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Analysis of the structure of lignin–carbohydrate complexes by the specific ^{13}C tracer method

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Abstract In the present study the specifically ^{13}C-enriched lignin precursors of biosynthesis (i.e., coniferin-[side chain-α-^{13}C], coniferin-[side chain-β-^{13}C] and coniferin-[side chain-γ-^{13}C]) were synthesized and administered exogeneously to ginkgo shoots (*Ginkgo biloba* L.) to obtain ^{13}C-enriched lignin–carbohydrate complexes (LCCs). The specifically ^{13}C-enriched LCCs were isolated from the newly formed xylem of ginkgo shoots administered with the ^{13}C-enriched precursors and degraded by enzymes. Lignin-rich fractions, so called enzyme-degraded LCCs (EDLCCs), were obtained. By determining their ^{13}C-NMR spectra, information related to the chemical structure of lignin building units and linkages between phenylpropane units of lignin and carbohydrates were obtained. It was found that these precursors were incorporated in natural lignin successfully. Three lignin–carbohydrates linkages (i.e., ether type, ester type, ketal type) were found at the C_α-position of the side chain of phenylpropane units in ginkgo LCC. No lignin–carbohydrate bond at the C_β- or C_γ-position of the lignin side chain was observed in the ^{13}C-NMR spectra of the ^{13}C-enriched LCCs. This fact indicates that a specific ^{13}C tracer technique can be useful in NMR study of the chemical structure of LCCs.

Key words Lignin–carbohydrate complexes · NMR · ^{13}C tracer · Coniferin · Ketal linkage

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Introduction

It is commonly assumed that lignin polymer is bound covalently to polysaccharides to form lignin–carbohydrate complexes (LCCs).[1–3] The lignin–carbohydrate covalent linkages were suggested as the most probable cause for the residual lignin to resist delignification during Kraft pulping and bleaching. Many experiments with LCCs strongly suggest that such bonds exist between lignin and hemicellulose.[4–12] The proposed linkage type is classified into the following six groups.

1. Ether linkage of the hydroxyl group at the α-position of the lignin side chain with alcoholic hydroxyl of sugar residue[4]
2. Ester linkage of the alcoholic OH of lignin with the carboxylic group of uronic acid[5]
3. Hemiacetal or acetal linkage of the carbonyl group located at β-position of lignin with carbohydrates[6]
4. Glycoside linkage with the primary alcoholic OH at the γ-position of the phenylpropane unit[7]
5. Glycosidic linkage at the phenolic OH of lignin[8–9]
6. Ester linkage of the carboxylic group of the cinnamic acid unit in lignin with the alcoholic OH of carbohydrates[10–12]

Much of the information about LCC bonds stated above was obtained by degradation analysis as acid hydrolysis and alkaline hydrolysis. However, it is almost impossible to depolymerize and degrade LCCs into their constituent units quantitatively by these methods. Furthermore, it is inevitable that many undesirable modifications occur during their degradation reactions. Therefore, little direct evidence of this linkage has been obtained so far.[13] It is advantageous to adopt a nondestructive analytical method for this purpose.

As a nondestructive analytical method, carbon 13 nuclear magnetic resonance (^{13}C-NMR) has proved to be a powerful method for elucidating the detailed chemical features of LCC because it reflects the nature of chemical bonding between lignin and carbohydrates.[14,15] However,

observation of the LCC bonds by ^{13}C-NMR is difficult because the strong signals arising from carbohydrates in LCC overlap with those from lignin. To overcome this difficulty, the method of specific ^{13}C-enrichment of lignin *in situ* can be applied successfully.[16–20] By ^{13}C-NMR analysis of ^{13}C-enriched LCC biosynthesized in a plant signals arising from the carbons with LCC bonds may be observed clearly, leading to elucidation of the chemical structure of LCC. In the present work, precursors of lignin biosynthesis with specifically ^{13}C-enriched side-chain carbons (i.e., coniferin-[side chain-α-^{13}C], coniferin-[side chain-β-^{13}C] and coniferin-[side chain-γ-^{13}C]) were synthesized and administered to cut shoots of ginkgo (*Ginkgo biloba* L.). LCCs specially enriched with ^{13}C were isolated from the newly formed xylem and treated with enzyme; they were then analyzed by ultraviolet photometry, infrared spectroscopy, and ^{13}C-NMR.

