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## Lignin fragments rich in detached side-chain structures found in water-soluble LCC

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**Abstract** The content of the glyceraldehyde-2-aryl ether-type structure in water-soluble lignin-carbohydrate complex (LCC) fractions, which were obtained from Japanese cedar and birch residual wood meal after milled wood lignin isolation, was determined by ozonation. Quite high amounts of the glyceraldehyde-2-aryl ether-type structure were found in water-soluble LCC fractions of both species; about 3–5 times higher than those of other fractions. This result, as well as the high content of the  $\beta$ -1 structure in these fractions shown in our previous reports, suggest that lignin in these fractions has the characteristics of endwise-type lignin, because the glyceraldehyde-2-aryl ether-type structure and  $\beta$ -1 structure are typical characteristics of this type of lignin fraction. These results are in good agreement with the generally accepted hypothesis that the glyceraldehyde-2-aryl ether-type structure and  $\beta$ -1 structure are generated at the same time by a radical coupling reaction. The results also indicated that these two structures are present in close proximity in the lignin.

**Key words** Endwise-type lignin · Lignin-carbohydrate complex · Glyceraldehyde-2-aryl ether-type structure ·  $\beta$ -1 Structure · Detached side-chain structures

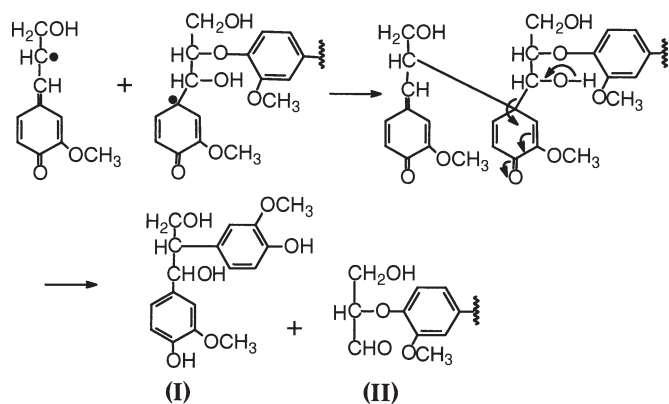
### Introduction

Several linkage types are known to be present in lignin. Although they are minor linkage types, the  $\beta$ -1 structure (I) and glyceraldehyde-2-aryl ether-type structure (II) are interesting because the presence of these structures is thought to be the typical of “endwise-type lignin”.<sup>1</sup> The

glyceraldehyde-2-aryl ether-type structure is believed to be generated as a counterpart of the  $\beta$ -1 structure during lignin biosynthesis. This structure is one of the “detached side-chain structures” and is only one structure of this type whose presence in lignin is accepted by many researchers. Even though these structures had been once regarded as major linkage types in lignin,<sup>2</sup> Lundquist and Olsson<sup>3</sup> denied their presence in spruce milled wood lignin (MWL) as a major linkage type, according to results of proton nuclear magnetic resonance (<sup>1</sup>H-NMR) analysis. Later, Matsumoto et al.<sup>4</sup> reported that the amount of this structure was only 0.016/C<sub>6</sub>-C<sub>3</sub> for spruce and 0.014/C<sub>6</sub>-C<sub>3</sub> for beech wood meals as determined by ozonation. By the same method, Eom et al.<sup>5</sup> reported an even lower amount of this structure in wood meal. In the case of the  $\beta$ -1 structure, the evidence for its presence was mainly obtained by several degradation analyses. Although degradation products corresponding to the  $\beta$ -1 structure are not minor products among those obtained by these degradation analyses, NMR analysis of MWL could not confirm the presence of this structure as a major linkage type (<5%).<sup>6,7</sup> Brunow et al.<sup>8</sup> reported that thioacidolysis of a lignin-like substance, which was released from suspension-cultured cells,<sup>9</sup> yielded significant amounts of dimeric degradation products from the  $\beta$ -1 structure,<sup>8</sup> but no evidence for the presence of this structure was obtained by NMR analysis.<sup>10,11</sup> As one of the reasons for these discrepancies between the degradation and NMR analyses, Lundquist<sup>12</sup> and Brunow et al.<sup>13</sup> speculated on the presence of hidden forms of this structure (such as cyclohexadienone). Recently, Ralph et al.<sup>14</sup> suggested the presence of a new  $\beta$ -1 structure with a six-membered ring containing an original side chain migrated to the 6 position of an aromatic ring from NMR analysis of pine MWL. The formation of such an isochroman structure suggests the presence of one of the cyclohexadienone-type hidden forms<sup>13</sup> as a progenitor of the  $\beta$ -1 structure.<sup>14,15</sup>

