Review Article

Structural Studies on the Covalent Bonds between Lignin and Carbohydrate in Lignin-Carbohydrate Complexes by Selective Oxidation of the Lignin with 2, 3-Dichloro-5, 6-dicyano-1, 4-benzoquinone*1

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Contents

Key words: lignin-carbohydrate complex (LCC), DDQ, binding-site analysis, akamatsu (*Pinus densiflora*)

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PREFACE

One of the major diffeculties in the chemical analysis of wood is due to the fact that intimate physical and chemical associations exist between macromolecules in the wood cell walls. In particular, the association occuring between lignin and polyoses is rather complicated and difficult to be elucidated. Since Erdman suggested the existence of "glycolignose" in 1866 [Erdman, 1866], considerable efforts have been made to prove the existence of covalent bonds between the lignin and carbohydrate in the wood cell walls, and the accumulated evidence strongly suggests that lignin-carbohydrate complexes (LCC) are not physically associated aggregates but are chemically linked compounds. At present, it is generally accepted that a part of the lignin is not simply deposited between the cell wall polysaccharides, but is chemically linked to them. Thus, major interest on this problem has gradually changed from their occurrence to the determination of binding-sites between both components.

So far, lignin-carbohydrate linkages of the ester [Morrison, 1973; Yaku, 1976; Eriksson, 1980; Obst, 1982; Meshitsuka, 1982; Lundquist, 1983; Das, 1984; Takahashi, 1988; Watanabe, 1988a], ether [Eriksson, 1977; Košíková, 1979; Eriksson, 1980; Yaku, 1981; Koshijima, 1984, Watanabe, 1986, 1987b, 1989b], glycoside [Kawamura, 1952; Smelstorius, 1974; Yaku, 1976, Joseleau, 1986] and acetal [Bolker, 1963] types have been proposed, based on the chemical and/or enzymatic treatment of LCCs. In particular, the existence of benzyl ether and ester types has often been suggested, not only from the formation of dehydrogenation polymer (DHP)-sugar complexes [Freudenberg, 1959, 1965; Tanaka, 1976, 1979; Hemmingson, 1979; Katayama, 1980; Tanahashi,

1981; Leary, 1983; Koshijima, 1989], but also from chemical analyses of the LCCs [Košhíková, 1979; Koshijima, 1984; Watanabe, 1986, 1987b, 1988a, 1989b]. For instance, Freudenberg at first demonstrated the formation of an addition compound of sucrose into quinonemethide intermediates by dehydrogenation of coniferyl alcohol in a concentrated sucrose solution [Freudenberg 1959, 1965]. Tanaka *et al.* also reported addition compounds from a quinonemethide and D-glucuronic acid or D-glucose and proved the formation of an ester or ether linkage between the sugar and the α-position of the dilignol [Tanaka, 1976, 1979]. Furthermore, Leary reported that the etherification of vanillyl alcohol with sugars proceeded both at primary and secondary hydroxyl groups of the sugars even in neutral aqueous solution [Leary, 1983]. Likewise, the formation of DHP-polysaccharide complexes has also been proved by dehydrogenative polymerization of coniferyl alcohol in the presence of pectin, mannan [Tanahashi, 1981], xylan and acetylglucomannan [Koshijima, 1989]. As for the chemical analyses of the benzylic bonds in native LCCs, Minor concluded that lignin linked to hexoses favorably at C-6 position, because Hakomori methylation of loblolly pine holocelluloses gave a larger amount of 2,3-di-*O*-methyl hexoses than those from cellulase-degraded milled wood lignin [Minor, 1982, 1986]. Košíková and others showed the existence of the benzyl ether bonds in LCCs, based on their stabilities against alkali, and calculated their frequency to be one benzyl ether linkage between lignin and hemicelluloses per 50 methoxyl groups [Košhíková, 1979]. Eriksson also reported that neutral sugar residues in hemicellulose were, to some extent, bound to lignin probably by benzyl ether bonds [Eriksson, 1980]. Thus, at present, the existence of the benzyl ether and ester linkages between lignin and carbohydrate is generally accepted. However, it should be pointed out that previous methods for determining their binding-sites cannot directly specify what kind of substance was bound to the carbohydrate components, because the chemical reactions involved in the previous methods were non-specific to lignin skeleton. For example, Hakomori methylation of LCCs gave information on branch points of sugar residues; however, by this method, lignin-carbohydrate bonds could not be discriminated from glycosidic bonds between sugar residues, or from covalent bonds between sugar and other kinds of chemical substances. Smith degradation of LCCs is the same as for this point. Thus, previous studies on the binding-site analysis of lignin-carbohydrate linkages stand on the assumption that chemical linkages existed between lignin and sugars. Furthermore, it should be noted that strong alkali media used in the previous methylation analysis destroys both ester and *p*-hydroxybenzyl ether bonds which are indispensable for the overall architecture of LCCs [Enoki, 1983; Joniak, 1987].

In this paper, the author mentions a direct evidence for the binding site between lignin and carbohydrate, based on a specific reaction toward lignin skeleton. To achieve this, the author developed a new method which involves an oxidative cleavage of lignin-carbohydrate bonds by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).

In chapter I, a new convenient method was proposed to isolate lignin-carbohydrate complexes. This method made it possible to isolate a large quantity of LCCs in a short period.

In chapter II, a new method to prove the ether linkages between lignin and carbohydrate was developed by using the DDQ-oxidation and methylation by the method of Prehm [Prehm, 1980].

In chapter III, existence of the ester linkages between the lignin and 4-*O*-methylglucuronic acid residue in hemicelluloses was evidenced by a new method using an oxidative cleavage of the esters with DDQ.

All these new analytical methods were applied to the LCCs from akamatsu (*Pinus densiflora* Sieb. et Zucc.) wood.

Chapter I Isolation of water-soluble lignin-carbohydrate complexes

I-1 Introduction

The association occurring between lignin and carbohydrate in lignified plant cell walls has been the subject of controversy for more than a century. One of the major difficulties in the characterization of lignin-carbohydrate complexes (LCC) has been in the isolation of them in a homogenious state. In 1957, Björkman presented a method for preparing a milled wood lignin (MWL) and lignin-carbohydrate complexes (Björkman LCC) and suggested the chemical linkages between both components [Björkman, 1957a~d]. In previous studies on Björkman LCC [Tanaka, 1972; Koshijima, 1971, 1972 and 1976], it was shown that N,N-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) extraction of the residual wood meal previously extracted with aqueous dioxane, resulted in isolation of the LCC. Furthermore, it was reported that approximately the same amount of LCC could be extracted with hot water from the residual wood meal after extraction of Björkman LCC [Yaku, 1976]. This LCC, designated as HWF, was composed of LCC similar to Björkman LCC. A number of studies have been made to elucidate the existence of lignin-carbohydrate linkages by using the Björkman LCC or HWF [Matsukura, 1969; Morohoshi, 1971; Yaku, 1971, 1976 and 1981; Yamaguchi, 1973; Koshijima, 1974; Eriksson, 1977, 1980; Teratani, 1977; Azyma, 1985a; Kato, 1987].

On the other hand, it should be noted that LCCs were prepared from the aqueous dioxane extract of finely divided wood by multitudinous purification steps [Azuma, 1981, 1983a, 1985b, 1985c; Takahashi, 1982; Kato, 1984], and the resulting LCC (LCC-W) has given information on the molecular properties of the amphipathic substance. However, this isolation process, which comprized of dialysis, centrifugation, dissolution in pyridine-acetic acid-water (9:1:4), chloroform-extraction, addition of water, centrifugation, and re-dialysis [Azuma, 1981], was rather troblesome compared with the isolation of Björkman LCC.

Björkman presented an excellent procedure for the preparation of LCC. However, this method was disadvantageous for the large scale operation in a short period, because it required complete removal of nonvolatile organic solvents by dialysis. To solve this problem, a convenient new method for preparing LCCs has been developed in this chapter. Chemical properties of the resulting LCC is described [Watanabe, 1987a].

I-2 Experimental

General methods

Carbon-13 n.m.r. spectra were recorded on a Varian XL-200 Spectrometer

(50.3 MHz) in D_2O at 70°C. Chemical shifts were measured relative to internal 1,4-dioxane, which was assigned the value of 67.4 ppm. G.l.c. was conducted with a Shimadzu GC-4CM with a flame ionization detector (FID) using packed glass column (0.3 cm×200 cm) of (a) 3% ECNSS-M on Gaschrom Q, (b) 3% PPE-21 on Supelcoport, and (c) 20% tetramethylcyclobutanediol adipate 4% phosphoric acid on Chromosorb W. U.v. spectra were measured on a UVDEC-420 spectrophotometer as 80% aqueous dioxane solution. Analytical ultracentrifugation was performed at 42,040 r.p.m. in a Spinco model E Ultracentrifuge equipped with schlieren optics. G.c.-m.s. was done with a Shimadzu LKB-9000 system (70 eV) using column (b) at 200°C and 195°C.

Isolation of lignin-carbohydrate complexes

The finely divided wood meal (*Pinus densiflora* Sieb. et Zucc.), previously extracted with an ethyl alcohol-benzene mixture (1:2, v/v), was treated with 0.25 % aqueous potassium acetate to remove pectin. Lignin was extracted from the wood meal with 80% aqueous dioxane for 48 hr at room temperature. LCCs were extracted with cold water (20°C) for 12 hr and then with hot water (80°C) for 5 hr from the residual wood meal. Both extracts were combined and precipitated from five times its volume of ethyl alcohol to give a water-soluble LCC (LCC -WE). This LCC was separated into neutral (C-1-M), acidic (C-1-A), and lignin-rich (C-1-R) subfractions by anion-exchange chromatography on DEAE-Sephadex A-50 (CO_3^{2-} form).

Determination of reducing-end group of C-1-M

Reducing-end group of C-1-M was determined as follows: C-1-M (200 mg) was dissolved in 20 ml of water and reduced with $NaBH_4$ (400 mg) for 18 hr at 40 °C with continuous stirring. After the reaction, 3 ml of 10 M acetic acid was added, and the terminal reduced C-1-M was precipitated twice from 57 ml of ethyl alcohol. The polysaccharide was then hydrolyzed with 1 N H_2SO_4 at 100°C for 5 hr and neutralized with barium carbonate. The hydrolyzates were deionized with a Dowex 50×8 (H^+ form), concentrated, and analyzed by h.p.l.c. on a column of Aminex HPX-87 P.

Methylation analysis

Methylation was done by the method of Hakomori [Hakomori, 1964] followed by the method of Kuhn [Wallenfels, 1963]. The methylated sample was hydrolyzed with 90% aqueous formic acid followed by 0.5 N sulfuric acid. The partially methylated monosaccharides obtained were then converted to alditol acetates and analyzed by g.l.c. on a column (a), and by g.c.-m.s. on a column (b).

I-3 Results and Discession

In most studies on Björkman LCC, DMF or DMSO has been used as the extraction solvent of the LCC. However, extraction with the nonvolatile organic solvents was time-consuming because dialysis of the extracts is necessary to remove the solvent. This disadvantage was hindrance for a large-scale preparation of the LCCs. To solve this problem, direct extraction of LCCs with water from the wood meal previously extracted with 80% aqueous dioxane was examined for the first time, and then chemical properties of the resulting water-soluble LCC were characterized.

The water-soluble LCCs were isolated from the finely divided wood meal previously extracted with 80% aqueous dioxane, by sequential extraction with cold and hot water. Both extracts were combined and poured into ethyl alcohol to give LCC-WE (Fig. 1). The yield of LCC-WE was 9.3% of the wood meal previously extracted with 80% aqueous dioxane. The yield is approaximately twice as much of the original Björkman LCC (5.31%) [Koshijima, 1976]. LCC-WE was then fractionated into neutral (C-1-M), acidic (C-1-A) and lignin-rich (C-1-R)

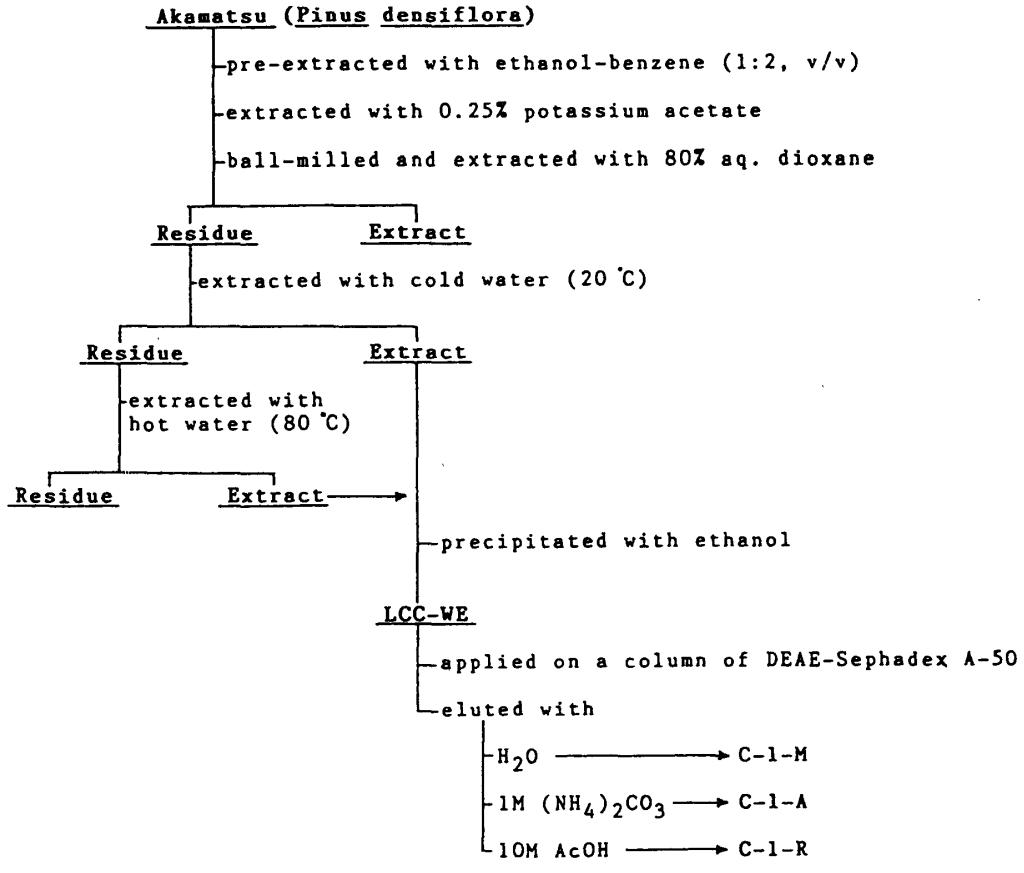


Fig. 1. Isolation of the water-soluble lignin-carbohidrate complex (LCC-WE) from *Pinus densiflora* wood.

subfractions by anion-exchange chromatography on DEAE-Sephadex A-50. The yields of these three subfractions were 43.3%, 48.7% and 2.1% of the original LCC-WE, respectively (Table 1). The yield of the C-1-A fraction was about twice that of Björkman LCC (24~26%) [Koshijima, 1976]. The chemical properties of the LCC-WE fractions are summarized in Table 1. The neutral sugar compositions are shown separately in Table 2. The neutral sugar composition of C-1-M indicates that the carbohydrate moieties of this fraction consists of glucomannan as in the case of Björkman LCC [Tanaka, 1972] and HWF [Yaku, 1981]. The other chemical properties also support this suggestion. The reducing terminal units of C-1-M consisted of 70.9% mannose and 29.1% glucose. No other reducing sugar residue could be detected (Table 3). In the methylation analysis, 2,3,6-tri-*O*-methyl mannose and 2,3,6-tri-*O*-methyl glucose were detected as the major compo-

Table 1. Chemical composition and properties of the LCC-WE fractions

Components	Lignin-carbohydrate complexes			
	LCC-WE	C-1-M	C-1-A	C-1-R
Recovery (%)	9.3[a]	43.3[b]	48.7[b]	2.1[b]
Carbohydrate content (%)				
Neutral sugar	80.0	95.5	76.0	41.5
Uronic acid	4.2	N.D.[c]	6.4	1.9
Lignin content (%)	17.9	3.7	26.6	43.6
Acetyl content (%)	3.3	7.6	N.D.[c]	N.D.[c]
$[\alpha]_D^{20}$	−15.5°	−28.2°	−11.4°	−8.0°
S(s)	N.D.[c]	0.9[d]	0.8[d]	N.D.[c]
$\overline{M}w$	1.2×10^4	1.2×10^4	1.1×10^4	N.D.[c]
$\overline{M}n$	7.6×10^3	7.5×10^3	6.7×10^3	N.D.[c]

a: Values are expressed as weight percentages of the wood meal extracted with 80% aqueous dioxane. b: Values are expressed as weight percentages of LCC-WE. c: Not determined. d: Sedimentation coefficient at infinite dilution.

Table 2. Neutral sugar composition of the LCC-WE fractions.[a]

Monosaccharides	Lignin-carbohydrate complexes			
	LCC-WE	C-1-M	C-1-A	C-1-R
L-Arabinose	3.8	0.0	6.7	5.2
D-Xylose	21.0	0.0	45.6	24.9
D-Mannose	52.3	74.0	29.8	33.0
D-Galactose	7.0	4.6	7.9	16.8
D-Glucose	15.9	21.5	10.0	20.0

a: Values are expressed as weight percentages of the total neutral sugar.

Table 3. Reducing-end group of C-1-M.[a]

Monosaccharides	Lignin-carbohydrate complex
	C-1-M
D-Mannose	70.9
D-Galactose	0.0
D-Glucose	29.1

a: Values are expressed as weight percentages of the total reducing-end group of C-1-M.

Table 4. Methyl ether from the hydrololyzates of the methylated LCC fractions.[a]

Methylaed sugars[b]	Lignin-carbohydrate complexes	
	C-1-M	C-1-A
2, 3, 4, 6-Glc or Man	5.9	1.4
2, 3, 4, 6-Gal	4.3	2.5
2, 3, 5-Ara	0.0	7.4
2, 3, 4-Xyl	0.0	1.9
2, 3, 6-Man	63.0	19.6
2, 3, 6-Glc	26.9	10.6
2, 3-Xyl	0.0	56.7

a: Values are expressed as relative molar percentages of the total partially methylated sugars identified. b: 2, 3, 4, 6-Glc=1-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and so forth.

nents of C-1-M. Galactose was recovered only as a single side chain (Table 4). Furthermore, C-1-M contained 7.6% of acetyl group, and optical rotation of this fraction showed a good accordance with that of Björkman LCC [Tanaka, 1972]. Carbon-13 n.m.r. spectrum of C-1-M verified that sugar moiety of this fraction consisted of glucomannan (Fig. 2). Galactose was assigned only as a nonreducing end group. Furthermore, existence of 3-*O*-acetylated mannopyranosyl residues was verified from a distinguished peak at 69.5 ppm [Tanaka, 1985]. The existence of acetyl group was also verified by a strong absorption at 1,735 cm^{-1} in its i.r. spectrum (Fig. 3). Because C-1-M contained 3.7% lignin, an interaction between lignin and acetyl glucomannan was analyzed by gel filtration on Sephadex G-100 (Fig. 4). The molecular weight distribution of C-1-M observed by the gel filtration, was similar to that of a previous report on the neutral fraction from HWF [Yaku, 1981].

The carbon-13 n.m.r. spectrum (Fig. 2), methylation analysis (Table 4), and neutral sugar analysis (Table 2) of C-1-A indicated that the carbohydrate moiety of the LCC consists of a mixture of coniferous hemicelluloses such as glucoman-

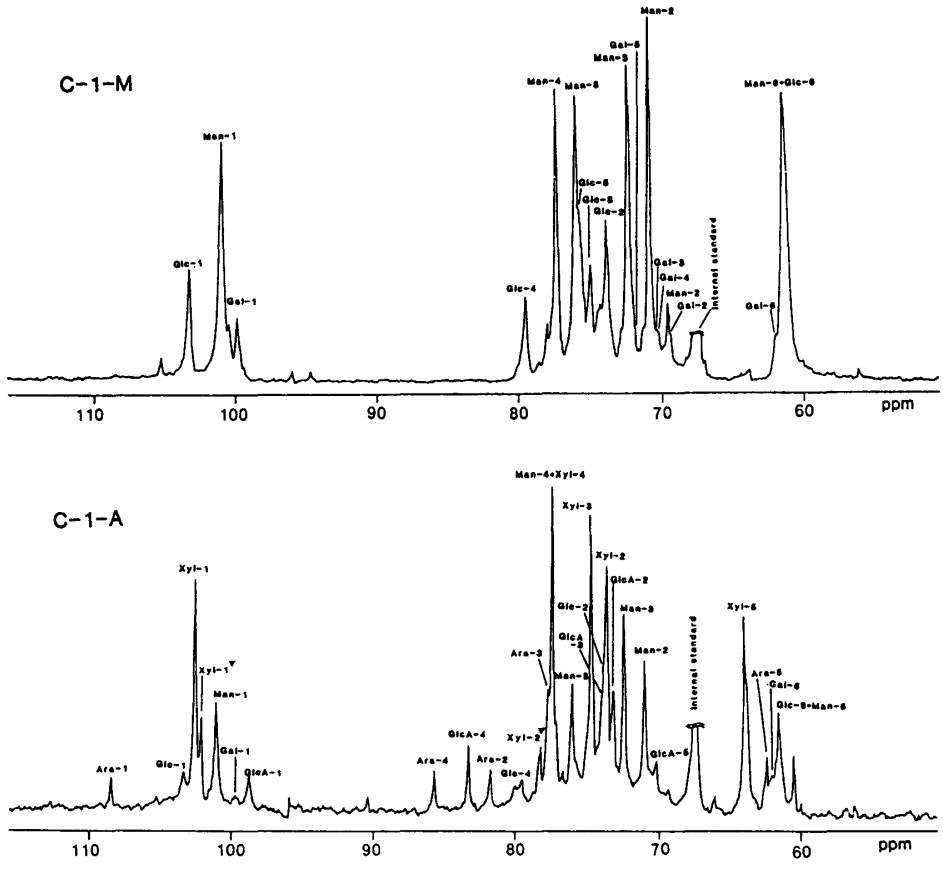


Fig. 2. Carbon-13 n.m.r. spectra of the LCC-WE fractions. Notes: Chemical shifts in ppm are expressed downfield from TMS with dioxane as an internal standard (67.4 ppm from TMS). ▲--- This signal was due to the C-2 substituted xylopyranosyl residues.

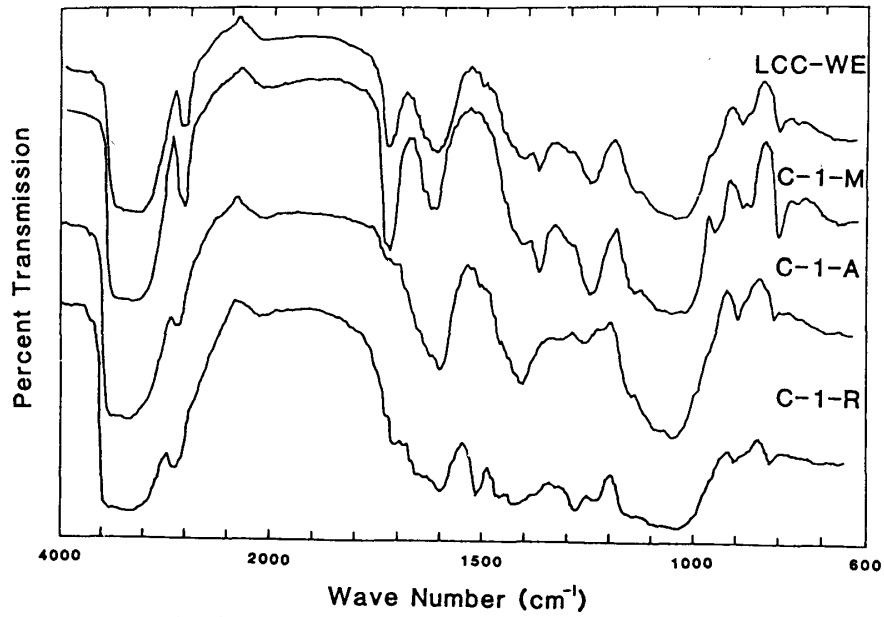


Fig. 3. I.r. spectra of the LCC-WE fractions.

nan and arabinoglucuronoxylan. Existence of glucomannan in this acidic fraction, suggested that the neutral polysaccharide, glucomannan formed single molecules with arabinoglucuronoxylan through covalent bonds with lignin moiety. The chemical properties of C-1-A were analogous to those from Björkman LCC [Koshijima, 1976] and from HWF [Yaku, 1981]. However, the lignin content was much higher in the fraction from LCC-WE (26.6%) compared with those from Björkman LCC (13.5%) [Koshijima, 1976] and from HWF (5.4%) [Yaku, 1976]. The apparent weight average molecular weight [Mw) of this fraction was estimated to be 1.1×10^4 on the basis of gel filtration on Sephadex G-100 (Fig. 4). In a sedimentation analysis, both C-1-M and C-1-A fractions showed individual single peak (Fig. 5), and extraporation of sedimentation coeficients of C-1-M and C-1-A at infinite dilution gave 0.9 s and 0.8 s, respectively. The large yield of the acidic fraction indicates that the water-extraction was the most effective in preparing the LCC containing uronic acid.

C-1-R was obtained in a yield of 2.1% based on the weight of LCC-WE. This value is considerably smaller than that of Björkman LCC (9.7%) [Koshijima, 1976]. However, this fraction was found to be rich in lignin (43.6%) as was Björkman LCC (50.7%) [Koshijima, 1976].