Materials and methods

Synthesis of ^{13}C-enriched lignin precursors

Coniferin-[side chain-α-^{13}C] (**I**) (Fig. 1), coniferin-[side chain-β-^{13}C] (**II**), and coniferin-[side chain-γ-^{13}C] (**III**) were synthesized according to the reported methods.[16,18] Their estimated ^{13}C-enrichment was 99%. As shown in Fig. 2, only the resonance signals from the enriched carbons could be observed in their ^{13}C-NMR spectra.

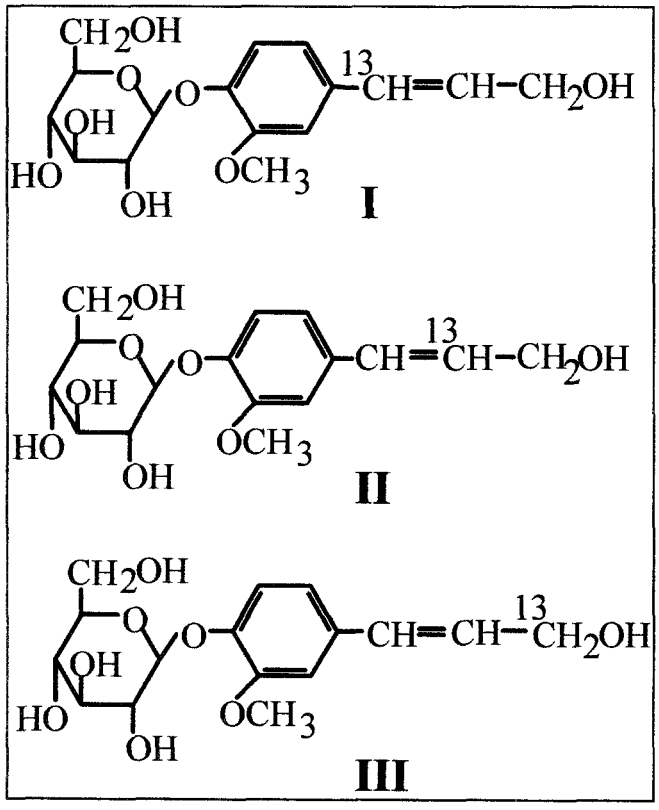

Fig. 1. Structure of ^{13}C-enriched Coniferins

Administration of lignin precursor to ginkgo shoots

Cut shoots with 25–30 leaves (about 35 cm long) of a 4-year-old ginkgo tree were obtained in June and put in vials containing a solution of coniferin (2.5 mg/ml), with water as control. The shoots were allowed to stand at 25°C in a plant-growth chamber for 4 weeks, and 250 mg of each kind of coniferin was administered.

Preparation of LCC and enzyme-degraded LCC from ginkgo xylem

A ginkgo shoot, treated with coniferin, was debarked, and 100 μm thick tangential sections were cut on a sliding microtome from cambium to pith by a procedure described earlier.[16] The dried xylem sections of the ginkgo were milled to the size of 100 mesh by a Wiley mill and then extracted thoroughly with ethanol-benzene (1:2, v/v) and hot water, successively. The extractive-free wood meals were ground in a vibratory ball mill for 72 h, and then the wood powder was used to prepare LCCs by the procedure of Björkman.[1]

A sample of 600 mg of LCC was suspended in an enzyme solution containing 0.4% cellulase (Onozuka RS, Yakult Pharmaceutical Chemical Industries), 0.4% hemicellulase (Sigama), and 0.4% pectinase (macerozyme R-10; Yakult). The buffer (pH 4.6), composed of 0.5 M sodium acetate and 0.5 M acetic acid, was applied; and a few drops of toluene were added as a preservative. The mixture was agitated gently on a shaker at 50°C for 2 days. After centrifugation and washing with distilled water, the precipitate of the enzyme-degraded LCC (EDLCC) was collected by freeze-drying. The yield was about 17.0% from LCC.

Alkali treatment of EDLCC

The EDLCC (100 mg) was dissolved in 1 N NaOH. The solution was stirred at 25°C in N_2 atmosphere for 12 h. After acidification with diluted HCl, the precipitate was collected by centrifugation and freeze dried.

