Studies on p-Hydroxyphenyl- and Syringyl Lignins

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STUDIES ON p-HYDROXYPHENYL- AND SYRINGYL LIGNINS

Toru Yamasaki

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References
I. Preface

Lignin occurs widely in the tissues of terrestrial vascular plants in great amounts equal to starch and next to cellulose. It is a natural high molecular compound produced by peroxidase-catalyzed dehydrogenative polymerization of coniferyl, sinapyl, and p-coumaryl alcohols. Lower terrestrial plant (club mosses and ferns)- and gymnosperm lignins are mostly composed of guaiacylpropane units, whereas angiosperm lignin is composed of guaiacylpropane and syringylpropane units, and grass and bamboo lignins, the both units and p-hydroxyphenylpropane units. No lignin has been found in bacteria, fungi, algae, lichens, and bryophytes.

Knowledge of lignins has been developed by numerous scientific workers, especially, by P. Klason, K. Freudenberg, H. Erdtman, H. Hibbert, and E. Adler in relation to the pulp and paper industry. Freudenberg confirmed that lignin is a dehydrogenation polymer of coniferyl alcohol or its related alcohols as suggested by Klason and Erdtman, and established the chemical scheme of spruce lignin over almost 30 years of his life. Neish and other plant biochemists have been trying to elucidate the biosynthesis of lignin monomers in higher plants.

However, the systematic investigation of angiosperm lignins is in rather slow progress. This may be due to the following two reasons that the pulp and paper industry was in need of softwoods in former, and that hardwood lignin is a complex dehydrogenation copolymer of coniferyl and sinapyl alcohols.

The present investigation comprises the following subjects.
1. Chemical properties of dehydrogenation polymer of p-coumaryl alcohol
2. Characterization of p-hydroxyphenyl component in the polymeric system of grass and bamboo lignins
3. Dehydrogenation polymer of sinapyl alcohol by peroxidase and hydrogen peroxide
4. Possible occurrence of syringyl lignin in nature

The amount of p-coumaric acid esterified to alcoholic hydroxyl groups of the side chain of grass and bamboo lignins is estimated to be 5 to 10% of these lignins. On the other hand p-hydroxyphenylglycerol-β-aryl ether moiety of these lignins is found to be quite minor. Therefore it is conceivable that various condensed p-hydroxyphenyl components in the polymeric system of the lignins probably occur in a considerable amount. However the condensed p-hydroxyphenyl moieties of grass and bamboo lignins have not yet been investigated. In the present investigation a considerable amount of a biphenyl and two diphenyl ether carboxylic acids composed of p-hydroxyphenyl and guaiacyl units, which could not be found from soft- and hardwoods lignins, were detected in the permanganate degradation products by gas-liquid chromatography. And a peculiar structural feature of grass and bamboo lignins is discussed in relation to chemical properties of dehydrogenation polymer of p-coumaryl alcohol (p-DHP).

It has been known that angiosperm lignins show the variation of the ratio of syringyl unit to guaiacyl within the same species, by tissue and cell wall. It is conceivable that
the variation is due to the modes of biosynthesis of sinapyl alcohol and coupling of the radicals of the lignin monomers, especially sinapyl alcohol. For the former problems roles of S-adenosylmethionine-catechol O-methyltransferase(6, 7) and ferulic acid 5-hydroxylase(8) is being actively studied. For the latter Sarkanen(9) has proposed the view that the nature of lignins (end-wise- and bulk polymers) is effected by the stationary concentration of monomer radicals, on the basis of difference between the two dehydrogenation procedures (Zutropf- and Zulauf methods) by Freudenberg(10).

The latter half of the present investigation has been performed to establish the occurrence of syringyl lignin in angiosperm tissues. Firstly formation of dehydrogenation polymer from sinapyl alcohol alone (s-DEIP) was confirmed. Secondly a distinct difference of the solubility in acetic acid between mercurated s-DHP and dehydrogenation polymer of coniferyl alcohol (c-DHP) could be found, and s-DHP was separated from a mercurated mixture of s- and c-DHPs. Finally syringyl unit-rich lignin could be isolated and characterized from beech and Yamamomo woods by the application of mercuration.

II. \( p \)-Hydroxyphenylpropane unit of grass lignin

1. Introduction

As early as 1943 Hachihama and Jodai(11) isolated and identified 1-propyl-4-hydroxybenzene in addition to 1-propyl-3-methoxy-4-hydroxybenzene and 1-propyl-3, 5-dimethoxy-4-hydroxybenzene from hydrogenolysis products of bagasse hydrochloric acid lignin, and proposed the view that \( p \)-hydroxyphenylpropane unit is one of main building units of grass lignin. At the same time Creighton and Hibbert(12) obtained \( p \)-hydroxybenzaldehyde from products of alkaline nitrobenzene oxidation of corn stalks, and proposed the similar view. \( p \)-Hydroxybenzaldehyde was also detected in the oxidation products of gymnosperms and angiosperms by Nord et al.(13), Leopold et al.(14) and Bland(15). On the other hand Goldschmid(16) and Higuchi(17) found a small amount of \( p \)-hydroxycinnamaldehyde in aqueous hydrolysis products of western hemlock and bamboo, respectively. Furthermore Kratzl and Schweers(18) obtained a small amount of \( p \)-hydroxyphenylpropanones from ethanolysis products of beech cuproxam lignin and p-DHP. Thus \( p \)-hydroxyphenylpropaone unit has been confirmed to be one of the building units of all higher plants.

It has been shown as a characteristic feature of grass and bamboo lignins that they contain 5 to 10\% of ester of \( p \)-coumaric acid accompanied by ferulic acid in small amounts(19). Occurrence of the ester of \( p \)-hydroxybenzoic acid in aspen lignin was first demonstrated by Smith(20) who indicated that the acid is linked to aliphatic hydroxyl group. Vanillic and syringic acids have been also known to occur as an ester with lignins(21). The occurrence of the esters is widely distributed over softwoods, hardwoods, and tropical woods(22). Nakano et al.(23) presumed that a part of \( p \)-hydroxybenzoic acid is esterified to alcoholic hydroxyl group at \( \alpha \)-position of the side chain of aspen lignin molecule. Okabe and Kratzl(24) proposed a possibility that \( p \)-hydroxybenzoic acid might be linked to the \( \alpha \)-position on the basis of the nucleophilic attack by the acid on \( \alpha \)-carbon atom of intermediate quinomemethide during dehydrogenation of coniferyl alcohol. On the other hand Pew and Connors(25) suggested
another possible esterification to phenolic hydroxyl group during intramolecular rearrangement of the side chain on the ground that new types of a dimer and a trimer containing an ester were obtained on dehydrogenation of \( p \)-hydroxypropiophenone by peroxidase and hydrogen peroxide. Shimada et al.\(^{25} \) and Nakamura et al.\(^{26} \) confirmed, on the basis of spectral and analytical data obtained by methanolysis, thioglycolation, hydrogenation and acidolysis using models and MWLs, that the majority of \( p \)-coumaric and \( p \)-hydroxybenzoic acids are linked to the alcoholic hydroxyl groups at \( \tau \)-carbon atoms of the side chains of lignin molecules, and that the formation of the esters may be biochemically controlled, i.e., by the mediation of an acylating enzyme. Kratzi and Claus\(^{27} \) obtained a small amount of 1-(4-hydroxyphenyl)-propane-1,2-dione from ethanolysis oil of bamboo and rye plants, and they confirmed that a part of \( p \)-hydroxyphenyl component of grass lignin is composed of \( p \)-hydroxyphenylglycerol-\( \beta \)-aryl ether structure. Higuchi and Kawamura\(^{4} \) showed that the \( p \)-hydroxyphenylglycerol-\( \beta \)-aryl ether moiety is not specific to grass lignin but occurs in various plant lignins, and the amount of the moiety between conifers and grasses is almost equal and a little less in dicotyledonous trees. Furthermore Adlerz\(^{8} \) obtained a small amount of 3-hydroxy-1-(4-hydroxyphenyl)-propane-2-one from acidolysis oil of spruce lignin, and they confirmed the occurrence of \( p \)-hydroxyphenylglycerol-\( \beta \)-aryl ether moiety in conifer lignin.

Aromatic carboxylic acids produced by permanganate oxidation of methylated lignin were first obtained by Freudenberg et al.\(^{29} \)–\(^{31} \) to investigate the condensed C—C linked aromatic moieties of lignin. Veratric, isohemipinic and 5,5'-dehydrodiveratric acids were obtained from spruce lignin, and trimethylgallic acid in addition to the acids from beech. Furthermore numerous aromatic compounds of anisole, veratrole and trimethoxybenzene series were isolated and identified by Richtzenhain\(^{12} \), Freudenberg et al.\(^{32} \)–\(^{35} \), and Larsson et al.\(^{36} \).

2. Enzymic dehydrogenation polymer of \( p \)-coumaryl alcohol (pc-DHP)\(^{37} \)

Grass and bamboo lignins are believed to be a polymer composed of \( p \)-hydroxyphenyl, guaiacyl and syringyl units. While, several workers\(^{38} \)–\(^{40} \) have proposed the view that \( Sphagnum \) cell walls contain a low methoxyl lignin which is basically a highly condensed polymer by double condensations at 3- and 5-positions of \( p \)-hydroxyphenyl nuclei.

In the present investigation enzymic dehydrogenation polymers (DHPs) prepared from \( p \)-coumaryl, coniferyl, and sinapyl alcohols by the Zulauf method were subjected to nitrobenzene and permanganate oxidation, and acidolysis, and their chemical properties, especially the degree and pattern of nucleus substitution of \( p \)-hydroxyphenyl component of grass and bamboo lignins were studied. \( p \)-DHP was first prepared by Freudenberg et al.\(^{11} \). However little information is available concerning chemical properties of the \( p \)-DHP, except an ethanolysis experiment by Kratzi et al.\(^{10} \).

Results and discussion

Yield of each aromatic aldehyde produced by alkaline nitrobenzene oxidation of the DHPs is shown in Table 1. The amount of \( p \)-hydroxybenzaldehyde from \( p \)-DHP was approximately equal to that of vanillin from c-DHP, and molar ratio of \( p \)-hydroxybenzaldehyde to vanillin for DHP of a 1:1 mixture of \( p \)-coumaryl alcohol and coniferyl alcohol (pc-DHP) was
Table 1. Yields of aromatic aldehydes on alkeline nitrobenzene oxidation of DHPs.

<table>
<thead>
<tr>
<th></th>
<th>p-HB (%)*</th>
<th>V (%)*</th>
<th>S (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-DHP</td>
<td>11.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-DHP</td>
<td></td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>pc-DHP</td>
<td>12.3</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>ps-DHP</td>
<td>10.4</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>pcs-DHP</td>
<td>6.9</td>
<td>12.4</td>
<td>15.0</td>
</tr>
<tr>
<td>cs-DHP</td>
<td></td>
<td>13.3</td>
<td>26.5</td>
</tr>
</tbody>
</table>


somewhat higher than one. The molar ratio for DHP of a 1:1:1 mixture of p-coumaryl, coniferyl and sinapyl alcohols (pcs-DHP), on the other hand, was about 2/3 of that for the pc-DHP, while the yield of p-hydroxybenzaldehyde from DHP of a 1:1 mixture of p-coumaryl and sinapyl alcohols (ps-DHP) was similar to that from the p-DHP and pc-DHP, although the reason is still unknown. Thus it may be considered that free radicals of p-coumaryl and sinapyl alcohols do not effect an apparent condensation pattern of coniferyl alcohol, and that the condensation pattern of p-coumaryl alcohol is very similar to that of coniferyl alcohol except the case of copolymerization of the three alcohols.

Table 2. Yields of acidolysis products of DHPs.

<table>
<thead>
<tr>
<th>Products (%)*</th>
<th>p-DHP</th>
<th>c-DHP</th>
<th>pc-DHP</th>
<th>ps-DHP</th>
<th>pcs-DHP</th>
<th>cs-DHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-CHO</td>
<td>0.4</td>
<td>0.5</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-CH₂COCH₃**</td>
<td>++</td>
<td>0.7</td>
<td>1.0</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-COCOCH₃</td>
<td>++</td>
<td>++</td>
<td>0.3</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-COOH</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-CHOHCOCH₃**</td>
<td>++</td>
<td>++</td>
<td>0.3</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-COOHCH₃**</td>
<td>0.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-CH₂COCH₂OH</td>
<td>+</td>
<td>0.3</td>
<td>0.3</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-CHO</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-CH₂COCH₂</td>
<td>0.3</td>
<td>0.3</td>
<td>+</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-COOCOCH₃</td>
<td>+</td>
<td>0.2</td>
<td>++</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-COOH</td>
<td>+</td>
<td>0.3</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-CHOHCOCH₃</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-COOCH₂OH</td>
<td>0.6</td>
<td>1.0</td>
<td>0.5</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R'-CH₂COCH₂OH</td>
<td>0.3</td>
<td>1.6</td>
<td>0.2</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: Per cent of DHP. **: These components were identified by gas chromatography-mass spectrometry using a Shimadzu LKB-9000. R-; p-Hydroxyphenyl group, R'; Guaicaryl group, R'"; Syringyl group, +; Small, +; Trace.
Table 2. shows yields of acidolysis products of the DHPs. A considerable amount of 2-hydroxy-1-(4-hydroxyphenyl)-propane-1-one and a small amount of 1-(4-hydroxyphenyl)-propane-2-one, 1-(4-hydroxyphenyl)-propane-1,2-dione, 1-hydroxy-1-(4-hydroxyphenyl)-propane-2-one and 3-hydroxy-1-(4-hydroxyphenyl)-propane-2-one, suggesting participation of 2-hydroxybenzaldehyde and 2-hydroxybenzoic acid were detected in the acidolysis products of the DHP of p-coumaryl alcohol alone. These p-hydroxyphenylpropanones which are ascribed to p-hydroxyphenylglycerol-β-aryl ether structure were also found from pc-, ps-, and pcs-DHPs.

