OAc)	—	—	12%	5–8%
5-5 (OR)	17%	27%	15%	19%
(Total) 5-5	—	—	27%	23–27%
4-O-5 (OH)	4%	5%	—	—
4-O-5 (OAc)	—	—	2%	—
4-O-5 (OR)	21%	14%	13%	—
(Total) 4-O-5	24%	19%	15%	5–8%

Ac, acetylation; NMR, nuclear magnetic resonance; T_{CT} , contact time.
^aValue ranges for softwood (mainly spruce).

Figure 5: Liquid-state quantitative ^{13}C NMR spectra of (a) UE-ELAc and (b) G5-ELAc as well as (c) the difference between (a) and (b). The signal assignments correspond to the structures shown in Figure 3 as follows: A', [a]; B', [b]; C', [c]-1; C'', [c]-2; D', [d]; E', [e]; and F', [f].

reports and are listed in Table 1. As mentioned earlier, the signal assignments agreed in principle with those of solid-state ^{13}C NMR.

As discussed earlier, we used the solid-state ^{13}C NMR spectra of EL due to the better signal resolution. Recent studies have reported that ball-milled cell walls or ELs can dissolve or swell in DMSO-containing solvent systems, and it is possible to acquire NMR spectra without acetylation (Lu and Ralph 2003; Kim et al. 2008; Kim and Ralph 2010). Based on these points, it might be possible to measure EL/DMSO by liquid-state NMR. In this study, however, we used the ELAc/ CDCl_3 system to quantify the G5 structures. The major reasons are the small amount of sample available, the lower viscosity of this system and the better solubility of the sample in the ELAc/ CDCl_3 system than in the EL/DMSO system.

The resultant peak area ratios are summarized in Table 2. The ratio of uncondensed G5 carbons was $17 + 23 = 40\%$, G5 carbons having carbon-carbon bonds (β -5 and 5-5) was $18 + 12 + 15 = 45\%$, and G5 carbons having an ether linkage (4-O-5) was $2 + 13 = 15\%$. According to recent studies evaluating lignin structures (Chen 1996; Capanema et al. 2004; Zhang and Gellerstedt 2007; Sette et al. 2011), nearly half of the interunit linkages in lignin are uncondensed structures (β -O-4, β - β and β -1), and other typical linkages are as follows: β -5 $\approx 10\%$, 5-5 $\approx 25\%$ and 4-O-5 $< 10\%$, as listed in Table 2 (see Supplementary Table S2 for details).

Discussion of condensed G5 structures in lignin as evaluated by NMR

Liquid-state NMR spectroscopy provides high-resolution spectra suitable for both qualitative and quantitative studies. In particular, recent progress using multidimensional NMR techniques has revealed the precise structures of plant lignin. The sensitivity of NMR for quaternary carbons is quite low, and they are difficult to resolve and quantify from ^1H - ^{13}C 2D NMR spectra. To determine these carbons by ^{13}C NMR spectroscopy, many researchers have synthesized fine model compounds and assigned their chemical shifts. However, their actual abundance and structural variety in native lignin have remained unclear.

To address these points, in this study, we used selective ^{13}C -enrichment and ^{13}C NMR difference spectra to focus on the G5 carbons in lignin. The obtained ^{13}C -enriched EL was examined by solid-state ^{13}C CP/MAS NMR measurements with the contact time extrapolation method. In comparison with the results of the liquid-state quantitative ^{13}C NMR measurements, most of the signal

assignments were consistent, and the G5 carbon signals were successfully specifically assigned. For the quantity, the ratio of G5 carbons in the condensed structures was found to be larger by solid-state CP/MAS NMR than by liquid-state NMR spectroscopy. Using the solid-state CP/MAS NMR contact time extrapolation method, although the quantification quality might not be as good as that of liquid-state NMR, we would like to note that the condensed G5-structures such as 5-5 (OR) might be underestimated using the general measurement conditions because of the entangled structure (Table 1).