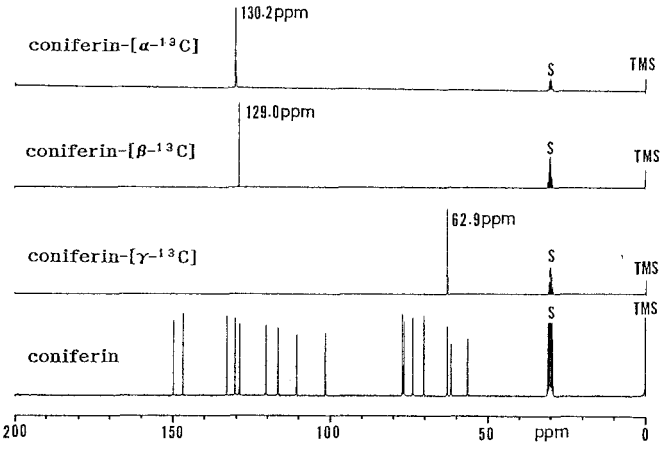

Fig. 2. ^{13}C-NMR spectra of coniferins. *S*, sovent, acetone-d_6/D_2O (7:3, v/v); TMS, reference

Determination of lignin content

A sample of 6 mg of LCC or 3 mg of EDLCC was dissolved in 100 ml dimethyl formamide (DMF). The ultraviolet (UV) absorbance at 280 nm (A_{280}) was determined with a Shimadzu 2201 UV-VIS spectrometer. The lignin content was calculated with the following formula.[21]

Lignin content (%) = $A_{280} \times 0.1 / DW$

where D (absorptivity) is 20.71 $g^{-1} cm^{-1}$, and W is the sample weight in grams.

Determination of infrared spectra

Infrared (IR) spectra were determined on a Hitachi model 260-10 infrared spectrometer. The spectra of the LCC and (MWL) samples were measured by the KBr technique. Each KBr disc contained 1.3 mg of sample in 180 mg of KBr.

Determination of ^{13}C-NMR spectra

An 80-mg sample of EDLCC was dissolved in 0.5 ml DMSO-d_6 and put in a 5 mm probe tube. The ^{13}C-NMR spectra were recorded on a JEOL JNM-EX 270 FT NMR spectrometer at 67.8 MHz under total proton decoupled conditions at 25°C. Tetramethylsilane (TMS) was added to the solution as an internal reference. Pulse width was 3.9 μs (45° pulse) with a 2.2 s pulse delay, and 3×10^4 scans were accumulated.

Results and discussion

Chemical composition of LCC and EDLCCs

As shown in Table 1, the EDLCCs recovered from the treatment of LCCs with cellulase, hemicellulase, and pectinase were lignin-rich fractions. This result indicates that the enzymatic treatment was effective in shortening the hemicellulose chain in the LCCs and reducing the sugar content. This result also is in satisfactory agreement with the experiment by Eriksson et al.[3] Because the strong signals from carbohydrate disturb the correct assignment of signals from phenylpropane units in LCCs when determined with ^{13}C-NMR, it is necessary to enrich the lignin content before NMR analysis.

Table 1. Lignin content in ginkgo LCC and EDLCCs

Sample	Lignin content (%)
LCC-control	31.8
EDLCC-control	84.5
EDLCC-[α-^{13}C]	86.6
EDLCC-[β-^{13}C]	85.7
EDLCC-[γ-^{13}C]	86.3

LCC, lignin–carbohydrate complex; EDLCC, enzyme-degraded LCC

Figure 3 shows the IR spectra of LCC, EDLCC, alkali-treated EDLCC, and MWL from intact ginkgo wood. In the LCC spectrum, the absorbance in the vicinity of 1730 cm^{-1} due to the esterified carbonyl groups, which may be the acetyl group attached to xylose and esters of glucuronic acid, was strong compared with that of MWL.[22] This indicates that LCC contained a high content of carbohydrates, as expected. However, the intensity of the band of 1730 cm^{-1} was greatly reduced after the enzyme treatment, as shown in the EDLCC spectrum, which was similar to that of MWL generally. This fact indicates that most of the carbohydrate was hydrolyzed by enzymes, and it agrees well with the determination by UV photometry. All ester linkages in EDLCC were saponified completely under the alkaline condition used, as the band at 1730 cm^{-1} has disappeared from the spectrum of alkali-treated EDLCC.