It is unknown whether the  $\beta$ -1 structure and glyceraldehyde-2-aryl ether-type structure are present in close proximity. These two structures are thought to be generated at the same time by the coupling reaction between a cinnamyl alcohol monomer radical and an already

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**Fig. 1.** Formation mechanism for the  $\beta$ -1 structure (I) and the glyceraldehyde-2-aryl ether-type structure (II)

formed lignin polymer (higher than dimer) radical during lignin biosynthesis (Fig. 1).<sup>1</sup> According to its formation mechanism, the glyceraldehyde-2-aryl ether-type structure remains at the end of the lignin polymer and the  $\beta$ -1 structure is released as a dimer. It is not known whether the  $\beta$ -1 dimer is incorporated again in the lignin polymer in the vicinity of the glyceraldehyde-2-aryl ether-type structure by a further coupling reaction.

In order to form detached side-chain structure, it is necessary to have a hydroxyl group at the  $\alpha$ -position of the polymer radical site during its biosynthesis (Fig. 1). As a candidate for such a structure at the polymer site in lignin, the  $\beta$ -O-4 structure is the most probable. Based on this consideration, the glyceraldehyde-2-aryl ether-type structure could be most abundant among detached side chain-type structures because it is generated from a  $\beta$ -O-4 type structure (Fig. 1). The glyceraldehyde-2-aryl ether-type structure and  $\beta$ -1 structure are expected to be accumulated in the lignin where the  $\beta$ -O-4 structure is abundant, namely in endwise-type lignin.

In previous studies,<sup>16,17</sup> the water-soluble lignin-carbohydrate complexes (LCCs) were isolated from Japanese cedar and birch residual wood meals after MWL isolation. Ozonation analysis suggested that relatively high content of the  $\beta$ -1 structure was present in these fractions when compared with other fractions. In order to confirm this, the same water-soluble LCCs were subjected to the determination of the glyceraldehyde-2-aryl ether-type structure. Structural characteristics of lignin in these fractions are discussed in this article.

## Experimental

### Preparation of samples

Detailed procedures for the preparation of the materials used in this experiment were described in previous reports.<sup>16,17</sup>

Crude MWL (CMWL) was obtained by dioxane-water (96:4, v/v) extraction of ball-milled Japanese cedar (*Cryp-*

*toemia japonica*) and birch (*Betula maximowicziana*) wood meals. The residues of the CMWL extraction were designated as MWLR. Purified MWL was prepared from CMWL according to Björkman's method.<sup>18</sup> The water-soluble part of birch CMWL (MWLW) was prepared according to the previous report.<sup>17</sup>

Fractions WS and DMF were prepared from water and dimethylformamide extracts of MWLR, respectively, and the residue of the dimethylformamide extraction were designated as DMFR.

### Ozonation and determination of glycerol

Samples containing 1–10 mg of lignin were suspended or dissolved in 10 ml of water and treated with the same amount of  $\text{NaBH}_4$  at room temperature overnight. Acetic acid was added to decompose the excess  $\text{NaBH}_4$  and the mixture was evaporated to dryness. The steps of an addition of a small amount of methanol and evaporation to dryness were repeated five times. Each sample was dissolved or suspended in 30 ml of ozonation solvent consisting of acetic acid/water/methanol (16:3:1 v/v/v) and bubbled with oxygen containing ozone (ca. 3%) at a rate of 0.5 l/min for 3 h at 0°C with stirring. After the ozone treatment, residual ozone was removed by bubbling with oxygen. The reaction mixture was evaporated to dryness and the trace amount of acetic acid was removed by repeated evaporation with water. The ozonation products were saponified with 20 ml of 0.1 M NaOH solution at room temperature overnight. After neutralization with about 2.0 ml of 1 M acetic acid solution, a calculated amount of erythritol was added as aqueous solution as an internal standard and the insoluble material was removed by filtration. The filtrate was evaporated to dryness and dried in vacuo at room temperature for 1 h. The residue was acetylated with a mixture of pyridine (5 ml) and acetic anhydride (5 ml) at room temperature overnight. The solvent was removed by evaporation and the residue was extracted with chloroform several times. After the removal of the insoluble residue by filtration, the extract was washed with water. The organic layer was concentrated to a minimum volume by evaporation and with a dry  $\text{N}_2$  stream. A part of the solution was analyzed for glycerol by gas chromatography.<sup>4</sup> The calibration curve for glycerol was prepared separately.