Faix and Schweers have recently reported that no p-hydroxybenzaldehyde was found in the products of alkaline nitrobenzene oxidation of various DHPs prepared from mixtures of p-coumaryl, coniferyl, and sinapyl alcohols in various ratios by laccase obtained from Psalliota campestris, and presumed that p-coumaryl alcohol gives a highly condensed C—C linked moieties during the formation of DHP. Their findings are in sharp contrast to the results in the present work. It is probably due to autoxidation of their DHPs by oxygen molecule, since radicals are formed by laccase much slower than by peroxidase.

Total yield of guaiacylpropanones, such as 1-(4-hydroxy-3-methoxyphenyl)-propane-2-one, 1-(4-hydroxy-3-methoxyphenyl)-propane-1,2-dione, 1-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propane-2-one, 2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propane-1-one, and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propane-2-one, from the c-DHP was several times higher than that of p-hydroxyphenylpropanones from the p-DHP. The results suggest whether the occurrence of β-O-4 coupling of radicals derived from p-coumaryl alcohol by peroxidase and hydrogen peroxide is rather deficient as compared to that of radicals from coniferyl alcohol or p-hydroxyphenylglycerol moiety of β-O-4 structure is subsequently condensed with other lignin units. Kratzl et al. also reported similar results with ethanolysis of p- and c-DHPs prepared by mushroom laccase.

However the yield of p-hydroxyphenylpropanones increased considerably in the case of the DHP in which p-coumaryl alcohol was copolymerized with coniferyl alcohol or sinapyl alcohol, indicating that dehydrogenation copolymers of p-coumaryl alcohol and other lignin monomers are sufficient in p-hydroxyphenylglycerol-β-aryl ether structure as compared to p-DHP.

The yields of guaiacylpropanones from the pc-DHP and DHP of a 1:1 mixture of coniferyl and sinapyl alcohols (cs-DHP) were also much higher than that from the c-DHP, whereas the difference in the yield between p-hydroxyphenylpropanones and guaiacylpropanones seems to lessen in the pcs-DHP. Kratzl and Buchtel reported that DHP prepared from 3 g of coniferyl alcohol and 1.5 g of sinapyl alcohol-(3-14C) by the Zulauf method using crude

* Peroxidase is widely distributed in plants, whereas the occurrence of laccase in higher plants is limited. Higuchi et al. found that radish peroxidase catalyzes the formation of DHP from coniferyl alcohol, and proposed that peroxidase plays a more important role than laccase in lignin biosynthesis. Nakamura demonstrated that plant laccase purified is incapable of catalyzing the formation of DHP from lignin monomers. Harkin and Obst have recently confirmed that peroxidase is unequivocally responsible for dehydrogenative polymerization of lignin monomers in plants. From these results is strongly supported a view that peroxidase principally participates in the formation of lignin in higher plants.
laccase obtained from mushroom, gave a considerable amount of 1-(4-hydroxy-3,5-dimethoxyphenyl)-propane-1,2-dione-(3-¹⁴C) as well as 1-(4-hydroxy-3-methoxyphenyl)-propane-1,2-dione by ethanolysis. In the present investigation, however, the yields of syringylpropanones from the ps-DHP, cs-DHP and pcs-DHP were quite small, indicating that, in the DHP prepared by the Zulauf method, a participation of syringylglycerol-β-aryl ether structure is of minor importance as compared to that in hardwood MWL. Studies on the enzymic dehydrogenation of sinapyl alcohol by Zutropf method should be still performed with reference to the Zulauf method.

Yields of methylesters of aromatic carboxylic acids in the potassium permanganate and hydrogen peroxide oxidation products of methylated DHPs are shown in Table 3. Yield of anisic acid from the DHP of $p$-coumaryl alcohol alone was 2.96%, that of 4-methoxyisophthalic acid was 1.05%, and that of 3,3'-dehydrodianisic acid was 2.43%, respectively. In addition a small amount of 4-methoxy-o-phthalic and 2-methoxydiphenyl ether-5,4'-dicarboxylic acids were found. However methoxytrimesic acid was scarcely detected in the degradation products of the DHP from $p$-coumaryl alcohol alone. On the other hand a considerable amount of veratric, isohepinic and 5,5'-dehydrodiveratric acids, and a small amount of metahepinic and 2,3,2'-trimethoxydiphenyl ether-5,4'-dicarboxylic acids were found from the DHP of coniferyl alcohol. These findings make it reasonable to believe that $p$-coumaryl alcohol, of which carbon atoms at 3- and 5-positions in the aromatic nucleus

<table>
<thead>
<tr>
<th>Methylester (%)*</th>
<th>p-DHP</th>
<th>c-DHP</th>
<th>pc-DHP</th>
<th>ps-DHP</th>
<th>pcs-DHP</th>
<th>cs-DHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisic acid</td>
<td>2.96</td>
<td>0.92</td>
<td>1.35</td>
<td>1.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veratric acid</td>
<td>1.75</td>
<td>1.09</td>
<td></td>
<td>1.90</td>
<td>6.89</td>
<td></td>
</tr>
<tr>
<td>Trimethylgallic acid</td>
<td>2.30</td>
<td>1.91</td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Methoxyisophthalic acid</td>
<td>1.05</td>
<td>0.16</td>
<td>0.45</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Methoxy-o-phthalic acid</td>
<td>0.10</td>
<td>0.05</td>
<td>?</td>
<td>?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isohepinic acid</td>
<td>0.60</td>
<td>0.14</td>
<td>0.25</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metahepinic acid</td>
<td>0.03</td>
<td>0.06</td>
<td>0.07</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methoxytrimesic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Methoxydiphenyl ether-5,4'-dicarboxylic acid</td>
<td>0.16</td>
<td>0.03</td>
<td>0.10</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,3'-Dehydrodianisic acid</td>
<td>2.43</td>
<td>0.33</td>
<td>0.79</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,3'-Dimethoxydiphenyl ether-5,4'-dicarboxylic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2'-Dimethoxydiphenyl ether-5,4'-dicarboxylic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2',3,3'-Trimethoxydiphenyl-5,5'-dicarboxylic acid</td>
<td>0.31</td>
<td>0.37</td>
<td>0.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,2',3'-Trimethoxydiphenyl ether-5,4'-dicarboxylic acid</td>
<td>0.29</td>
<td>-</td>
<td>-</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,5'-Dehydrodiveratric acid</td>
<td>1.42</td>
<td>0.43</td>
<td>0.46</td>
<td>1.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,2',5'-Tetramethoxydiphenyl ether-5,4'-dicarboxylic acid</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>1.20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*; Per cent of methylated DHP. ++; Small. +; Trace.
are unsubstituted for methoxyl groups, polymerizes in a similar manner with coniferyl alcohol, and a participation of a considerable amount of dehydrodi-\(p\)-coumaryl alcohol, \(p\)-coumarylresinol, and dehydrobis-\(p\)-coumaryl alcohol, and of a small amount of dehydrodi-\(p\)-coumaryl ether involving diphenyl ether are suggested in the DHP of \(p\)-coumaryl alcohol. In fact these dimers were isolated and identified from the dehydrogenation products of \(p\)-coumaryl alcohol\(^4\).

Molar ratio, calculated on the yield shown in Table 3, of each methylester of the main acids of anisole and veratrole series to methyl anisate and methyl veratrate, respectively is shown in Table 4. The molar ratio of dimethyl 4-methoxyisophthalate to methyl anisate is very similar to that of dimethyl isohepininate to methyl veratrate. The same thing is recognized for the molar ratio of dimethyl 3,3'-dehydrodianisate to methyl anisate and for that of dimethyl 5,5'-dehydrodiveratrate to methyl veratrate.

The above findings in the present investigation suggest that no difference of condensation pattern between \(p\)-coumaryl alcohol and coniferyl alcohol substantially occurs in their DHPs formation by the Zulauf method.

**Experimental**

*Preparation of enzymic dehydrogenation polymers (DHPs)*

**DHP of \(p\)-coumaryl alcohol (\(p\)-DHP):** To a solution containing 3 g of \(p\)-coumaryl alcohol in 3 liters of 1/15 M sodium phosphate buffer solution (pH 6.0) were added 0.45 mg of crude horseradish peroxidase (\(RZ\); approximately 0.3, Sigma) and 40.8 ml of 0.1% hydrogen peroxide ten times at intervals of 12 min. On stirring the mixture solution at room temperature, it became milky within about 30 min, and amorphous precipitates were found to be formed gradually. The same amounts of peroxidase and 0.1% hydrogen peroxide were added every day by the same procedure, and the mixture was stirred for another 5 days. The reaction solution was evaporated to 500 ml under nitrogen at 35-40°C, and the precipitates were collected by centrifugation, washed with water, and dried in vacuo over calcium chloride. The crude p-DHP was dissolved in a small amount of a mixture solution of dichloroethane/ethanol (2:1), filtered to remove a trace of insoluble material, and precipitated into ether with stirring. Yield of the purified p-DHP was 1.8 g (60%).

**DHP of coniferyl alcohol (\(c\)-DHP):** To a solution containing 1.8 g of coniferyl alcohol in 1.8 liters of 1/15 M sodium phosphate buffer solution (pH 6.0) were added a total 17 mg of horseradish peroxidase and 510 ml of 0.1% hydrogen peroxide for 5 days, and precipitates
formed were purified by the same procedure. Yield of the c-DHP was 1.2 g (67%).

**DHP of p-coumaryl alcohol and coniferyl alcohol (1:1) (pc-DHP):** To a solution containing 0.6 g of p-coumaryl alcohol and 0.72 g of coniferyl alcohol in 1.5 liters of 1/15 M sodium phosphate buffer solution (pH 6.0) were added a total 9 mg of the peroxidase and 408 ml of 0.1% hydrogen peroxide for 5 days, and precipitates formed were purified by the same procedure. Yield of the pc-DHP was 0.99 g (75%).

**DHP of p-coumaryl alcohol and sinapyl alcohol (1:1) (ps-DHP):** To a solution containing 0.6 g of p-coumaryl alcohol and 0.84 g of sinapyl alcohol in 2.5 liters of 1/15 M sodium phosphate buffer solution (pH 6.0) were added a total 9 mg of the peroxidase and 408 ml of 0.1% hydrogen peroxide for 5 days, and precipitates formed were purified by the same procedure. Yield of the ps-DHP was 1.55 g (72%).

**DHP of p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (1:1:1) (pcs-DHP):** To a solution containing 0.6 g of p-coumaryl alcohol, 0.72 g of coniferyl alcohol, and 0.84 g of sinapyl alcohol in 3.5 liters of 1/15 M sodium phosphate buffer solution (pH 6.0) were added a total 13.65 mg of the peroxidase and 612 ml of 0.1% hydrogen peroxide for 5 days, and precipitates formed were purified by the same procedure. Yield of the pcs-DHP was 1.36 g (70%).

**Nitrobenzene oxidation**

DHPs were subjected to alkaline nitrobenzene oxidation for 2 hr at 170°C. The reaction solution was diluted with water, washed with ether, and adjusted to pH 2-3. Aromatic aldehydes produced were extracted with ether, and the ether solution was dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was dissolved in 0.1 ml of pyridine, and 0.1 ml of hexamethyldisilazane and 0.05 ml of trimethylchlorosilane were added. The reaction mixture was shaken, and, after 5 min, analyzed by gas-liquid chromatography. A flame ionization detector was used for analyses of the trimethysilyl ethers of the products: Stainless steel column (1 m, 3 mm ID) packed with 5% SE-30 on Chromosorb W(AW). Column temperature; 175°C. Injector temperature; 250°C. Carrier gas; nitrogen, 0.8 kg/cm².