Incorporation of coniferin into ginkgo LCC

Figure 4 shows the ^{13}C-NMR spectra of EDLCCs prepared from ^{13}C-enriched ginkgo wood and control wood. Tentative assignment of the signals shown in Table 2 were made by comparisons with signal chemical shifts reported for the main structures of monolignols, dilignols, LCC models and carbohydrates in lignin preparations.[23–27]

As shown in Fig. 4, in the aromatic region of 111–149 ppm, there was no appreciable qualitative difference

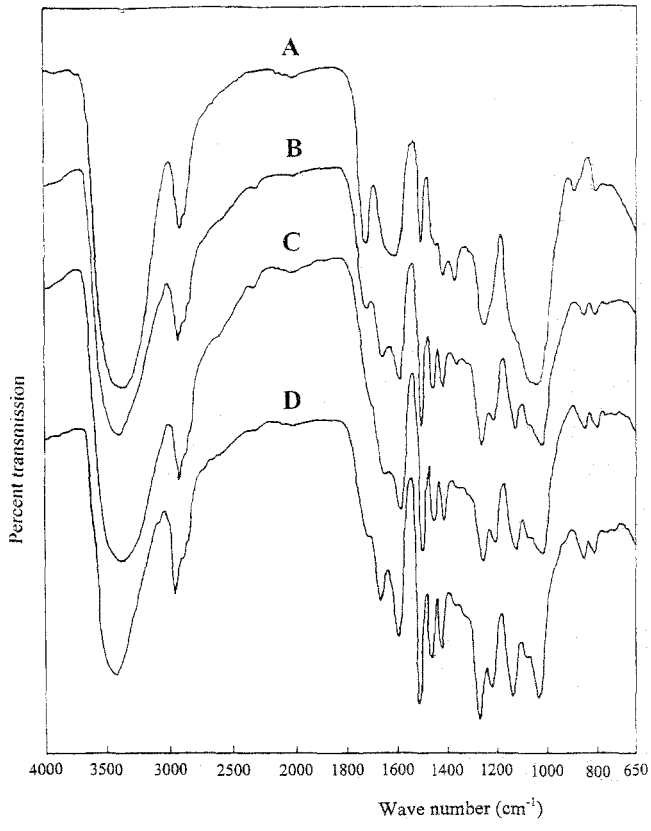

Fig. 3. Infrared spectra of lignin-carbohydrate complex (LCC), enzyme-degraded LCCs (EDLCCs), and MWL from intact ginkgo wood. *A*, LCC; *B*, EDLCC; *C*, EDLCC treated with alkali; *D*, MWL