Here, it should be mentioned that the resultant value of the condensed G5 ratio by liquid-state NMR was somewhat higher than the previously reported values for softwoods, as suggested in Table 2. The difference might be related to variations in the species, the sample preparation procedure, and so on. With this sample preparation procedure, excessive ball milling may alter the cell wall structure and decrease the yield of thioethylated monomeric compounds by thioacidolysis and GC-MS measurements (Shipponen et al. 2014). This report suggests that ball milling may increase the ratio of condensed structures in lignin. In this study, we regarded the solubility of the samples in CDCl_3 for liquid-state NMR as important for comparing the samples using solid- and liquid-state NMR, and thus, the milling step was of great importance. For the precise quantification of the structure of lignin in its natural state using solid-state NMR, further studies using moderately prepared samples and molecular dynamics discussions are required.

Conclusions

The aromatic carbon at the G-5 position in lignin was investigated using specifically ^{13}C -enriched ginkgo lignin by solid- and liquid-state NMR difference spectra. The liquid-state NMR measurements provide precise signal assignments and fine structural information. The solid-state NMR measurements showed the T_{CT} -dependent variation in the signal intensities and suggested that the condensed structures might be underestimated because of their entangled structures. Most of the signal assignments in the two types of NMR spectra agreed with each other. The complementary use of solid- and liquid-state NMR techniques would facilitate the elucidation of the native structure and molecular properties of lignin in plant cell walls.

Acknowledgments: The authors thank K. Koga and Y. Maeda, Technical Centre of Nagoya University, for performing NMR measurements.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This study was financially supported by the Japan Society for the Promotion of Science KAKENHI (Nos. 25252032, Funder Id: <http://dx.doi.org/10.13039/501100001691>, 15K07510, Funder Id: <http://dx.doi.org/10.13039/501100001691>, 17H03842, and 18H03959) and the Joint Usage/Research Program on Zero-Emission Energy Research, Institute of Advanced Energy, Kyoto University (ZE30A-17).

Employment or leadership: None declared.

Honorarium: None declared.