**Acidolysis**

Ten mg of DHP was dissolved in 1 ml of a mixture solution of dioxane and water (9:1) containing 0.2 N hydrogen chloride in a glass tube and the solution was sealed and heated at 120°C for 4 hr. The solution was added dropwise into 10 ml of water under stirring, and adjusted to pH 3-4 with 0.4 N sodium bicarbonate. Precipitates were centrifuged off and the acidolysis products in a supernatant solution were extracted with chloroform, and the chloroform solution was dried over anhydrous sodium sulfate. After evaporating the chloroform, the residue was dissolved in 0.1 ml of pyridine, and 0.1 ml of hexamethyldisilazane and 0.05 ml of trimethylchlorosilane were added. The reaction mixture was
shaken, and, after 5 min, dried in vacuo over phosphorous pentoxide. The trimethylsilyl derivatives of the acidolysis products were dissolved in 1 ml of n-hexane and analyzed by gas-liquid chromatography. A flame ionization detector was used for analyses: Stainless steel column (2 m, 3 m ID) packed with 3% SE-52 on Chromosorb W; Column temperature; 195°C. Injector temperature; 240°C. Carrier gas; helium, 0.9 kg/cm².

Permanganate and hydrogen peroxide oxidation
Methylated DHPs were hydrolyzed and subjected to permanganate and hydrogen peroxide oxidation successively according to the procedure described by Larsson et al.⁵⁰: Each 0.5 g of DHPs was methylated twice with dimethyl sulfate and sodium hydroxide in dioxane/water (5:3) at 65°C under nitrogen gas, and the methylated DHP was hydrolyzed with 1 M sodium hydroxide for 3 hr at 168-170°C, and followed by remethylation in the same procedure. The methylated DHP thus obtained was oxidized with 5% potassium permanganate at pH 11-12, 90-100°C, and the ether soluble-acid fraction of the permanganate oxidation products was then treated with 30% hydrogen peroxide at pH 9-10. A mixture of aromatic carboxylic acids thus obtained was methylated with diazomethane in methanol, and analyzed by gas-liquid chromatography. A flame ionization detector was used for analyses at following conditions: Stainless steel column (1 m, 3 mm ID) packed with 5% SE-30 on Chromosorb G (AW, DMCS). Column temperature; 165-265°C. 7.5°C or 4°C/min up, then isotherm. Injector temperature; 310°C. Carrier gas; nitrogen, 30 ml/min.

Syntheses

p-Coumaryl alcohol: Acetate of ethyl p-coumarate was reduced with lithium aluminum hydride in absolute ether at -20°C⁴⁵, and the crude alcohol obtained was recrystallized from ethyl acetate. Mp 122-124°C.

Coniferyl alcohol: The alcohol was synthesized from ethyl ferulate by lithium aluminum hydride reduction⁵¹, and recrystallized from dichloromethane. Mp 74°C.

Sinapyl alcohol: Acetate of ethyl sinapinate was reduced with lithium aluminum hydride⁵¹, and the crude alcohol obtained was recrystallized from ether/petroleum ether. Mp 65-67°C.

3-Hydroxy-1-(4-hydroxyphenyl)-propane-2-one: The ketol was synthesized from p-acetoxyphenylacetic acid according to the procedure described by Lundquist⁵². Mp 71-72°C. Anal. Calcd. for C₈H₁₀O₃: C, 65.03; H, 6.07. Found: C, 64.81; H, 5.99.

3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propane-2-one: The ketol was prepared from acetyl homovanillic acid according to the procedure by Fischer et al.⁵⁵. Mp 81-82°C. Anal. Calcd. for C₁₀H₁₂O₄: C, 61.20; H, 6.17. Found: C, 61.08; H, 6.17.


Trimethylgallic acid: Gallic acid was methylated with dimethyl sulfate and sodium hydroxide⁵⁶. Mp 167°C.

4-Methoxyisophthalic acid: as. m-Xylenol was methylated with dimethyl sulfate and sodium hydroxide, and then oxidized with potassium permanganate⁵⁶ to the acid. Mp 245°C.
4-Methoxy-o-phthalic acid: o-Xylenol was methylated with dimethyl sulfate and sodium hydroxide, and then oxidized with potassium permanganate. Mp 162-163°C.

Isohemipinic acid: 5-Allylacetovanillone was methylated with dimethyl sulfate and sodium hydroxide, and then oxidized with potassium permanganate. Mp 261-262°C.

Metahemipinic acid: 4,5-Dimethoxy-2-methylbenzaldehyde was oxidized with potassium permanganate to the acid. Mp 202-204°C.

Methoxytrimesic acid: The acid was synthesized from p-cresol, Mp 248°C.

Dimethyl 3,3'-dehydrodianisate, Dimethyl 2,3,2'-trimethoxybiphenyl-5,5'-dicarboxylate, and Dimethyl 5,5'-dehydrodianisate: The esters were synthesized according to the procedure described by Larsson et al. A mixture of methyl 3-iodoanisate (3.54 g), methyl 5-iodoveratrate (3.87 g) and copper powder (15 g) was heated at 220-230°C for 30 min, and the reaction products were extracted with ethyl acetate. The solvent was evaporated and the residue was fractionated by vacuum distillation. Dimethyl 5,5'-dehydrodianisate crystallized first from the distillate at 130-190°C (0.01 mmHg). The crude crystals were recrystallized from ethyl acetate. Mp 129-130°C. Anal. Calcd. for C₁₈H₁₈O₆: C, 61.52; H, 5.69. Found: C, 61.78; H, 5.77.

Dimethyl 3,3'-dehydrodianisate crystallized by concentration of the mother liquor. The ester was recrystallized from ethyl acetate. Mp 173-174°C. Anal. Calcd. for C₁₈H₁₈O₆: C, 65.44; H, 5.50. Found: C, 65.27; H, 5.28.

Dimethyl 2,3,2'-trimethoxybiphenyl-5,5'-dicarboxylate crystallized finally by addition of n-hexane to the mother liquor. The ester was recrystallized from ethyl acetate/n-hexane. Mp 106-107°C. Anal. Calcd. for C₁₉H₂₀O₇: C, 63.32; H, 5.61. Found: C, 63.54; H, 5.70.

2-Methoxydiphenyl ether-5,4'-dicarboxylic acid: To a solution of potassium metal (0.34 g) in 16 ml of absolute methanol, a solution of methyl p-hydroxybenzoate (1.24 g) in 4 ml of absolute methanol was added. After evaporating the solvent, methyl 3-bromo-4-methoxybenzoate (2 g), copper powder (0.2 g) and anhydrous cupric acetate (0.2 g) were added and heated at 195-205°C for 4.5 hr. The reaction products were extracted with ether, and the ether extract was washed with 1% sodium hydroxide and water, and separated by a preparative TLC using benzene/methanol (40:0.3) as solvent. The oily substance giving the RF value of 0.38 was isolated and subjected to saponification with 1 N methanolic potassium hydroxide. After evaporating the solvent, the residue was dissolved in water and washed with ether. The aqueous solution was acidified to pH 2, and then extracted with ether. The free acid thus obtained was recrystallized from methanol. Mp 311.5 to 312.5°C. Anal. Calcd. for C₁₈H₁₈O₆: C, 62.49; H, 4.20. Found: C, 62.52; H, 4.45.

2,3-Dimethoxydiphenyl ether-5,4'-dicarboxylic acid: A mixture of potassium salt of methyl p-hydroxybenzoate (2.16 g), methyl 5-bromoveratrate (3 g), copper powder (0.3 g), and anhydrous cupric acetate (0.3 g) was heated at 195-205°C for 4.5 hr. The reaction products were extracted with ether and the ether solution was washed with 1% sodium hydroxide and water, and after evaporating the solvent, the residue was fractionated by column chromatography (celite 545, AW, DMCS, 2.4×30 cm) using methanol/water (500 ml of 60% then 1000 ml of 70% methanol). The oily substance from fractions 85-89 (each 8 ml) was subjected to saponification with 1 N methanolic potassium hydroxide. The free acid thus
obtained was recrystallized from methanol. Mp 215–217°C. *Anal.* Calcd. for C_{16}H_{14}O_{7}: C, 60.37; H, 4.44. Found: C, 60.49; H, 4.76.

*Dimethyl 2, 2'-dimethoxydiphenyl ether-5, 4'-dicarboxylate:* A mixture of potassium salt of methyl 4-hydroxy-3-methoxybenzoate (0.91 g), methyl 3-bromo-4-methoxybenzoate (1 g), copper powder (0.1 g), and anhydrous cupric acetate (0.1 g) was heated at 195–200°C for 4.5 hr. The reaction mixture was extracted with ether, and the ether solution was washed with 1% sodium hydroxide and water, and after evaporating the solvent, the residue was separated by preparative TLC using benzene/methanol (20:0.3). The substance giving the Rf value of 0.33 was isolated and recrystallized from methanol. Mp 96–96.5°C. *Anal.* Calcd. for C_{18}H_{18}O_{7}: C, 62.41; H, 5.25. Found: C, 62.45; H, 5.04.

*Dimethyl 2, 3, 2', 6'-tetramethoxydiphenyl ether-5, 4'-dicarboxylate:* The ester was synthesized according to the procedure described by Inubushi et al. *Anal.* Calcd. for C_{30}H_{22}O_{9}: C, 59.10; H, 5.46. Found: C, 59.42; H, 5.65.

3. *p*-Hydroxyphenyl component of grass lignin*<sup>64</sup>)

Studies on condensed *p*-hydroxyphenyl components of grass and bamboo lignins have not yet been performed, although the uncondensed structures have been investigated by hydrogenolysis, alkaline hydrolysis, nitrobenzene oxidation, ethanolysis, and acidolysis as described previously. According to a recent study*<sup>65</sup>), polymeric system of the bamboo lignin is probably composed of about 10:68:22 of *p*-coumaryl, coniferyl, and sinapyl alcohols, and *p*-coumaric acid esterified to the lignin is estimated to be 0.07/C_{6}-C_{9}. Then for the bamboo lignin is finally given a possible composition of 20:60:20 of *p*-hydroxyphenyl, guaiacyl, and syringyl propane units. While Bland et al.*<sup>66</sup>,<sup>67</sup>) reported that the artificial lignin prepared from *p*-coumaric acid and *Sphagnum* "MWL" are highly condensed polymers containing double condensations at 3- and 5-positions in *p*-hydroxyphenyl nuclei. However the data obtained with the DHP of *p*-coumaryl alcohol in the present investigation have shown that no difference of condensation pattern between *p*-coumaryl and coniferyl alcohols occurs substantially in the formation of DHP.

The present investigation was undertaken to establish the structural feature of *p*-hydroxyphenyl component in the polymeric system of grass and bamboo lignins.

**Results and discussion**

Gas chromatograms and yields of methylesters of aromatic carboxylic acids in the permanganate and hydrogen peroxide oxidation products of various MWLs and of saponified bamboo MWL are shown in Fig. 1 and Table 5, respectively. Both Jyuzudama and bamboo
MWLs gave apparently very similar chromatographic feature, except that the amount of anisic acid (I) from the former lignin was much higher than that of the latter. It has been shown that Jyuzudama lignin contains almost twice amounts of \( p \)-coumaric acid esterified to the lignin as compared to that of bamboo lignin\(^{3}\). The higher yield of anisic acid from Jyuzudama lignin may be ascribed to the higher amount of \( p \)-coumaric acid of that lignin. Hachihama \( et \ al.\)\(^{67}\) reported that no anisic acid was found in the permanganate oxidation products of methylated beech powder. Small amounts of anisic acid, however, was detected from beech as well as Japanese red pine lignins. Yield of veratric acid (II) from the grass and bamboo lignins is 5 to 6\% of the lignins and is higher than that from beech lignin, whereas yield of trimethylgallic acid (III) is 2.5 to 3\% of the lignins and much lower than that from beech lignin. MWL of Japanese red pine, which was used for comparison, gave 8.8\% of veratric acid as well as a small amount of trimethylgallic acid. The yield of anisic acid from the bamboo MWL was quite high (7\%), and even in the MWL, in which \( p \)-coumaric acid esterified was previously removed by saponification with alkali, still gave about 1/4 of the amount of the acid from the untreated MWL, and the result was comparable to that of \( p \)-hydroxybenzaldehyde on alkaline nitrobenzene oxidation of the saponified MWL. As the structure producing anisic acid from the saponified bamboo MWL, dehydrodi-\( p \)-coumaryl alcohol, \( p \)-coumarylresinol, and the similar compounds composed of \( p \)-coumaryl and coniferyl alcohols or \( p \)-coumaryl and sinapyl alcohols are suggested. The possibility of the participation of \( p \)-hydroxyphenylglycerol-\( \beta \)-aryl ether structure is, however, minor as shown by Higuchi \( et \ al.\)\(^{4}\). Isohemipinic (V), metahemipinic (VI) and 4-methoxysiphaspheric (IV) acids which are produced from the condensed units of guaiacyl and \( p \)-hydroxyphenyl groups in lignin were obtained from

![Gas chromatograms of methylesters of aromatic carboxylic acids in permanganate and hydrogen peroxide oxidation products of methylated MWL.](image-url)
Table 5. Yields of methylesters of aromatic carboxylic acids in permanganate and hydrogen peroxide oxidation products of methylated MWLs.