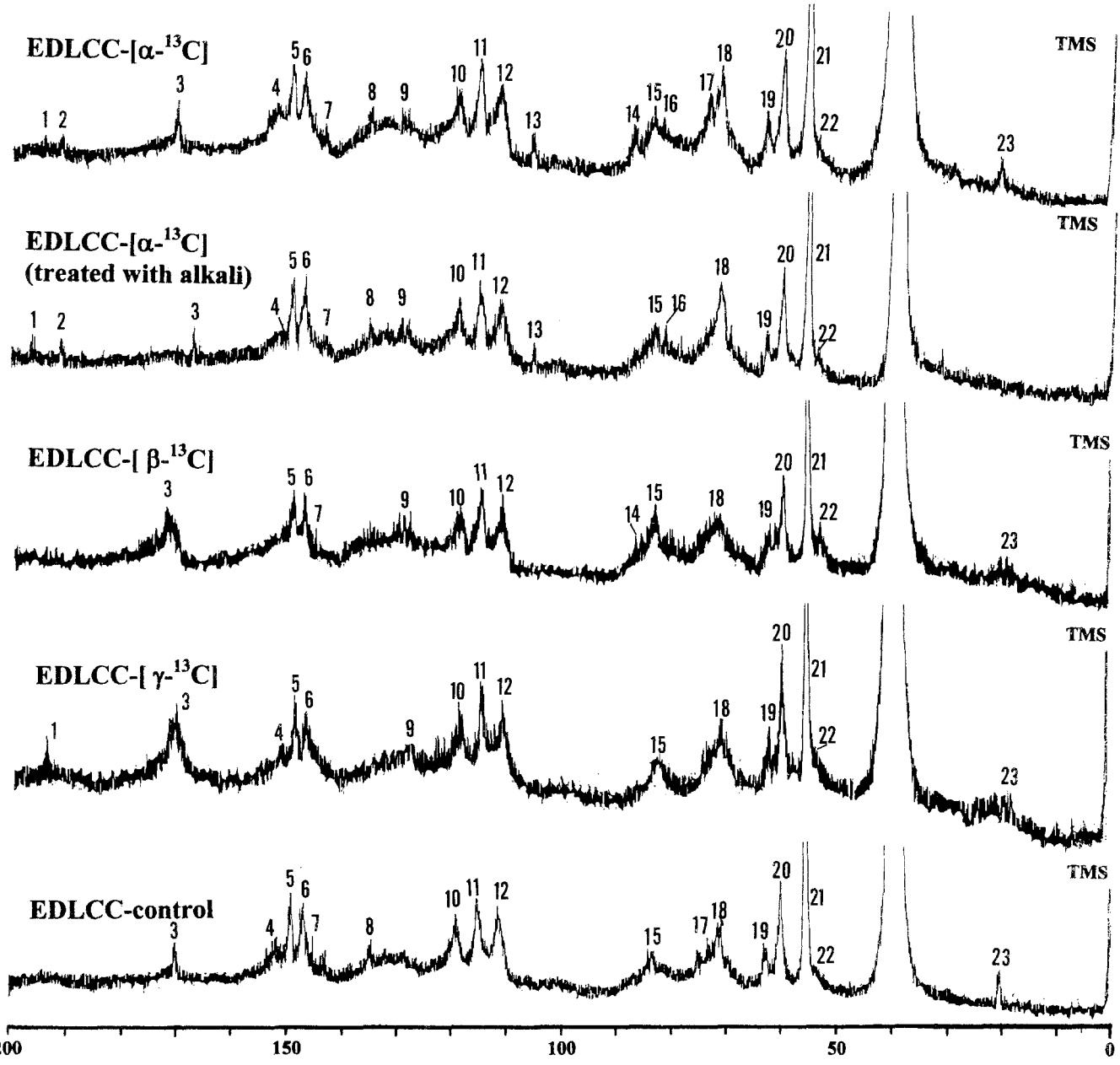

Fig. 4. ^{13}C-NMR spectra of EDLCCs from ginkgo wood. *TMS*, reference

between the spectra of EDLCC-control and ^{13}C-enriched EDLCCs. However, in the regions of aliphatic carbons (53–105 ppm and 191–194 ppm), some signals were enhanced greatly by ^{13}C enrichment at the side-chain position. This indicates that coniferin administered exogeneously is incorporated into the LCCs with no disturbance of the normal formation of LCCs.

^{13}C-NMR spectra of EDLCC-[α-^{13}C]

If the spectrum of EDLCC-[α-^{13}C] was compared with that of EDLCC-control, the intensities of signal 1 (194.4 ppm) and signal 2 (191.0 ppm) were enhanced in the spectrum of EDLCC-[α-^{13}C], and could be assigned respectively to α-carbonyl (**IV**) (Fig. 5), γ-cinnamaldehyde group (**V**), and α-aldehyde group (**VI**), respectively. Signal 13 at 105.6 ppm was enhanced by the α-^{13}C enrichment. This signal was assigned to the α-carbon of the lignin side chain with ketal linkage to carbohydrates as shown in Fig. 5 (**VII**) according to the ^{13}C-NMR spectra of model compound.[28] By contrast, signal 13 still existed in the spectrum of alkali-treated EDLCC-[α-^{13}C], a result that agreed well with the stability of ketal linkage in alkaline solution. Bolker and Sommerville[6] thought that hemiacetal or acetal linkage of the carbonyl group is located at the β-position of lignin with carbohydrates. Our work gives evidence of ketal linkage and proves that this kind of LCC bond exists at the α-