These results lead to the conclusion that a large amount of LCC (LCC-WE)

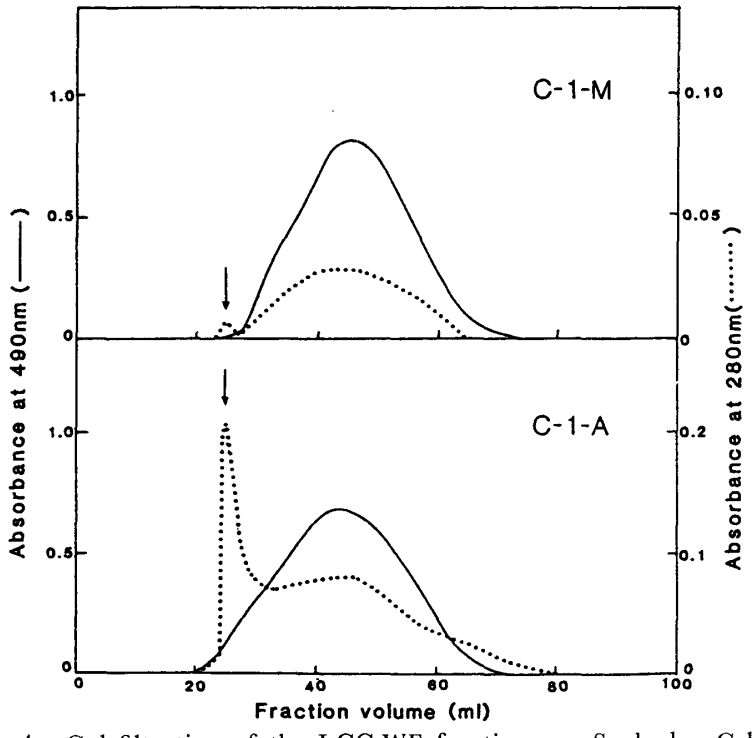


Fig. 4. Gel filtration of the LCC-WE fractions on Sephadex G-100. Notes: The arrows represent void volumes. Each fraction was analyzed for carbohydrate (—) and lignin (···).

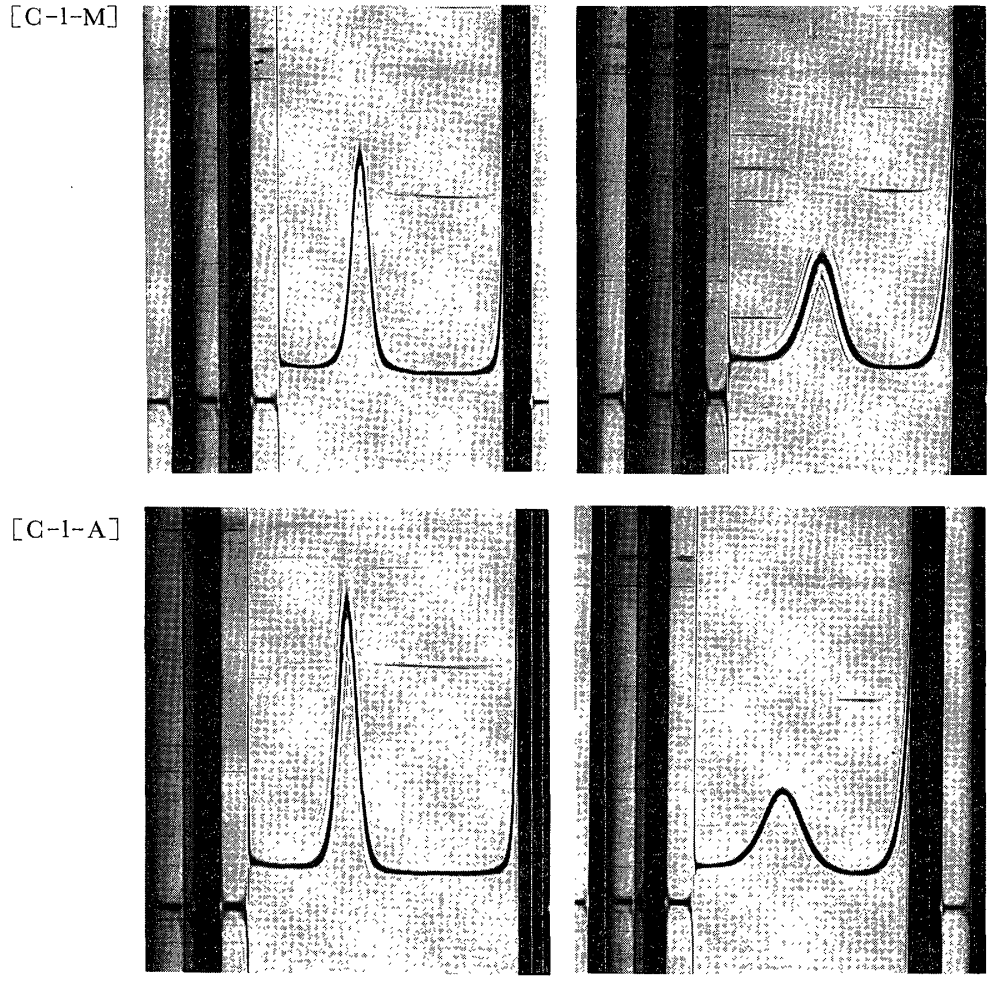


Fig. 5. Velocity sedimentation patterns of C-1-M and C-1-A.
Notes: The patterns were photographed at 10 min. and 84 min. in C-1-M, and 16 min. and 80 min. in C-1-A after reaching maximum speed. Concentration of C-1-M and C-1-A were 1.0 mg/ml.

can be extracted with water from the finely divided wood meal previously extracted with 80% aqueous dioxane, and that the chemical properties of the water-soluble LCC (LCC-WE) was similar to those of Björkman LCC and of HWF. This easy method of preparation is recomended to be used in place of the original Björkman method because of its convenience [Watanabe, 1987a]. Analyses of the ether and ester linkages between the lignin and carbohydrate existing in LCC-WE, are described in chapter II and III, respectively.

I-4 Summary

A new convenient method was developed to isolate LCCs from finely divided wood meal previously extracted with 80% aqueous dioxane. A large amount of water-soluble LCC was easily prepared from the wood meal by sequential extraction

with cold and hot water. The resulting water-soluble LCC (LCC-WE) was further fractionated into neutral, acidic and lignin-rich subfractions by anion-exchange chromatography. Because the chemical properties of the subfractions were similar to those of Björkman LCC, this convenient method for LCC preparation is recomended to be used in place of the original Björkman method.

Chapter II Binding-site analysis of ether linkages between lignin and carbohydrate

A number of attempts have been made to prove the existence of covalent bonds between lignin and carbohydrate. However, no one have succeeded in determining structural formula of depolymerized LCC molucules by instrumental analysis. The major reasons for the failure lie in the following two facts, that is, the lability and heterogeneity of LCC structures made their isolation considerably difficult and no one could exclude a possibility of recombination between both components during the depolymerization. Therefore, various indirect approaches, such as alkali degradation [Wang, 1967; Merewether, 1972; Kosíková, 1979; Smelstorius, 1974; Yaku, 1976; Eriksson, 1980; Obst, 1982; Lundquist, 1983; Takahashi, 1988], acid degradation [Eriksson, 1977 and 1980], sodium borohydride reduction [Košíková, 1979; Eriksson, 1980; Takahashi, 1988], Smith degradation [Eriksson, 1980; Yaku, 1981] and methylation analysis under strong alkaline media [Koshijima, 1976; Minor, 1982 and 1986; Iversen, 1986], have been made to prove the existence of lignin-carbohydrate linkages. However, it should be pointed out that the previous methods for determining their binding-sites could not directly specify what kind of substance was bound to the carbohydrate component, because the chemical reactions involved in the previously proposed methods were non-specific to the lignin skeleton. For example, a usual methylation analysis of LCCs gave only information on branch points of the sugar residues, however, this method, which is also known to be ineffective for the alkali labile linkages, has often been used for the determination of the binding-sites between lignin and carbohydrate [Minor, 1982, 1986].

In order to avoid this ambiguousness, the author has developed a new method giving direct evidence for the ether linkages between lignin and carbohydrate. The characteristic of this method lies in the fact that oxidation by hydride ion transfer mechanism was adopted for the linkage analysis of LCCs, and that all main reactions involved in the method were performed in a mild medium where acetyl group and glycosidic linkages were stable.

In the former part of this chapter (II-1), the author established a new method to raise the frequency of lignin-carbohydrate bonds so that their chemical structures can be correctly analyzed. Next, the new analytical method for the ether type of lignin-carbohydrate bonds has been developed. As shown in Fig. 6, the major parts of this method consist of the following three parts; (1) protection of hydroxyl groups in LCCs with acetyl groups under the mild condition which does not cleave the original lignin-carbohydrate linkages, (2) oxidative cleavage of lignin-carbohydrate bonds with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and (3) methylation of the newly formed hydroxyl groups by the method of Prehm

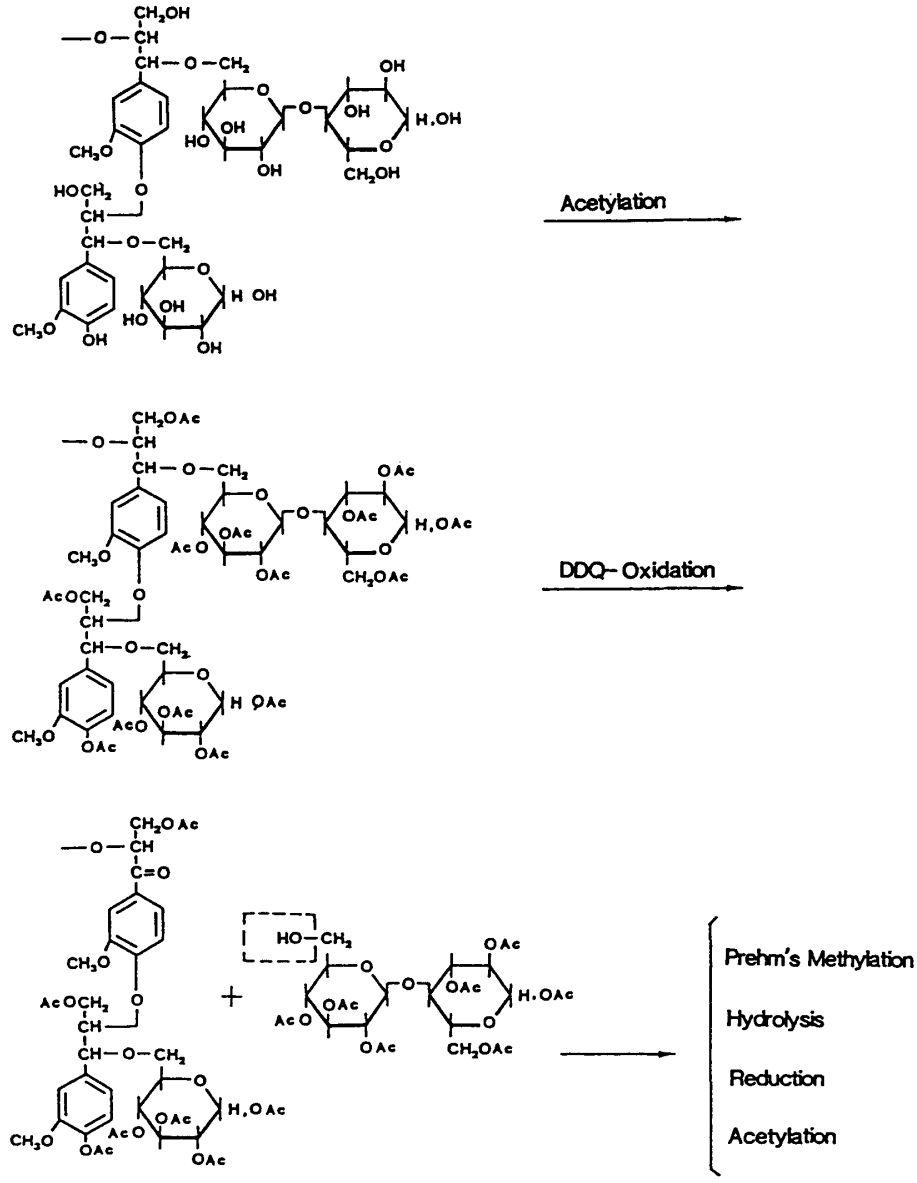


Fig. 6. New method for the binding-site analysis of ether linkages between lignin and carbohydrate.

[Prehm, 1980]. In order to establish this method, the author clarified the reactivities of DDQ with acetylated lignin (II-2), and then examined stabilities of the acetyl-protecting group during the DDQ-oxidation and Prehm methylation (II-3). Finally, the new method was applied to the cellulase-degraded LCC fragments isolated from *Pinus densiflora* wood (II-4) by the method described in section II-1.

II-1 Isolation of cellulase-degraded LCC fragments

II-1-1 Introduction

Cellulase-digestion of LCCs has often been done prior to the binding-site analysis of the LCCs, because frequency of lignin-carbohydrate bonds in intact LCCs is considerably lower than that of their glycosidic bonds between sugar residues. However, isolation of the cellulase-degraded LCC fragments from the enzymatic hydrolyzates of water-soluble LCCs was considerably difficult. As reported by Feckel, usual gel filtration method was useless to isolate LCCs [Feckl, 1982]. This has been one of the obstacles to clarify the binding-sites between lignin and carbohydrate. In this section, the author describes a new method for isolating cellulase-treated LCC fragments from the enzymatic digests of water-soluble LCCs. Application of this method to the analysis of LCCs from *Pinus densiflora* wood is mentioned [Watanabe, 1985, 1989b].

II-1-2 Experimental

General methods

p-Nitrophenyl glycosides, Avicel and sodium salt of CMC were used for activity assay of aryl glycosidases, Avicelase and CMCase, respectively. The incubation was done at 40°C in 0.1 M sodium acetate buffer (pH 4.8) with purified commercial cellulase preparations. The reducing sugars formed from the Avicel and sodium salt of CMC by the incubation, were determined by DNS method [Borel, 1953], and the *p*-nitrophenol from *p*-nitrophenyl glycosides were determined from the measurement of the absorbance at 405 nm. Activity for the formation of ether linkages between lignin and carbohydrate was examined by incubating veratryl alcohol and glucose with a mixture of the purified cellulase preparations at 40°C for 72 hr in a 0.1 M sodium acetate buffer (pH 4.8). Carbon-13 n.m.r. spectrum of the cellulase-treated LCC fragments was recorded in a CD_3CN/D_2O mixture (7:3, v/v) or in an acetone-d_6/D_2O mixture (3:1, v/v) at room temperature. T.l.c. was done on Merck Kieselgel 60 plates using ethyl ether/*n*-hexane (10:1, v/v) mixture as developing solvent. G.p.c. was done with a Waters 600 model on a TSK-GEL G 2000H+G 4000H at 50°C with dimethylformamide at a flow rate of 0.7 ml/min

using standard polystylene series (Pressure Chemical Co. Ltd.) of No. 493-82 ($\overline{M}w$: 929000, $\overline{M}n$: 892000), No. 493-81 ($\overline{M}w$: 591000, $\overline{M}n$: 573000), No. 487-01 ($\overline{M}w$: 169000, $\overline{M}n$: 166000), No. 493-78 ($\overline{M}w$: 99600, $\overline{M}n$: 86700), No. 493-77 ($\overline{M}w$: 47500, $\overline{M}n$: 45500), No. 493-75 ($\overline{M}w$: 9000, Mn: 9050) and No. 493-73 ($\overline{M}w$: 2500, $\overline{M}n$: 2300). Neutral sugar composition of LCC fraction was determined by hydrolyzing each fraction with 2M trifluoroacetic acid for 3 hr at 100°C, and then converting the hydrolyzates to the corresponding alditol acetates. Determination of the alditol acetates was done by g.l.c. on a Ulbon HR-SS-10 (25 m×0.25 mm) column at 210°C. Uronic acid content of an acidic LCC fraction was determined by carbazole sulfuric acid method [Bitter, 1962].

Purification of cellulase

Two types of commercial cellulase, Cellulosin AC (*Aspergillus niger*, Ueda Kagaku Kogyo Co. Ltd.) and Meicelase (*Trichoderma viride*, Meiji Seika Co. Ltd.) were dissolved in water, and then the water-insoluble materials were removed by centrifugation. The supernatant was then poured into 80% aqueous ammonium sulfate with stirring, and the precipitates formed were washed twice with the same solution. To remove stabilizers and lower molecular weight impurities, the precipitates were further purified by gel filtration on Bio-Gel P-2. The higher molecular weight fractions were collected and lyopilized.

Isolation of a LCC

An acidic LCC (com-C-1-A) was isolated from compression wood of *Pinus densiflora* according to the method described in chapter I. Carbon-13 n.m.r. spectrum of the acidic fraction was recorded in D_2O at 70°C. All chemical shifts were measured relative to internal 1,4-dioxane (67.4 ppm downfield from TMS).

Enzymatic degradation of LCCs

A neutral LCC from normal wood (nor-C-1-M) and an acidic LCC from compression wood (com-C-1-A) were hydrolyzed at 40°C for 72 hr in 0.1 M acetate buffer (pH 4.8) with the two cellulase preparations. The enzymatic hydrolyzates formed were heated for 5 min. in a boiling water bath to inactivate the enzymes and then centrifuged to remove water-insoluble materials.

Isolation of cellulase-degraded LCC fragments

The isolation of cellulase-degraded LCC fragments from the hydrolyzates of nor-C-1-M and com-C-1-A was carried out by a new method of adsorption chromatography on Toyopearl HW-40 S (4.0×45 cm) and HW-50 F (3.0×45 cm), respectively. Namely, the enzymatic hydrolyzates were applied to a Toyopearl HW-40 S or HW-50 F column, and then the water-soluble materials were thoroughly eluted with water from the column. Thereafter, the eluent was changed to 50% aqueous

dioxane to recover the adsorbed LCC fragments (M-ESD and A-ESD).

II-1-3 Results and Discussion

A number of investigators have paid attention to the sugar moiety of LCCs and degraded its glycosidic bonds with cellulase preparations in order to raise the frequency of lignin-sugar bonds [Yaku, 1976; Obst, 1982; Minor 1982 and 1986; Eriksson, 1977 and 1980]. This is because usual chemical degradation of lignin moiety is always accompanied by the possibilities of the cleavage of original lignin-sugar bonds or recombination of the lignin with carbohydrate *via* ion, radical or quinonemethide intermediates. In the case of cellulase-digestion of the sugar moiety, there is also one possibility that the sugars may be linked to the lignin glycosidically by transferase or reversed hydrolysis activities of the glycosidases. In fact, this type of recombination has often been reported [Fujimoto, 1988; Kondo, 1988a, 1988b]. However, on the analyses of ether and ester types of lignin-carbohydrate linkages, such condensation or transfer of sugars can be neglected because both activities are not involved in the formation of lignin-sugar bonds at non-anomeric positions. In this thesis, activity for the formation of ether linkages between lignin and carbohydrate was also examined.

Usually, the enzymatic degradation of LCCs is easily carried out by incubation of the LCCs with cellulases in a buffer solution. However, isolation of the cellulase -degraded LCC fragments from the hydrolyzates was considerably difficult because of amphipathic properties of the molecules. Usual chromatographic techniques were useless to solve this problem. In this section, the author described a new systematic procedure for the isolation of cellulase-degraded LCC fragments from the enzymatic hydrolyzates of water-soluble LCCs.

Neutral (nor-C-1-M) and acidic (com-C-1-A) LCC fractions were isolated from normal and compression woods of *Pinus densiflora*, respectively, according to the method described in chapter I. Carbon-13 n.m.r. spectrum of the acidic LCC fraction (Fig. 7) revealed that the sugar moiety of the LCC consisted of arabinoglucuronoxylan, glucomannan and β-1,4-galactan, as previously reported by Mukoyoshi [Mukoyoshi, 1981]. Neutral sugar and uronic acid content of com-C-1-A was 64.1% and 10.1%, respectively. Chemical properties of the neutral fraction (nor-C-1-M) were described in detail in chapter I.

In order to raise the frequency of lignin-sugar bonds, both LCC fractions were first hydrolyzed with two different types of purified cellulase preparations. Namely, the LCC fractions were hydrolyzed with a mixture of Cellulosin AC (*Aspergillus niger*) and Meicelase (*Trichoderma viride*) preparations which had been purified by means of salting-out from aqueous ammonium sulfate and gel filtration

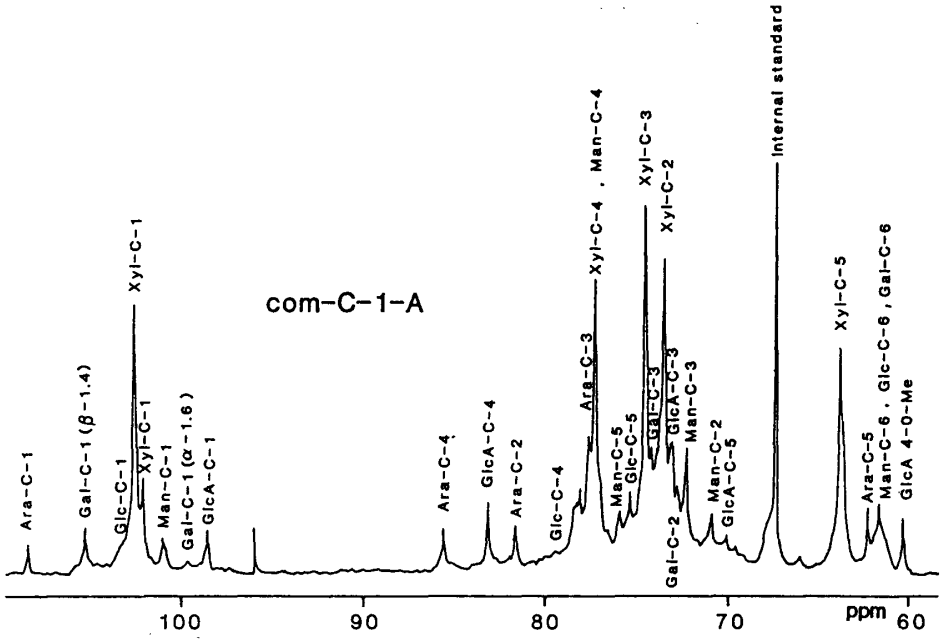


Fig. 7. Carbon-13 n.m.r. spectrum of the acidic fraction isolated from compression wood of *Pinus densiflora*.

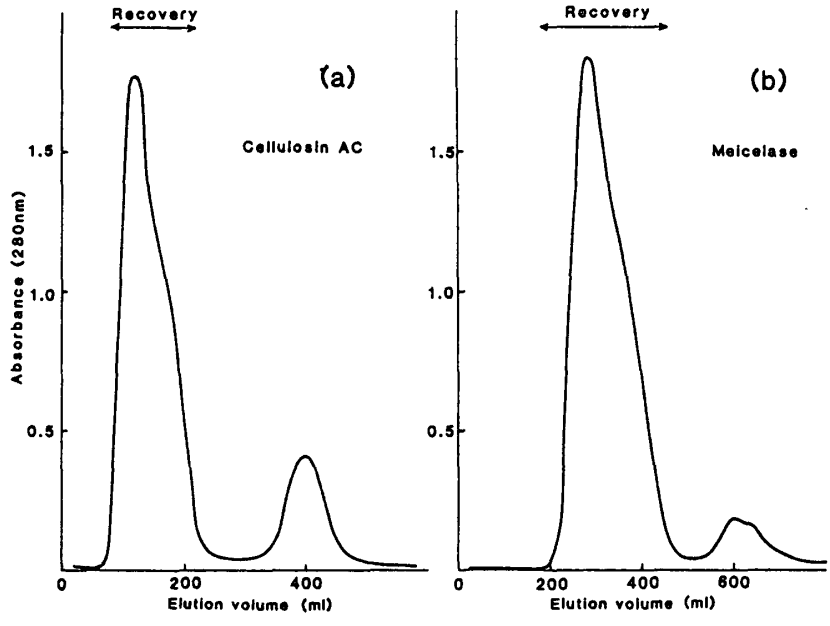


Fig. 8. Gel filtration profiles of the salting-out products from cellulases on Bio-Gel P-2.
Notes: The eluate indicated by the arrows were recovered, lyophilized, and used for the enzymatic hydrolysis of the LCC fractions.

on Bio-Gel P-2 to remove stabilizer and lower molecular weight impurities (Fig. 8). Activities of both cellulase preparations are tabulated in Table 5 and Table 6. Activity for the formation of ether linkages between lignin and carbohydrate

Table 5. Activities of Avicelase and CMCase in the cellulase praparations from Aspergillus niger and Trichoderma viride.[c]

Enzymes[b]	Avicicelase	CMCase
Aspergillus niger	N.D.[c]	3.86
Trichoderma viride	0.24	5.88

a: Values are expressed as units per mg of protein. b: Values were determined using the cellulase preparations purified by the salting-out and gel filtration on Bio-Gel P-2. c: Not determined.

Table 6. Aryl glycoside-hydrolyzing activities of the cellulase preparations.

Enzymes	Activities of aryl glycosidases[a]								
	α-Glc	β-Glc	α-Gal	β-Gal	α-Man	β-Man	α-Xyl	β-Xyl	α-Ara
Aspergillus niger	0.02	1.09	3.19	0.13	0.01	0.12	0.01	0.15	0.59
Trichoderma viride	0.00	1.22	0.02	0.00	0.01	0.00	0.01	0.16	0.48

a: Values are expressed as units per mg of protein.; p-Nitrophenyl glycosides were used as substrate.

was examined by incubating veratryl alcohol and glucose with the cellulases under the same condition as in the enzymatic degradation of the LCCs. By t.l.c with the developing solvent system of ethyl ether/ *n*-hexane mixture (10:1, v/v), none of addition compounds from both components could be detected, because no u.v. absorption was observed in their Rf region between 0.0 and 0.4 (Fig. 10).

The enzymatic hydrolysis of the LCCs was done by using these cellulase preparations at 40°C in a sodium acetate buffer (pH 4.8) for 72 hr. After inactivation of the enzymes in a boiling water bath, the enzymatic digests obtained were centrifuged to remove water-insoluble materials. Isolation of the cellulase-treated LCC fragments from the hydrolyzates was carried out by a new method using affinity of lignin to a polyvinyl gel. Namely, the author developed adsorption chromatography using affinity of lignin component on Toyopearl HW-40 S or HW-50 F gel which has been used for gel filtration of polysaccharides or proteins.