References

- Alves, V.L., Drumond, M.G., Stefani, G.M., Chen, C.-L., Piló-Veloso, D. (2000) Synthesis of new trimeric lignin model compounds containing 5-5' and β -O-4' substructures, and their characterization by 1D and 2D NMR techniques. *J. Braz. Chem. Soc.* 11:467–473.
- Aoki, D., Hanaya, Y., Akita, T., Matsushita, Y., Yoshida, M., Kuroda, K., Yagami, S., Takama, R., Fukushima, K. (2016) Distribution of coniferin in freeze-fixed stem of *Ginkgo biloba* L. by cryo-TOF-SIMS/SEM. *Sci. Rep.* 6:31525.
- Atalla, R.H., Gast, J.C., Sindorf, D.W., Bartuska, V.J., Maciel, G.E. (1980) ^{13}C NMR spectra of cellulose polymorphs. *J. Am. Chem. Soc.* 102:3249–3251.
- Bardet, M., Gerbaud, G., Trân, Q.-K., Hediger, S. (2007) Study of interactions between polyethylene glycol and archaeological wood components by ^{13}C high-resolution solid-state CP/MAS NMR. *J. Archaeol. Sci.* 34:1670–1676.
- Campbell, G.C., Crosby, R.C., Haw, J.F. (1986) ^{13}C chemical shifts which obey the curie law in CP/MAS NMR spectra. The first CP/MAS NMR chemical-shift thermometer. *J. Magn. Reson.* 69:191–195.
- Capanema, E.A., Balakshin, M.Y., Kadla, J.F. (2004) A comprehensive approach for quantitative lignin characterization by NMR spectroscopy. *J. Agric. Food Chem.* 52:1850–1860.
- Chen, C.-L. (1996) Characterization of milled wood lignins and dehydrogenative polymerisates from monolignols by carbon-13 NMR spectroscopy. In: ACS Symposium Series 697 Lignin and Lignan Biosynthesis. Eds. Lewis, N.G., Sarkanen, S. American Chemical Society, Washington, DC. pp. 255–275.
- Dimmel, D. (2010) Overview. In: Lignin and Lignans: Advances in Chemistry. Eds. Heitner, C., Dimmel, D.R. Schmidt, J.A. CRC Press, Boca Raton. pp. 1–10.
- Drumond, M., Aoyama, M., Chen, C.-L., Robert, D. (1989) Substituent effects on C-13 chemical shifts of aromatic carbons in biphenyl type lignin model compounds. *J. Wood Chem. Technol.* 9:421–441.
- Gansow, O.A., Burke, A.R., Vernon, W.D. (1972) Temperature-dependent carbon-13 nuclear magnetic resonance spectra of the h^5 -cyclopentadienyliron dicarbonyl dimer, an application of a shiftless relaxation reagent. *J. Am. Chem. Soc.* 94:2550–2552.
- Hatcher, P.G. (1987) Chemical structural studies of natural lignin by dipolar dephasing solid-state ^{13}C nuclear magnetic resonance. *Org. Geochem.* 11:31–39.
- Hatfield, G.R., Maciel, G.E., Erbatur, O., Erbatur, G. (1987) Qualitative and quantitative analysis of solid lignin samples by carbon-13 nuclear magnetic resonance spectrometry. *Anal. Chem.* 59:172–179.