Table 2. ^{13}C chemical shifts and assignments for EDLCCs from ginkgo wood

Signal no.	Chemical shifts (δ, ppm)					Assignments
	α-^{13}C	α-^{13}C (treated)	β-^{13}C	γ-^{13}C	Control	
1	194.4	194.4	–	194.4	–	α-CO, and γ-CHO in cinnamaldehyde
2	191.0	191.0	–	–	–	α-CHO
3	170.2	167.4	172.0	170.6	170.1	Unknown
4	152.2	152.2	–	152.3	151.7	C-4 in G with α-ether; C-α in cinnamaldehyde
5	149.3	149.0	149.1	149.0	149.1	C-4 in G; C-3 in G with α-CO
6	147.2	147.1	147.2	147.2	146.9	C-3 in G
7	143.1	143.1	143.1	–	143.3	C-α in cinnamic acid; C-4 in phenylcoumaran
8	134.9	134.9	–	–	134.7	C-1 in G
9	129.5	129.5	130.0	130.0	129.5	C-1 in G with C-α in HC=C; C-2 and C-6 in H; C-β in cinnamaldehyde
10	118.8	118.9	118.9	118.9	119.1	C-6 in G
11	115.2	115.1	115.2	115.2	115.2	C-6 in phenylcoumaran; C-5 in G; C-3 and C-5 in H
12	111.2	111.2	111.3	111.2	111.3	C-2 in G
13	105.6	105.6	–	–	–	C-α with ketal linkage; C-1 in β-D-glucuronate
14	87.4	–	87.1	–	–	C-α in phenylcoumaran
15	83.4	83.0	83.4	83.0	83.0	C-β in β-arylether; C-α in pinoresinol; C-4 in Ara
16	81.7	81.6	–	–	–	C-α etherified to carbohydrates; C-4 in 4-*O*-MeGlcA; C-2 in Ara
17	73.6	–	–	–	73.4	C-α esterified to carbohydrates; C-2 in Xyl
18	71.6	71.4	71.5	71.2	71.5	C-α in β-arylether; C-2 in 4-*O*-MeGlcA
19	63.1	63.0	63.0	62.5	63.3	C-α and C-β in β-1 dilignols; C-γ in phenylcoumaran and cinnamylalcohol; C-5 in Xyl
20	60.1	59.7	60.1	60.1	60.0	C-γ in β-arylether; C-6 in Glc; C-5 in 4-*O*-MeGlcA
21	55.5	55.5	55.5	55.5	55.5	$-OCH_3$
22	53.2	53.2	53.2	53.1	53.2	C-β in β-β and β-5 units
23	20.1	–	20.1	20.0	20.1	$-CH_3$ in units in acetyl group

G, guaiacyl; H, *p*-hydroxyphenyl; Ara, arabinose; 4-*O*-MeGlcA, 4-*O*-methyl glucuronic acid; Xyl, xylose; Glc, glucose

position but not at the β-position of phenylpropane units. Enhanced signals were noted at 87.4 ppm (signal 14) and 81.7 ppm (signal 16) in the spectrum of EDLCC-[α-^{13}C] and assigned to the C-α in phenylcoumaran (**VIII**) and C-α etherified to carbohydrates (**IX**), respectively.[25,29] However, signal 14 became weak, but signal 16 remained in the spectrum of alkali-treated EDLCC-[α-^{13}C]. This result strongly supports the assignment of these two signals because phenolic-type phenylcoumaran is easily degraded by hydrolysis, and the benzyl ether-type LCC bond is stable during alkali treatment.[25,30] An enhanced signal at 73.6 ppm (signal 17) may arise from C-α in phenylpropane units containing ester linkage with carbohydrates (**X**) and C-2 in xylose,[31] and the existence of ester linkage could be proved by the alkali treatment experiment as the signal could not be observed in the spectrum of alkali-treated EDLCC-[α-^{13}C]. This fact also agrees well with the IR determination of ginkgo EDLCC, as mentioned above. The great enhancement of signal 18 indicates the high content of β-arylether structure (**XI**) in the newly formed LCC.