<table>
<thead>
<tr>
<th>Methylesters (%)</th>
<th>Bamboo MWL, NaOH treated</th>
<th>Grass (Coix lacryma)</th>
<th>Red pine</th>
<th>Beech</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisic acid (I)</td>
<td>6.98</td>
<td>1.71</td>
<td>11.60</td>
<td>0.10</td>
</tr>
<tr>
<td>Veratric acid (II)</td>
<td>6.04</td>
<td>5.00</td>
<td>5.13</td>
<td>8.84</td>
</tr>
<tr>
<td>Trimethylgallic acid (III)</td>
<td>3.19</td>
<td>4.81</td>
<td>2.48</td>
<td>0.16</td>
</tr>
<tr>
<td>4-Methoxyisophthalic acid (IV)</td>
<td>0.11</td>
<td>0.42</td>
<td>0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>Isohemipinic acid (V)</td>
<td>0.72</td>
<td>0.81</td>
<td>0.51</td>
<td>1.94</td>
</tr>
<tr>
<td>Metahemipinic acid (VI)</td>
<td>0.12</td>
<td>0.15</td>
<td>0.14</td>
<td>1.12</td>
</tr>
<tr>
<td>Methoxytrimesic acid (VII)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2-Methoxydiphenyl ether-5,4'-dicarboxylic acid (VIII)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3,3'-Dehydrodianisic acid (IX)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,3-Dimethoxydiphenyl ether-5,4'-dicarboxylic acid (X)</td>
<td>0.09</td>
<td>+</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>2,2'-Dimethoxydiphenyl ether-5,4'-dicarboxylic acid (XI)</td>
<td>?</td>
<td>+</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>2,3,2'-Trimethoxybiphenyl-5,5'-dicarboxylic acid (XII)</td>
<td>0.19</td>
<td>0.16</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td>2,3,2'-Trimethoxybiphenyl ether-5,4'-dicarboxylic acid (XIII)</td>
<td>0.32</td>
<td>0.21</td>
<td>0.22</td>
<td>1.36</td>
</tr>
<tr>
<td>5,5'-Dehydrodimeritric acid (XIV)</td>
<td>0.43</td>
<td>0.26</td>
<td>0.46</td>
<td>2.14</td>
</tr>
<tr>
<td>2,3,2',6'-Tetramethoxydiphenyl ether-5,4'-dicarboxylic acid (XV)</td>
<td>0.65</td>
<td>0.58</td>
<td>0.59</td>
<td>-</td>
</tr>
</tbody>
</table>

*: Based on lignin weight. +: Trace.

all the MWL. The yield of 4-methoxyisophthalic acid was highest in beech MWL, being in agreement with the data obtained by Hachihama et al.\textsuperscript{67}. Bland et al.\textsuperscript{49,66} reported that an artificial lignin prepared from \(p\)-coumaric acid on potato parenchyma and \textit{Sphagnum} "MWL" gave hydroxytrimesic acid as well as 4-hydroxyisophthalic acid on permanganate oxidation, and they proposed a view that the artificial lignin and \textit{Sphagnum} "MWL" are basically highly condensed C-C linked polymer of \(p\)-hydroxyphenyl unit through double condensations at 3- and 5-positions in aromatic nuclei.

However in the present investigation methoxytrimesic acid was scarcely detected in the degradation products of grass and bamboo lignins, and the possibility of double condensations at the 3- and 5-positions of the \(p\)-hydroxyphenyl nuclei was quite small.

2,3,2'-Trimethoxydiphenyl ether-5,4'-dicarboxylic acid (XIII) and 5,5'-dehydrodimeritric (XIV) acids were found in all the MWLs tested in the present investigation, whereas 2,3,2',6'-tetramethoxydiphenyl ether-5,4'-dicarboxylic acid (XV), which originated from a structural moiety composed of a guaiacyl and a syringyl groups, was found in grass, bamboo, and beech MWLs except Japanese red pine. Furthermore 2,3,2'-trimethoxybiphenyl-5,5'-dicarboxylic (XII), 2,3-(X) and 2,2'-dimethoxydiphenyl ether-5,4'-dicarboxylic (XI) acids, which are a biphenyl and diphenyl ether composed of a guaiacyl and a \(p\)-hydroxyphenyl ether groups, were found only in the degradation products of grass and bamboo lignins, indicating a peculiar structural feature of the grass and bamboo lignins.

Diphenyl ether and biphenyl carboxylic acids composed of two \(p\)-hydroxyphenyl groups
such as 2-methoxydiphenyl ether-5,4’-dicarboxylic (VIII) and 3,3’-dehydrodianisic (IX) acids could not be detected in any MWL, suggesting a difficult condensation between two \( p \)-hydroxyphenyl groups. Any dimeric acid composed of a \( p \)-hydroxyphenyl and a syringyl groups was not detected.

In view of the above experimental results, it is concluded that the grass and bamboo lignins are qualitatively but not quantitatively similar to hardwood lignin in the occurrence of the structural moieties composed of \( p \)-hydroxyphenyl and guaiacyl, of two guaiacyl, and of guaiacyl and syringyl units through phenylcoumaran ring, diphenyl ether and biphenyl, and that the structural moieties composed of two \( p \)-hydroxyphenyl units through diphenyl ether and biphenyl may not participate.

Experimental

Preparation of milled wood lignin (MWL)

The following plant stems; bamboo (\textit{Phyllostachys heterocycla} Matsum. var. \textit{pubescens} Ohwi), Jyuzudama (\textit{Coix lacryma-jobi} Linn.), Japanese red pine (\textit{Pinus densiflora} Sieb. et Zucc.), and beech (\textit{Fagus crenata} Blume) were used in the present experiment. Ten grams of the extractive-free powder of the plant stems were milled for 48 hr by using a vibratory ball mill (Hinodekoki, Gifu). The resulting fine powder was extracted with acetone/water (8 : 2), and the extracted crude lignin was purified according to the standard method of Björkman\textsuperscript{66}.

Saponification of bamboo MWL

Two grams of a bamboo MWL dissolved in 20 ml of 1 N sodium hydroxide and the solution was kept for 24 hr at room temperature. The saponified lignin was separated by acidifying to pH 2 with hydrochloric acid, and purified by dropping a solution of the lignin in dichloroethane/ethanol (2 : 1) into ether with stirring.

Permanganate and hydrogen peroxide oxidation

The MWLs and the saponified bamboo MWL were methylated, hydrolyzed, and remethylated, and then subjected to permanganate and hydrogen peroxide oxidation successively by the procedure as described previously. A mixture of aromatic carboxylic acids thus produced was methylated with diazomethane in methanol, and analyzed by gas-liquid chromatography.

4. Occurrence of diphenyl ether structure in lignin\textsuperscript{57,64}

Diphenyl ethers composed of \( p \)-hydroxyphenyl and guaiacyl groups such as 2,3- and 2,2’-dimethoxydiphenyl ether-5,4’-dicarboxylic acids, and those of two guaiacyl groups such as 2,3,2’-trimethoxydiphenyl ether-5,4’-dicarboxylic acid which were detected in the permanganate and hydrogen peroxide oxidation products of the c-DHP and cs-DHP could not be detected in the pc-DHP and pcs-DHP\textsuperscript{57}.

As described previously\textsuperscript{64}, molar ratio of dimethyl 2,3,2’-trimethoxydiphenyl ether-5,4’-dicarboxylate to methyl 5,5’-dehydrodiveratrate was 0.66, 0.17, 0.77, and 0.55 in the degradation products of methylated MWLs of Japanese red pine, beech, bamboo, and Jyuzudama (\textit{Coix lacryma-jobi}), respectively, whereas the molar ratio for the both methyl-esters from the c-DHP and cs-DHP was 0.21 and 0.22, respectively.
Furthermore the yield of 2,3,2',6'-tetramethoxydiphenyl ether-5,4'-dicarboxylic acid from bamboo, Jyuzudama, and beech MWLs tested in the present investigation was quite higher than that of 5,5'-dehydrodiferatrate, while the yield of the former acid was lower than that of the latter acid in the cs-DHP and pcs-DHP.

It is concluded from the experimental results, that, in the DHP, the participation of diphenyl ether moiety is of minor importance as compared with that of biphenyl moiety, indicating a peculiar structural feature of the DHP, whereas the participation of the diphenyl ether moiety is not less major than that of biphenyl one in all the tested lignin.

Freudenberg et al.\textsuperscript{60} proposed a view that at oligolignol stages during lignin formation a predominant amount of biphenyl component occurs, and followed with formation of diphenyl ether component during lignin formation. The present experimental results seem in harmony with his view.

Thus it is confirmed in the present investigation that the occurrence of diphenyl ether moiety is characteristic of the structure of mature lignin.

It has been long discussed whether \textit{Sphagnum} contains a certain lignin, especially a \textit{p}-hydroxyphenyl lignin\textsuperscript{70}. Erickson and Miksche\textsuperscript{71,72} recently found 4-methoxyisophthalic and 3,3'-dehydrodianisic acids in permanganate and hydrogen peroxide oxidation products of methylated sodium hydroxide-sodium monosulfid-soluble fractions of \textit{Ptilium crys-ta-castrensis} and \textit{Plagiochila asplenoides}, and 4,7,9-trimethoxy-2-dibenzofuran carboxylic acid from \textit{Dicranum bergeri}, \textit{Leptobryum pyriforme}, \textit{Pogonatum urnigerum}, \textit{Polytricum commune}, and \textit{Scapania undulata}, and also 3-(4,7,9-trimethoxy-2-dibenzofranyl)-propionic acid from \textit{Pogonatum} and \textit{Polytrichum}, in addition to anisic, veratric, isohemipinic and metahemipinic acids from all tested 8 species of bryophytes.

In the present investigation\textsuperscript{73,64}, it has been established that permanganate and hydrogen peroxide oxidation of the methylated DHP of \textit{p}-coumaryl alcohol generally gives anisic, 4-methoxyisophthalic, 4-methoxy-o-phthalic, 3,3'-dehydrodianisic and 2-methoxydiphenyl ether-5,4'-dicarboxylic acids, and that the occurrence of diphenyl ether moiety is characteristic of the structure of mature lignin. Erickson et al.\textsuperscript{75} also confirmed that the occurrence of diphenyl ether structure in lignin is an important indicator of the criterion for lignin by the permanganate oxidation method. From the absence of the diphenyl ether component in the permanganate oxidation products of all tested species, and also no detection of even 4-methoxyisophthalic and 3,3'-dehydrodianisic acids from \textit{Sphagnum} in spite of detection of a considerable amount of anisic acid, they have proposed a view that any lignin is present in none of bryophytes species.
trifurca, Gnetum indicum and Welwitschia mirabilis), and syringaldehyde and vanillin in the ratio of 1–3 : 1 from angiosperms, resulting in harmony with Mäule reaction. Kawamura and Higuchi\(^7\) studied comparatively on the properties of lignins of plants in various taxonomical positions on the basis of the Mäule tests, methoxyl contents, molar ratios of syringaldehyde to vanillin (S/V) on alkaline nitrobenzene oxidation and patterns of UV and IR spectra, and they classified lignins into various types.

Sarkanen et al.\(^7\) have developed the achievements obtained by Kawamura et al., and confirmed that absorptivities of maxima dominated by syringyl nuclei at 1130, 1235, 1335, 1430, 1470 and 1600 cm\(^{-1}\) all show a linear ascending relationship with methoxyl/C\(_6\)-C\(_3\) values, whereas the maximum at 1275 cm\(^{-1}\), typical of guaiacyl nuclei, shows a linear descending relationship with the value. Furthermore many investigations have revealed that ratios of syringyl unit to guaiacyl in lignins differ from tissues to tissues\(^7\)–\(^8\).

It has been known that methoxyl contents of Brauns lignins from certain species of angiosperms are generally lower than those of MWLs from the same species, being suggestive of for the Brauns lignin to be a guaiacyl component-rich lignin\(^8\). This was supported by low syringaldehyde yields on nitrobenzene oxidation of the Brauns lignins (1.7% for birch and 4.0% for oak lignins)\(^8\). A clear interpretation of these findings is hardly permissible, since the Brauns lignins may sometimes be contaminated with certain polyphenolic components and other extractives.

The heterogeneity was also found within the lignosulfonic acid from the same hardwood. Stone\(^8\) reported that initial lignin derivative fractions obtained with neutral sulphite cooking of aspen chips gave low syringaldehyde yields on nitrobenzene oxidation when compared with the overall yield of the aldehyde for the chips. Similar results were obtained by Marth\(^9\) with sulphite-bisulphite cooking of the chips. Furthermore Kyogoku and Hachihama\(^9\) found that low molecular weight fractions, obtained by fractional precipitation, of barium lignosulfonate from beech wood powder showed ascending syringaldehyde yields and methoxyl contents. Iwamida et al.\(^9\) reported the similar results with sulphite cooking of the beech chips.

The variation of the relative amounts of syringyl units in lignins of species in the various taxonomical positions and their tissues is probably due to different substrate specificity of O-methyltransferase and the presence or absence of ferulic acid 5-hydroxylase. And the occurrence of these enzymes may be closely related to polyphyliesis\(^9\) of the angiosperms and diverse differentiation of a primitive tissue.