The enzymatic hydrolyzates from nor-C-1-M and com-C-1-A were first applied to the Toyopearl column and the water-soluble materials were washed out from the column with distilled water. After absence of carbohydrate component in the eluate was comfirmed, the eluent was changed from water to 50% aqueous dioxane to recover the adsorbed LCC fragments. The elution profiles of the enzymatic digests from nor-C-1-M and com-C-1-A on the column are shown in Fig. 11 and 12, respectively. As can be seen from both figures, the water-soluble

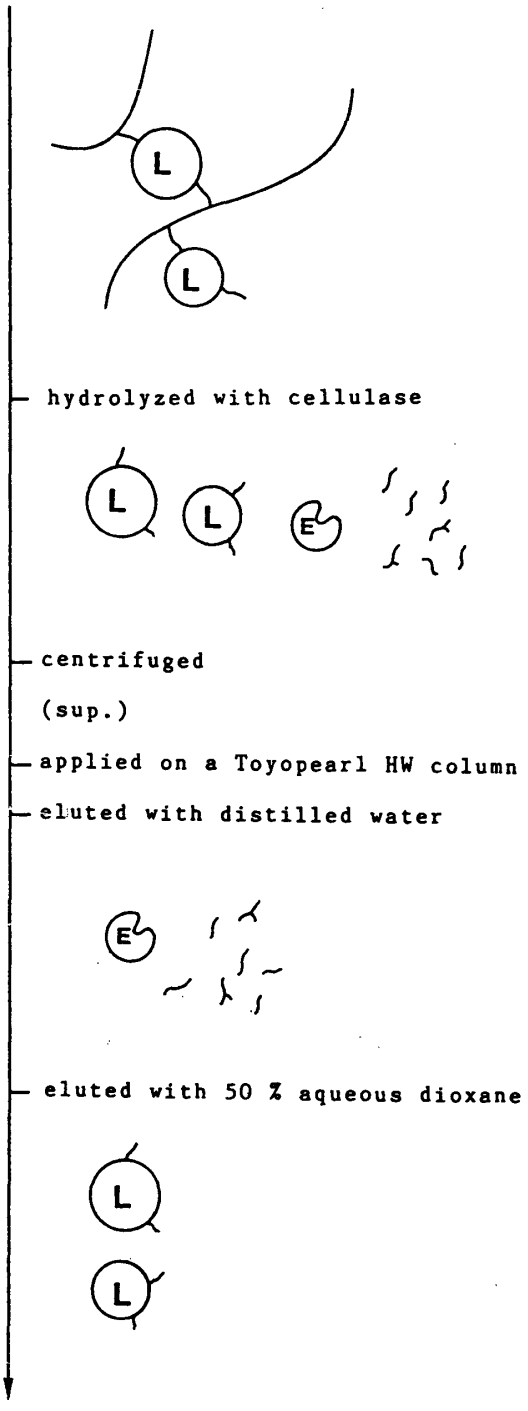


Fig. 9. Schematic representation of the new method for the isolation of cellulase-degraded LCC fragments from the enzymatic digests of water-soluble LCCs.

materials including oligosaccharides and the enzyme preparations used were first eluted from the column, and then the adsorbed LCC fragments were recovered

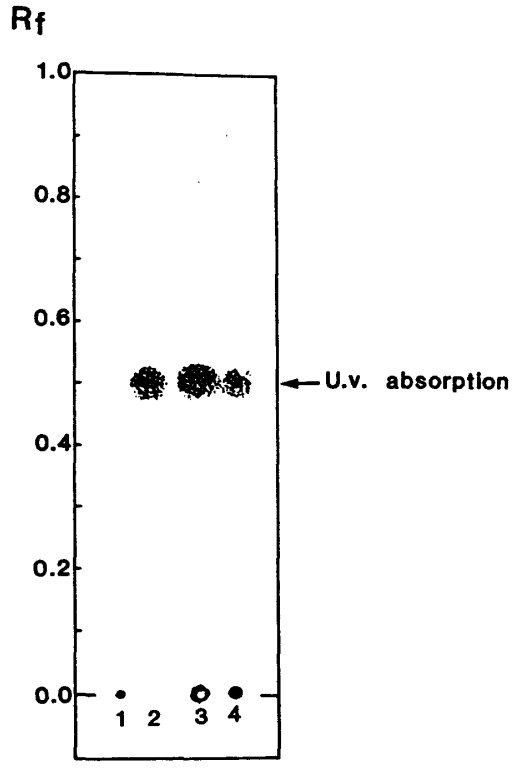


Fig. 10. T.l.c. of the aqueous solution containing the cellulase preparations, veratryl alcohol and D-glucose after the incubation at 40°C for 72 hr in a 0.1 M sodium acetate buffer (pH 4.8).
Notes: The t.l.c. was developed with ethyl ether-*n*-hexane mixture (10:1, v/v). The spots were visualized by spraying 10% sulfuric acid and heating to 110°C. Aromatic substances were detected under u.v. light.; Addition compounds from D-glucose and veratryl alcohol, which had u.v. absorption in the Rf region between 0.0 and 0.4, could not be detected after the incubation.; Line 1: D-glucose; Line 2: Veratryl alcohol; Line 3: Reaction mixture+ Veratryl alcohol; Line 4: Reaction mixture.

successfully with 50% aqueous dioxane (M-ESD and A-ESD). Absence of protein component in the adsorbed fraction was confirmed by coomassie blue reagent. Weight average molecular weight of the main fractions involved in M-ESD and A-ESD were 13,000 and 9,000, respectively. Neutral sugar analysis of the adsorbed LCC fraction, A-ESD, showed that the enzymatic treatments increased the relative composition of mannose and galactose, whereas those of xylose decreased (Table 7), suggesting that chemical linkages between lignin and the hexoses are favored. Furthermore, carbon-13 n.m.r. spectra of M-ESD and A-ESD showed that the main component of the LCC fragments consisted of guaiacyl lignin (Figs. 13, 14). In the spectrum of A-ESD, several distinguished peaks could be detected in the anomeric region of reducing-end xylose (α: 92.8 ppm; β: 97.5 ppm), mannose (α: 94.8 ppm; β: 94.6 ppm) and galactose (α: 93.2 ppm; β: 97.5 ppm) residues, together

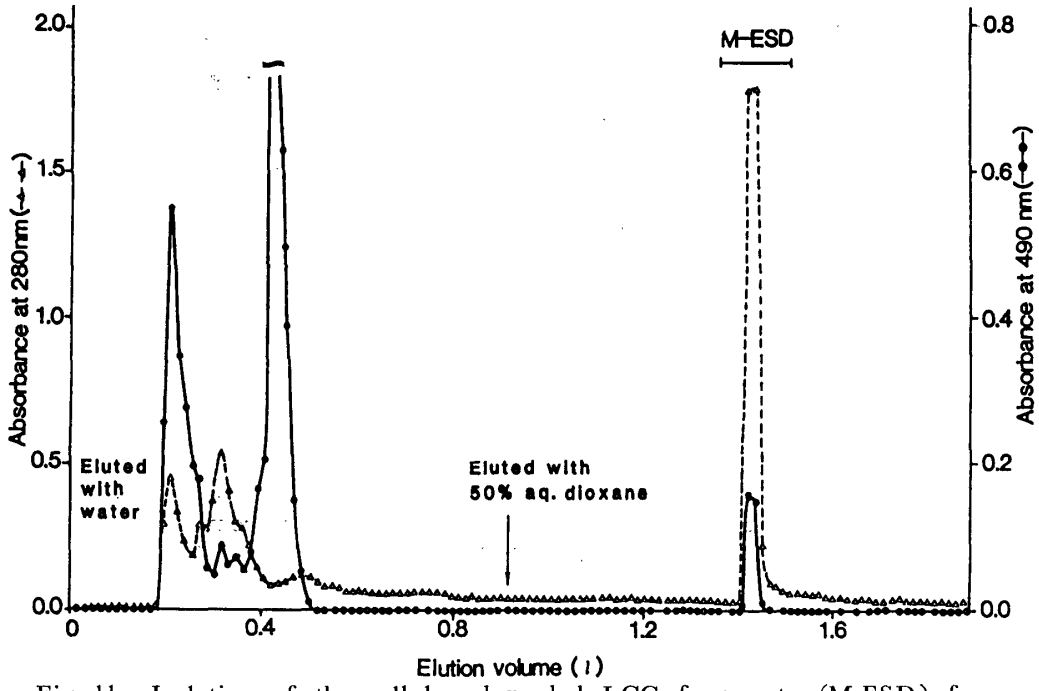


Fig. 11. Isolation of the cellulase-degraded LCC fragments (M-ESD) from the enzymatic hydrolyzates of the lignin-acetylglucomannan complex (nor-C-1-M) by adsorption chromatography on Toyopearl HW-40S.
Notes: Each fraction was analyzed for carbohydrate (—) and lignin (-△-△-)

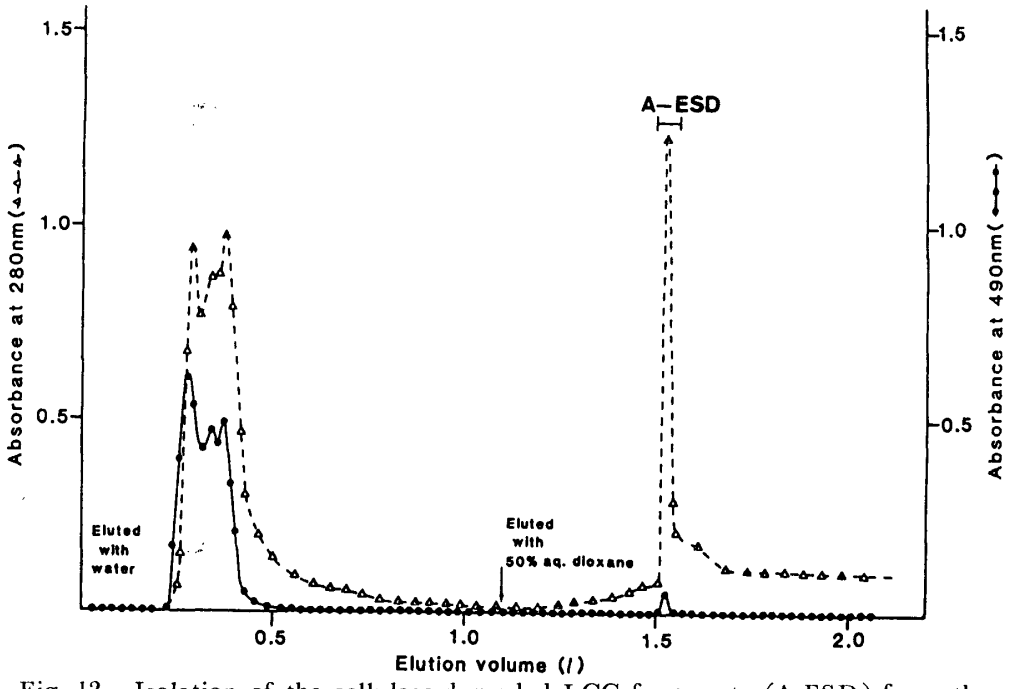


Fig. 12. Isolation of the cellulase-degraded LCC fragments (A-ESD) from the enzymatic hydrolyzates of the acidic LCC (com-C-1-A) by adsorption chromatography on Toyopearl HW-50F.
Notes: Each fraction was analyzed for carbohydrate (—) and lignin (-△-△-).

Table 7. Neutral sugar composition of the LCC fractions before and after cellulase-digestion.[a]

Components	Lignin-carbohydrate complexes			
	nor-C-1-M[b]	M-ESD[c]	com-C-1-A[d]	A-ESD[e]
Rhamnose	0.0	0.0	trace	trace
Arabinose	0.0	0.0	6.6	9.1
Xylose	0.0	0.0	47.5	27.4
Mannose	74.0	75.0	18.1	31.5
Galactose	4.6	4.9	13.5	24.5
Glucose	21.5	20.1	11.4	7.5

a: Values are expressed as relative weight percenages. b: Lignin-acetylglucomannan complex isolated from normal wood. c: Cellulase-treated LCC fragments originating from nor-C-1-M. d: Acidic LCC isolated from compression wood. e: Cellulase-treated LCC fragments originating from com-C-1-A.

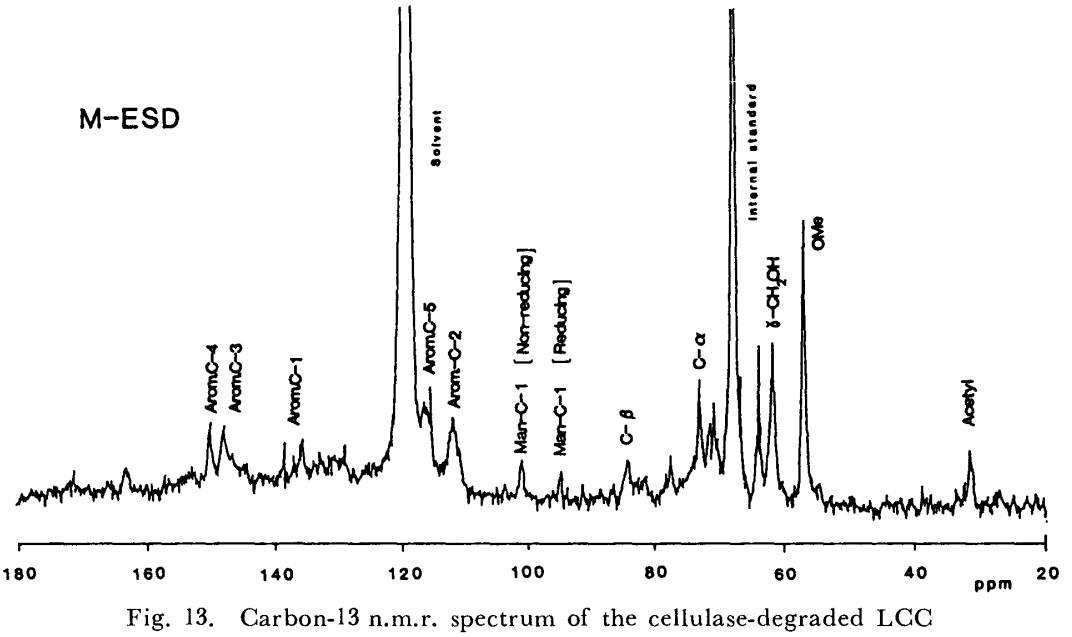


Fig. 13. Carbon-13 n.m.r. spectrum of the cellulase-degraded LCC fragments (M-ESD) isolated from the enzymatic digests of the lignin-acetylglucomannan complex (nor-C-1-M) by adsorption chromatography on Toyopearl HW-40S.

with those of non-reducing arabinose (180.5 ppm), xylose (102.8 ppm), mannose (101.0 ppm), β-1,4-galactose (105.7 ppm), α-1,6-galactose (99.5 ppm) and 4-*O*-methyl glucuronic acid (98.4 ppm) residues, in good accordance with their relative composition (Table 7) and authentic chemical shifts [Pfeffer, 1979; Hirsch, 1982; McCleary, 1982; Azuma, 1983b, 1988; Utille, 1986; Excoffier, 1986]. In the spectrum of M-ESD, signals were also detected in the anomeric region of mannose residues. The signals appearing in the reducing-end region suggest the existence

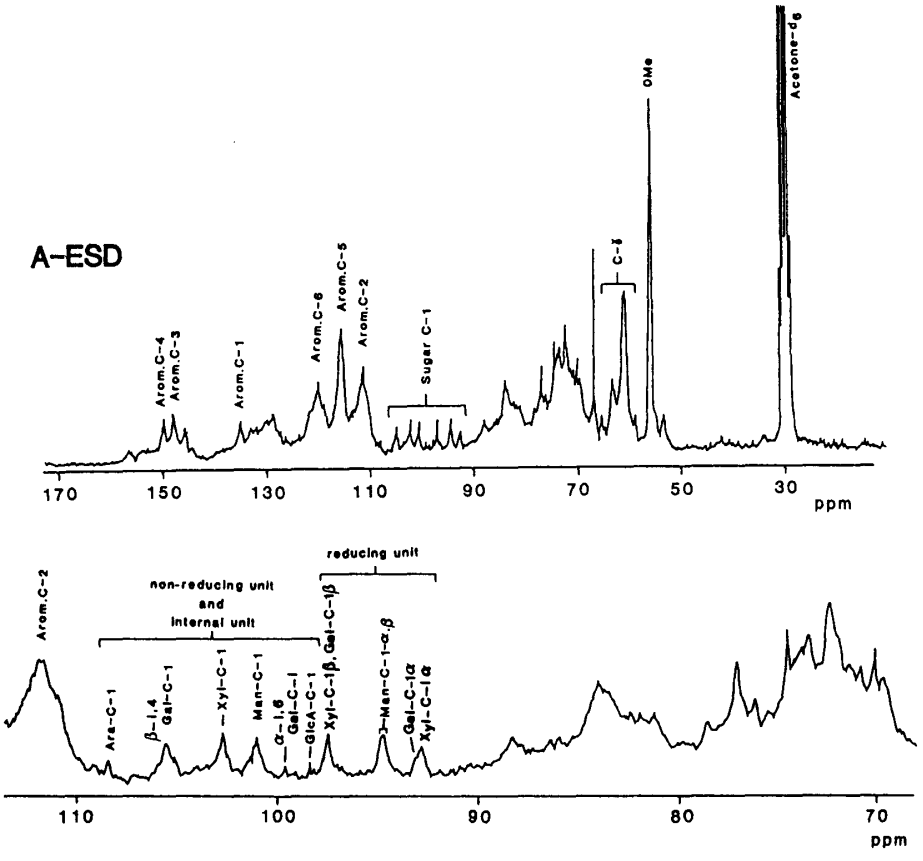


Fig. 14. Carbon-13 n.m.r. spectra of the cellulase-degraded LCC fragments (A-ESD) isolated from the enzymatic digests of the acidic LCC (com-C-1-A) by adsorption chromatography on Toyopearl HW-50F.

of non-glycosidic type of lignin-sugar bonds. If all of the lignin-sugar bonds in the adsorbed LCC fragments were glycosidic type, none of reducing-end sugar residues could be detected.

Because Nakakuki and Kainuma reported that gel filtration of oligosaccharides on the polyvinyl gel could be accomplished in a 100% total yield [Nakakuki, 1982], the co-existence of lignin and carbohydrate components in the adsorbed LCC fraction strongly suggests that lignin is linked covalently to the carbohydrate component in the LCCs. Linkage analysis of these LCC fragments is described in the section II-4 [Watanabe, 1985, 1989b].

II-1-4 Summary

A new method of adsorption chromatography has been developed for the isolation of cellulase-degraded LCC fragments from the enzymatic hydrolyzates of water-soluble LCCs. After water-soluble materials were eluted with water from Toyopearl HW column, the LCC fragments adsorbed on the gel were successfully

recovered with 50% aqueous dioxane. Chemical and spectroscopic analyses of the adsorbed LCC fractions showed a co-existence of both lignin and carbohydrate components in the molecules, suggesting the existence of chemical bonds between these two components.

II-2 Reaction of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone with wood components

II-2-1 Introduction

In this section, the author describes reactivities of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) toward wood components in order to develop the new method for the bindeng-site analysis of LCCs.

DDQ was first synthesized by Thiele and Günther in 1906 [Thiele, 1906]. Thereafter, numerous papers have been published on the side chain dehydrogenation or oxidation of alkylaromatic compounds by the oxidant [Becker, 1961, 1965, 1969 and 1980a, 1980b; Findlay, 1971; Sadler, 1969; Lutz, 1970a; Kiefer, 1972; Nakamura, 1976; Iwamura 1978; Ohki, 1979; Brown, 1975; Oikawa, 1976, 1979, 1982a, 1982b, 1983a, 1983b, 1984a, 1984b, 1985a, 1985b, 1985c; Eberson, 1979; Lee, 1983; Kim, 1985]. Typical reactions of DDQ observed both in anhydrous and hydrous media are oxidation of benzyl alcohols [Becker, 1961, 1965 and 1980; Brown, 1975], whereas some other reactions take place characteristically for the solvent system used. For example, oxidative cleavage of benzyl ethers [Becker, 1965; Oikawa, 1982a, 1984a, 1984b, 1985a, 1985b, 1985c] and esters [Kim, 1985] have been observed mainly in hydrous media because this reaction needs nucleophiles to attack the carbocation intermediate formed by hydride ion transfer from the benzyl position to DDQ. Similarly, arylalkanes were easily oxidized into aryl ketones and aldehydes in hydrous media by addition of water to the carbonium ion intermediates and the subsequent oxidation of the newly formed benzyl alcohols with excess amount of DDQ [Lee, 1983]. On the other hand, under anhydrous conditions, dehydrogenation of saturated compounds such as formation of α,β-unsaturated compounds from ketones [Murata, 1972], and aromatization of cyclic alkanes [Braude, 1954; Muller, 1971] have been observed. Thus reaction of DDQ should be understood dependent on the solvent system used. In particular, existence of water and alcohol in the reaction media must be taken into account. In this chapter, the author discusses the DDQ-reaction in hydrous media of dichloromethane-water system.

The reactivities of DDQ in the hydrous solvent system have been studied in detail by Oikawa *et al.* [Oikawa, 1982a, 1982b, 1984a, 1984b, 1985a, 1985b, 1985c].

They established that DDQ oxidatively degraded 4-methoxybenzyl and 3,4-dimethoxybenzyl ethers in the dichloromethane-water mixture, while other protecting groups, including isopropylidene, methoxymethyl, benzyloxymethyl, tetrahydropyranyl, acetyl, benzyl, tosyl, epoxide, double bond and ketone, remained unchanged [Oikawa, 1982a, 1982b, 1984a, 1984b, 1985a, 1985b, 1985c]. In this case, a low solubility (0.4 g/1,000 ml) of 2,3-dichloro-5,6-dicyanohydroquinone (DDHQ) in dichloromethane contributes to maintaining the reaction media almost in neutral pH region, which results in the above high selectivity. Even acid-sensitive acetal compounds were stable to the DDQ-exidation [Oikawa, 1984a]. Thus, selective reactivities of the DDQ-reaction in the hydrous solvent system is already established. In this media, it is known that the reaction of DDQ toward benzyl ethers proceeds by formation of a charge transfer (CT) complex, hydride ion transfer from the benzyl ether to DDQ and subsequent attack by water to the cation intermediate [Oikawa, 1984a]. As expected from the properties as hydride anion donors, β and γ positions in arylalkanes have not been oxidized by DDQ in the solvent system. However, in the case of conjugated arylpropenes, DDQ oxidizes the γ position both under anhydrous and hydrous conditions. This is because the carbocation intermediate from the arylpropenes can be stabilized by conjugation [Lutz, 1970b; Carpenter, 1956; Kiefer, 1972; Nakamura, 1976]. In the reaction under hydrous condition, the hydride anion transfer to DDQ takes place directly at the conjugated γ position, and the resulting γ-carbonium ion intermediate reacts rapidly with water as in the same manner of the DDQ-oxidation of the benzyl ethers. Thus, methoxymethyl isoeugenol was oxidized into methoxymethyl coniferyl aldehyde with DDQ in a water-saturated benzene [Nakamura, 1976]. In this case, the oxygen atom introduced into the γ position of the methoxymethyl isoeugenol is undoubtedly derived from the water in the solvent system used.

In any event, the DDQ-oxidation of alkylaromatic compounds proceeds *via* formation of initial CT complexes. Therefore, the solvents to stabilize the CT complexes accelerate the oxidation [Ohki, 1979], and electron-donative substituents in arylalkanes facilitate the oxidation, whereas electron-withdrawing substituents render the reaction difficult [Becker, 1980a]. This substituent effect is mainly due to the delocalization of cation charge in the carbonium intermediate. As expected from the resonance substituent constant, the effect of the electron donative substituents on the DDQ-oxidation is greater at *o* and *p* positions than at *m* position, which is important to understand the difference of reaction rates between various types of substituted arylalkanes. The benzylic oxidation by DDQ is reviewed by Becker and Turner [Becker, 1980b], and the mechanisms for the hydride ion transfer involved in the DDQ-oxidation are summarized by Ohki *et al.* [Ohki,

1979]. Concerning the solubility of DDHQ in organic solvents, a low solubility of DDHQ in benzene (0.6 g/1,000 ml) is also reported by Kiefer [Kiefer, 1972]. In general, heterogeneous sovent system, such as the dichloromethane-water or benzene-water mixture, are advantageous to proceed the DDQ-reaction selectively.

With respect to application of the DDQ-oxidation to wood chemistry, Becker first demonstrated that DDQ oxidizes benzyl alcohols in a guaiacylglycerol-β-guaiacyl ether to give corresponding α-carbonyl compounds. In the study, he confirmed that β and γ positions of the compound remained unchanged during the oxidation as expected from the reaction mechanism for DDQ [Becker, 1961]. However, application of this useful reaction to wood chemistry is, so far, limited to several investigations, such as determination of benzyl alcohol groups [Adler, 1966; Košíková, 1979], synthesis of lignin model compounds [Nakamura, 1976; Nakatsubo, 1981; Umezawa, 1982] and studies on cocking meshanism during a kraft process [Gierer, 1982] and an oxygen-alkali pulping [Aoyagi, 1980]. In particular, it should be noted that the oxidative cleavage of benzyl ethers with DDQ in hydrous media has never been utilized in this field.

In this chapter, the author has proposed a new method for analyzing the binding-site between lignin and carbohydrate by using the oxidative cleavage of benzyl ethers with DDQ in hydrous media (Fig. 6). To establish this method, reactivities of coniferous milled wood lignin (MWL) acetate and model compounds with DDQ in the dichloromethane-water mixture were investigated and the results obtained were discussed based on the theoretical background established by the above organic chemists.

II-2-2 Experimental

Preparation of acetylated milled wood lignin (MWLa)

Milled wood lignin from akamatsu (*Pinus densiflora* Sieb. et Zucc.) was prepared according to the method of Björkman [Björkman, 1957a]. Acetylation of the MWL was carried out with pyridine and acetic anhydride (1:1, v/v) at 40°C. After 18 hr, the acetylated sample was recovered by precipitation from diethyl ether, washed successively with the same solvent and dried *in vacuo*.