## Results and discussion

In this study, the glyceraldehyde-2-aryl ether-type structure was determined as glycerol by ozonation method according to the method of Matsumoto et al.<sup>4</sup> (Fig. 2) with some modification.

The results for Japanese cedar and birch are listed in Tables 1 and 2, respectively. The ratio of *erythro* to *threo* types of 3-hydroxy-2-hydroxymethyl butanedioic acid ( $E'/T'$ ) and estimated yield of these acids from lignin ( $E' + T'$ ), both of which were reported in previous studies,<sup>16,17</sup> are

**Table 1.** Amount of glycerol generated from each fraction of Japanese cedar by ozonation treatment

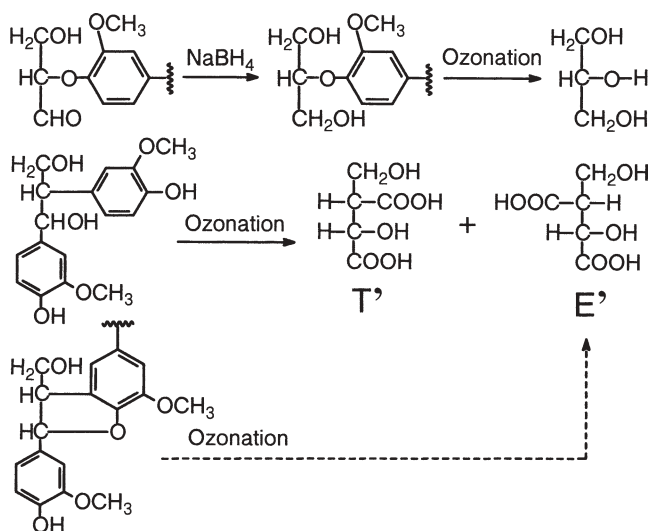
Sample	Glycerol (%) <sup>a</sup>	E'/T' <sup>b</sup>	E' + T' <sup>c</sup>
MWLR	0.32	3.89	0.94
WS	1.20	0.98	2.32
WSH	0.91	1.11	2.56
WSL	1.12	1.34	0.85
DMF	0.32	3.52	1.04
DMFR	0.33	4.02	0.85
MWL	0.45	3.35	1.07

MWLR, residue from dioxane – water extraction of crude milled wood lignin; WS, water-soluble extract of MWLR; WSH, high molecular weight fraction of WS; WSL, low molecular weight WS; DMF, dimethylformamide-soluble extract of MWLR; DMFR, residue from dimethylformamide extraction of MWLR; MWL, purified milled wood lignin

<sup>a</sup> On Klason lignin

<sup>b</sup> The ratio of *erythro* and *threo* types of 3-hydroxy-2-hydroxymethyl butanedioic acid<sup>16</sup>

<sup>c</sup> Estimated amount of *erythro* and *threo* types of 3-hydroxyl-2-hydroxymethyl butanedioic acid produced from lignin<sup>17</sup>

**Fig. 2.** Reaction products by ozonation of glycerinaldehyde-2-aryl ether-type structure (*top*),  $\beta$ -1 structure (*middle*), and  $\beta$ -5 structure (*bottom*)

also presented in Tables 1 and 2. These acids are ozonation products of *erythro* and *threo* structures carrying a C-aryl linkage at the  $\beta$ -position, of which  $\beta$ -5 and  $\beta$ -1 structures are known to be present in lignin (Fig. 2). As shown in Tables 1 and 2, the WS fraction series (fractions WS, WSH, WSL) of both species exhibit about 3–5 times higher yields of glycerol than other fractions.