- Haw, J.F., Schultz, T.P. (1985) Carbon-13 CP/MAS NMR and FT-IR study of low-temperature lignin pyrolysis. *Holzforchung* 39:289–296.
- Hawkes, G.E., Smith, C.Z., Utley, J.H.P., Vargas, R.R., Viertler, H. (1993) A comparison of solution and solid state ^{13}C NMR spectra of lignins and lignin model compounds. *Holzforchung* 47:302–312.
- Heikkinen, S., Toikka, M.M., Karhunen, P.T., Kilpeläinen, K.A. (2003) Quantitative 2D HSQC (Q-HSQC) via suppression of J -dependence of polarization transfer in NMR spectroscopy: application to wood lignin. *J. Am. Chem. Soc.* 125:4362–4367.
- Iiyama, K., Wallis, A.F.A. (1990) Determination of lignin in herbaceous plants by an improved acetyl bromide procedure. *J. Sci. Food Agric.* 51:145–161.
- Kim, H., Ralph, J. (2010) Solution-state 2D NMR of ball milled plant cell wall gels in DMSO- d_6 /pyridine- d_5 . *Org. Biomol. Chem.* 8:576–591.
- Kim, H., Ralph, J., Akiyama, T. (2008) Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO- d_6 . *Bioenerg. Res.* 1:56–66.
- Lapierre, C., Monties, B., Rolando, C. (1985) Thioacidolysis of lignin: comparison with acidolysis. *J. Wood Chem. Technol.* 5:277–292.
- Lapierre, C., Pollet, B., Monties, B., Roland, C. (1991) Thioacidolysis of spruce lignin: GC-MS analysis of the main dimers recovered after Raney nickel desulphuration. *Holzforchung* 45:61–68.
- Li, Y., Akiyama, T., Yokoyama, T., Matsumoto, Y. (2016) NMR assignment for diaryl ether structures (4-O-5 structures) in pine wood lignin. *Biomacromolecules* 17:1921–1929.
- Lu, F., Ralph, J. (2003) Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. *Plant J.* 35:535–544.
- Lundquist, K. (1980) NMR studies of lignins. 4. Investigation of spruce lignin by ^1H NMR spectroscopy. *Acta Chem. Scand.* B34:21–26.
- Maciel, G.E., O'Donnell, D.J., Ackerman, J.J.H., Hawkins, B.H., Bartuska, V.J. (1981) A ^{13}C NMR study of four lignins in the solid and solution states. *Makromol. Chem.* 182:2297–2304.
- Mansfield, S.D., Kim, H., Lu, F., and Ralph, J. (2012) Whole plant cell wall characterization using solution-state 2D NMR. *Nat. Protocol.* 7:1579–1589.
- Mao, J., Holtman, K.M., Scott, J.T., Kadla, J.F., Schmidt-Rohr, K. (2006) Differences between lignin in unprocessed wood, milled wood, mutant wood, and extracted lignin detected by ^{13}C solid-state NMR. *J. Agric. Food Chem.* 54:9677–9686.
- Nimz, H. (1974) Beech lignin-proposal of constitutional scheme. *Angew. Chem. Int. Ed.* 13:313–321.
- Nimz, H.H., Robert, D., Faix, O., Nemr, M. (1981) Carbon-13 NMR spectra of lignins 8. Structural differences between lignins of hardwoods, softwoods, grasses and compression wood. *Holzforchung* 35:16–26.