DDQ-oxidation of acetylated MWL

The fully acetylated MWL (MWLa) was reacted with an equal weight of DDQ in a refluxing dichloromethane-water mixture (18:1, v/v) for 2 hr. After the DDQ-oxidation, the DDQ-treated MWLa was partitioned between chloroform and water. After vigorous shaking, the chloroform solution was separated, washed five times with distilled water and then concentrated to dryness under reduced pressure.

NMR spectra

Proton decoupled carbon-13 n.m.r. spectra were obtained from 15 to 20% solutions in acetone-d_6 on a Varian XL-200 Spectrometer operating at 50.3 MHz (10 mm tubes; TMS as an internal standard) using four conventional techniques, complete decoupling (MLEV16 pulse sequence: COM), Attached Proton Test (APT) [Patt, 1982], Intensive Nuclei Enhancement by Polarization Transfer (INEPT) [Padwa, 1980] and Weak Noise Decoupling (WND) techniques. Recording conditions of each spectrum mode are as follows [Abbreviations: Flip angle (PWh, degree), Pulse delay time (PD, sec.), τ Delay time (TD, m sec.), Aquisition time (AT, sec.), Coupling constant (J, Hz), Decoupler offset (DO, Hz)]; COM: PW1 =40, PD=1.0; AT=0.666, APT: PW1=75, PW2=180, PD=0.0, DT=6.0, AT= 0.666, INEPT: PW1=90, PW2=180, PD=2.0, DT=1.6, J=155, AT=0.666, WND: PW1=40, PD=1.0, DO=−4000, AT=0.666.

Model compounds

Methyl 6-*O*-[1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-3-propanol-1-yl] α-*D*-glucopyranoside (I) was synthesized according to the method described in literatures [Ralph, 1983; Taneda, 1987]. Methyl 6-*O*-[1-(4-acetoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-3-acetoxypropane-1-yl] 2,3,4-tri-*O*-acetyl-α-D-glucoside (II) was prepared by acetylating the compound I with acetic anhydride and pyridine at room temperature for 12 hr. Methyl 6-*O*-[1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-3-acetoxypropane-1-yl] 2,3,4-tri-*O*-acetyl-α-D-glucopyranoside (III) was prepared from the compound I by diazomethane-methylation and subsequent acetylation with acetic anhydride and pyridine. Methyl 6-*O*-[1-(4-benzyloxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-3-acetoxypropane-1-yl] 2,3,4-tri-*O*-acetyl-α-D-glucopyranoside (IV) was prepared from the compound I by benzylation with benzyl chloride in the presence of pottasium carbonate and subsequent acetylation with acetic anhydride and pyridine at room temperature. Carbon-13 n.m.r. spectra and assignments of the model compound I and its *p*-methylated (III') and *p*-benzylated (IV') compounds were shown in Fig. 15. Acetates of lactose (V), maltose (VI), gentiobiose (VII) and raffinose (VIII) were prepared from the corresponding oligosacchrides by acetylation with acetic anhydride and pyridine. Cellobiose octaacetate (IX) was purchased from Nakalai Tesque Co. Ltd. and used without further purification.

Reaction of model compounds with DDQ

Each model compound (30 mg) was reacted with DDQ (50 mg) in a refluxing dichloromethane-water mixture for 2 hr. The reflux was done in a oil bath below 55°C. After the oxidation, the reaction mixture was fractionated by t.l.c. and

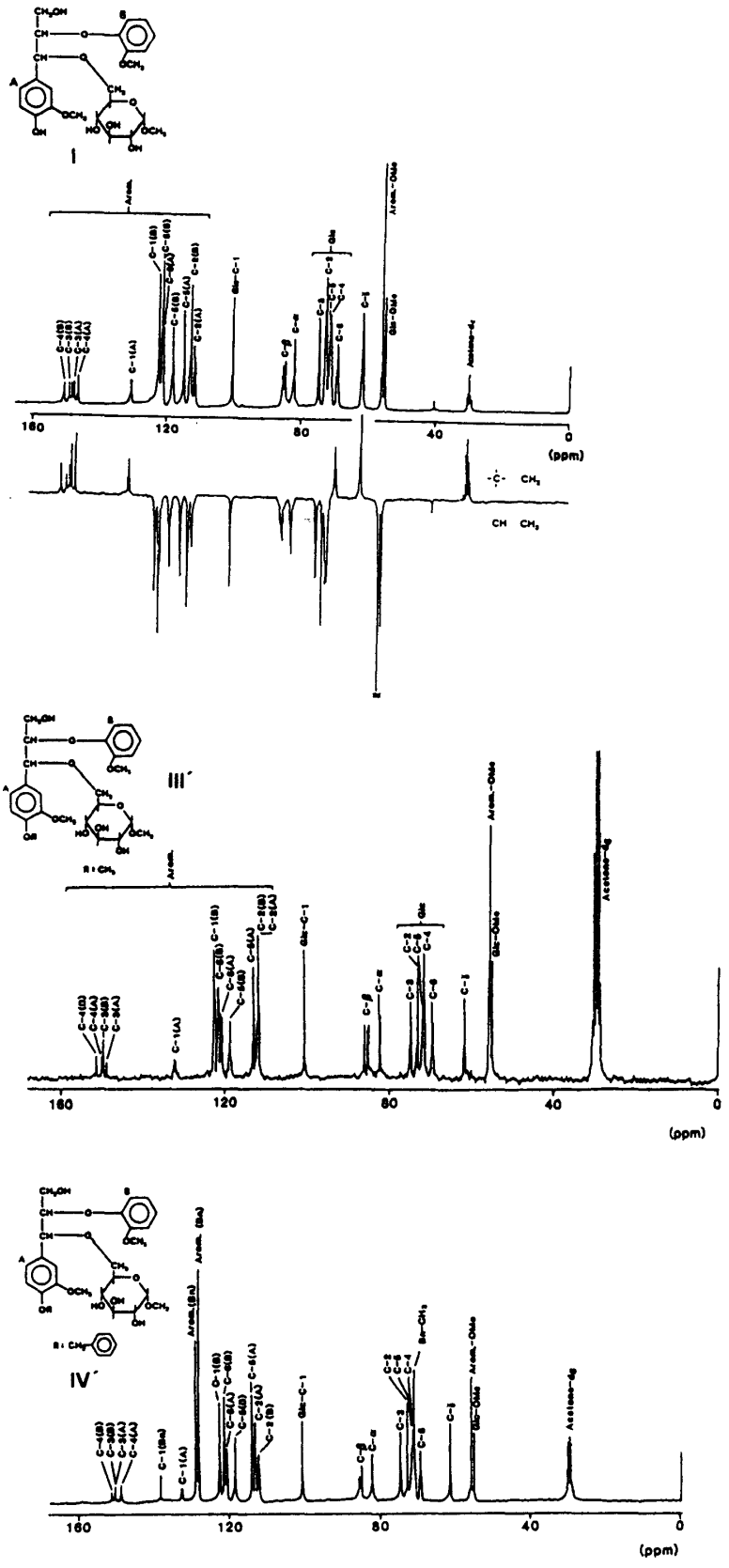
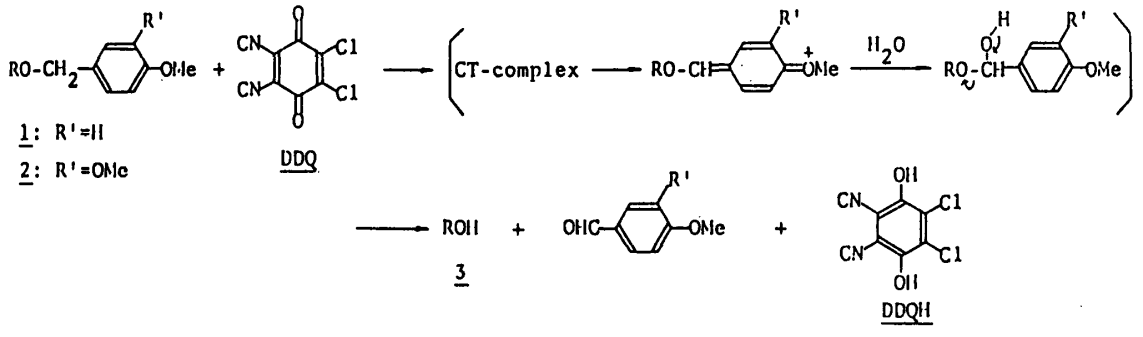


Fig. 15. Carbon-13 n.m.r. spectra of the LCC model compounds.

analyzed by proton-n.m.r. spectroscopy.

II-2-3 Results and Discussion

It has already been confirmed by u.v. spectroscopy that DDQ reacted with lignin in dry dioxane to produce α-carbonyl groups [Gierer, 1982; Aoyagi, 1980]. In this process, it is evident that the carbonyl group formed was derived from oxidation of phenylpropane subunits at benzyl position (Scheme 1), because methoxybenzyl alcohols [Becker, 1961] were shown to be oxidized by DDQ to yield α-cabonyl compounds under anhydrous condition. Recently, it was demonstrated that dimethoxybenzyl ethers [Oikawa, 1982a, 1982b, 1984a, 1984b, 1985a, 1985b, 1985c] and esters [Kim, 1985] were oxidatively cleaved by DDQ in the presence of water to produce α-carbonyl compounds. The latter type of reaction is known to proceed by the consecutive reaction steps including formation of a charge transfer (CT) complex, hydride ion transfer from the benzyl ether to DDQ and addition of water to the resulting benzyl cation intermediates [Oikawa, 1984a] (Fig. 16).

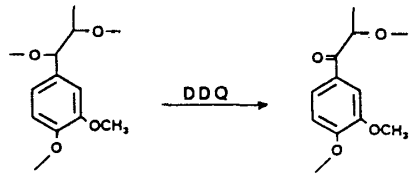


Y. Oikawa et al., Tetrahedron Lett., 25, 5393 (1984).

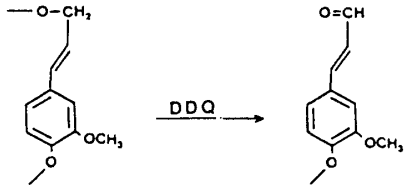
Fig. 16. Oxidative cleavage of benzyl ethers by DDQ in the presence of water.

Because it has been reported DDQ oxidized a γ position of 1-(*p*-methoxyphenyl)-1-propene [Kiefer, 1972], it is expected that DDQ oxidizes the γ-positions of conjugated arylpropene unit in lignin to yield cinnamaldehydes as shown in Scheme 2. In fact, this type of reaction in lignin model compounds has been observed in a water-saturated benzene [Nakamura, 1976].

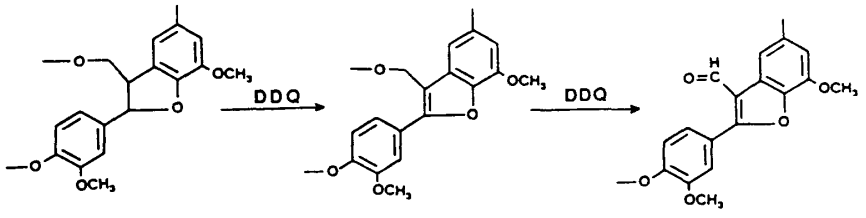
Phenylcoumaran is known to be dehydrogenated by DDQ in a refluxing benzene [Nakatsubo, 1981] or in ethyl acetate at room temperature [Umezawa, 1982] to yield phenylcoumarone under anhydrous conditions. The resulting phenylcoumarone was further oxidized to a γ-aldehyde compound in the presence of excess amount of DDQ (Scheme 3). However, in the presence of water, the formation of the double bond could be inhibited owing to nucleophilic addition of water to



Scheme 1. Oxidation of the benzyl position of phenylpropane units by DDQ.



Scheme 2. Oxidation of the γ-position of conjugated arylpropene units by DDQ.



Scheme 3. Oxidation of phenylcoumaran uniits by DDQ under anhydrous condition.

the benzyl cation intermediate formed by hydride anion transfer to DDQ.

In this section, the author recorded carbon-13 n.m.r. spectra of milled wood lignin acetate from *Pinus densiflora* wood before and after the the DDQ-oxidation in the dichloromethane-water mixture in order to clarify the above reactivity of DDQ toward the coniferous lignin. In this study, assignments of n.m.r. signals from the lignin acetate were done by referring recent articles, in which n.m.r. spectra were measured for acetone-d_6 solutions [Nimz, 1981; Sorvari, 1986]. Supplementary information was obtained from the original papers using an acetone-d_6-D_2O mixture [Nimz, 1984; Lüdemann, 1974] and chloroform-d [Mörck, 1985]. The n.m.r. spectra of the DDQ-untreated MWL fraction (MWLa) measured by conventional complete decoupling (COM) and Attached Proton Test (APT) [Patt, 1982] methods, are shown in Fig. 17 [Watanabe, 1988b]. Assignments of each signal are tabulated in Table 8.

The APT spectra of the untreated (MWLa) and DDQ-treated (D-MWLa) lignin fractions (Fig. 18) gave information on the DDQ-oxidation of the lignin acetates. As shown in Fig. 18, marked changes of β-carbon atoms in β-*O*-4 linkages in the lignin could not be observed as expected from the oxidizing mechanism by DDQ. However, structural changes at the benzyl and the γ-positions of conjugated

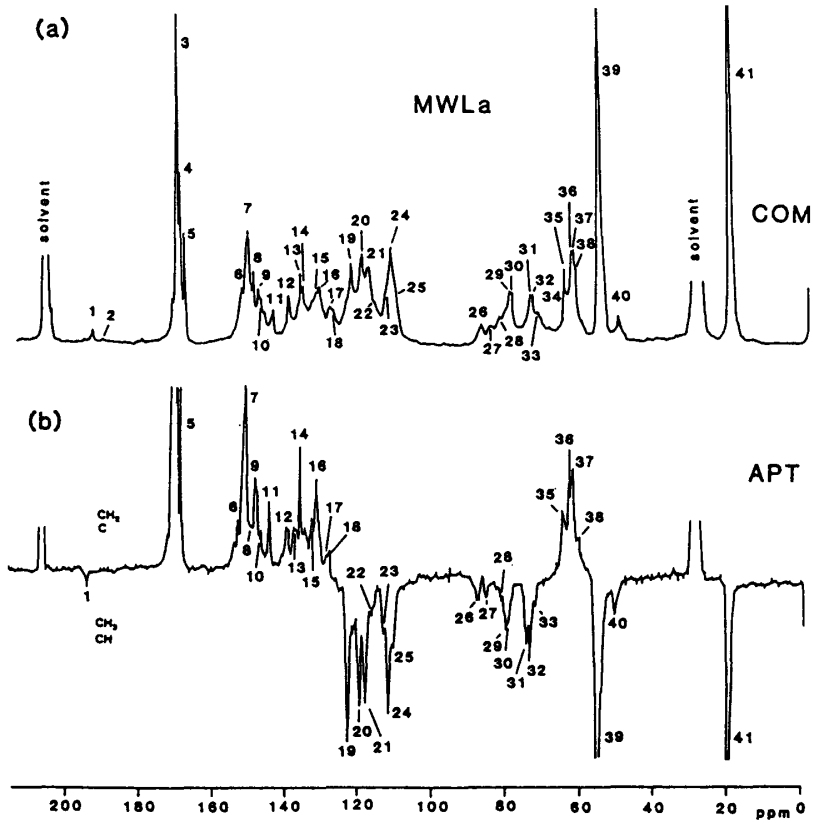


Fig. 17. Carbon-13 n.m.r. spectra of the acetylated milled wood lignin fraction (MWLa) from *Pinus densiflora* wood.
Notes: (a) Conventional complete decoupling method (COM)
(b) Atached Proton Test (APT) method.

arylpropene units were clearly shown by the signal intensities of two carbonyl signals at 194.2 ppm and 191.1 ppm (Fig. 19). The signal at 191.1 ppm has been assigned to the carbonyl groups in α-aldehyde, while the signal at 194.2 ppm is known to originate both from the carbonyl group of cinnamaldehyde and the α-ketone group of guaiacylpropane unit [Lüdemann, 1974; Sorvari, 1986; Nimz, 1981]. The author confirmed this assignment by discriminating carbon multiplicities. Contribution of the α-ketone to this signal was confirmed by broadening methine and methane signals by means of WND technique, while the existence of cinnamaldehyde was verified from the INEPT spectrum (Fig.19), in which quarternary carbon signals were eliminated. Because intensities of the downward signal at 194.2 ppm in the APT spectrum remarkably increased by the DDQ-oxidation (Fig. 19), it is evident that the γ-position of conjugated guaiacylpropene unit in the lignin acetate were oxidized to yield cinnamaldehydes by the action of DDQ. Furthermore, it has been clarified that remarkable changes of α, β and γ carbon atoms in phenylcoumaran could not be observed (Fig. 20) under this condition.

Regarding the cleavage of benzyl ether linkages in etherified guaiacylalkane

Table 8. Chemical shifts and main assignments for the acetylated milled wood lignin fraction (MWLa) from *Pinus densiflora* wood.

Signal No.	ppm	Main assignments[a]	Intensity	Atomic group	Ref.[b]
1	194.2	α-CO and γ-CHO in cinnamaldehyde	m	C, CH	1
2	191.1	α-CHO	w	CH	1
3	179.0	Primary acetoxy-CO	vs	C	1
4	170.1	Secondary acetoxy-COv	vs	C	1
5	169.1	Phenolic acetoxy-CO	s	C	1
6	153.3	Ge-4 (α=0); Cα in cinnamaldehyde; Ge-4 (α-CHO)	m	C, CH	1, 2
7	151.3	Ga-3 (α-OAc)	s	C	1
8	150.0	Ge-3 (α-OR)	m	C	1
9	148.8	Ge-3 (α-OAc); Ge-4 in β-O-4 (threo)	m	C	1, 3
10	148.2	Ge-4 in β-O-4 (erythro)	m	C	3
11	144.9	C-4′ in phenylcoumaran	m	C	1
12	140.5	Ga-4, Ga-1 (α-OR)	m	C	1
13	137.1	Ga-1 (α-OAc)	w	C	1
14	136.3	Ga-1 (α-OAc) in β-arylethers	m	C	3
15	132.8	Ge-1 (α-OR)	m	C	1
16	132.0	Ge-1 (α-OAc); Cβ in cinnamaldehyde	m	C, CH	1
17	130.0	C-1 in phenylcoumaran; H2/6	m	C, CH	1, 2
18	128.8				
19	123.4	Ga-5	s	CH	1
20	120.5	Ge-6	s	CH	1
21	118.9	Ge-5 (α-OAc); Ga-6	s	CH	1
22	117.6	C-6′ in phenylcoumaran; Ge-5 (α-OR)	w	CH	1, 2
23	113.9	Ge-2 (α=0)	w	CH	2
24	112.4	Ge-2; Ga-2	s	CH	1
25	111.2				
26	88.0	Cα in phenylcoumaran	m	CH	1
27	85.8	Cα in pinoresinol	m	CH	1
28	83.2	Cα in β-ethers	w	CH	3
29	80.5	Cβ in β-O-4 (threo)	m	CH	4
30	80.0	Cβ in β-O-4 (erythro)	m	CH	4
31	75.1	Cα in β-O-4 (threo)	m	CH	4
32	74.5	Cα in β-O-4 (erythro)	m	CH	4
33	72.9	unknown	w	CH	-
34	72.2	Cγ in pinoresinol	w	CH_2	1
35	65.9	Cγ in phenylcoumaran and cinnamyl alcohol	s	CH_2	2, 3
36	63.9	Cγ in β-1 and α,β-bis-O-4	s	CH_2	1
37	63.5	Cγi n β-O-4 (threo)	s	CH_2	4
38	63.0	Cγ in β-O-4 (erythro)	m	CH_2	4
39	56.2	OMe	vs	CH_3	1
40	51.0	Cβ in β-1 and phenylcoumaran	m	CH	1
41	20.7	CH3 in acetyl	vs	CH_3	1

a: Abbreviations: guaiacyl (G), acetylated (a), etherified (e), very strong (vs), strong (s), medium (m), weak (w). b: References; 1: [Nimz, 1981], 2: [Lüdemann, 1974], 3: [Mörck, 1985], 4: [Nimz, 1984].

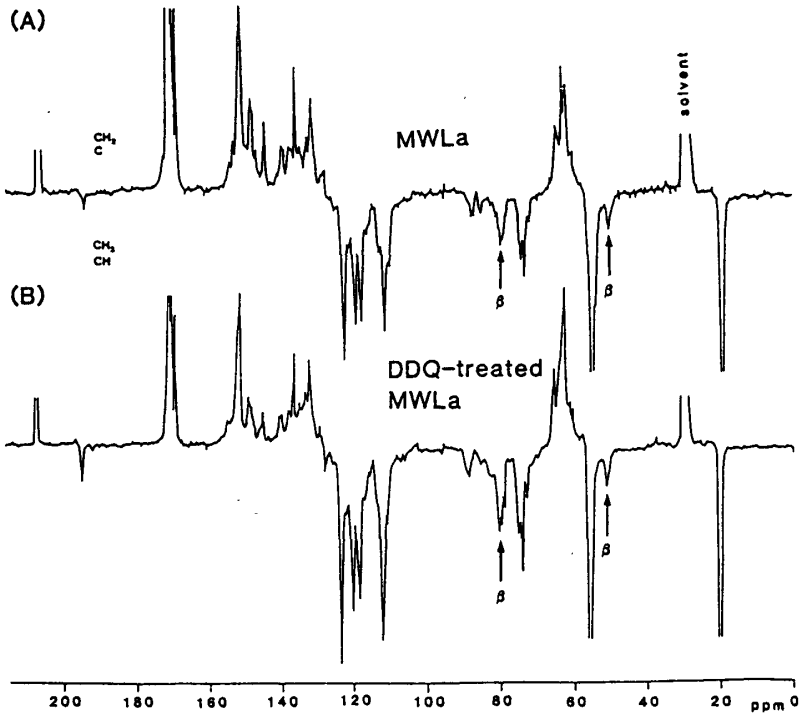


Fig. 18. 50.3 MHz APT spectra of the acetylated MWL fracion (MWLa) before and after the DDQ-oxidation.
Notes: (A) Untreated MWLa; (B) DDQ-treated MWLa.

units by DDQ, it was proved by spectral changes of the signals ascribed to *p*-etherified phenylpropane structure. The signal at 150.0 ppm (Fig. 21), which originates from C-3 in etherified guaiacyl nuclei accompanied by benzyl ether linkages (Maple acetylated lignin: 150.1 ppm, acetone-d_6, Nimz) [Nimz, 1981] disappeared by the DDQ-oxidation, while the signal originating from *p*-etherified guaiacyl nuclei with α-carbonyl group [Lüdemann, 1974] was enhanced by the treatment (Fig. 21). This result strongly suggests that the benzyl ether linkages with *p*-etherified guaiacyl nuclei were oxidized by DDQ to give the α-carbonyl groups, which appear at 194.2 ppm in the carbon-13 n.m.r. spectra (Fig. 19). Actually, LCC model compounds of 3-methoxy-4-hydroxybenzyl ether I and 3-methoxy-4-benzyloxybenzyl ether IV were oxidatively decomposed by DDQ to yield the α-carbonyl commounds, while a *p*-acetoxy LCC model compound II was inert to the oxidation because of electron-withdrawing inductive effect of the *p*-acetoxyl group. The reaction rate of the *p*-benzyloxy model compound IV was considerably higher than that of the *p*-methoxy model compound III (Table 9), demonstrating contribution of electron-donating inductive effect to the reaction rate. This is in accordance with the theory described by Becker [Becker, 1980b]. However, both compounds could not be decomposed quantitatively. Considering the structures of *p*-substiuents in lignins, it is expected that the secondary *p*-substituents such as β-ether raise the

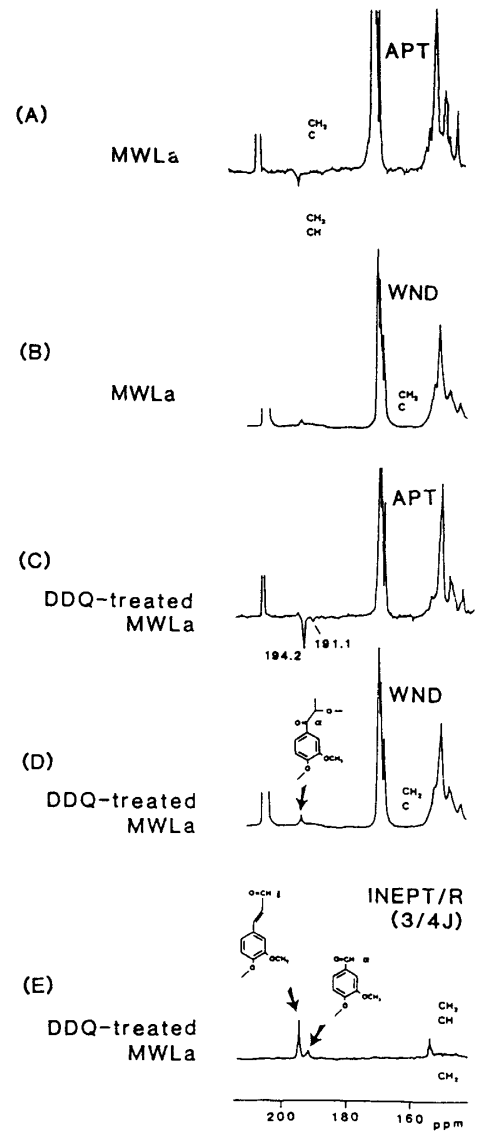


Fig. 19. Carbonyl signals of the acetylated MWL fractions. Notes: (A) Untreated fraction (APT); (B) Untreated fraction (WND); (C) DDQ-treated fraction (APT); (D) DDQ-treated fraction (WND); (E) DDQ-treated fraction (INEPT).

reaction rate drastically. However, the heterogeneity of *p*-substituents in lignin, which have often been neglected on the structural analyses of LCCs, makes it impossible to cleave all types of lignen-sugar bonds quantitatively. This reaction should be used for giving direct evidence for the occurence of lignin-sugar bonds based on its selective reactivity.