The glycerinaldehyde-2-aryl ether-type structure and  $\beta$ -1 structure are thought to be generated at the same time by the mechanism proposed in Fig. 1, but there is no evidence to support this hypothesis to date. The results obtained here could confirm this generally accepted hypothesis, because enrichment of both the glycerinaldehyde-2-aryl ether-type structure and  $\beta$ -1 structure, which is suggested from a low E'/T' ratio and high E' + T'<sup>16,17</sup> in Tables 1 and 2, occurs in the same fraction, namely WS and WSH fractions. It was

**Table 2.** Amount of glycerol generated from each fraction of birch by ozonation treatment

Sample	Glycerol (%)	E'/T'	E' + T'
Wood meal	0.27	3.42	0.37
MWLR	0.27	2.76	0.36
WS	1.42	1.30	0.47
WSH	1.48	1.65	0.56
WSL	1.15	0.71	0.37
DMF	0.35	2.10	0.35
DMFR	0.26	2.85	0.38
MWL	0.39	3.10	0.32
CMWL	0.27	2.45	0.35
MWLW	0.45	2.13	0.48

CMWL, crude milled wood lignin; MWLW, water-soluble extract of CMWL

also unknown whether the  $\beta$ -1 structure and the glycerinaldehyde-2-aryl ether-type structure are present in close proximity, because the glycerinaldehyde-2-aryl ether-type structure remains at the end of the lignin polymer while the  $\beta$ -1 structure is released as a dimer after its coupling reaction. It is very interesting to note that the WS fraction series were rich not only in the glycerinaldehyde-2-aryl ether-type structure but also in the  $\beta$ -1 structure. If the  $\beta$ -1 structure remains as a dimer or very low molecular weight compound, these compounds must have been already lost at the preceding MWL preparation stage. The fact that both the  $\beta$ -1 structure and glycerinaldehyde-2-aryl ether-type structure are found in higher amounts in fractions of the WS series than other fractions indicates that these structures exist in close proximity in lignin.

As stated in the introduction, the  $\beta$ -O-4 structure might be much more frequently involved in the formation of detached side-chain structures than other linkage types, and thus, the glycerinaldehyde-2-aryl ether-type structure and  $\beta$ -1 structure could be found more frequently in endwise-type lignin. On the other hand, the mechanism that is widely accepted for the formation of lignin-carbohydrate (L-C) linkages is nucleophilic addition of hydroxyl groups in carbohydrate to  $\beta$ -O-4 type quinone methides. Therefore, it is expected that endwise-type lignin could have more chances to form L-C linkages. On the basis of these considerations, it seems reasonable to suggest that lignin in the WS fraction has endwise characteristics, because lignin in this fraction was demonstrated to be the small fragments attached to high molecular weight polysaccharides.<sup>16,17</sup> However, there are several points that have to be solved. These are the molecular weight of lignin in the WS fractions and the frequency of  $\beta$ -O-4 structures. In previous studies,<sup>16,17</sup> it was suggested that the lignin in the WS fraction is present as small lignin fragments. This is not consistent with the general idea of endwise-type lignin. It has to be assumed that lignin in WS fractions was produced in a circumstance favorable for the formation of endwise-type lignin, but for some reason could not grow to a high molecular weight, or was separated from the main body of endwise lignin during the milling process.

Regarding the frequency of the  $\beta$ -O-4 structure in WS fractions, although the critical evidence for the enrichment

of the  $\beta$ -O-4 structure in these fractions could not be obtained using ozonation analysis,<sup>17</sup> it should be noted that the WSH fraction showed relatively high yields of the main oxidation products for both species<sup>16,17</sup> and a high syringyl/guaiacyl (S/V) ratio for birch by alkaline nitrobenzene oxidation.<sup>17</sup> If these results could be taken as an indication of the enrichment of the  $\beta$ -O-4 structure, all the structural data become consistent with the concept of endwise-type lignin.

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