- Nishimura, H., Kamiya, A., Nagata, T., Katahira, M., Watanabe, T. (2018) Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls. *Sci. Rep.* 8:6538.
- Ottenbours, B., Adriaenssens, P., Carleer, R., Vanderzande, D., Gelan, J. (1998) Quantitative carbon-13 solid-state NMR and FT-Raman spectroscopy in novolac resins. *Polymer* 39:5293–5300.
- Parkås, J., Paulsson, M., Terashima, N., Westermark, U., Ralph, S. (2004) Light-induced yellowing of selectively ^{13}C -enriched dehydrogenation polymers (DHPs). Part 2. NMR assignments and photoyellowing of aromatic ring 1-, 3-, 4-, and 5- ^{13}C DHPs. *Nord. Pulp Pap. Res. J.* 19:44–52.
- Ralph, J., Landucci, L.L. (2010) 5 NMR of Lignins. In: *Lignin and Lignans: Advances in Chemistry*. Eds. Heitner, C., Dimmel, D.R., Schmidt, J.A. CRC Press, Boca Raton, FL, USA. pp. 137–244.
- Ralph, S., Ralph, J. (2009) NMR database of lignin and cell wall model compounds. Available from https://www.glbc.org/databases_and_software/nmrdatabase/ (Jan 2019 accessed).
- Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P.F., Marita, J.M., Ralph, S.A., Srinivasa Reddy, M.S., Chen, F., Dixon, R.A. (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. *J. Biol. Chem.* 281:8843–8853.
- Rokhin, A.V., Kanitskaya, L.V., Kushnarev, D.F., Kalabin, G.A., Smirnova, L.S., Abduazimov, Kh.A., Pulatov, B.Kh. (1994) Quantitative ^1H and ^{13}C NMR spectroscopies of cottonplant dioxane lignin. *Chem. Nat. Comp.* 30:745–753.
- Rolando, C., Monties, B., Lapierre, C. (1992) Thioacidolysis. In: *Methods in Lignin Chemistry*. Eds. Lin, S.Y., Dence, C.W. Springer-Verlag, Berlin, Germany. pp. 334–349.
- Saito, K., Fukushima, K. (2005) Distribution of lignin interunit bonds in the differentiating xylem of compression and normal woods of *Pinus thunbergii*. *J. Wood Sci.* 51:246–251.
- Samuels, A.L., Rensing, K.H., Douglas, C.J., Mansfield, S.D., Dharmawardhana, D.P., Ellis, B.E. (2002) Cellular machinery of wood production: differentiation of secondary xylem in *Pinus contorta* var. *latifolia*. *Planta* 216:72–82.
- Sette, M., Wechselberger, R., Crestini, C. (2011) Elucidation of lignin structure by quantitative 2D NMR. *Chem. Eur. J.* 17:9529–9535.
- Sipponen, M.H., Laakso, S., Baumberger, S. (2014) Impact of ball milling on maize (*Zea mays* L.) stem structural components and on enzymatic hydrolysis of carbohydrates. *Ind. Crops Prod.* 61:130–136.
- Terashima, N., Fukushima, K. (1988) Heterogeneity in formation of lignin – XI: an autoradiographic study of the heterogeneous formation and structure of pine lignin. *Wood Sci. Technol.* 22:259–270.
- Terashima, N., Seguchi, Y., Robert, D. (1991) Selective ^{13}C -enrichment of side chain carbons of guaiacyl lignin in pine. *Holzforschung* 45(Suppl.):35–39.
- Terashima, N., Fukushima, K., He, L-F., Takabe, K. (1993) Comprehensive model of the lignified plant cell wall. In: *Forage Cell Wall Structure and Digestibility*. Eds. Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J. Am. Soc. Agron. Crop Sci. Soc. Am. Soil Sci. Soc. Am. Madison, pp. 247–270.
- Terashima, N., Atalla, R.H., VanderHart, D.L. (1997) Solid state NMR spectroscopy of specifically ^{13}C -enriched lignin in wheat straw from coniferin. *Phytochemistry* 46:863–870.
- Terashima, N., Hafrén, J., Westermark, U., VanderHart, D.L. (2002) Nondestructive analysis of lignin structure by NMR spectroscopy of specifically ^{13}C -enriched lignins. Part 1. Solid state study of ginkgo wood. *Holzforschung* 56: 43–50.
- Terashima, N., Evtuguin, D.V., Neto, C.P., Parkås, J. (2003) Synthesis of coniferins ^{13}C -enriched at Position 4 or 5 of the guaiacyl ring. *Holzforschung* 57:485–488.
- Terashima, N., Akiyama, T., Ralph, S., Evtuguin, D., Neto, C.P., Parkås, J., Paulsson, M., Westermark, U., Ralph, J. (2009) 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. *Holz-forschung* 63:379–384.
- Terashima, N., Ko, C., Matsushita, Y., Westermark, U. (2016) Monolignol glucosides as intermediate compounds in lignin biosynthesis. Revisiting the cell wall lignification and new ^{13}C -tracer experiments with *Ginkgo biloba* and *Magnolia liliiflora*. *Holzforschung* 70:801–810.
- Wheeler, H.L., Soong, R., Courtier-Murias, D., Botana, A., Fortier-McGill, B., Maas, W.E., Fey, M., Hutchins, H., Krishnamurthy, S., Kumar, R., Monette, M., Stronks, H.J., Campbell, M.M., Simpson, A. (2015) Comprehensive multiphase NMR: a promising technology to study plants in their native state. *Magn. Reson. Chem.* 53:735–744.
- Whetten, R.W., MacKay, J.J., Sederoff, R.R. (1998) Recent advances in understanding lignin biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:585–609.
- Xia, Z., Akim, L.G., Argyropoulos, D.S. (2001) Quantitative ^{13}C NMR analysis of lignins with internal standards. *J. Agric. Food Chem.* 49:3573–3578.
- Xie, Y., Terashima, N. (1991) Selective carbon 13-enrichment of side chain carbons of ginkgo lignin traced by carbon 13 nuclear magnetic resonance. *Mokuzai Gakkaishi* 37:935–941.
- Yue, F., Lu, F., Ralph, S., Ralph, J. (2016) Identification of 4-O-5-Units in softwood lignins via definitive lignin models and NMR. *Bio-macromolecules* 17:1909–1920.
- Zhang, L., Gellerstedt, G. (2007) Quantitative 2D HSQC NMR determination of polymer structures by selecting suitable internal standard references. *Magn. Reson. Chem.* 45:37–45.

Supplementary Material: The online version of this article offers supplementary material (<https://doi.org/10.1515/hf-2019-0011>).