It is important to note that the low solubility of 2,3-dichloro-5,6-dicyanohydroquinone in dichloromethane (0.4 g/1,000 ml) makes it possible to maintain the reaction media almost in neutral pH region during the course of DDQ-oxidation.

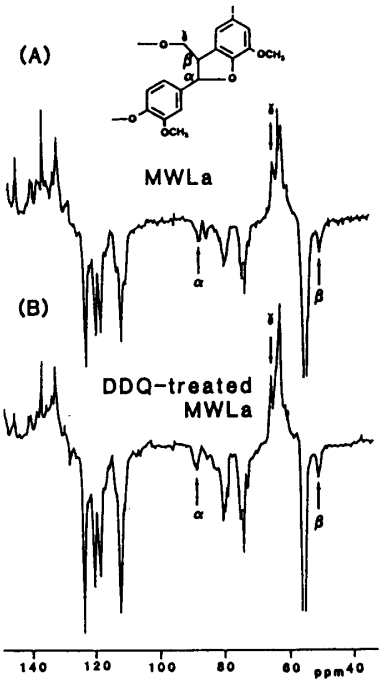


Fig. 20. Signals of carbon atoms in the phenylcoumaran units before and after the DDQ-oxidation. Notes: (A) Untreated fraction; (B) DDQ-treated fraction.

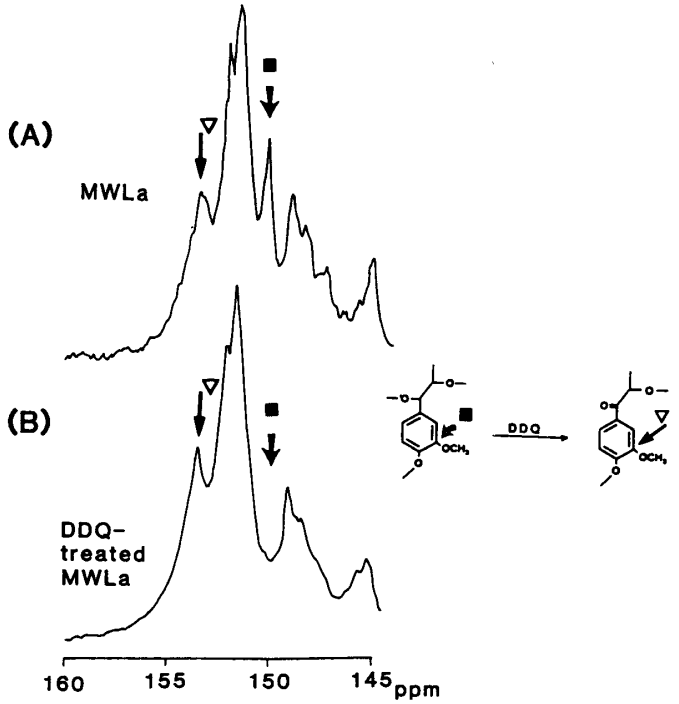


Fig. 21. Cleavage of the benzyl ether linkages in the acetylated lignin from *Pinus densiflora* wood by DDQ.
Notes: The spectrum was recorded by COM method.; (A) Untreated fraction; (B) DDQ-treated fraction.; ■····· C-3 of *p*-etherified phenylpropane unit accompanied by *α*-ether, contributes to this signal. [Nimz, 1981]; △····· C-3 of *p*-etherified phenylpropane unit accompanied by *α*-carbonyl group, contributes to this signal, together with C-*α* in cinnamaldehyde, etc. [Lüdemann, 1974].

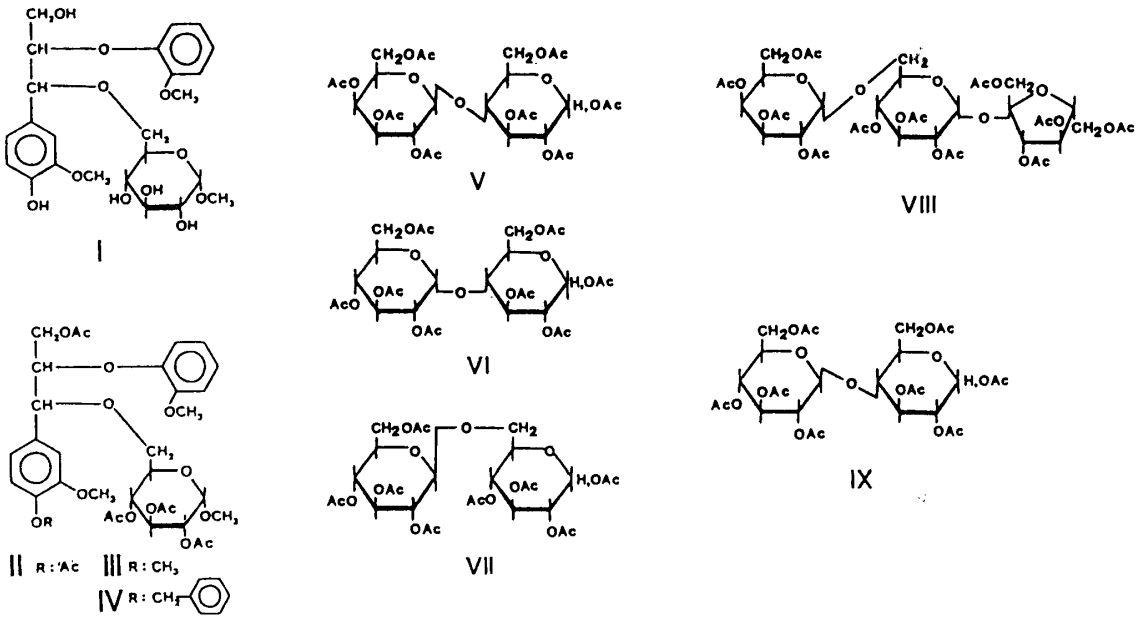


Table 9. Oxidative cleavage of the benzyl ether linkages in the LCC model compounds with DDQ.[a]

p-Substituents of model compounds			
Hydroxy (I)	Acetoxy (II)	Methoxy (III)	Benzyloxy (IV)
100.0	0.0	Trace	50.1

a: Values are expressed as the yield of the corresponding carbonyl compounds.

Owing to the properties of the solvent, acid-sensitive acetal compounds were inert to the DDQ-oxidation in dichloromethane-water mixture [Oikawa, 1982a, 1984a]. Actually, glycosidic bonds in the acetates of lactose (V), maltose (VI), gentiobiose (VII), raffinose (VIII) and cellobiose (IX) were inactive to the DDQ-oxidation, indicating the stabilities of glycosidic bonds to the DDQ-oxidation.

From these results, it is concluded that the acetylated lignin from *Pinus densiflora* wood and the LCC model compounds can be oxidized with DDQ according to the reaction pathway described by Oikawa [Oikawa 1984a], and that glycosidic bonds between sugar residues were inert to the DDQ-oxidation [Watanabe, 1989a, 1989b].

II-2-4 Summary

Acetylated milled wood lignin from akamatsu (*Pinus densiflora* Sieb. et Zucc.) wood was oxidized with DDQ in a dichloromethane-water mixture. Carbon-13 n.m.r. spectra of the lignin revealed that the benzyl position of guaiacylpropane

and the γ-position of 1-guaiacyl-1-propene units were oxidized to carbonyl groups. The spectra also gave unequivocal evidence for the oxidative cleavage of *p*-etherified benzyl ether linkages by DDQ. Furthermore, benzyl ether linkages of LCC model compounds were oxidatively cleaved by DDQ, while glycosidic linkages between sugar residues were inert to the oxidation in the solvent.

II-3 Stabilities of acetyl group during DDQ-oxidation and methylation by the method of Prehm

II-3-1 Introduction

Acetyl derivatives of sugars have been employed extensively as intermediates in sugar synthesis and for the isolation and identification of the sugars. Their advantage for these purposes arises from the fact that acetylation could be done under mild conditions and that the acetyl groups could be easily removed. The basis for these utilization, of course, lies in the stabilities of the acetyl group during the synthetic or analytical processes. Actually, it has been shown that methylation of partially acetylated glucose derivatives including 1,2,3,4-tetra-*O*-acetylglucose with diazomethane and boron trifluoride etherate proceeded without liberation and migration of the acetyl groups to yield the expected sugar derivatives [Mastronardi, 1966]. However, it is also undoubted fact that acyl groups migrate in partially acylated sugars under some external conditions. In particular, the acyl migration from *O*-4 to *O*-6 positions in partially acylated glucopyranose derivatives have often been reported both in acidic and weakly alkaline media [Takano, 1987; Haworth, 1931; Yoshimoto, 1983; Bouveng, 1957]. For example, Haworth reported that methylation of methyl 2,3,4-tri-*O*-acetyl-α-D-glucoside with Purdie's reagents introduced a methyl group at position 2, which was attributable to a consecutive *O*-2→*O*-3→*O*-4→*O*-6 acyl migration in the sugar, to yield the 3,4,6-tri-*O*-acetyl-2-*O*-methyl derivative [Haworth, 1931]. Thus, stabilities of acetyl groups depend on the reaction conditions. In this section, the author examined the stabilities of acetyl groups in partially acetylated sugars during the new analytical procedures in order to apply the acetylation to the initial reaction for the new binding-site analysis of LCCs [Watanabe, 1989b].

II-3-2 Experimental

General methods

H.p.l.c. analysis was performed on a Waters μ-porasil column with an eluent system of *n*-hexane/ethyl alcohol/ acetic acid (200:20:1, v/v/v) using a Waters M-600 system. N.m.r. spectra were recorded on a Varian XL-200 Spectrometer in

$CDCl_3$ at room temperature. T.l.c. was done on Merck Kieselgel 60 plates using a ethyl ether/ *n*-hexane mixtuue (10:1, v/v).

Preparation of partially acetylated glucose

All types of tetra-*O*-acetylglucose derivatives were prepared to discriminate

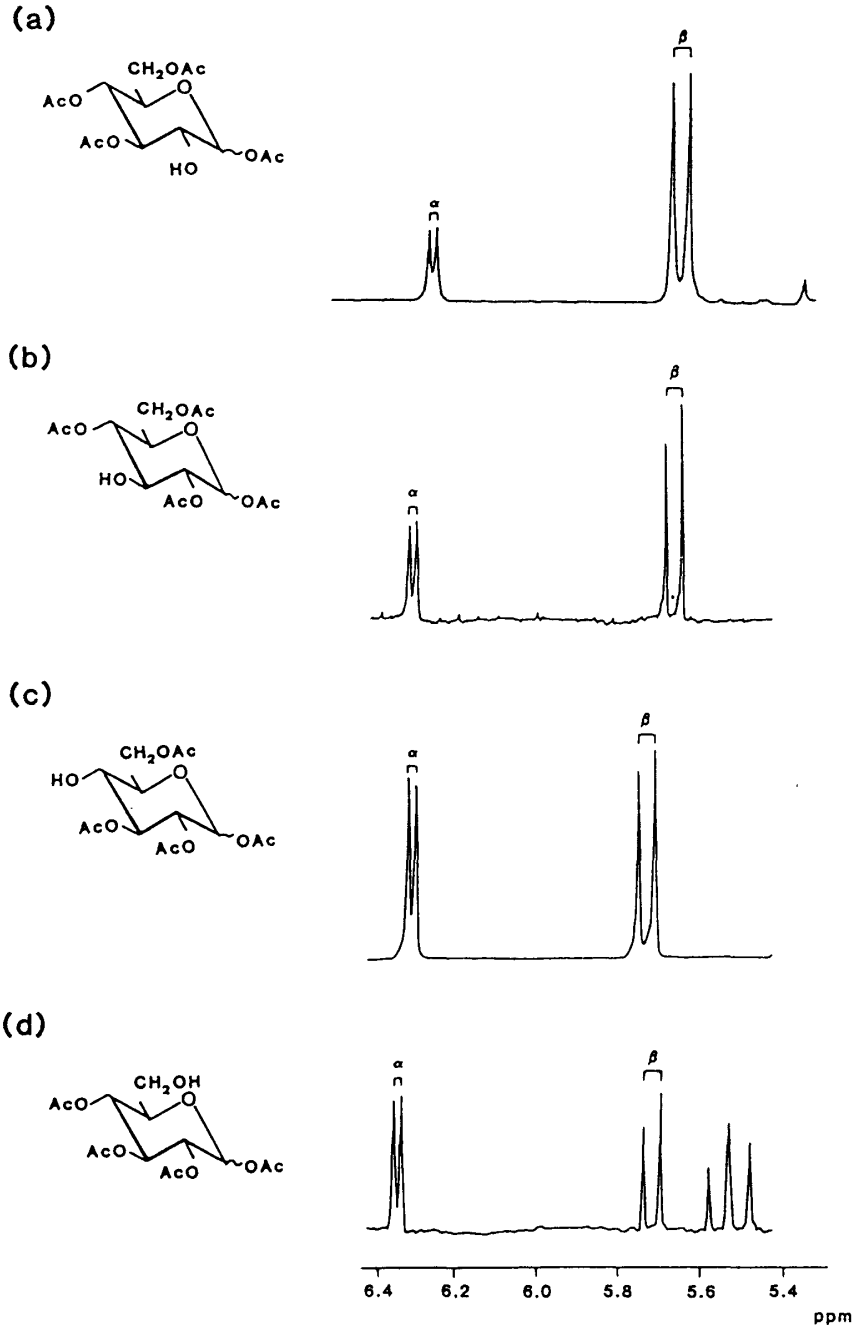


Fig. 22. Proton-n.m.r. spectra of anomeric region of the partially acetylated glucose. Notes: (a) 1,3,4-6-Tetra-*O*-acetyl-D-glucose; (b) 1,2,4,6-Tetra-*O*-acetyl-D-glucose; (c) 1,2,3,6-Tetra-*O*-acetyl-D-glucose; (d) 1,2,3,4-Tetra-*O*-acetyl-D-glucose.

each isomer by n.m.r. spectroscopy. 1,2,3,4-tetra-*O*-acetylglucose was synthesized from D-glucose by a method described in a literature [Reynolds, 1965]. 2,3,4,6-Tetra-*O*-acetyglucose was prepared by detritylation of 1,2,3,4-tetra-*O*-acetyl-6-*O*-trityl -*D*-glucose with excess amount of hydrogenbromide in acetic acid at room temperature. 1,2,4,6,-Tetra-*O*-acetyl-D-glucose and 1,2,3,6-tetra-*O*-acetyl-D-glucose were prepared by acetyl migration of 1,2,3,4-tetra-*O*-acetyl-D-glucose in methyl alcohol. 1,3,4,6-Tetra-*O*-acetyl-D-glucose was synthesized by the method of Lemieux [Lemieux, 1953]. All these sugar acetates obtained were identified by n.m.r. spectrocopy by comparing the resonance lines with those authentic values [Komura, 1978]. The spectroscopic data were summarized in Fig. 22, Table 10 and Table 11.

Table 10. Carbon-13 n.m.r spectral lines (δ) and their assignments for the tetra-*O*-acetyl-D-glucose.[a]

Acetylated α-D-glucopyranose	C-1	C-2	C-3	C-4	C-5	C-6
2,3,4,6-*O*-Ac-D-Glc	90.0	71.1	69.9	68.5	67.0	62.0
1,3,4,6-*O*-Ac-D-Glc	91.3	69.8*	73.1	67.3	69.6*	61.5
1,2,4,6-*O*-Ac-D-Glc	89.4	71.8	70.0*	70.3*	70.0*	61.7
1,2,3,6-*O*-Ac-D-Glc	89.3	69.3	72.3	68.5	72.1	62.6
1,2,3,4-*O*-Ac-D-Glc	89.1	69.3	69.6	68.2	72.1	60.8
Acetylated β-D-glucopyranose	C-1	C-2	C-3	C-4	C-5	C-6
2,3,4,6-*O*-Ac-D-Glc	95.4	73.0	72.3	68.5	72.0	62.0
1,3,4,6-*O*-Ac-D-Glc	93.8	71.3	75.2	67.5	72.7	61.5
1,2,4,6-*O*-Ac-D-Glc	91.6	72.8*	73.9	70.3	72.9*	61.7
1,2,3,6-*O*-Ac-D-Glc	91.8	70.3	75.0	68.3	74.9	62.6
1,2,3,4-*O*-Ac-D-Glc	91.7	70.4	72.4	68.2	75.0	60.8

a: Assignments of the resonances indicated by * may be interchanged.

Table 11. Chemical shifts (δ) and coupling constants ($J_{1,2}$) for anomeric protons in the tetra-*O*-acetyl-D-glucose.

Acetylated α-D-glucopyranose	Chemical shift (ppm)	$J_{1,2}$ (Hz)
1,3,4,6-*O*-Ac-D-Glc	6.23	3.6
1,2,4,6-*O*-Ac-D-Glc	6.30	3.7
1,2,3,6-*O*-Ac-D-Glc	6.29	3.8
1,2,3,4-*O*-AcD--Glc	6.34	3.8
Acetylated β-D-glucopyranose	Chemical shift (ppm)	$J_{1,2}$ (Hz)
1,3,4,6-*O*-Ac-D-Glc	5.63	8.2
1,2,4,6-*O*-Ac-D-Glc	5.67	8.3
1,2,3,6-*O*-Ac-D-Glc	5.72	8.1
1,2,3,4-*O*-Ac-D-Glc	5.72	8.2

Reection of partially acetylated glucose with DDQ

Tetra-*O*-acetylglucose derivatives were reacted with DDQ for 2 hr in a refluxing dichloromethane-water mixture (18:1, v/v). The reaction mixture was then transferred into a separatory funnel and partitioned between chloroform and distilled water. The organic layer was washed with water, concentrated to a small volume and then applied to a preparative t.l.c. to remove hydroquinones. The silicagel containing sugar acetates (Rf=0.3~0.8) was collected and extracted with chloroform. The chloroform solution was concentrated below 30°C and directly applied to the h.p.l.c. and n.m.r. spectroscopy.

Methylation partially acetylated glucose by the method of Prehm

Tetra-*O*-acetylglucose derivatives were methylated with methyl trifluoromethanesulfonate and 2,6-di-(*tert*-butyl) pyridine in trimethyl phosphate at 50°C for 3 hr according to the method of Prehm [Prehm, 1980]. The reaction mixture was shaken with chloroform and distilled water in a separatory funnel. The organic layer separated, was concentrated, and applied to a preparative t.l.c. to remove 2,6-di-(*tert*-butyl) pyridine. The silicagel containing sugar acetates (Rf=0.3~0.8) was collected and extracted with chloroform. The chloroform solution was concentrated and subjected to the h.p.l.c. and n.m.r. analysis.

II-3-3 Results and Discussion

In this chapter, the author has proposed a new method for the binding-site analysis of LCCs which involves a series of reactions including protection of hydroxyl groups, oxidative cleavage of lignin-carbohydrate bonds with DDQ and methylation of the newly formed hydroxyl groups by the Prehm method [Prehm, 1980] (Fig. 6). In order to obtain correct information by this method, the first protection step must proceed in a mild media where original lignin-carbohydrate bonds are not cleaved. Furthermore, the protecting groups must be stable during the DDQ-oxidation and the methylation. Acetylation, which has been employed extensively both in organic sinthesis and in identification of natural macromolecules, is one of the mildest methods to satisfy these limitations, though stability of the introduced acetyl groups must be checked prior to the binding-site analysis.

In this section, the stability of acetyl groups in partially acetylated sugars during the new analytical procedures was cheked by recording high performance liquid chromtograms (h.p.l.c.) and n.m.r. spectra of tetra-*O*-acetylglucopyranose derivatives before and after the DDQ-oxidation or Prehm methylation [Prehm, 1980]. Prior to the experiment, eluting condition for the h.p.l.c. was examined by using a Waters μ-porasil column with 8 solvent system listed in Table 12. As a result, a mixture of *n*-hexane/ethyl alcohol/acetic acid (200/20/1, v/v/v) was found

Table 12. Separation of tetra-*O*-acetyl-D-glucose isomers by high performance liquid chromatography (HPLC) on a μ-porasil column with several eluent systems.

Eluents	Separation of isomers[a]
n-Hexane/acetone (8:1)	±
Benzene/ethyl alcohol (7:1)	−
Benzene/ethyl alcohol (20:1)	−
Ethyl acetate/*n*-hexane (1:5)	−
Ethyl acetate/*n*-hexane (5:1)	−
Ethyl ether/*n*-hexane (1:2)	−
n-Hexane/benzene/ethyl alcohol (10:15:1)	±
n-Hexane/benzene/ethyl alcohol (15:5:1)	±
n-Hexane/ethyl alcohol/acetic acid (200:20:1)	+

a: Separation of isomeric mixture was examined using 1, 2, 3, 4-tetra-*O*-acetyl-D-glucose at a flow rate of 2 ml/min.; +: separable, ±: partially separable, −: inseparable.

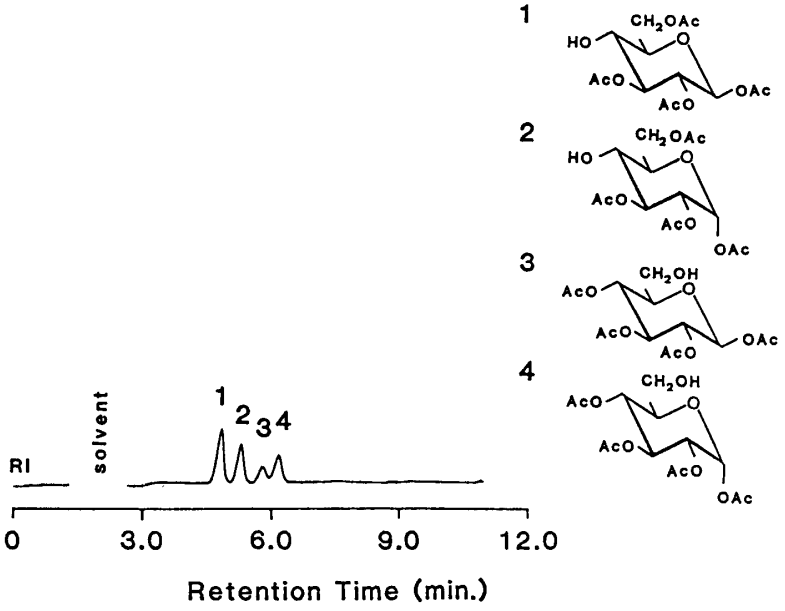


Fig. 23. High performance liquid chromatogram of the partially acetylated D-glucose derivatives on Waters μ-porasil with *n*-hexane/ethyl ahcohol/acetic acid (200:20:1, v/v/v).
Notes: 1: 1,2,3,6-Tetra-*O*-acetyl-β-D-glucose; 2: 1,2,3,6-Tetra-*O*-acetyl-α-D-glucose; 3: 1,2,3,4-Tetra-*O*-acetyl-β-D-glucose; 4: 1,2,3,4-Tetra-*O*-acetyl-α-D-glucose.

to be the most effective mobile phase to separate each isomer. The elution profile of a mixture of 1,2,3,4- and 1,2,3,6-tetra-*O*-acetylglucose is shown in Fig. 23.

The migration of acyl groups was first discovered by Fischer in 1920 [Fisher, 1920]. Thereafter, it has been generally accepted that some kinds of reaction

conditions cause the acyl migration between vicinal hydroxyl groups in polyhydric alcohols. The migration of acyl groups in partially acylated polyhydric alcohols proceeds through orthoacid intermediates, and it is known that the acyl migration *via* intermediates having $^1C_4$ (D) conformation is a minor pathway [Yoshimoto, 1983] and that the acyl groups generally rearrange from secondary to primary positions and migrate away from *O*-1 to *O*-6 of an aldohexose. Particularly, acyl migration from *O*-4 to *O*-6, in other words, secondary to primary hydroxyl group in partially acylated glucose derivatives has been frequently observed both in acidic and weakly alkaline media because the strainless intermediate of 4,6-orthoacetate can be easily formed from the sugars. The facility of the migration from the *O*-4 to *O*-6 positions in partially acetylated glucose derivatives is well established based on a reasonable stereochemical basis [Wood, 1956]. From this reason, stabilities of the labile compound of 1,2,3,4-tetra-*O*-acetylglucopyranose was primarily examined in this study.

Stabilities of acetyl groups during the DDQ-oxidation or Prehm methylation was investigated by using tetra-*O*-acetylglucose derivatives. To identify each isomer by n.m.r. spectroscopy, all of the sugar derivatives were prepared beforehand. The spectroscopic data are summarized in Fig. 22, Tables 10 and 11. H.p.l.c and n.m.r. spectra of 1,2,3,4-tetra-*O*-acetylglucose before and after the DDQ-oxidation revealed that the sugar acetate was stable to the DDQ-oxidation (Figs. 24, 25) because no significant spectral changes could be detected after the oxidation. Similarly, stabili-

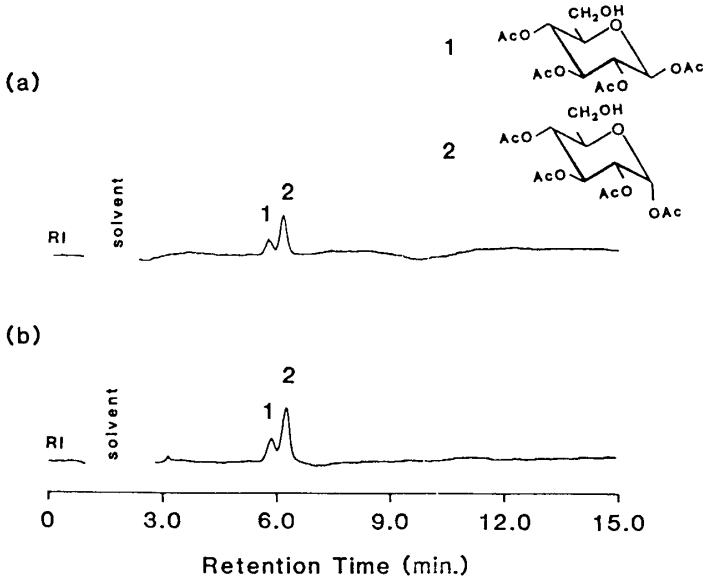


Fig. 24. High performance liquid chromatogram of 1,2,3,4-tetra-*O*-acetyl-D-glucose before and after the DDQ-oxidation in the dichloromethane-water mixture.
Notes: (a): Untreated; (b): DDQ-treated.