^{13}C-NMR spectra of EDLCC-[β-^{13}C]

In the spectrum of EDLCC-[β-^{13}C] (Fig. 4), the intensities of signals 15 and 22 were enhanced by ^{13}C enrichment compared with that of EDLCC-control. Signal 15 could be assigned to C-β in the β-arylether structure shown in Fig. 5 (**XI**) and to C-4 in arabinose. Signal 22 was assigned to C-β in the pinoresinol (**XII**) and phenylcoumaran (**VIII**) subunits in LCCs.[26,27] However, no LCC linkage was observed throughout the β-^{13}C tracer experiment.

^{13}C-NMR spectra of EDLCC-[γ-^{13}C]

The ^{13}C-NMR spectrum of the EDLCC-[γ-^{13}C] prepared from the ^{13}C-enriched ginkgo cut shoot treated with coniferin-[γ-^{13}C] is shown in the Fig. 4. Enhanced signal 1 at 194.4 ppm was assigned the C-γ carbonyl group in the cinnamylaldehyde structure (Fig. 5) (**V**) in ginkgo LCC. As could be expected, the intensities of signals 18 at 71.2 ppm, 19 at 62.5 ppm, and 20 at 60.1 ppm, mainly from γ-carbons in pinoresinol (**XII**), phenylcoumaran (**VIII**), and β-arylether structure (**XI**), were enhanced, respectively, by ^{13}C enrichment at the γ-position. We observed no signal related to linkages between lignin moieties and carbohydrates in the γ-^{13}C-enrichment experiment.

Conclusions

We reached the following conclusions.

1. Coniferin-[side chain-α-^{13}C], coniferin-[side chain-β-^{13}C], and coniferin-[side chain-γ-^{13}C] administered exogenously to ginkgo shoots were incorporated into natural LCC successfully.

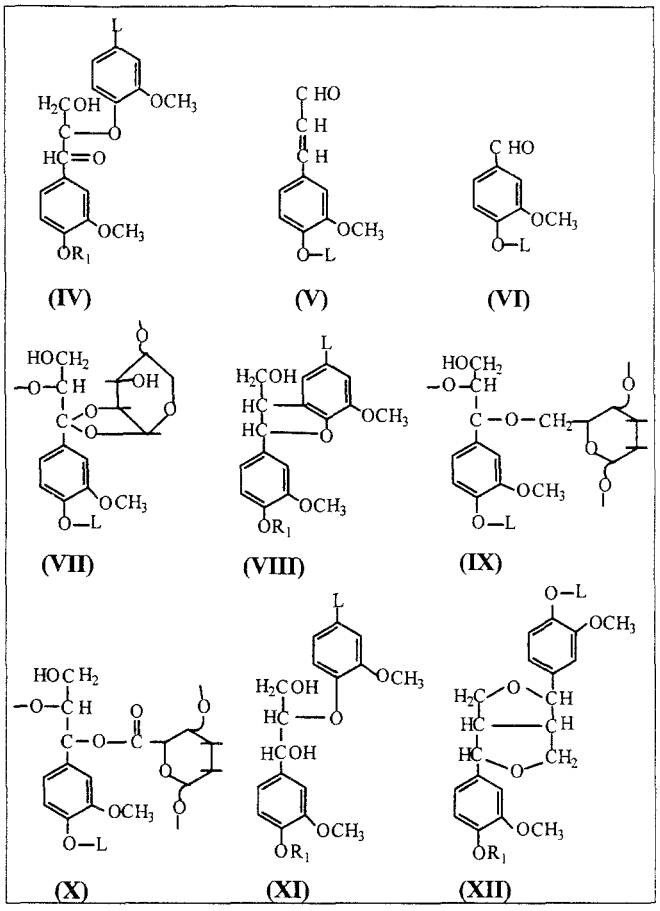

Fig. 5. Substructures found in ginkgo LCCs. R_1, H, alkyl or aryl; L, polylignol

2. EDLCCs recovered from the treatment of ginkgo LCCs with cellulase, hemicellulase, and pectinase were lignin-rich and useful in the ^{13}C-NMR analysis.

3. The ^{13}C-NMR spectra of ^{13}C-enriched EDLCC provided definite information on the structure of LCC.

4. Three kinds of lignin-carbohydrate linkage (i.e., ether type, ester type, and ketal type) were found in the α-position of the side chain of phenylpropane units in ginkgo LCC.

5. No lignin–carbohydrate bond was observed at the β- or γ-position of the phenylpropane units.

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