However any methoxyl-rich lignin corresponding to syringyl lignin has never been isolated. Freudenberg\(^9\) has described that coniferyl and sinapyl alcohols are copolymerized to angiosperm lignin by the similar dehydrogenative principle as conifer lignin is formed, and that the syringyl units may occur in a random distribution spread within the angiosperm lignin molecule. Many compounds, monomers to tetramers, have been recently isolated and identified by Nimz et al.\(^9\) and Sakakibara et al.\(^9\) from the products of hydrolysis and hydrogenolysis of wood powder. These results almost entirely agree with the above Freudenberg's theory for the formation of angiosperm lignin. A constitutional scheme for beech lignin composed of 25 units has been proposed by Nimz\(^9\) on the basis of elementary
analysis, methoxyl content, UV and IR spectra, PMR and CMR spectra, and the yields of degradation products.

A finding of syringylglycerol-β-syringylglycerol ether in hydrolysis products of Fraxinus mandshurica wood powder, however, is of interest in relation to a possibility that a different scheme from the Nimz's may be conceivable in angiosperm lignin.

As early as 1951 Nord et al described, during the course of his study on Braun's lignins of hardwoods such as oak and birch, that a possibility of the existence of "syringyl lignin" should not be overlooked, and predicted the possibility that the "syringyl lignin" may be obtained, if fractionation procedure, e.g., a continuation of enzymic decay of hardwoods, are specific to isolate the "lignin".

2. Enzymic dehydrogenation polymer of sinapyl alcohol (s-DHP)\(^{88,99}\)

A. Formation of s-DHP

Freudenberg and coworkers reported that DL-syringaresinol is easily produced by dehydrogenation of sinapyl alcohol\(^{100-102}\) and that continued dehydrogenation of this alcohol does not lead to a lignin-like polymer but instead to 2,6-dimethoxy-1,4-benzoquinone and other degradation products\(^{101,103}\). They also reported that a 1:1 mixture of coniferyl and sinapyl alcohols is dehydrogenated to a lignin-like polymer\(^{101}\), and that if sinapyl alcohol component predominates in the mixture, the excess is not incorporated into the polymer\(^{104}\). From these results Freudenberg\(^{83}\) has expressed doubts about the existence of syringyl lignin in nature. However Sarkane\(^{96}\) has suggested a possibility of formation of s-DHP on the basis of his theory for end-wise- and bulk polymer formation.

In the present investigation it has been established that a considerable amount of a lignin-like polymer from sinapyl alcohol alone was formed in peroxidase and hydrogen peroxide system.

Results and discussion

Molecular size distribution of the polymer, which was determined by gel filtration on a column of Sephadex G-50 using dioxane/water (1:1) as eluent, is shown in Fig. 2. The location of the elution peak of vitamin B\(_{12}\) (\(M_w=1355\)) which was used as a marker is also given in the diagram. Methoxyl content of the polymer was 28.56% indicating almost no demethoxylation or demethylation during dehydrogenation. Phenolic hydroxyl content of the polymer determined by Goldschmid method\(^{108}\) was 0.38 per methoxyl group. UV spectrum of the polymer which is shown in Fig. 3 indicated a characteristic feature of "syringyl lignin" and differed from those of sinapyl alcohol and DL-syringaresinol. \(\lambda_{\text{max}}\) of methyl cellulose nm: 273. IR spectrum also showed characteristics of the "lignin". \(\nu_{\text{max}}\) cm\(^{-1}\): 3400, 2920, 2820, 1605, 1510, 1460, 1420, 1365, 1325, 1220, 1135 and 1100 (Fig. 4). The signal for guaiacyl nuclei at 1275 cm\(^{-1}\) was missing. Acidolysis of the polymer gave 16% of Hibbert's monomers which were identified by gas chromatography-mass spectrometry using authentic Hibbert's monomers as reference. 3-Hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-propane-2-one; MS(TMS) \(m/e\): 370(M\(^+\)), 255(M-15), 239(M-COCH\(_2\)-TMS), 209 (239-CH\(_2\)=O), 103(CH\(_2\)=O-TMS). 73 (TMS\(^+\)). 1-Hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-propane-2-one; MS (TMS) \(m/e\): 370 (M\(^+\)), 355 (M-15), 327 (M-COCH\(_3\)),...
Eluate Fig. 3

Spectra of s-DHP (-), Fig. 2 Gel filtrations of s-DHP (-----) and vitamin B₁₂ (-----).

298 (M-TMS), 73 (TMS⁺). 2-Hydroxy-1-(4-hydroxy-3, 5-dimethoxyphenyl)-propane-1-one; MS(TMS) m/e: 370(M⁺), 355(M-15), 257(M-CH₃CHOTMS), 117(CH₃C'OHTMS), 73(TMS⁺).

1-(4-Hydroxy-3, 5-dimethoxyphenyl)-propane-2-one; MS(TMS) m/e: 282(M⁺), 267(M-15), 252 (M-O=CH₂), 239(M-COCH₃), 209(252-COCH₃), 179(209-O=CH₂), 73(TMS⁺).

1-(4-Hydroxy-3, 5-dimethoxyphenyl)-propane-1, 2-dione; MS (TMS) m/e: 296(M⁺), 281 (M-15), 253 (M-COCH₃), 223(253-O=CH₂), 73(TMS⁺). The acidolysis results indicated that the polymer contains a considerable amount of β-O-4 linkage which is a most important structural moiety in the growing of lignin polymer. The polymer and its methyl derivative afforded 8-10%
of syringaldehyde and 15–16% of trimethylgallic acid by alkaline nitrobenzene and permanganate-hydrogen peroxide oxidation, respectively.

The data in the present investigation clearly show that radicals formed enzymically coupled not only by $\beta$-$\beta$ to form syringaresinol but also by $\beta$-$O$-$4$ to make growth of syringyl lignin via syringylglycerol-$\beta$-sinapyl ether, and a possible occurrence of syringyl lignin in the cell walls of hardwoods.

Experimental

*Preparation of s-DHP*

To a solution containing 10 mg of crude horseradish peroxidase ($RZ$; approximately 0.3, Sigma) in 1 liter phosphate buffer solution (1/60 M, pH 6.9) were added dropwise at an equal rate using a microtube pump 3.4 mmoles of sinapyl alcohol in 2 liters of the same buffer and 3.4 mmoles of hydrogen peroxide in 0.58 liters of the buffer over a period of 30 hr at 22°C with stirring. After addition of the solutions, the reaction mixture was stirred for another 24 hr, and then evaporated to 0.4–0.5 liters under nitrogen gas at 40°C. Precipitates formed were collected by centrifugation, washed with water, dried *in vacuo* over phosphorus pentoxide and sodium hydroxide. The crude s-DHP was dissolved in a mixture of dichloroethane/ethanol (2:1), and the solution was added dropwise into 200 to 300 times volume of ether with stirring. Yield of the purified milky s-DHP powder was 20%.

*Nitrobenzene oxidation*

S-DHP was subjected to alkaline nitrobenzene oxidation at 170°C for 3 hr and the yield of syringaldehyde was determined by gas-liquid chromatography as described previously.

*Acidolysis*

Ten mg of s-DHP was subjected to acidolysis as described previously and TMS derivatives of the products were analyzed by means of a Shimadzu LKB-9000 Gas Chromatograph-Mass Spectrometer.

*Permanganate and hydrogen peroxide oxidation*

S-DHP was methylated, hydrolyzed and remethylated, and then subjected to permanganate and hydrogen peroxide oxidation successively by the same procedure as described previously, and the yield of trimethylgallic acid was determined by gas-liquid chromatography.

B. Constitutional model of s-DHP

In the previous study a considerable amount of DHP was found to be formed from sinapyl alcohol alone by peroxidase and hydrogen peroxide. The s-DHP has been confirmed to be a synthetic syringyl lignin on the basis of methoxyl and phenolic hydroxyl group contents, UV and IR spectra, and further the amounts of syringaldehyde, trimethylgallic acid, and Hibbert's monomers produced by alkaline nitrobenzene oxidation, methylation-permanganate oxidation, and acidolysis, respectively.

In the present study a structural feature of s-DHP was studied analytically and spectroscopically to establish a constitutional model, and discussed in relation to syringyl lignin in nature.
Results

Nitrobenzene oxidation

Yield of syringaldehyde in the oxidation products of s-DHP is shown in Fig. 5. Behavior of s-DHP in the oxidation was different from that of MWL, suggesting that molecular weight of s-DHP is low and that s-DHP may be composed of certain simple structural elements, e.g., ones outstandingly resistant towards the oxidation such as syringaresinol, and submissive such as β-O-4.

Acidolysis and mild acidolysis

Ten% of syringaresinol was obtained from acidolysis products of s-DHP, and 3% of that from mild acidolysis. On calculating from recovery yield of authentic compound, syringaresinol may be yielded in 26% and in 7% by the acidolysis and the mild acidolysis, respectively.

Estimation of functional groups of s-DHP

Content of free phenolic hydroxyl group was 0.24/methoxyl which corresponds to 0.48/C₆-C₃. The content was a little higher than that (0.40/methoxyl)\(^{106}\) of a DHP of coniferyl alcohol, and much higher than that (0.28/methoxyl)\(^{106}\) of MWL from wood of Thuja Standishii. The higher phenolic hydroxyl content may be due to lower molecular weight of s-DHP. Content of p-hydroxybenzyl alcohol group was 0.08/methoxyl, being much higher than that (0.06/methoxyl)\(^{106}\) of the DHP of coniferyl alcohol and that (0.05/methoxyl)\(^{106}\) of the MWL of T. Standishii. Content of α-carbonyl group was 0.02/methoxyl. Content of p-alkoxybenzyl alcohol group was 0.07/methoxyl, being a little higher than that (0.10/methoxyl)\(^{106}\) of the DHP of coniferyl alcohol and that (0.09/methoxyl)\(^{106}\) of the MWL. The content of p-alkoxybenzyl alcohol group was almost equal to that of p-hydroxybenzyl alcohol.
group, suggesting that molecular weight of s-DHP is low. The benzyl alcohol group is formed by nucleophilic attack of hydroxyl ion to a β-quinonemethide intermediate during dehydrogenation of sinapyl alcohol. Therefore the higher content of benzyl alcohol group should indicate a higher content of β-O-4 and β-1 linkages in s-DHP. In fact content of β-O-4 linkage of the s-DHP was estimated to be 0.13/methoxyl, which may be much lower than the true value, since various side reactions are known to occur during acidolysis. The content of α-O-4 linkage of the s-DHP was 0.09/methoxyl.

**Molecular weight of s-DHP**

Molecular weight (Mn) of s-DHP was 1172 ± 60, being 1/2-1/3 of that of MWL and similar to that of c-DHP.

**CMR spectrum of s-DHP**

Chemical shifts of *threo*-1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2',6'-dimethoxyphenoxy)-propane-1,3-diol (V) and syringaresinol (VIII), a CMR spectrum of s-DHP, and assignments of absorption peaks of the s-DHP are shown in Table 6, Fig. 6, and Table 7, respectively. The absorption peaks of the s-DHP could be assigned, expect very weak obscure peaks. The data clearly show that structural moieties of syringylglycerol-β-syringyl ether and syringaresinol are predominant in s-DHP. Lüdemann and Nimz\(^{107,108}\) reported that a chemical shift of β-C of 1,2-diarylpropane-1,3-diol is 63 to 65 ppm from TMS. The peak at 63 to 65 ppm is missing in the s-DHP, indicating that a possibility of occurrence of 1,2-disyringylpropane-1,3-diol during the formation of s-DHP is quite small. In agreement with the fact, stilbene derivative was not detected, by TLC, in the alkali degradation