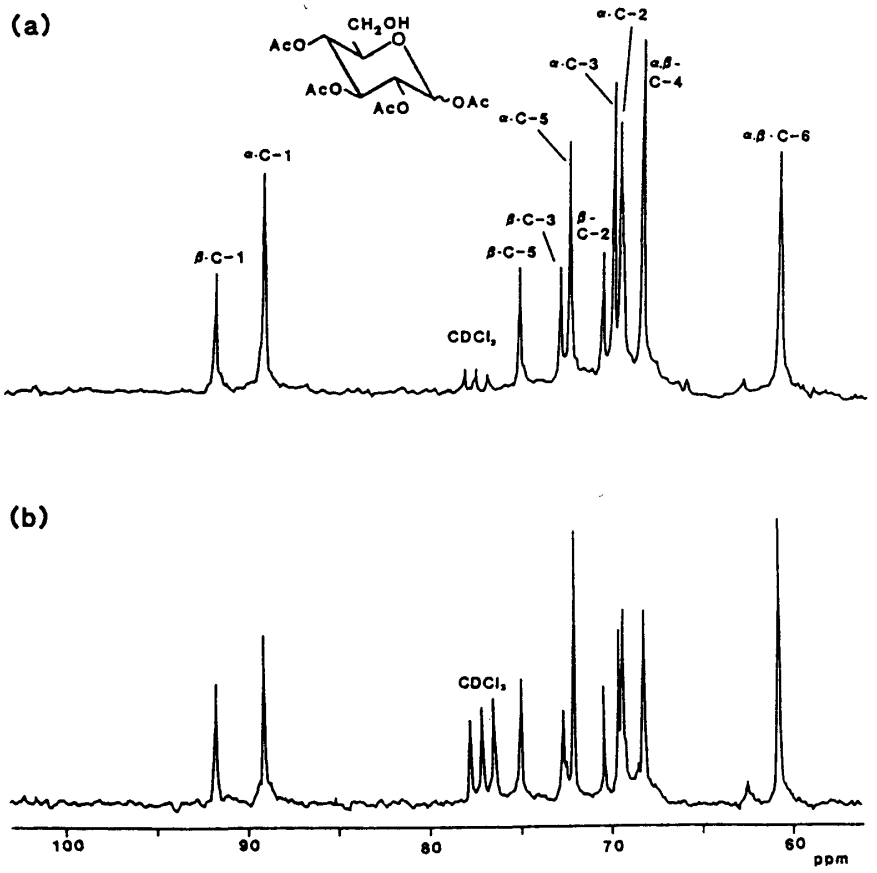


Fig. 25. Carbon-13 n.m.r. spectra of 1,2,3,4-tetra-*O*-acetyl-D-glucose before and after the DDQ-oxidation in the dichloromethane-water mixture.
Notes: (a): Untreated; (b): DDQ-treated.

ties of 1,2,3,6 and 2,3,4,6-derivatives during the DDQ-oxidation were confirmed by n.m.r. spectra (Figs. 26, 27). Methylation of 1,2,3,4-tetra-*O*-acetylglucose was found to produce quantitatively 1,2,3,4-tetra-*O*-acetyl-6-*O*-methyl-D-glucose because the starting material disappeared by the metylation (Fig. 28) and a n.m.r. signal ascribed to C-6 of the 1,2,3,4- derivative showed downfield shift by the methylation (Fig. 29). Similarly, the selectivity in the methylation of 1,3,4,6-tetra-*O*-acetyl-D-glucose could be confirmed by the Prehm methylation and subsequent deacetylation. N.m.r. spectrum of the resulting mono-methylated sugar demonstrated the steric compression effect between 1-α-OH group and the introduced 2-*O*-methyl group (Fig. 30) as reported by Usui [Usui, 1973]. Thus, no liberation and migration of acetyl groups were observed during the DDQ-oxidation in the dichloromethane-water mixture and methylation by the method of Prehm [Prehm, 1980.].

Summary

Partially acetylated glucose having vicinal trans or cis centers between acetoxyl

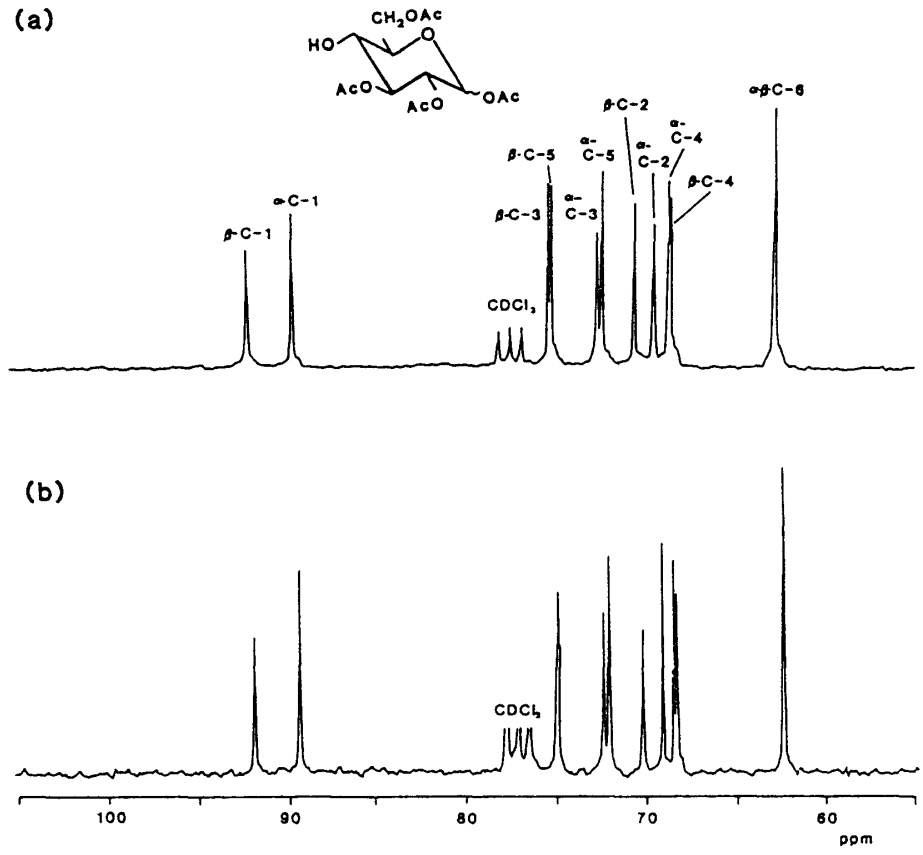


Fig. 26. Carbon-13 n.m.r. spectra of 1,2,3,6-tetra-*O*-acetyl-D-glucose before and after the DDQ-oxidation in the dichloromethane-water mixture.
Notes: (a): Untreated; (b): DDQ-treated.

and hydroxyl groups, showed stability to the DDQ-oxidation and Prehm methylation, demonstrating the appropriateness of the process for the initial reaction of the binding-site analysis between lignin and carbohydrate.

II-4 Binding-site analysis of lignin-carbohydrate linkages in cellulase-degraded LCC fragments

II-4-1 Introduction

A number of studies have been made to prove the existence of covalent bonds between lignin and carbohydrate. However, previous methods for determining their binding-sites could not directly sepcify what kind of substance was bound to the carbohydrate component, because chemical reactions involved in the previous methods were non-specific to lignin skeleton. To overcome this problem, the author proposed a new method for proving the existence of ether linkages between lignin and carbohydrate by using the reactivity of DDQ described in the section II-2.

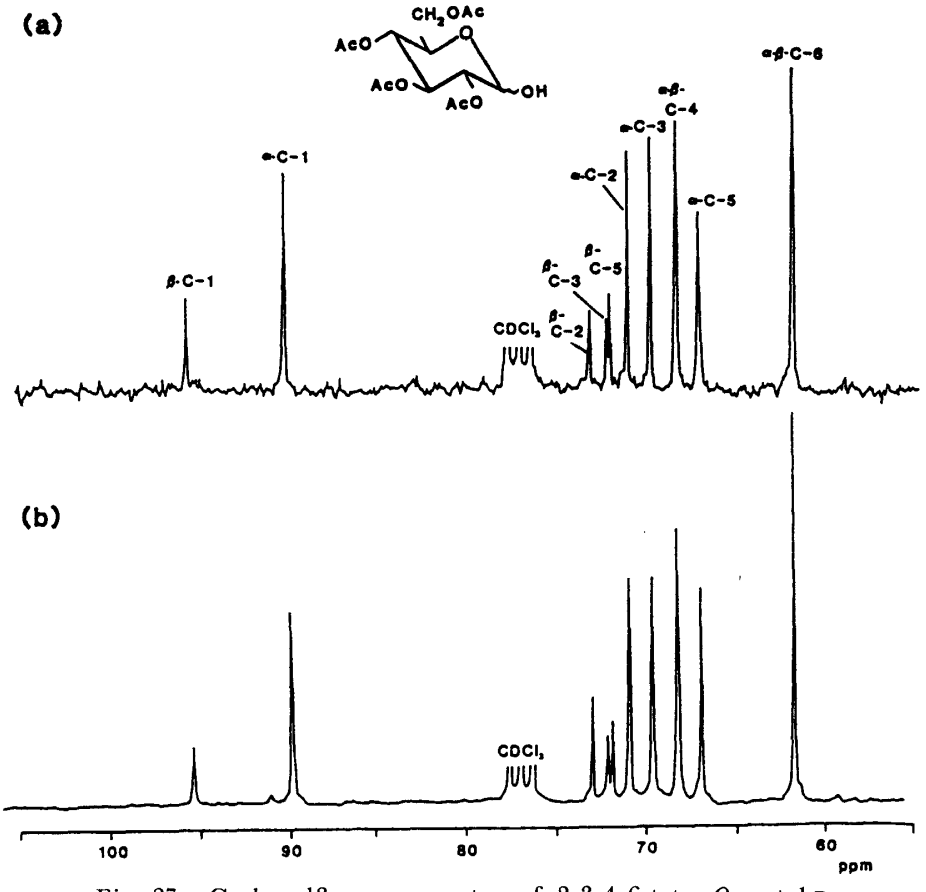


Fig. 27. Carbon-13 n.m.r. spectra of 2,3,4,6-tetra-*O*-acetyl-D-glucose before and after the DDQ-oxidation in the dichloromethane-water mixture.
Notes: (a): Untreated; (b): DDQ-treated.

In this section, the new method was actually applied to the cellulase-degraded LCC fragments which were isolated by means of the adsorption chromatography on Toyopearl HW gel (section II-1), and obtained the evidence for the ether linkages between lignin and carbohydrate.

II-4-2 Experimental

DDQ-Oxidation of LCC

The LCC fragments (M-ESD, A-ESD) described in the section II-1 were acetylated with pyridine and acetic anhydride at 40°C for 18 hr. The acetylated LCC fragments were then oxidized with DDQ in a refluxing dichloromethane-water mixture (18:1, v/v) for 2 hr. After the reaction, the reaction mixture was partitioned between chloroform and distilled water ten times and the chloroform solution was passed through a sodium sulfate column, evaporated below 30°C and then dried *in vacuo*.

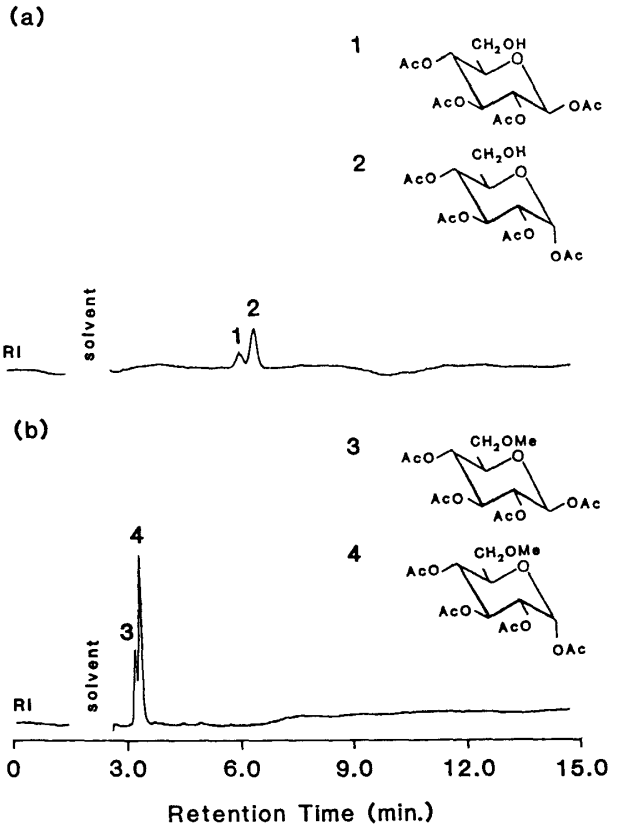


Fig. 28. High performance liquid chromatograms of 1,2,3,4-tetra-*O*-acetyl-D-glucose and its methylation products by the method of Prehm.
Notes: (a): 1,2,3,4-tetra-*O*-acetyl-D-glucose; (b): Methylation products from 1,2,3,4-tetra-*O*-acetyl-D-glucose by the method of Prehm

Methylation analysis of LCC fragments

The DDQ-oxidized LCC fragments were methylated for 3 hr with methyl trifluromethanesulfonate and 2,6-di-(*tert*-butyl) pyridine at 50°C for 3 hr in trimethyl phosphate according to the method of Prehm [Prehm, 1980]. The reaction mixture was partitioned between chloroform and water. The organic layer was separated, washed with water five times, and then evaporated thoroughly with a vacum pump. The methylated sample thus obtained was then hydrolyzed with 2M trifluoroacetic acid at 100°C for 3 hr in a sealed tube. The hydrolyzates were evaporated thoroughly, and partitioned between chloroform and water. Next, the upper aqueous solution was recovered, concentrated to a small volume, and reduced with sodium borohydride for 12 hr at room temperature. The alditol fraction obtained was evaporated in the presence of methyl alchol, and dried in vacuo for 3 hr. Acetylation of the alditol fraction was carried out with acetic anhydride and pyridine for 1 hr at 100°C. The methylated alditol acetates thus obtained were separated by

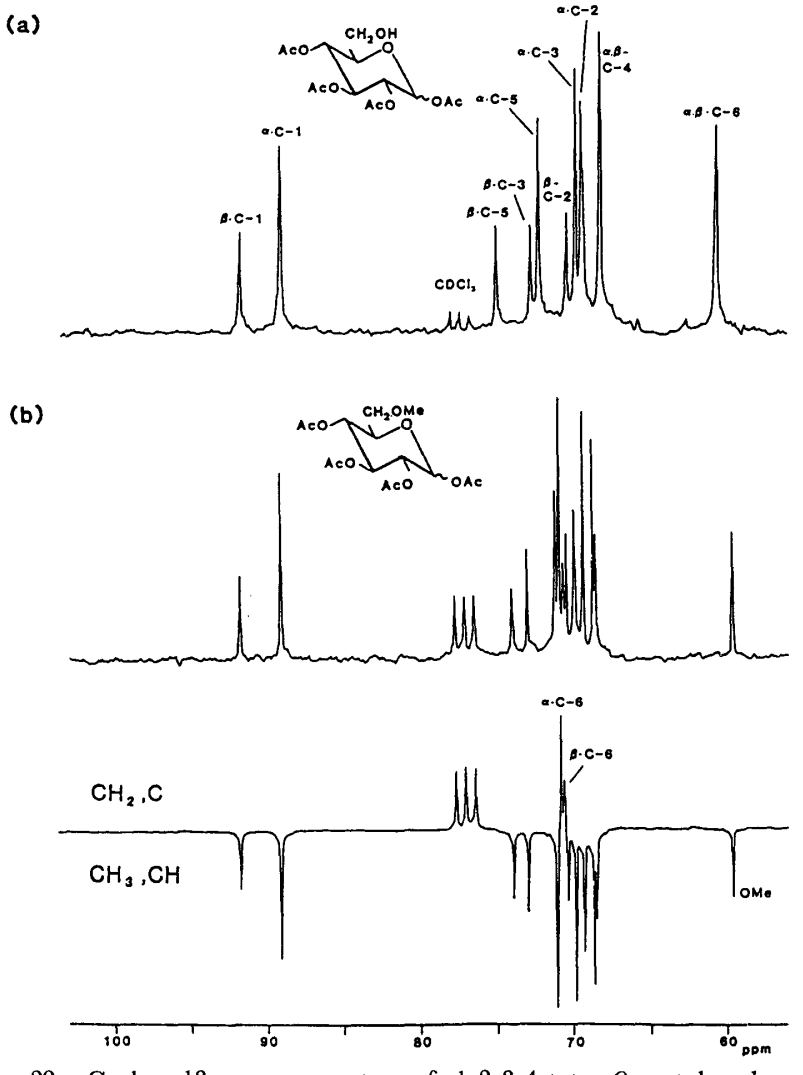


Fig. 29. Carbon-13 n.m.r. spectra of 1,2,3,4-tetra-*O*-acetyl-D-glucose and its methylation products by the method of Prehm.
Notes: (a): 1,2,3,4-tetra-*O*-acetyl-D-glucose; (b): Methylation products from 1,2,3,4-tetra-*O*-acetyl-D-glucose by the method of Prehm.

g.l.c by using capillary columns coated with (a) silicon OV-225 (50 m×0.22 mm) and (b) silicon OV-101 (50 m×0.22 mm) at 210°C. Component sugars corresponding to each peak were identified by g.c.-m.s. on a Shimadzu QP-1000 using mass chromatography at m/z 45, 59, 71, 85, 87, 89, 99, 101, 113, 115, 117, 129, 139, 145, 161, 189, 205, 233 and 261 on the column (a).

Control experiment

The LCC fragments M-ESD and A-ESD were acetylated with acetic acid and pyridine at 40°C for 18 hr. The acetylated fractions were directly methylated by the method of Prehm [Prehm, 1980], hydrolyzed, converted to aldiol acetate and

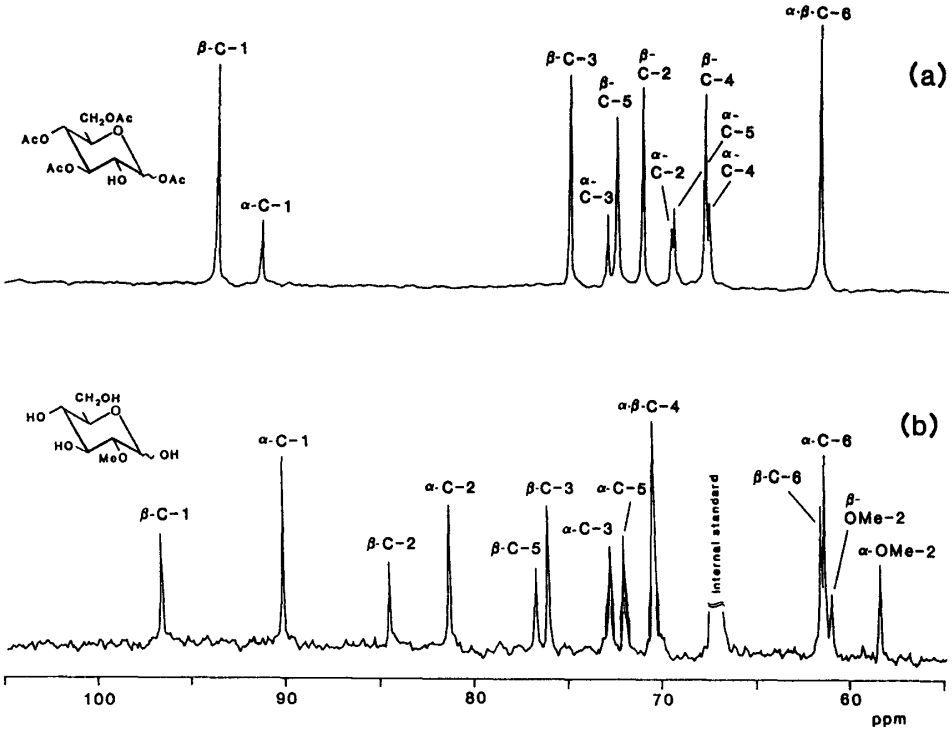


Fig. 30. Carbon-13 n.m.r. spectra of 1,3,4,6-tetra-*O*-acetyl-D-glucose and its methylated derivative.
Notes: (a): 1,3,4,6-tetra-*O*-acetyl-D-glucose; (b): Deacetylated sugar derived from the methylation products of 1,3,4,6-tetra-*O*-acetyl-D-glucose.

analyzed by g.l.c on the column (a).

II-4-3 Results and Discussion

In lignin-carbohydrate complexes, the frequency of lignin-carbohydrate linkages is extremely fewer than that of glycosidic bonds between sugar residues. Therefore, it is evident that enzymatic hydrolysis of their carbohydrate moiety is necessary for the correct analysis of lignin-carbohydrate linkages. Based on this concept, the author prepared cellulase-degraded LCC fragments (M-ESD, A-ESD) from the enzymatic hydrolyzates of lignin-acetyglucomannan complex (nor-C-1-M) and from an acidic LCC (com-C-1-A) by means of a new method described in the section II-1. Carbon-13 n.m.r. spectra of the isolated LCC fragments suggested the existence of non-glycosidic type of lignin-carbohydrate linkages.

In this section, binding-sites of ether linkages between lignin and carbohydrate in the cellulase-treated LCC fragments were analyzed by a new method using acetylation, DDQ-oxidation and Prehm's methylation techniques. As described in the section II-2, the oxidative cleavage of benzyl ethers with DDQ in a dichloromethane-water mixture proceeds by way of formation of an initial charge transfer

(CT) complex, hydride ion transfer from the benzyl ether to DDQ, and addition of water to the benzyl cation intermediate. As a result, the oxidation occurs both at the α position of arylalkane and at the γ position of conjugated arylpropene accompanied by electron-donating substituents. This is because the carbocation intermediates from those compounds can be stabilized by conjugation and the electron-donating substituents. It makes possible to discriminate lignin skelton from other kind of chemical substances. For example, a β ether compound of 6-*O*-(1-phenylpropane-3-yl)-D-galactose which resembles LCC structure but lacks 3,4-dialkoxy substituents and the conjugation system, could not be cleaved by the action of DDQ [Koshijima, 1984]. Furthermore, glycosidic bonds in acetylated oligosaccharides were stable to the DDQ-oxidation (section II-2), demonstrating that the cleavage of benzyl ether bonds with DDQ does not proceed by acid hydrolysis mechanism. Thus, a number of reports have demonstrated the oxidation mechanism for the DDQ-reaction. In fact, MWL acetate from *Pinus densiflora* wood could be oxidized selectively at the α and γ positions as described in section II-2. Furthermore, alkali-labile substituent of acetyl group was stable to the DDQ-oxidation and Prehm's methylation (section II-3).

Based on these results, the author applied the new method to the cellulase-degraded LCC fragments from the lignin-acetylglucomannan complex (nor-C-1-M) and those from the acidic lignin-carbohydrate complex (com-C-1-A) whose sugar moiety consised of arabinoglucuronoxylan, glucomannan and β-1,4-galactan, as described in the section II-2. To elucidate the binding-sites between lignin and carbohydrate, the cellulase-degraded LCC fragments from nor-C-1-M (M-SED) and com-C-1-A (A-ESD) were first acetylated with acetic acid and pyridine, and the acetylated fractions were oxidized with DDQ in the dichloromethane-water mixture. In this experiment, completion of the first acetylation step was confirmed by a control experiment in which the DDQ-oxidation step was eliminated. Namely, the LCC fragments of M-ESD and A-ESD were acetylated and then directly methylated with methyl trifluoromethanesulfonate by the method of Prehm [Prehm, 1980]. After the reaction, the resulting LCC fractions were hydrolyzed, converted to alditol acetates and analyzed by g.l.c. Because none of methyl ether could be detected on the g.l.c. of both fractions, it is evident that the first acetylation was accomplished completely (Fig. 31).

After completion of the acetylation was confirmed, the binding-sites between lignin and carbohydrate was analyzed by using the DDQ-oxidation. The DDQ-oxidation of the fully acetylated LCC fragments were performed in a refluxing dichloromethane-water mixture for 2 hr below 55°C. The DDQ-oxidized fractions were then methylated by the method of Prehm [Prehm, 1980], hydrolyzed and

converted to alditol acetates (Fig. 32). G.c-m.s. analysis of the resulting methylated sugar derivatives revealed that alditol acetates from 6-*O*-methyl mannose, 6-*O*-methyl

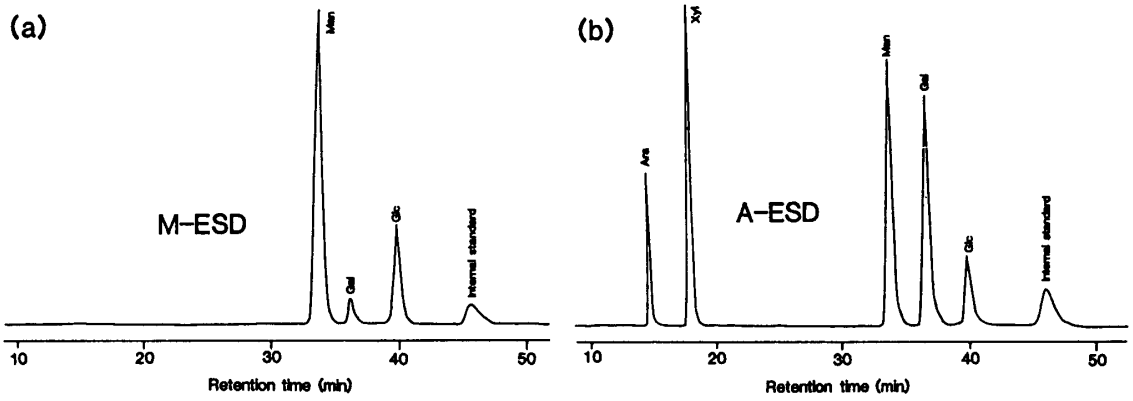


Fig. 31. Gas-liquid chromatograms of the alditol acetates originating from the LCC fragments, acetylated, methylated, and hydrolyzed, successively. Notes: (a) M-ESD; (b) A-ESD; The LCC fragments were acetylated, methylated by the mithod of Prehm, and then hydrolyzed with TFA. The resulting sugars were then converted to alditol acetate form and analyzed by g.l.c. in order to clarify whether the first acetylating process achieved completely.

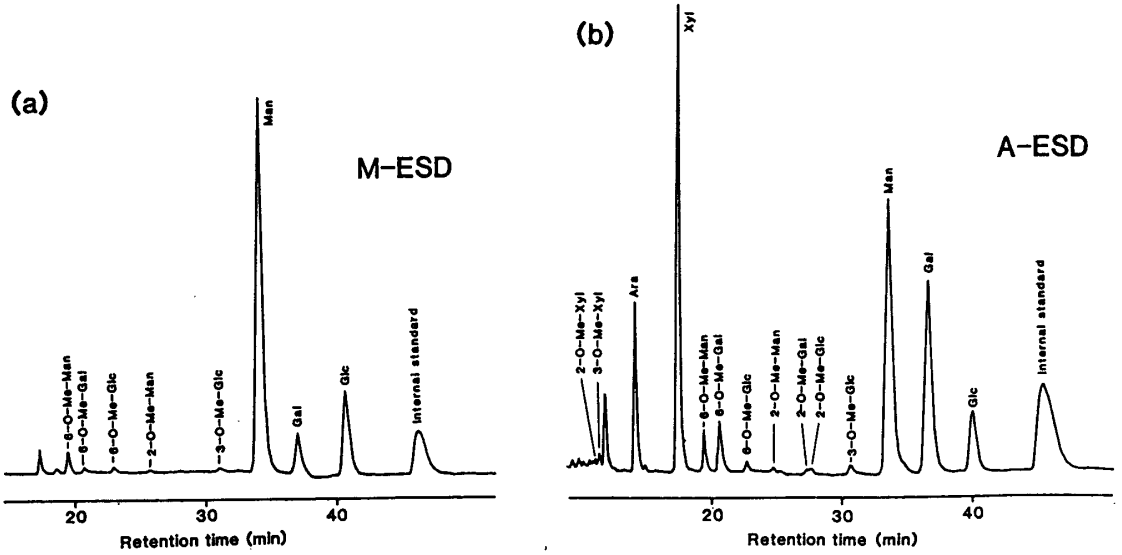


Fig. 32. Gas-liquid-chromatograms of the alditol acetates originating from the LCC fragments, acetylated, DDQ-decomposed, methylated by the method or Prehm, and hydrolyzed, successively.
Notes: (a) LCC fragments (M-ESD) from a lignin-acetylglucomannan complex (nor-C-1-M); (b) LCC fragments (A-ESD) from an acidic LCC (com-C-1-A): Non-methylated alditol acetates appeared on the chromatograms are derived from the following two kinds of carbohydrate residues; (1) carbohydrate residues glycosidically linked to the other sugar residues, (2) carbohydrate residues linked to lignin moiety, whose chemical linkages did not undergo the oxidative cleavage with DDQ, but underwent the acid hydrolysis with TFA.

Table 13. Methylated monosaccharides originating from lignin-carbohydrate bonds in LCCs isolated from normal and compression parts of *Pinus densiflora* wood.[a]

Methylated sugars	Lignin-carbohydrate complexes	
	Lignin-acetylglucomannan complex (nor-C-1-M)	Acidic lignin-hemicellulose complex (com-C-1-A)
2-Me-Xylose	0.0	2.3
3-Me-Xylose	0.0	4.5
Total xylose	0.0	6.8
2-Me-Mannose	6.0	2.3
6-Me-Mannose	44.0	28.4
Total mannose	50.0	30.7
2-Me-Galactose	0.0	2.3
6-Me-Galactose	10.0	44.3
Total galactose	16.0	46.6
2-Me-Glucose	8.0	2.3
3-Me-Glucose	12.0	4.5
6-Me-Glucose	20.0	9.1
Total glucose	40.0	15.9

a: Values are expressed as weight percentages of the total methylated sugars identified.

galactose, 6-*O*-methyl glucose and a small amount of their 2-*O*- or 3-*O*-methyl isomers existed in both methylated fractions (Table 13). 2-*O*-Methyl xylose and 3-*O*-methyl xylose were also identified in the fraction from the acidic LCC (com-C-1-A), indicating the chemical bonds between the lignin and C-2 and C-3 positions of xylose units in arabinoglucuronoxylan. Since it has been shown that 3,4-dimethoxybenzyl alcohol linked to secondary alcohols were more susceptible to the DDQ-oxidation than those linked to primary alcohols [Oikawa, 1984a], the predominance of 6-*O*-methyl hexoses indicates that acetylglucomannan and β-1,4-galactan were preferably bound to the lignin at C-6 position of the sugars. Judging from the oxidizing mechanism for DDQ, the binding position of lignin moiety is concluded to be the α position of guaiacylpropane or γ position of conjugated guaiacylpropene units [Watanabe, 1989b].

Summary

Based on the studies on DDQ-oxidation and acetyl migration, a new analytical method has been developed to determine the binding sites between lignin and carbohydrate. Successive treatment of cellulase-treated LCC fragments (M-ESD and A-ESD) with acetic anhydride/pyridine, DDQ and methyl trifluoromethanesul-

fonate gave partially methylated carbohydrates which were derived from carbohydrate moiety linked to the α position of guaiacylpropane or γ position of conjugated guaiacylpropene units accompanied by electron-donative *p*-substituents. Application of this methods to the cellulase-degraded LCC fragments from *Pinus densiflora* wood gave 9 types of monomethylated carbohydrates, indicating that acetylglucomannan and β-1,4-galactan were preferably bound to the lignin through their primary hydroxyl group, while arabinoglucuronoxylan was linked to the lignin through C-2 and C-3 positions of the xylan main chain.

Chapter III Binding-site analysis of ester linkages between lignin and carbohydrate

III-1 Introduction

Ester linkages between the carboxyl group of glucuronic acid and lignin in lignified plant cell walls have often been demonstrated by alkali degradation [Wang, 1967; Yaku, 1976; Obst, 1982; Lundquist, 1983], sodium borohydride reduction [Eriksson, 1980; Takahashi, 1988], and i.r. spectroscopy [Das, 1984] of acidic LCCs. However, each of these methods has one serious disadvantage that the binding-site in lignin units is indeterminate. Moreover, these previously proposed methods did not utilize specific reaction toward lignin skeleton. For example, existence of some kind of ester linkages in 4-*O*-methylglucuronic acid residues could be demonstrated by the sodium borohydride reduction of acidic LCCs. However, it is impossible to clarify what kind of substance was bound to the 4-*O*-methylglucuronic acid residues by this method because the reduction could proceed in any kind of 4-*O*-methyl glucuronate. Based on this concept, the author has developed a novel method to give a direct evidence for the ester linkages between the glucuronic acid and lignin by adopting a selective cleavage of substituted benzyl glucuronate with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).

In chapter II, ether type of lignin-carbohydrate linkages were analyzed by successive treatments of LCC with acetic anhydride/pyridine, DDQ and methyl trifluoromethanesulfonate. In this chapter, the author extended the DDQ-reaction to the oxidative cleavage of methoxybenzyl glucuronate in order to obtain a direct evidence for the ester linkages between the lignin and 4-*O*-methylglucuronic acid residue in wood hemicelluloses, because Kim *et al.* have already demonstrated that 2,4-dimethoxybenzyl and 2,4,6-trimethoxybenzyl esters were oxidatively decomposed by DDQ in a dichloromethane-water mixture to yield corresponding benzaldehydes [Kim, 1985]. As described in section II-2, this reaction proceeds by way of the

formation of an initial CT complex, hydride anion transfer from the benzyl ester to DDQ and subsequent attack of water to the carbonium ion intermediate. As a result, the reaction takes place in the compounds which produce those intermediate. This theory was proved by the difference of reaction rate between 4-methoxy, 2,4-dimethyoxy and 2,4,6-trimethoxybenzyl esters with DDQ [Kim, 1985]. As described in the previous section (II-2), electron-donative substituents attached to the aromatic ring of arylalkanes facilitate the DDQ-oxidation because of their delocalization effect of cation charge. In fact, the 4-methoxybenzyl ester did not undergo the oxidative cleavage while the 2,4-6-trimethoxybenzyl ester was oxidized imediately with DDQ [Kim, 1985].

Based on the reactivity of DDQ, the author developed a new analytical method for the ester linkages between lignin and the 4-*O*-methylglucuronic acid residue, and applied it to an acidic LCC fraction (C-1-A) isolated from *Pinus densiflora* wood [Watanabe, 1988a].

III-2 Experimental

General methods

Carbon-13 n.m.r. spectra of LCC model compounds were recorded at 50.3 MHz in $CDCl_3$ at room temperature. Carbon-13 n.m.r. spectrum of a DDQ-oxidized LCC fraction was recorded at 50.3 MHz in D_2O at 70°C using dioxane as an internal standard (67.4 ppm from TMS). Potentioelectric titration was done with a TOA HM-18B digital pH meter using 0.01 N NaOH (Factor 1.000) at the sample concentration of 100 mg/10 ml (C-1-A), 100 mg/10 ml (C-1-Am), and 15 mg/5 ml (Am-DH). The ambient temperature was automatically corrected. T.l.c. was done on Whatman K 5F silicagel plates with a ethyl ether/ *n*-hexane mixture (10:1, v/v) as developer.

Reaction of model compounds with DDQ

3,4-Dimethoxybenzyl (1,2,3,4-tetra-*O*-acetyl)-D-glucuronate (I) was synthesized by reaction of veratryl alcohol with 1,2,3-4-tetra-*O*-acetyl-D-glucuronic acid in the presence of N,N-dimethylforamide dineopentylacetal according to the method by Brechbuhler [Brechbuhler, 1965], ^{1}H-n.m.r. ($CDCl_3$) δ (ppm): 1.78~2.08 (12H, CH_3-CO-); 3.87, 3.89 (6H, OCH_3); 5.07 (α); 4.09~5.55 (4H, C2-C5); 5.78 (C-1α, $J_{1,2}$=7.6 Hz); 6.39 (C-1β, $J_{1,2}$=3.5 Hz); 6.82~6.94 (3H, Arom.), ^{13}C-n.m.r. ($CDCl_3$) δ (ppm): 20.2~20.7 (CH_3-CO-); 55.9 (OCH_3); 68.1~72.9 (C2~C5, Cα); 88.7 (C-1α); 91.3 (C-1β); 110.9 (Arom.-5); 112.3 (Arom.-2); 121.9 (Arom.-6); 127.1 (Arom.-1); 148.9 (Arom.-3); 149.4 (Arom.-4).

[3-Methoxy-4-(methoxymethoxyl)]-1-phenyl-2-propene-3-yl (1,2,3,4-tetra-*O*-acetyl)-D-glucuronate (II) was synthesized from methoxymethylisoeugenol [Nakamura, 1976] and 1,2,3,4-tetra-*O*-acetyl-D-glucuronic acid as in the case of the compound

(I); ^{1}H-n.m.r. ($CDCl_3$) δ (ppm): 1.96~2.19 (12H, CH_3-CO-); 3.50 (2H, -OCH_2O-); 3.91 (6H, OCH_3); 6.42 (C-1α, $J_{1,2}$=3.8 Hz); 5.78 (C-1β, $J_{1,2}$=7.6 Hz); 4.76 (2H, γ, $J_{1,2}$=6.5 Hz); 4.06~5.58 (4H, C2~C5); 6.09~6.20 (1H, β); 6.60 (1H, α, $J_{1,2}$=15.8 Hz); 6.88~7.12 (3H, Arom.), ^{13}C-n.m.r. ($CDCl_3$) δ (ppm): 20.1~20.7 (CH_3-CO-); 55.5, 55.8 (OCH_3); 66.5~71.5 (C2-C5); 72.6, 72.7 (γ); 88.4 (C-1α); 90.7 (C-1β); 94.9 (-OCH_2O-); 108.7 (Arom.-2); 109.1 (Arom.-5); 115.3, 115.8 (β); 119.8 (Arom.-6); 129.9 (Arom.-1); 135.0 (α); 146.3 (Arom.-3); 149.3 (Arom.-4).

Each of synthesized model compounds I, II, methyl glucuronate (III) and methyl 1,2,3,4-tetra-*O*-acetylglucuronate (IV) was allowed to react with its twice molar amount of DDQ in a refluxing dichloromethane-water mixture (18:1, v/v) or a refluxing benzene-water-mixture (18:1, v/v) for 5 hr and 2 hr, respectively. The reaction mixture was then fractionated and analyzed by ^{1}H-n.m.r. spectroscopy.

Binding-site analysis of a LCC

An acidic LCC (C-1-A) from normal wood of *Pinus densiflora*, whose chemical properties were described in chapter I, was methylated with diazomethane at room temperature for 48 hr in ethyl ether. After evaporation, the methylated LCC (C-1-Am) was refluxed with the same amount (mg) of DDQ for 2 hr in a benzene-water mixture (18:1, v/v), and then poured into ten times its volume of ethyl alchol. The precipitate formed was washed with the same solvent, recovered and fractionated into unadsorbed and adsorbed acidic (Am-DH) fractions by anion-exchange chromatography on Dowex 1×8 (AcO^- form). Structural analysis of the DDQ-treated acidic fraction (Am-DH) was accomplished by means of n.m.r. spectroscopy and titrimetry.

III-3 Results and Discussion

It has already been shown that DDQ decomposed 2,4-dimethoxybenzyl esters in the presence of water to yield corresponding benzaldehydes and that the cleavage of the ester proceeded by consecutive reaction steps including formation of a charge transfer (CT) complex, hydride ion transfer from the ester to DDQ and addition of water to the benzylcation intermediate [Kim, 1985] as in the case of the ether cleavage (chapter II). Therefore, this reaction must be promoted by electron-donating functional groups attached to the aromatic ring, and that the oxidation occurs selectively at such positions where those intermediates can be stabilized. The difference of reaction rates between 2,4,6-trimethoxybenzyl, 2,4-dimethoxybenzyl and 4-methoxybenzyl esters clearly supported this reaction mechanism. Actually, 3,4-dimethoxybenzyl (1,2,3,4-tetra-*O*-acetyl)-D-glucuronate (I) and [3-methoxy-4-(methoxymethoxy)]-1-phenyl-2-propene-3-yl (1,2,3,4-tetra-*O*-acetyl)-D-glucuronate (II) were decomposed by DDQ in a refluxing benzene-water mixture (18:1, v/v)

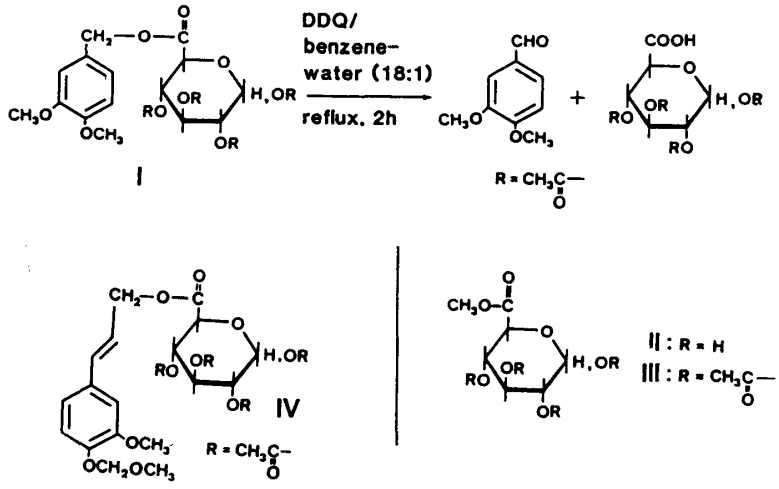


Table 14. Reaction of the glucuronates with DDQ in the presence of water.[a]

Solvents	Temp. (°C)	Reaction time (hr)	Model compounds	
			I	IV
Dichloromethane-water (18:1)	reflux	5	12.6	95.0
Benzene-water (18:1)	reflux	2	40.0	96.0

a: Values are expressed as molar percentages of the aldehydes formed by the action of DDQ.

to yield 3,4-disubstituted benzaldehydes (Table 14), while methyl glucuronate (II) and its acetate (III) were unreactive to the oxidation. The contribution of the electron-donating substituents to the DDQ-oxidation was also proved from the fact that the 2,4-disubstituted benzyl esters [Kim, 1985], were more susceptible to the DDQ oxidation than the 3,4-disubstituted benzyl ester (I). This is due to the difference of resonance substituent effect between the *o* and *m* positions. Thus, the present results supported the oxidation pathway by the hydride ion transfer mechanism.

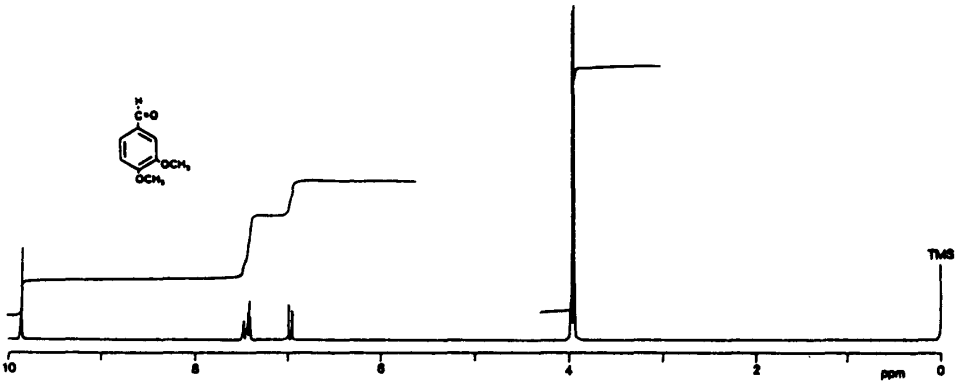


Fig. 33. Proton-n.m.r. spectrum of the aldehyde formed from 3,4-dimethoxybenzyl (1,2,3,4-tetra-*O*-acetyl)-D-glucuronate by the action of DDQ in the presence of water.

Based on the reactivities of DDQ, the author has developed a new qualitative analytical method for proving the existence of ester linkages between lignin and glucuronic acid residue in wood hemicelluloses. As shown in Fig. 34, the basis of this new method lies in the difference of reactivities between methyl glucuronate and 3,4-disubstituted benzyl glucuronate with DDQ. Because methyl glucuronate was unreactive to the DDQ-oxidation, formation of free carboxyl groups in the 4-*O*-methylglucuronic acid residues from initially diazomethane-methylated LCCs should indicate the existence of the ester linkages which were cleaved by DDQ.

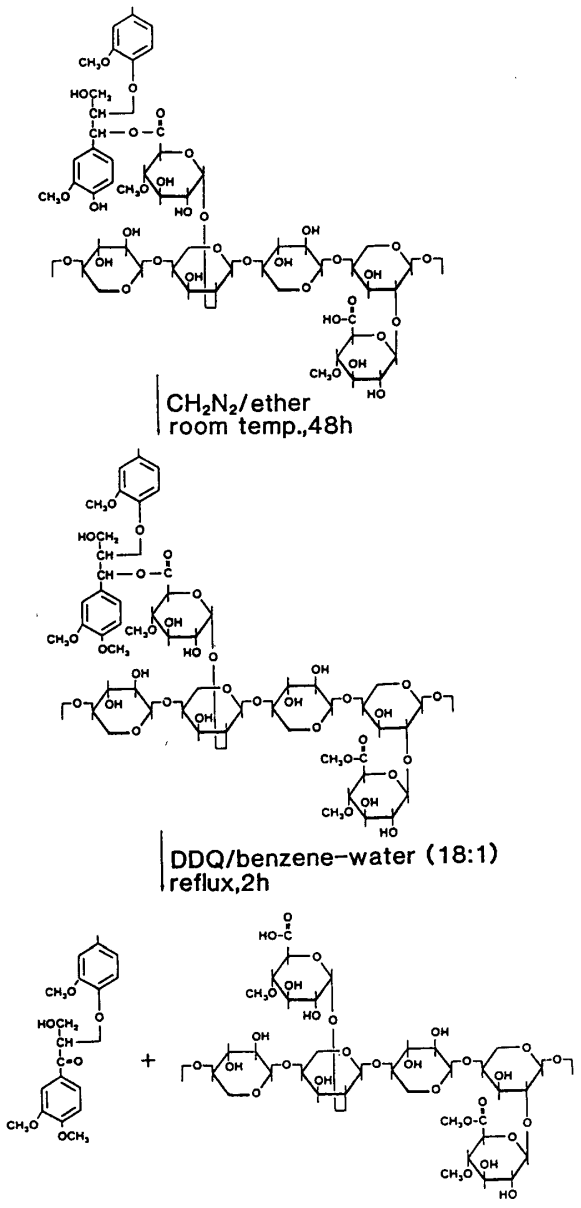


Fig. 34. Schematic representation of the new method to prove the existence of ester linkages between lignin and 4-*O*-methylglucuronic acid residue in hemicelluloses.

Based on the concept, an acidic LCC from *Pinus densiflora* wood (C-1-A), whose chemical properties were described in detail in chapter I, was methylated with diazomethane at room temperature for 48 hr. The resulting methylated LCC fraction (C-1-Am) was then oxidized with DDQ for 2 hr in the benzene-water mixture where methyl glucuronate was inert. After the reaction, an acidic fraction (Am-DH) was separated from the DDQ-treated fraction by anion-exchange chromatography on Dowex 1×8. The adsorbed acidic fraction (Am-DH) was then subjected to the structural analysis by n.m.r. spectroscopy and titrimetry. The yield of the acidic fraction (Am-DH) was 12.8% based on the diazomethane-methylated LCC fraction (C-1-Am).

Binding-site analysis of ester linkages between lignin and glucuronic acid

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C-1-A (acidic lignin-carbohydrate complex) [pH=4.84]

- CH_2N_2/ ethyl ether, room temp., 48 hr

C-1-Am [pH=7.26] (200 mg)

- DDQ/ benzene-water (18:1, v/v), reflux, 2 hr
- precipitated from ethyl alcohol
  (ppt.)
- Dowex 1 x 8 (AcO^- form)
  - .eluted with water (141.3 mg)
  - .eluted with 30% aqueous acetic acid

Am-DH [pH=2.84] (25.5 mg)

The carbon-13 n.m.r. spectrum of the DDQ-treated fraction (Am-DH) is shown in Fig. 35. Assignments of the signals indicated that most of the carbohydrate moiety in the DDQ-treated acidic fraction (Am-DH) consisted of arabinoglucuronoxylan as shown by chemical analysis (Table 15). Furthermore, it has been found that a part of 4-*O*-methylglucuronic acid residue in the DDQ-oxidized LCC fraction existed in the form of free carboxylic acid because the signals ascribed to 4-*O*-methylglucuronic acid residues were markedly observed, while the signal of

Table 15. Neutral sugar compositions of the LCC fractions.[a]

LCCs	L-Ara	D-Xyl	D-Man	D-Gal	D-Glc
C-1-A	6.7	45.6	29.8	7.9	10.0
Am-DH	3.4	78.6	6.8	5.3	5.9

a: Values are expressed as weight percentages of the total neutral sugar.

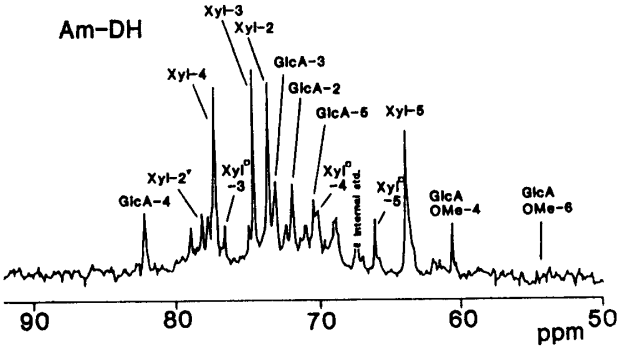


Fig. 35. Carbon-13 n.m.r. spectrum of the acidic fraction (Am-DH) formed from the diazomethane-methylated LCC (C-1-Am) by the action of DDQ.
Notes: ▲···, C-2 substituted xylose residue; □···, non-reducing xylose residue.

the methyl ester in methyl 4-*O*-methylglucuronate (54~54.5 ppm) residue [Utille, 1986; Excoffier, 1986] was indistinguishable from background noise (Fig. 35).

A titrimetric analysis of the three fractions, original LCC (C-1-A), diazomethane-methylated LCC (C-1-Am) and the DDQ-treated acidic fraction (Am-DH) indicated the structural changes of the glucuronic acid residues. The diazomethane-methylation of C-1-A changed the pH value from the acidic to neutral range, and extinguished the neutral equivalent point, indicating the complete masking on free carboxyl group of the glucuronic acid residues, while the subsequent DDQ-treatment changed the pH back to the acidic range (Fig. 36). Because methyl glucuronate was inactive to the DDQ-oxidation, the change of pH to acidic range indicates the oxidative cleavage of substituted benzyl glucuronate by DDQ.

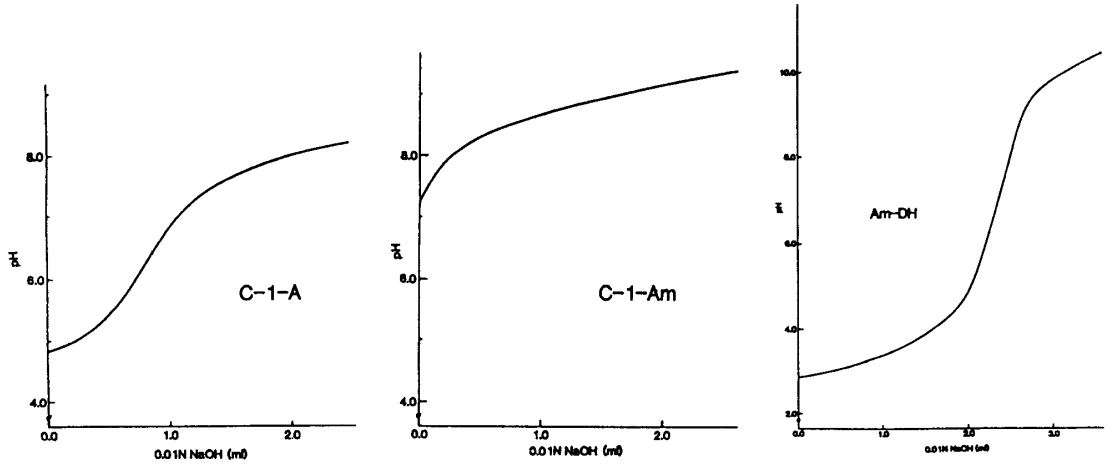


Fig. 36. Titration curves for the LCCs from *Pines densiflora* wood.
Notes: C-1-A: Acidic LCC (chapter I); C-1-Am: Diazomethane-methylated C-1-A; Am-DH: Acidic fraction formed from the diazomethane-methylated LCC (C-1-Am) by the action of DDQ.