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Assignment</th>
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<tbody>
<tr>
<td>55.3</td>
<td>β, β'-C in VIII</td>
</tr>
<tr>
<td>56.5-57.1</td>
<td>OCH(_3)</td>
</tr>
<tr>
<td>61.2</td>
<td>γ-C in V</td>
</tr>
<tr>
<td>72.7</td>
<td>γ, γ'-C in VIII</td>
</tr>
<tr>
<td>73.8</td>
<td>α-C in V</td>
</tr>
<tr>
<td>87.2</td>
<td>α, α'-C in VIII</td>
</tr>
<tr>
<td>88.8</td>
<td>β-C in V</td>
</tr>
<tr>
<td>104.8</td>
<td>2, 6-C in VIII</td>
</tr>
<tr>
<td>105.1</td>
<td>2, 6-C in V</td>
</tr>
<tr>
<td>106.5</td>
<td>3', 5'-C in V</td>
</tr>
<tr>
<td>125.0</td>
<td>4'-C in V</td>
</tr>
<tr>
<td>132.3</td>
<td>1-C in V</td>
</tr>
<tr>
<td>133.4</td>
<td>1-C in VIII</td>
</tr>
<tr>
<td>135.8</td>
<td>1'-C in V</td>
</tr>
<tr>
<td>136.5-136.7</td>
<td>4-C in V and VII</td>
</tr>
<tr>
<td>149.3</td>
<td>3', 5'-C in V</td>
</tr>
<tr>
<td>153.7</td>
<td>2', 6'-C in V</td>
</tr>
</tbody>
</table>
products of s-DHP with 2 N sodium hydroxide at 100°C for 6 hr, according to the same procedure described by Nimz\textsuperscript{109}. However a weak absorption peak corresponding to that of α-C or β-C of 1,2-diarylpropane-1,3-diol, or of γ-C of cinnamyl alcohol or Ar-CO-CH\textsubscript{2}OH appeared in CMR spectrum of a s-DHP (Ph-OH content, 0.11/OCH\textsubscript{3}) which was obtained in 45% yield. Two 1,2-disyringylpropane elements of 25 phenyl units are inserted into a constitutional scheme of beech lignin, which has been recently proposed by Nimz\textsuperscript{40}. 1,2-Disyringylpropane-1,3-diol moiety may be formed at maturation stages during s-DHP formation and lignification.
Fig. 7 shows a PMR spectrum of s-DHP which was mildly hydrogenated with \( \text{H}_2\text{-Pd/C} \) and then acetylated. The spectrum was sharp, indicating that molecular weight of s-DHP was low. The signal for \( \alpha\)-CH in diacetate of guaiacylglycerol-\( \alpha\)-guaiacyl-\( n\)-propyl-\( \beta\)-guaiacyl diether is a doublet at 5.40 ppm\(^{10)}\). A doublet at 5.40 ppm in the spectrum of s-DHP must reflect the presence of syringylglycerol-\( \alpha\),\( \beta\)-disyringyl ether moiety. The spectrum also shows the presence of syringylglycerol-\( \beta\)-syringyl ether and syringaresinol moieties in the s-DHP. PMR (20% acetate in CDCl\(_3\)) \( \delta \): 2.00 ppm (0.17, singlet, \( \gamma\)-OAc as in V or Ar-CH(\( \text{O}-\text{C}^\gamma\'))-CH(\( \text{O}-\text{C}^\gamma\'))-CH\(_2\)OH, or \text{threeo-} \alpha\text{-OAc as in V}), 2.15 ppm (0.14, singlet, \text{erythro-} \alpha\text{-OAc as in V}), 2.33 ppm (0.22, singlet, Ph-OAc), \text{ca} 3.06 ppm (multiplet, \( \beta\),\( \beta\')-CH as in VIII), 3.72, 3.78 and 3.82 ppm (1, singlet, OCH\(_3\)), 3.90-4.70 ppm (multiplet, \( \gamma\),\( \gamma\')-CH\(_2\) as in VIII, and \( \gamma\)CH\(_2\) and \( \beta\)-CH as in V and Ar-CH(\( \text{O}-\text{C}^\gamma\'))-CH(\( \text{O}\text{-C}^\gamma\'))-CH\(_2\)OH), 4.76 ppm (0.10, doublet, \( \alpha\),\( \alpha\')-CH as in VIII), 5.40 ppm (0.02-0.03, doublet, \( \alpha\)-CH as in Ar-CH(\( \text{O}-\text{C}^\gamma\'))-CH(\( \text{O}-\text{C}^\gamma\'))-CH\(_2\)OH), 6.06 ppm (0.05, doublet, \( \alpha\)-CH as in V), 6.51, 6.60 and 6.63 ppm (0.27, singlet, Ar-H).

![Fig. 7. PMR spectrum of hydrogenated and acetylated s-DHP.](image)

The intensity ratio of each peak to methoxyl signals is shown above by the boldface. The intensity ratios of \( \alpha\)-CH in Ar-CH(\( \text{O}-\text{C}^\gamma\'))-CH(\( \text{O}-\text{C}^\gamma\'))-CH\(_2\)OH to \( \alpha\)-CH in the type V, \( \alpha\)-CH in the type V to \( \alpha\),\( \alpha\')-CH in the type VIII, and \( \alpha\)-CH in Ar-CH(\( \text{O}-\text{C}^\gamma\'))-CH(\( \text{O}-\text{C}^\gamma\'))-CH\(_2\)OH to \( \alpha\),\( \alpha\')-CH in the type VIII were 1/2, 1/2, and 2-3/10, respectively.

**Discussion**

Freudenberg\(^{9)}\) reported that laccase-catalyzed dehydrogenation of sinapyl alcohol always favors \( \beta\)-\( \beta\) coupling, and any lignin-like polymer is not formed from sinapyl alcohol. Sarkanen\(^{9)}\) has proposed, however, that an important factor during formation of DHP or lignin is the stationary concentration of lignin monomer radicals, \textit{i.e.}, the radicals formed...
through the Zutropf method couple predominantly by β-O-4 to make growth of the end-wise polymer, whereas the radicals through the Zulauf method couple mostly by 5-5 and 5-O-4 to make the bulk polymer. And he suggested a possibility of formation of s-DHP on the basis of his theory for the formation of end-wise- and bulk polymers.

In the present investigation the formation of s-DHP by peroxidase and hydrogen peroxide gives the foundation on a possible occurrence of syringyl lignin in hardwood tissues. β-Quinonemethide and phenoxy radicals formed in the peroxidase-hydrogen peroxide system play an important role during polymerization. Syringaresinol is formed by β-β coupling between β-quinonemethide radicals at the initial stages during enzymic dehydrogenation, and followed by the formation of β-O-4 structure by coupling between a phenoxy radicals of syringaresinol thus formed and another β-quinonemethide radical of sinapyl alcohol.

Thus s-DHP is predominantly composed of syringylglycerol-β-syringyl and -α,β-disyringyl ethers and syringaresinol moieties. The molecular weight (Mn) of the s-DHP is similar to that of the DHP of coniferyl alcohol. On the assumption that the representative of s-DHP is a heptasyringyl lignol, a constitutional model was established as shown in Fig. 8. The model is almost satisfied with the analytical and spectrometric data.

![Fig. 8 A constitutional model of s-DHP.](image)

Sakakibara et al. have recently isolated and identified syringylglycerol-β-syringylglycerol and -β-syringaresinol ethers from hydrolysis products of *Fraxinus mandshurica*. 
wood meal, and syringylglycerol-β-dihydrosinapyl alcohol ether from hydrogenolysis products of the wood. Tanahashi et al. have recently found that sinapyl alcohol gives almost quantitatively syringylglycerol-β-sinapyl ether in FeCl₃-dioxane system and that continued dehydrogenation of this alcohol produces a polymer. Fergus and Goring, on the basis of the spectral analysis with a UV microscope, have suggested that birch lignin deposited in the secondary layers of wood fiber and ray parenchyma cell walls is mostly composed of syringylpropane units, whereas the lignin distributed in the vessel secondary cell walls and middle lamellae of the xylem is predominantly of guaiacyl units. Furthermore Sakai et al. presumed a possibility of the existence of syringyl lignin on the basis of the variation of S/V values on nitrobenzene oxidation of dioxane lignin treated with acetyl hydroperoxide.

The formation of DHP of sinapyl alcohol in vitro described in the present investigation and the other findings above mentioned make it reasonable to believe that the radicals formed enzymically from sinapyl alcohol couple not only by β-β to form syringaresinol but also by β-O-4 to make growth of syringyl lignin in vivo. Thus the formation of syringyl lignin may very possibly occur in the cell walls of hardwoods.

Experimental

Preparation and purification of s-DHP

S-DHP was prepared from aqueous solution of sinapyl alcohol by the Zutropf method using horseradish peroxidase (RZ; approximately 0.3, Sigma) as described previously. The reaction mixture was extracted with dioxane/chloroform (1:1) and then the organic solution was washed with water and dried over anhydrous sodium sulfate. After evaporation of the solvent under reduced pressure at 35°C, the residue was dissolved in a small amount of a mixture of dichloroethane/ethanol (2:1) and then the solution was added dropwise to 200 times volume of ether with stirring. Yield of the milky powder precipitated was 20-28%. The yield of s-DHP was depended on concentration of the enzyme, hydrogen peroxide and sinapyl alcohol, e.g., 45% yield for 10 mg/liter and 2 mg/liter of initial and final concentration of the enzyme. One g of the powder was fractionated by gel filtration on Sephadex G-10 (4.5×93 cm, 860 g) with dioxane/water (1:1) as solvent. The fractions 64-74 (each 10 ml), checking the homogeneity by preparative TLC developed with benzene/ethyl acetate (1:1), were combined and evaporated in vacuo at 35°C to dryness and then the residue was purified with the system of dichloroethane/ethanol (2:1)-ether. Methoxyl content of the s-DHP which was used for analyses was 28.60% indicating that almost no demethylation or demethoxylation occurred during the formation of s-DHP.

Isolation and identification of syringaresinol and dimethoxyquinone

The soluble dehydrogenation products in ether were separated by preparative TLC using isopropyl ether/chloroform/benzene (1:2:1) as solvent. Syringaresinol was obtained in 5% yield after recrystallization from methanol. Mp 170-171°C. MS m/e: 418 (M⁺). PMR (10% diacetate in CDCl₃) δ: 2.32 ppm (6H, singlet, 4,4'-OAc), ca. 3.07 ppm (2H, multiple, β,β'-CH), 3.90-4.38 ppm (4H, multiple, γ,γ'-CH₂), 3.79 ppm (12H, singlet, OCH₃), 4.70 ppm (2H, doublet, α,α'-CH), 6.53 ppm (4H, singlet, Ar-H). 2,6-Dimethoxy-1,4-
benzoquinone was also obtained in 0.3% yield after recrystallization from ethanol/ethyl acetate. Mp 258.5-260°C. Mixed Mp with the authentic quinone synthesized from syringaldehyde according to the procedure by Ioffe et al.\textsuperscript{114} for the synthesis of 2-methoxy-1,4-benzoquinone was 257.5-259°C.

**Nitrobenzene oxidation of s-DHP**

S-DHP was subjected to various conditions to alkaline nitrobenzene oxidation, and syringaldehyde was converted to its TMS derivative and determined by gas-liquid chromatography as described previously.

**Acidolysis and mild acidolysis**

One hundred mg of s-DHP was dissolved in 10 ml of a mixture of dioxane/water (9:1) containing 0.2 N hydrogen chloride in a glass tube. The tube was sealed after flushing with nitrogen gas and heated at 120°C for 4 hr, or 50°C for 24 hr. Three ml of water was added to the reaction mixture and pH of the mixture was adjusted to 4 with 0.4 N sodium bicarbonate. After being concentrated to 3 ml in vacuo at 30°C, the mixture solution was dropped into 120 ml of water with vigorous stirring and precipitates formed were taken off. The supernatant solution was extracted with chloroform and the extract was subjected to preparative TLC using benzene/ethyl acetate (1:1). As the acidolysis products gave a tailing band on the plate, the part of Rf value of 0.2-0.4 was separated. Mild acidolysis products gave a main band corresponding to Rf value (0.35) of syringaresinol and three weakly bands (Rf: 0.6, 0.44 and 0.11) on the plate by a UV lamp. The compound extracted from the band which gave the Rf value corresponding to that of syringaresinol was purified by preparative TLC developed 8 times with chloroform/benzene/isopropyl ether (1:1:1). The yields of syringaresinol were 10% for acidolysis and 3% for mild acidolysis. Diacetae of syringaresinol obtained was recrystallized from methanol. Mp 180-181°C. MS m/e: 502(M+).

Synthetic syringaresinol was also subjected to acidolysis and mild acidolysis by the same procedure and the reaction mixtures were separated by preparative TLC. Recoveries of syringaresinol were 35% for acidolysis and 42% for mild acidolysis.

*Estimation of α- and β-O-4 linkages of s-DHP*

Alternatively the reaction mixtures in the acidolysis and mild acidolysis glass tubes were used directly for determination of increased free phenolic hydroxyl group based on δε value at 290 nm given with a model compound (V) according to the Goldschmid method\textsuperscript{105}, and amounts of α-O-4 and β-O-4 linkages in s-DHP were estimated.

*Estimation of functional groups in s-DHP*

Content of free phenolic hydroxyl group of s-DHP was determined spectrometrically by the method described by Goldschmid\textsuperscript{105} based on a calibration curve at 290 nm obtained with the model compound (V). Content of p-hydroxybenzyl alcohol group was determined by the method described by Gierer\textsuperscript{117} based on a calibration curve at 640 nm obtained with the V and quinonechloroimine synthesized from p-aminophenol hydrochloride according to the procedure described by Willstätter et al.\textsuperscript{118}. Contents of α-carbonyl and p-alkoxybenzyl alcohol groups were determined by the procedure described by Adler et al.\textsuperscript{119} based on a
calibration curve at 310 nm obtained with the model compound (VII).

**Determination of molecular weight of s-DHP**

A suitable amount of s-DHP was dissolved in DMF, and the molecular weight (Mn) of s-DHP was determined by means of a Hewlett Packard Model-302 Vapor Pressure Osmometer at 37°C.