These results lead to the conclusion that the 4-*O*-methylglucuronic acid residue in arabinoglucuronoxylan was bound to a part of the lignin by an ester linkage in *Pinus densiflora* wood. Judging from the oxidizing mechanism for DDQ, the linkage position of the glucuronic acid to lignin is deduced to be the α position of guaiacylalkane or γ position of conjugated guaiacylpropene units of the lignin skeleton.

III-4 Summary

Direct evidence for the existence of ester linkages between hydroxyl groups of lignin moiety and 4-*O*-methylglucuronic acid residue in wood hemicelluloses was obtained by a new method using a diazomethane-methylation and subsequent oxidative cleavage of substituted benzyl glucuronate with DDQ. A DDQ-treatment of a diazomethane-methylated LCC fraction from *Pinus densiflora* wood showed formation of free glucuronic acid residues. Because methyl glucuronate could not be decomposed by DDQ but the ester linkages between glucuronic acid residue and 3,4-disubstituted lignin units were cleaved by DDQ, it is concluded that lignin is bound to the hemicelluloses through a carboxyl group of the glucuronic acid residue in *Pinus densiflora* wood [Watanabe, 1988a].

Conclusion

To prove the existence of covalent bonds between lignin and carbohydrate in ligninfied plant cell walls, the author has developed several new methods for the isolation and binding-site analyses of lignin-carbohydrate complexes (LCC).

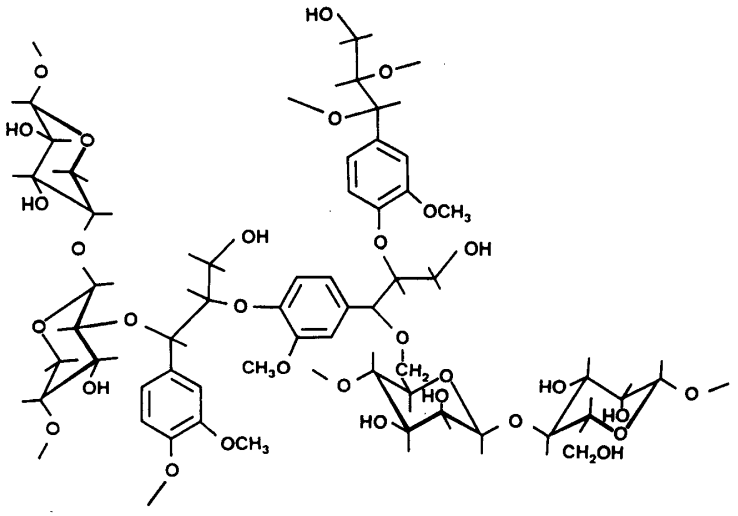
In chapter I, a new method for the isolation of water-soluble LCCs was developed. A large amount of LCC (LCC-WE) was easily extracted with cold and hot water from the wood meal previously extracted with 80% aqueous dioxane, and the chemical properties of the resulting LCC were found to be similar to those of Björkman LCC. Because this simple procedure for LCC praparation does not need the removal of nonvolatile organic solvent from the extract, the new method is recomended to be used in place of the original Björkman method [Björkman, 1957a].

In chapter II, a new method giving direct evidence for the ether linkages between lignin and carbohydrate has been developed by an oxidative cleavage of benzyl ethers with 2,3-dichloro5,6-dicyano-1,4-benzoquinone (DDQ).

Usually, frequency of lignin-carbohydrate bonds is extremely fewer than that of glycosidic bonds between sugar residues in native LCCs. Therefore, LCCs

were first hydrolyzed with cellulases, and the cellulase-degraded LCC fragments were isolated by a newly developed method, because previous separating methods including gel filtration, were ineffective to isolate LCC fragments from the enzymatic hydrolyzates of water-soluble LCCs. In this experiment, the author found the affinity of lignin component toward Toyopearl HW gel, and developed a new separating method for the isolation of the cellulase-degraded LCC fragments. As a result, two kinds of cellulase degraded LCC fragments, (1) M-ESD from lignin-acetylglucomannan complex and (2) A-ESD from an acidic LCC containing β-1,4 galactan chain, were successfully isolated by the new method of adsorption chromatography. Carbon-13 n.m.r. spectra of both LCC fragments suggested that a part of carbohydrates were linked to the lignin through non-anomeric carbon atoms of the sugars.

Existence of ether linkages between the lignin and carbohydrate in the cellulase degraded LCC fragments has been proved by a newly proposed method. Hydroxyl groups of both LCC fragments were first protected with acetyl group, and the fully acetylated LCC fragments were allowed to react with DDQ to cleave ether linkages between both components. The hydroxyl groups formed by the action of DDQ were methylated by the method of Prehm [Prehm, 1980], and the resulting methylated fraction was hydrolyzed with trifluoroacetic acid (TFA), reduced, and reacetylated. To establish this new method, the reactivities of DDQ toward a coniferous lignin acetate and the stabilities of acetyl group during the analytical procedures, were examined beforehand, by using the acetylated milled wood lignin from *Pinus densiflora* wood, LCC model compounds and partially acetylated glucose.



Scheme 5. Schemetic representation of the possible structure for the ether linkages between lignin and carbohydrate in *Pinus densiflora* wood.

Application of this method to the two types of cellulase-degraded LCC fragments, M-ESD and A-ESD, gave 9 types of mono methylated sugars, indicating that acetylglucomannan and β-1,4-galactan were preferably bound to the lignin through their primary hydroxyl groups, while arabinoglucuronoxylan was linked to the lignin through C-2 and C-3 positions of the xylan main chain.

In chapter III, a new method giving a direct evidence for the ester linkages between lignin and 4-*O*-methylglucuronic acid residue in hemicelluloses has been developed by applying the DDQ-reaction to the oxidative cleavage of substituted benzyl glucuronate. An acidic LCC (C-1-A) was first methylated with diazomethane in order to protect carboxyl groups of the glucuronic acid residues. The methylated LCC (C-1-Am) was then oxidized with DDQ, and an acidic (Am-DH) subfraction was separated from the DDQ-oxidized fraction by anion-exchange chromatography. Titration and carbon-13 n.m.r. analysis of the acidic fraction revealed that a part of the glucuronic acid residues in the DDQ-oxidized acidic fraction (Am-DH) existed in the form of free carboxylic acid. Because methyl glucuronate was not oxidized by DDQ but the ester linkages between 3,4-disubstituted benzyl alcohols and glucuronic acid were cleaved by DDQ, the formation of carboxyl group of the uronic acid from the diazomethane-methylated LCC indicates that a part of glucuronic acid residues were linked directly to the lignin through their carboxyl groups. Judging from the oxidizing mechanism for DDQ, the linkage positions in the lignin moiety are concluded to be the α position of guaiacylalkane or γ position of conjugated guaiacylpropene units of the lignin.

With respect to the frequency of lignin-sugar bonds, it should be noted that a vibratory ball-milling treatment for preparing wood components is suggested to cleave chemical linkages between wood components, because around 40% of MWL component was newly liberated from Björkman LCC by milling the LCC [Björkman, 1957d]. In other words, it is no exaggeration to say that this decomposing action enables the extraction of wood components. Therefore, frequency of lignin-sugar bonds in native wood cell walls cannot be determined by chemical analysis of extracted wood comonents. However, considering the results obtained by the water-extraction and DDQ-oxidation, it is evident that the various kinds of sugar residues are, to a considerably extent, taking part in the lignin-carbohydrate linkages through their primary hydroxyl, secondary hydroxyl and carboxyl groups, to form the amphipathic single molecules.

It is desired that some kind of estimation on the frequency of lignin-sugar bonds are to become feasible in the near future by extending this type of oxidative cleavage. To achieve this purpose, it is necessary to know the action of milling toward various types of lignin-carbohydrate linkages, and to develop more powerful

oxidant or oxidase which cleaves all types of lignin-sugar bonds quantitatively by the hydride ion transfer mechanism.

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REFERENCES

ADLER, E., BECKER, H.-D., ISHIHARA, T. and STAMVIK, A.: *Holzforschung,* **20**, 3 (1966).
AOYAGI, T., HOSOYA, S. and NAKANO, J.: *Mokuzai Gakkaishi,* **26**, 27 (1980).
AZUMA, J., TAKAHASHI, N. and KOSHIJIMA, T.: *Carbohydr. Res.,* **93**, 91 (1981).
AZUMA, J., KATO, A., WATANABE, T. and KOSHIJIMA, T.: *Proceedings of International Symp. Wood and Pulping Chem.,* **4**, 85 (1983a).
AZUMA,J. and KOSHIJIMA, T.: *Wood Res. Technical Notes,* **1983b**, 132.
AZUMA, J. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **31**, 383 (1985a).
AZUMA, J., NOMURA, T. and KOSHIJIMA, T.: *Agric. Biol. Chem.,* **49**, 2661 (1985b).
AZUMA, J., TAKAHASHI, N., ISAKA, M. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **31**, 587 (1985c).
AZUMA, J., SAKANAKA, M., MING, Z. and OKAMURA, K.: *Bull. of the Kyoto Univ. Forests,* **1988**, 319.
BITTER, T. and MUIR, H.M.: *Anal Biochem.,* **4**, 330 (1962).
BECKER, H.D. and ADLER, E.: *Acta Chem. Scand.,* **15**, 218 (1961).
BECKER, H.D.: *J.Org. Chem.,* **30**, 982 (1965).
BECKER, H.D.: *J. Org. Chem.,* **34**, 1203 (1969).
BECKER, H.D., BJÖRK, A. and ADLER, E.: *J. Org. Chem,* **45**, 1596 (1980a).

BECKER, H.D. and TURNER, A.B.: *Yukigoseikagaku,* **38**, 1163 (1980b).
BROWN, D.R. and TURNER, A.B.: *J. Chem. Soc., Perkin II,* **1975**, 1307.
BRAUDE, E.A., BROOK, A.G., and LINSTEAD, R.P.: *J. Chem. Soc.,* **1954**, 3569.
BOUVENG, H.O., LINDBERG, B. and THEANDER, O.: *Acta Chem. Scand.,* **11**, 1788 (1957).
BJÖRKMAN, A.: *Ind. Eng. Chem.,* **49**, 1395 (1957a)
BJÖRKMAN, A.: *Svensk Papperstidn.,* **59**, 477 (1957b).
BJÖRKMAN, A.: *Svensk Papperstidn.,* **60**, 158 (1957c).
BJÖRKMAN, A.: *Svensk Papperstidn.,* **60**, 243 (1957d).
BOREL, E. and DENKEL, H.: *Helv. Chim. Acta,* **36**, 801 (1953).
BOLKER, H.I. and SOMMERVILLE, N.G.: *Nature,* **197**, 489 (1963).
BRECHBUHLER, H., BUCHI, H., HALZ, E., SCHREIBER, J. and ESCHENMOSER, A.: *Helv. Chim. Acta,* **48** 1746 (1965).
CARPENTER, I., MCGARRY, E.J. and SCHEINMANN, F.: *J. Chem. Soc.,* **1956**, 3070.
DAS, N.N., DAS, S.C. and MUKHERJEE, A.K.: *Carbohydr Res.,* **127**, 345 (1984).
ENOKI,, A., YAKU, F. and KOSHIJIMA T.: *Holzforschung,* **37**, 135 (1983).
ERDMAN, J.: *Annalen,* **138**, 1 (1866).
ERIKSSON, Ö. and LINDGREN, B.O.: *Svensk Papperstidn.,* **2**, 59 (1977).
ERIKSSON, Ö. and GORING, D.A.I.: *Wood Sci. Technol.,* **14**, 267 (1980).
EBERSON, L., JONSSON, L. and WISTRAND, L.-G.: *Acta Chem. Scand.,* **33**, 413 (1979).
EXCOFFIER, G., NARDIN, R. and VIGNON, M.R.: *Carbohydr. Res.,* **149**, 319 (1986).
FECKEL, V.J. and FENGEL, D.: *Holzforschung,* **36**, 269 (1982).
FINDLAY, J.W.A. and TURNER, A.B.: *J. Chem. Soc. (C),* **1971**, 23.
FISCHER, E., BERGMANN,, M. and RABE, A.: *Chem. Ber.,* **53**, 2362 (1920).
FREUDENBERG, K. and GRION, G.: *Chem. Ber.,* **92**, 1355 (1959).
FREUDENBERG, K.: *Science,* **148**, 595 (1965).
FUJIMOTO, H., NISHIDA, H. and AJISAKA, K.: *Agric. Biol. Chem.,* **52**, 1345 (1988).
GIERER, J. and NORÉN, I.: *Holzforschung,* **36**, 123 (1982).
HAKOMORI, S.: *J. Biochem.,* **55**, 205 (1964).
HAWORTH, W.H., HIRST, E.L. and TEECE, E.G.: *J. Chem. Soc.,* **1931**, 2858.
HEMMINGSON, J.A.: *Aust. J. Chem.,* **32**, 225 (1979).
HIRSCH, J., KOVAC, P. and PETRAKOVA, E.: *Carbohydr. Res.,* **106**, 203 (1982).
IVERSEN, T. and WANNSTORM, S.: *Holzforschung,* **40**, 19 (1986).
IWAMURA, J.: *Nippon Kagakukaishi,* **1978**, 1307.
JONIAK, D., KOŠÍKOVÁ B. and MLYNAR, J.: *Cellulose Chem. Technol.,* **21**, 121 (1987).
JOSELEAU, J.P. and KESRAOUI, R.: *Holzforschung,* **40**, 163 (1986).
KATAYAMA, Y., MOROHOSHI, N. and HARAGUCHI, T.: *Mokuzai Gakkaishi,* **26**, 414 (1980).
KAWAMURA, K. and HIGUCHI, T.: *Seni Gakkaishi,* **8**, 335 (1952).
KATO, A, AZUMA, J. and KOSHIJIMA, T.: *Holzforschung,* **38**, 141 (1984).
KATO, A., AZUMA, J. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **33**, 487 (1987).
KIEFER, E.F. and LUTZ, F.E.: *J. Org. Chem.,* **37**, 1519 (1972).
KIM, C.U. and MISCO, P.F.: *Tetrahedron Lett.,* **26**, 2027 (1985).
KOMURA, H., MATSUMOTO, A., ISHIDO, Y., KUSHIDA, K. and AOKI, K.: *Carbohydr. Res.,* **65**, 271 (1978).
KONDO, R. and IMAMURA, H.: *Mokuzai Gakkaishi,* **34**, 609 (1988a)
KONDO, R., IIMORI, T. and IMAMURA, H.: *Mokuzai Gakkaishi,* **34**, 724 (1988b).
KOSHIJIMA, T. and TANAKA, R.: *Cellulose Chem. Technol.,* **6**, 609 (1971).
KOSHIJIMA, T., TANIGUCHI, T. and TANAKA, T.: *Holzforschung,* **26**, 211 (1972).
KOSHIJIMA, T., YAKU, F. and FUKUBE, K.: *Mokuzai Gakkaishi,* **20**, 238 (1974).
KOSHIJIMA, T., YAKU, F. and TANAKA, R.: *Applied Polymer Simposium,* **28**, 1025 (1976).
KOSHIJIMA, T., WATANABE, T. and AZUMA, J.: *Chem. Lett.,* **1984**, 1737.
KOSHIJIMA, T., OHNISHI, J. and WATANABE, T.: *Proceedings of International Symp. on Wood and Pulping Chem.,* 623 (1989).

Košíková, B., Joniak, D. and Kosáková, L.: *Holzforschung,* **33**, 11 (1979).
Leary, G.J., Sawtell, D.A. and Wong, H.: *Holzforschung,* **37**, 11 (1983).
Lee, H and Harvey, R.G.: *J. Org. Chem.,* **48**, 749 (1983).
Lemieux, R.U. and Huber, G.: *Can. J. Chem.,* **31**, 1040 (1953).
Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: *J. Biol. Chem.,* **193**, 265 (1951).
Lüdemann, H.-D. and Nimz, H.H.: *Macromol. Chem.,* **175**, 2393 (1974).
Lundquist, K., Simonson, R. and Tingsvik, K.: *Svensk Papperstidn.,* **86**, 44 (1983).
Lutz, F.E. and Kiefer, E.F.: *Tetrahedron Lett.,* **11**, 4851 (1970a).
Lutz, F.E. and Kiefer, E.F.: *J. Chem. Soc. (C),* **1970b**, 1722.
Mastronardi, I.O., Flemtti, S.M., Deferrari, J.O. and Gros, E.G.: *Carbohydr. Res.,* **3**, 177 (1966).
Matsukura, M. and Sakakibara, A.: *Mokuzai Gakkaishi,* **15**, 1969.
McCleary, B.V., Taravel, F.R. and Cheetham, N.W.H.: *Carbohydr. Res.,* **104**, 285 (1982).
Merewethr, J.W.T. and Samsuzzaman, L.A.M.: *Holzforschung,* **26**, 11 (1972).
Meshitsuka, G., Lee, Z.Z., Nakano, J. and Eda, S.: *J. Wood Chem. Technol.,* **2**, 251 (1982).
Minor, J.L.: *J. Wood Chem. Technol.,* **2**, 1 (1982).
Minor, J.L.: *J. Wood Chem. Technol.,* **6**, 185 (1986).
Mörck, R. and Kringstad, K.P.: *Holzforschung,* **39**, 109 (1985).
Morohoshi, N. and Sakakibara, A.: *Mokuzai Gakkaishi,* **17**, 354 (1971).
Morrison, I.M.: *Phytochem.,* **12**, 2979 (1973).
Mukoyoshi, S., Azuma, J. and Koshijima, T.: *Holzforschung,* **35**, 233 (1981).
Muller, J.F., Cagniant, D. and Cagniant, P.: *Tetrahedron Lett.,* **12**, 45 (1971).
Murata, I., Yamamoto, K., Hirotsu, T. and Morioka, M.: *Tetrahedron Lett.,* **13**, 331 (1972).
Nakakuki, T. and Kainuma, K.: *J. Jap. Soc. Starch Sci.,* **29**, 27 (1982).
Nakamura, Y. and Higuchi, T.: *Wood Res.,* **1976**, 101.
Nakatsubo, F., Kirk, T.K. and Shimada, M.: Higuchi, T.: *Arch. Microbiol.,* **128**, 416 (1981).
Nimz, H.H., Robert, D., Faix, O. and Nemr, M.: *Horzforschung,* **35**, 16 (1981).
Nimz, H.H., Tschirner, U., Stahle, M., Lehmann, R. and Schlosser, M.: *J. Wood Chem. Technol.,* **4**, 265 (1984).
Obst, J.R.: *Tappi,* **65**, 109 (1982).
Ohki, A., Nishiguchi, T. and Fukuzumi, K.: *Tetrahedron,* **35**, 1737 (1979).
Oikawa, Y. and Yonemitsu, O.: *Heterocycles,* **5**, 233 (1976).
Oikawa, Y., Yoshioka, T., Mohri, K. and Yonemitsu, O: *Heterocycles,* **11**, 1457 (1979).
Oikawa, Y., Yoshioka, T. and Yonemitsu, O: *Tetrahedron Lett.,* **23**, 885 (1982a).
Oikawa, Y., Yoshioka, T. and Yonemitsu, O.: *Tetrahedron Lett.,* **23**, 889 (1982b).
Oikawa, Y., Nishi, T. and Yonemitsu, O: *Tetrahedron Lett.,* **24**, 3635 (1983a).
Oikawa, Y., Nishi, T. and Yonemitsu, O.: *Tetrahedron Lett.,* **24**, 4037 (1983b).
Oikawa, Y., Tanaka, T., Horita, K., Yoshioka, T. and Yonemitsu, O: *Tetrahedron Lett.,* **25**, 5393 (1984a).
Oikawa, Y., Tanaka, T., Horita, K., Yoshioka, T. and Yonemitsu, O: *Tetrahedron Lett.,* **25**, 5397 (1984b).
Oikawa, Y., Nishi, T. and Yonemitsu, O.: *J. Chem. Soc. perkin Trans. I,* **1985a**, 7.
Oikawa, Y., Nishi, T. and Yonemitsu, O.: *J. Chem. Soc. perkin Trans. I,* **1985b**, 19.
Oikawa, Y., Nishi, T. and Yonemitu, O.: *J. Chem. Soc. perkin Trans. I,* **1985c**, 27.
Padwa, A. and Pulwer, M.: *J. Amer. Chem. Soc.,* **102**, 6388 (1980).
Patt, S.L. and Shooly, J.N.: *J. Magn. Reson.,* **46**, 535 (1982).
Pfeffer, P.E., Valentine, K.M. and Parrish, F.W.: *J. Am. Chem. Soc.,* **101**, 1265 (1979).
Prehm, P. *Carbohydr. Res.,* **78**, 372 (1980).
Ralph, J. and Young, R.A.. *J. Wood Chem. Technol.,* **3**, 161 (1983).
Reynolds, D.D. and Evans, W.L.: *Org. Syn. Coll. Vol.,* **3**, 432 (1965).
Sadler, I.H. and Stewardt, J.A.G.: *J. Chem. Soc. (C),* **1969**, 773.

SMELSTORIUS, J.A.: *Holzforschung,* **28**, 99 (1974).
SORVARI, J., SJOSTROM, E., KLEMOLA, A. and LAINE J.E.: *Wood Sci. Technol.,* **20**, 35 (1986).
TAKAHASHI, N., AZUMA, J. and KOSHIJIMA, T.: *Carbohydr. Res.,* **107**, 161 (1982).
TAKAHASHI, N. and KOSHIJIMA, T.: *Wood Sci. Technol.,* **22**, 231 (1988).
TAKANO, T.:NAKATSUBO, F. and MURAKAMI, K.: *Mokuzai Gakkaishi,* **33**, 667 (1987).
TANAHASHI, M., AOKI, T. and HIGUCHI, T.: *Mokuzai Gakkaishi,* **27**, 116 (1981).
TANAKA, K., NAKATSUBO, F. and HIGUCHI, T.: *Mokuzai Gakkalshi,* **22**, 589 (1976).
TANAKA, K., NAKATSUBO, F. and HIGUCHI, T.: *Mokuzai Gakkaishi,* **25**, 653 (1979).
TANAKA, R. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **18**, 403 (1972).
TANAKA, R., YAKU, F., IYODA, J., KOSHIJIMA, T. and AZUMA, J.: *Mokuzai Gakkaishi,* **31**, 859 (1985).
TANEDA, H., NAKANO J., HOSOYA, S. and CHANG, H.M.: *J. Wood Chem.* Technol., **7**, 485 (1987).
TERATANI, F. and YAMATANI, O.: *Bull. Fac. Agr. Shizuoka Univ.,* **1977**, 47.
THIELE, J. and GÜNTHER, F.: *Ann,* **349**, 45 (1906).
TROST, B.M.: *J. Am. Chem. Soc.,* **89**, 1847 (1969).
UMEZAWA, T., NAKATSUBO, F. and H IGUCHI, T.. *Arch. Microbiol,* **131**, 124 (1982).
USUI, T., YAMAOKA, N., MATSUDA, K., TSUJIMURA, K., SUGIYAMA, H. and SETO, S.: *J. Chem. Soc., Perkin Trans. I,* **1973**, 2425.
UTILLE, J.-P.: *Carbohydr. Res.,* **154**, 251 (1986).
WALLENFELS, K., BECHTLER, G., KUHN, R., TRISCHUMANN, H. and EGGE, C.: *Angew, Chem. Intern. Ed.,* **2**, 511 (1963).
WANG, P.Y., BOLKER, H.I. and PURVES, C.B.: *Tappi,* **50**, 123 (1967).
WATANABE, T., AZUMA, J. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **31**, (1985).
WATANABE, T., KAIZU, S. and KOSHIJIMA, T.: *Chem. Lett.,* **1986**, 1871.
WATANABE, T., AZUMA, J. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **33**, 798 (1987a).
WATANABE, T., KOSHIJIMA, T. and AZUMA, J.: *Proceedings of International Symp. on Wood and Pulping Chem.,* **2**, 45 (1987b).
WATANABE, T. and KOSHIJIMA, T. *Agric. Biol. Chem.,* **52**, 2953 (1988a).
WATANABE, T. and KOSHIJIMA, T.: *Wood Res.,* **1988b**, 13.
WATANABE, T. and KOSHIJIMA, T.: *Mokuzai Gakkaishi,* **35**, 130 (1989a).
WATANABE, T., OHNISHI, J., YAMASAKI, Y., KAIZU, S. and KOSHIJIMA, T.: *Agric. Biol. Chem.,* **53**, 2233 (1989b).
WOOD, H.B.Jr. and FLETCHER, H.G.Jr.: *J. Am. Chem. Soc.,* **78**, 207 (1956).
YAKU, F. and KOSHIJIMA. T.: *Mokuzai Gakkaishi,* **17**, 267 (1971).
YAKU, F., YAMDA, Y. and KOSHIJIMA, T.: *Holzforschung,* **30**, 148 (1976).
YAKU, F. TSUJI, S. and KOSHIJIMA, T.: *Holzforschung,* **33**, 54 (1979).
YAKU, F., TANAKA, R. and KOSHIJIMA, T.: *Holzforschung,* **35**, 177 (1981).
YAMAGUCHI, A.: *Mokuzai Gakkaishi,* **19**, 29 (1973).
YOSHIMOTO, K. and TSUDA, Y.: *Chem. Pharm. Bull.,* **31**, 4335 (1983).