**Mild hydrogenation and acetylation of s-DHP**

A mixture of 60 mg of s-DHP and 90 mg of 5% Pd/C in dioxane was stirred under hydrogen gas at room temperature for 24 hr and the hydrogenated s-DHP was acetylated by mixing with 40 ml of acetic anhydride and 12 ml of pyridine, allowing the mixture to stand for 24 hr at room temperature. The reaction mixture was added dropwise to water with cracked ice, and the precipitates were dissolved in a mixture of dichloroethane/ethanol (2:1) and purified by dropping the solution into ether with stirring.

**Spectrometric analyses**

PMR spectra were taken by using a Hitachi R-22 High Resolution NMR Spectrometer with TMS internal standard. CMR spectra were taken on 20% solution of samples in a mixture of deutoacetone/heavy water (9:1) at 50°C by means of a Nihondenshi FX-100 Spectrometer operating at 25.2 MHz by FT-13C{'H)-technique with TMS internal standard. Mass spectra were obtained by using a Nihondenshi 07 Mass Spectrometer.

**Syntheses**

**Acetosyringone (I):** The ketone was synthesized from acetyl dimethylpyrogallol according to Brit. Pat. 1 188 480\(^\text{\textsuperscript{120}}\). To a solution containing 325 g of acetyl dimethylpyrogallol in 1590 ml of absolute nitrobenzene, 220 g of anhydrous aluminum chloride was added for a period over 4 hr at 2-3°C with stirring. The reaction mixture was heated for 15 hr at 50°C and poured into a mixture of 20% hydrochloric acid (2 liters)-ice (1.3 kg), and then extracted with ether. The ether solution was washed with water and dried over anhydrous sodium sulfate. After being concentrated to 280-290 ml, the solution was allowed to stand overnight at -20°C. Crude crystals separated were wrap in sheets of filter paper and pressed at 300 kg/cm\(^2\) pressure for 15 min, and recrystallized from ether after decolorized with activated charcoal. Pale yellow crystals were obtained in 30% yield. Mp 125-127°C. MS m/e: 196 (M\(^+\)). PMR (20% in CDCl\(_3\)) \(\delta\): 2.57 ppm (3H, singlet, \(\alpha\)-CH\(_3\)), 3.95 ppm (6H, singlet, 3,5-OCH\(_3\)), 6.13 ppm (1H, singlet, 4-OH), 7.26 ppm (2H, singlet, Ar-H).

**4-Acetoxy-3,5-dimethoxy-\(\alpha\)-bromoacetophenone (II):** The compound was synthesized from acetate of I by the procedure described by Leopold\(^\text{\textsuperscript{121}}\) for the synthesis of 4-benzyloxy-3-methoxy-\(\alpha\)-bromoacetophenone. Colorless crystals from benzene/ligroin were obtained in 70% yield. Mp 125-128°C.

**4-Acetoxy-3,5-dimethoxy-\(\alpha\)-(2',6'-dimethoxyphenoxy)-acetophenone (III):** The compound was synthesized from II according to the procedure described by Miksch\(^\text{\textsuperscript{122}}\) for the synthesis of 4-acetoxy-3,5-dimethoxy-\(\alpha\)-(4-formyl-2',6'-dimethoxyphenoxy)-acetophenone. Pale yellow crystals from ethanol were obtained in 72% yield. Mp 118-120°C. MS m/e: 390 (M\(^+\)). PMR (20% in CDCl\(_3\)) \(\delta\): 2.36 ppm (3H, singlet, 4-OAc), 3.80 ppm (6H, singlet, 2',6'-OCH\(_3\)), 3.89 ppm (6H, singlet, 3,5-OCH\(_3\)), 5.12 ppm (2H, singlet, \(\alpha\)-CH\(_3\)), 6.58 ppm
(2H, doublet, Ar-H), 7.02 ppm (1H, triplet, Ar-H), 7.40 ppm (2H, singlet, 2,6-H).

1-(4-acetoxy-3,5-dimethoxyphenyl)-2-(2', 6'-dimethoxyphenoxy)-3-hydroxy-propane-1-one (IV): The ketol was synthesized from III by the procedure described by Mikosch for the synthesis of 1-(4-acetoxy-3,5-dimethoxyphenyl)-2-(4-formyl-2', 6'-dimethoxyphenoxy)-3-hydroxy-propane-1-one. Colorless crystals from ethyl acetate/n-hexane were obtained in 68% yield. Mp 121-122°C. MS m/e: 420 (M+). PMR (20% diacetate in CDCl₃) δ: 1.97 ppm (3H, singlet, r-OAc), 2.35 ppm (3H, singlet, 4-OAc), 3.74 ppm (6H, singlet, 2',6'-OCH₃), 3.85 ppm (6H, singlet, 3,5-OCH₃), 4.57 ppm (ZH, doublet, T-CH₂), 5.47 ppm (1H, triplet, P-CH), 6.55 ppm (2H, doublet, Ar-H), 6.98 ppm (1H, triplet, Ar-H), 7.49 ppm (2H, singlet, 2,6-H).

threeo-1-(4-Hydroxy-3,5-dimethoxyphenyl)-2-(2', 6'-dimethoxyphenoxy)-propane-1,3-diol (V): Fifteen g of IV was reduced with 6.5 g of lithium aluminum hydride in 510 ml of absolute tetrahydrofuran under nitrogen gas for 4 hr at 50°C with stirring. Colorless material obtained was crystallized from ethyl acetate/benzene. Colorless crystals from ethyl acetate/n-hexane were obtained in 61% yield. Mp 125-126°C. MS m/e: 380 (M+). PMR (20% triacetate in CDCl₃) δ: 2.00 ppm (6H, singlet, threo-a-OAc and r-OAc), 2.31 ppm (3H, singlet, 4-OAc), 3.76 ppm (6H, singlet, 2',6'-OCH₃), 3.80 ppm (6H, singlet, 3,5-OCH₃), ca. 3.98 ppm (2H, multiplet, 7-CH₂), ca. 4.45 ppm (1H, multiplet, β-CH), 6.11 ppm (1H, doublet, α-CH), 6.55 ppm (2H, doublet, Ar-H), 6.97 ppm (1H, triplet, Ar-H), 6.71 ppm (2H, singlet, 2,6-H).

1-(3,4,5-Trimethoxyphenyl)-2-(2', 6'-dimethoxyphenoxy)-propane-1,3-diol (VI): The diol (V) was methylated with diazomethane in absolute methanol at room temperature overnight. A colorless oil was obtained. MS m/e: 394 (M+).

1-(3,4,5-Trimethoxyphenyl)-2-(2', 6'-dimethoxyphenoxy)-3-hydroxy-propanone (VII): The diol (VI) was oxidized in absolute dioxane with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone for 24 hr at room temperature by the procedure described by Adler et al. The reaction mixture was filtered on a column of aluminum oxide (Woelm, neutral, activity grade 1, 2x10 cm, 34 g) using absolute dioxane. Yield of the ketol thus obtained was estimated UV spectrometrically to be 94%. The pale yellow oil was crystallized from methanol. Colorless crystals recrystallized from methanol were obtained in 75% yield. Mp 79-80°C. MS m/e: 392 (M+).

DL-Syringaresinol (VIII): To 3 g of sinapyl alcohol dissolved in a mixture of 60 ml of acetone and 240 ml of water, 60 ml of a solution containing 3.57 g of CuSO₄.5H₂O was added, and the mixture was vigorously stirred for 5 hr. The reaction mixture was extracted with dioxane/chloroform (1:1) and then the organic solution was washed with water and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was dissolved in a small amount of dioxane and then the solution was added dropwise to 200 times volume of ether with vigorous stirring. Syringaresinol was obtained in 49% yield from the ether soluble fraction. Melting point and Mass and PMR spectra of the synthetic syringaresinol were completely in agreement with those of that obtained from the enzymic dehydrogenation products.
In addition, milky polymer (DHP) was found to be formed in 8% yield on the cupric sulfate-catalyzed oxidation of sinapyl alcohol.

3. Syringyl lignin of hardwood

A. Separation of s-DHP from a mixture of c- and s-DHPs by mercuration

The most reactive sites in electrophilic substitution are 5- and 6-positions in any of the aromatic nuclei of lignin. As early as 1898 to 1902, Gimroth found the substitution reaction of acetoxymercuri group for hydrogen atom of benzene, phenols, methoxybenzenes, amines and aromatic hydrocarbons. Many studies on mercuration of lignin were performed in detail for a long time to establish the aromatic nature of lignin. However any information on the mercuration of hardwood lignin has not yet been offered.

Hibbert et al. confirmed that no loss of methoxyl group is involved during mercuration of lignin. Furthermore acetoxymercuri derivatives of aromatic compound containing free phenolic hydroxyl group are generally dissolved in acetic acid but hardly in neutral organic solvent. Therefore acetic acid may be specific to fractionation of mercurated hardwood lignin.

The present investigation was undertaken to separate s-DHP from a mixture of s-DHP and c-DHP by mercuration in order to establish the specific isolation procedure of syringyl lignin in nature.

Results and discussion

Table 8 shows proportions of each fraction obtained to the total of mercurated DHP. A half of the mercurated c-DHP remained in Fraction V and 1/3 was distributed in Fraction IV. On the other hand more than 60% and 15% of the mercurated s-DHP were distributed in Fraction III and Fraction I-II respectively, and no material distributed in Fraction V was observed. Thus the distinct difference of the solubility in acetic acid between the mercurated c-DHP and mercurated s-DHP was found. Three fourths of mercurated 1:1 mixture of s-DHP and c-DHP (1:1 DHP mixture) were dissolved in reaction solvent, and distributed in Fraction I to Fraction IV. One fourth of the total remained in Fraction V.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-DHP</td>
<td>0.9</td>
<td>3.0</td>
<td>8.5</td>
<td>32.4</td>
<td>52.0</td>
</tr>
<tr>
<td>s-DHP</td>
<td>3.6</td>
<td>12.4</td>
<td>61.5</td>
<td>18.7</td>
<td>0</td>
</tr>
<tr>
<td>1:1 DHP mixture</td>
<td>5.1</td>
<td>16.3</td>
<td>32.6</td>
<td>20.2</td>
<td>23.5</td>
</tr>
<tr>
<td>cs-DHP</td>
<td>1.1</td>
<td>6.0</td>
<td>10.5</td>
<td>4.4</td>
<td>74.1</td>
</tr>
</tbody>
</table>

Unexpectedly, in the case of dehydrogenation copolymer of a 1:1 mixture of coniferyl and sinapyl alcohols (cs-DHP), 1/4 of the total was dissolved in the reaction solvent, and 3/4 remained in Fraction V.

Mercury content of each fraction is shown in Table 9. Lautsch and Piazolo reported
that the mercury atom is substantially located at 5-position in guaiacyl nuclei of lignin. That the acetoxymercuri group is joined to 2-position in syringaldehyde was found from its quantitative conversion to 2-iodosyringaldehyde. Hence the acetoxymercuri group is probably located at 2-position in the syringyl nuclei of s-DHP.

Table 10 shows methoxyl contents of various fractions and DHPs treated with 15% hydrochloric acid. No significant difference between methoxyl contents of DHP and the DHP treated with the acid was found. Fraction II obtained from the mercurated 1:1 DHP mixture contained a maximum amount of methoxyl, 26%, of all the various fractions. The methoxyl content of Fraction I was 24%, that of Fraction III was 22.4%, and that of Fraction IV was 20% which was similar to that of 1:1 DHP mixture. Furthermore it is very interesting that Fraction V of the 1:1 DHP mixture contained 17.4% of methoxyl content which was very similar to that of c-DHP treated with the acid.

Thus it is evident from the methoxyl content that various fractions from s-DHP-rich fraction to c-DHP-rich one were separated from the mercurated 1:1 DHP mixture. On the other hand the methoxyl contents of all the fractions of the mercurated cs-DHP showed a level of 23%.

Yields of aromatic aldehydes produced by nitrobenzene oxidation of the various fractions and the DHPs treated with 15% hydrochloric acid are shown in Table 11. Molar ratio of syringaldehyde to vanillin (S/V) of Fraction II from the mercurated 1:1 DHP mixture was a maximum amount, 4.1, of all the fractions, and that of Fraction V was a minimum amount, 0.1, whereas the values of S/V of all the fractions from the mercurated cs-DHP were 1.4 to 1.5. Thus the molar ratios of the aldehyde in all the fractions from the
Table 11. Yields of aromatic aldehydes on nitrobenzene oxidation of various fractions and DHPs treated with HCl.

<table>
<thead>
<tr>
<th></th>
<th>V (%)</th>
<th>S (%)</th>
<th>Molar ratio (S/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>2.3</td>
<td>8.3</td>
<td>3.0</td>
</tr>
<tr>
<td>II</td>
<td>1.7</td>
<td>8.4</td>
<td>4.1</td>
</tr>
<tr>
<td>III</td>
<td>3.0</td>
<td>4.6</td>
<td>1.3</td>
</tr>
<tr>
<td>1:1 DHP mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3.5</td>
<td>3.8</td>
<td>0.9</td>
</tr>
<tr>
<td>V</td>
<td>5.6</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>DHP</td>
<td>5.8</td>
<td>6.0</td>
<td>0.9</td>
</tr>
<tr>
<td>DHP**</td>
<td>10.2</td>
<td>13.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

|                |       |       |                   |
| Fraction I     | —     | —     | —                 |
| II             | 3.8   | 6.8   | 1.5               |
| III            | 3.1   | 5.2   | 1.4               |
| cs-DHP         |       |       |                   |
| IV             | 3.3   | 5.6   | 1.4               |
| V              | 3.5   | 6.2   | 1.5               |
| DHP            | 4.2   | 7.0   | 1.4               |
| DHP**          | 9.8   | 17.3  | 1.5               |

*: Based on various fractions and DHPs treated with HCl. **: Untreated with HCl. V: Vanillin, S: Syringaldehyde.
mercurated 1:1 DHP mixture reflect reasonably their methoxyl contents.

The ratio of s-DHP to c-DHP of each fraction, which was untreated with 15% hydrochloric acid, obtained from the mercurated 1:1 DHP mixture is shown in Fig. 9. A calibration curve was obtained by using DHP mixture in various ratios of c-DHP and s-DHP. From the calibration curve, the ratio of s-DHP to c-DHP of Fraction II, which was untreated with the acid, was estimated to be 0.85 to 0.9, that of Fraction I to be 0.75, that of Fraction III to be 0.65, that of Fraction IV to be 0.45, and that of Fraction V to be less than 0.1.

Since mercuration is known as a substitution reaction for lignin not involving loss of methoxyl groups\(^{131}\), the present method must be an effective means for the selective isolation of syringyl lignin or syringyl unit-rich lignin as well as guaiacyl lignin or guaiacyl unit-rich lignin from hardwoods.

**Experimental**

**Preparation of DHPs**

S-DHP was prepared by the Zutropf method as described previously, and c-DHP and cs-DHP (DHP from a 1:1 mixture of coniferyl and sinapyl alcohols) were also prepared according to the procedure for the preparation of s-DHP.

**Mercuration of DHPs**

To a suspension of each 700 mg of s-DHP/, c-DHP/, cs-DHP/, or 1:1 DHP mixture in 10 ml of absolute ethanol was added a solution of 1.5 g of mercuric acetate in a mixture of 50 ml of absolute ethanol and 3 ml of acetic acid, and the mixture was refluxed for 4 hr. The progress of the reaction was generally accompanied with production of brown material, although acetoxymercuri derivative of the s-DHP was dissolved in the reaction solvent as the reaction proceeded.

**Fractionation of mercurated DHPs**

The hot reaction solution was immediately separated from the brown material by decantation, and the products were fractionated and purified according to the scheme summarized in Fig. 10.

**Demercuration of each fraction**

A suitable amount of each fraction was heated with 15% hydrochloric acid for 15 min, and then washed with boiling water according to the procedure described by Polčin\(^ {132}\). Mercuric chloride thus produced was reduced to metal mercury with stannous chloride, and the total content of mercury was determined by a Hiranuma Mercury Analyzer Model Hg-1\(^ {133}\).

**Nitrobenzene oxidation of demercurated DHP**

Ten mg of each fraction thus demercurated was subjected to alkaline nitrobenzene oxidation as described previously, and TMS derivatives of aromatic aldehydes obtained were analyzed by gas-liquid chromatography.

**Syntheses**

*Acetoxymercuriavanillin and 2-acetoxymercurisyringaldehyde*: To a solution of 0.031 moles of mercuric acetate in a mixture solution of 320 ml of absolute ethanol and 18 ml
of anhydrous acetic acid, was added 0.03 moles of vanillin/, or syringaldehyde. The mixture solution was refluxed for 1 hr. After removing mercurous salt produced, the solvent was evaporated to dryness. Recrystallization of crude acetoxymercurivanillin from acetic acid/water gave 7.2 g of colorless crystal which decomposed without melt. Analysis of the crystal showed 51.39% of mercury which corresponds to 1.16 Hg per vanillin.

Similar colorless crystal (7.8 g) of 2-acetoxymercurisyringaldehyde from acetic acid/water was obtained, and the crystal decomposed without melt. Anal. Calcd. for C_{11}H_{12}O_{6}Hg: Hg, 45.50. Found: Hg, 45.91.

5-Acetoxymercuri-3,4-dimethoxytoluene and 2-acetoxymercuri-3,4,5-trimethoxytoluene: Molar proportions of mercuric acetate and 3,4-dimethoxytoluene/, or 3,4,5-trimethoxytoluene were mixed as described above and the mixture was refluxed for 4 hr. Recrystallization of crude 5-acetoxymercuri-3,4-dimethoxytoluene from methanol/water gave 7 g of colorless crystal. Mp 162-165°C. Anal. Calcd. for C_{11}H_{14}O_{4}Hg: Hg, 48.82. Found: Hg, 48.35.
Colorless crystal (11 g) of 2-acetoxymercuri-3,4,5-trimethoxytoluene from methanol/water was obtained similarly. Mp 108-110°C. Anal. Calcd. for C_{12}H_{16}O_{5}Hg: Hg, 45.50. Found: Hg, 45.97.

Chloromercuri derivatives: To a solution containing 3 g of acetoxymercurivanillin, or 2-acetoxymercurisyringaldehyde in 150 ml of a 1:1 mixture solution of acetic acid and water, was added dropwise 50 ml of 20% aqueous sodium chloride solution. Precipitates formed were washed with water, added to 70 ml of 10% sodium chloride solution, and heated to boil. After cooling, the product was washed with water. Chloromercuri derivatives were quantitatively obtained.

5-Iodovanillin and 2-iodosyringaldehyde: To a suspension of 2 g of chloromercurivanillin, or 2-chloromercurisyringaldehyde in 150 ml of 5% aqueous potassium iodide solution, was gradually added iodine with stirring until a faint brown color persists for at least 10 min, and then the reaction mixture was kept at 75°C for 30 min. If iodine disappeared on heating, a small amount of iodine was added. After cooling the reaction mixture, a few milliliters of sodium thiosulfate solution were added to remove a small amount of free iodine, and the product was washed with water. 5-Iodovanillin was obtained in 90% yield from chloromercurivanillin. Colorless crystal from methanol/water. Mp 178°C.

Similarly 2-iodosyringaldehyde was produced quantitatively from 2-chloromercurisyringaldehyde. Colorless crystal from methanol/water. Mp 164-165°C. MS m/e: 308 (M^+). Anal. Calcd. for C_{12}H_{14}O_{2}I: C, 35.09; H, 2.95; I, 41.19. Found: C, 35.01; H, 3.08; I, 41.68.

B. Syringyl unit-rich lignin of hardwood

The distinct difference of the solubility in acetic acid between the mercurated s-DHP and c-DHP was found as described previously, and s-DHP and c-DHP contaminated in a small amount of c-DHP and s-DHP respectively could be separated from a mercurated 1:1 mixture of s-DHP and c-DHP. The present investigation was performed to isolate and characterize syringyl unit-rich lignin from hardwoods by the application of mercuration.

Results and discussion

Table 12 shows proportions of each fraction obtained to the total mercurated lignin preparations. One tenth to one fourth of all the mercurated lignin preparations was dissolved in the reaction solvent and nine tenths to three fourths were separated as insoluble material. As shown in Table 13, mercury contents of the fractions dissolved in
Table 13. Percentages of mercury contents of various fractions.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beech MWL</td>
<td>41.9</td>
<td>41.8</td>
<td>39.2</td>
<td>25.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Beech dioxane lignin</td>
<td>38.7</td>
<td>43.2</td>
<td>45.2</td>
<td>43.0</td>
<td>26.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Yamamomo MWL</td>
<td>38.7</td>
<td>38.5</td>
<td>40.0</td>
<td>41.9</td>
<td>41.5</td>
<td>44.2</td>
<td>23.9</td>
</tr>
<tr>
<td>Kiri MWL</td>
<td>43.0</td>
<td>43.7</td>
<td>39.4</td>
<td>23.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

the reaction solvent were higher than those of the insoluble ones.

Table 14 shows methoxyl contents of various demercurated fractions and lignin preparations treated with 15% hydrochloric acid.

Table 14. Percentages of methoxyl contents of various demercurated fractions and lignins treated with HCl.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>Lignin</th>
<th>Lignin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beech MWL</td>
<td>24.5</td>
<td>22.2</td>
<td>20.8</td>
<td>20.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>20.3</td>
<td>20.1</td>
</tr>
<tr>
<td>Beech dioxane lignin</td>
<td>27.8</td>
<td>25.8</td>
<td>23.5</td>
<td>22.3</td>
<td>20.7</td>
<td>—</td>
<td>—</td>
<td>20.8</td>
<td>20.3</td>
</tr>
<tr>
<td>Yamamomo MWL</td>
<td>23.9</td>
<td>25.2</td>
<td>24.9</td>
<td>23.1</td>
<td>21.7</td>
<td>21.1</td>
<td>19.0</td>
<td>21.8</td>
<td>21.7</td>
</tr>
<tr>
<td>Kiri MWL</td>
<td>19.0</td>
<td>18.4</td>
<td>17.8</td>
<td>17.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>17.8</td>
<td>17.7</td>
</tr>
</tbody>
</table>

*; Untreated with HCl.

No significant difference between the methoxyl contents of the original lignin preparation and the one treated with the acid was found. Fraction I, I, and II obtained from the mercurated beech MWL, beech dioxane lignin, and Yamamomo (Myrica rubra Sieb. et Zucc.) MWL, respectively, contained maximum amounts, 24.5%, 27.8%, and 25.2% of methoxyl group. Syringyl unit-rich fraction (methoxyl content; 19.0%) was obtained even from Kiri (Paulownia tomentosa Steud.) MWL (methoxyl content; 17.8%) which is composed of rather a small amount of syringyl unit; the result shows that the present method is the effective means for the selective isolation of syringyl unit-rich lignin from hardwoods. Furthermore it is noteworthy that Fraction VII from the mercurated Yamamomo MWL contained 19.0% of methoxyl which was lower than those of the original MWL (methoxyl content; 21.7%) and of the MWL treated with the acid (methoxyl content; 21.8%), suggesting the existence of guaiacyl unit-rich moiety in Yamamomo MWL.

Yields of aromatic aldehydes produced by nitrobenzene oxidation of the demercurated fractions and lignin preparations treated with the acid are shown in Table 15. For the molar ratios of syringaldehyde to vanillin (S/V), Fraction I, I, II, and I obtained from the mercurated beech MWL, beech dioxane lignin, Yamamomo MWL, and Kiri MWL, respectively, showed maximum ratios, 4.1, 4.7, 4.0 and 1.2, confirming reasonably their methoxyl contents.

Thus it is evident from the methoxyl content and the molar ratio of the aromatic aldehydes that hardwood lignins differ not only from species to species, but also within a lignin preparation from the same species. Fergus and Goring suggested on the basis of spectral analyses of lignin distributed in the cell walls of the xylem with a UV
Table 15. Syringaldehyde-vanillin ratios on nitrobenzene oxidation of various demercurated fractions and lignin preparations treated with HCl.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>Lignin</th>
<th>Lignin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beech MWL S/V</td>
<td>4</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>Total aldehyde(%)**</td>
<td>13.6</td>
<td>14.5</td>
<td>15.8</td>
<td>12.2</td>
<td>--</td>
<td>--</td>
<td>13.3</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>Beech dioxane lignin S/V</td>
<td>4.7</td>
<td>4.5</td>
<td>3.6</td>
<td>2.7</td>
<td>2.8</td>
<td>--</td>
<td>--</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Total aldehyde(%)**</td>
<td>12.6</td>
<td>13.6</td>
<td>15.4</td>
<td>14.9</td>
<td>10.0</td>
<td>--</td>
<td>--</td>
<td>14.8</td>
<td>20.4</td>
</tr>
<tr>
<td>Yamamomo MWL S/V</td>
<td>3.5</td>
<td>4.0</td>
<td>3.8</td>
<td>3.0</td>
<td>2.7</td>
<td>2.4</td>
<td>1.9</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Total aldehyde(%)**</td>
<td>17.0</td>
<td>17.7</td>
<td>18.3</td>
<td>17.9</td>
<td>19.3</td>
<td>17.3</td>
<td>19.0</td>
<td>17.9</td>
<td>23.6</td>
</tr>
<tr>
<td>Kiri MWL S/V</td>
<td>1.2</td>
<td>1.0</td>
<td>0.6</td>
<td>0.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Total aldehyde(%)**</td>
<td>14.2</td>
<td>13.7</td>
<td>12.0</td>
<td>12.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>13.3</td>
<td>17.1</td>
</tr>
</tbody>
</table>

*: Untreated with HCl. **: Per cent of fraction or lignin preparation.

microscope that birch lignin deposited in the secondary layers of wood fibers and ray parenycma cell walls is composed mostly of syringylpropane units. However a reservation must be made in the interpretation on the existence of syringyl and guaiacyl units-rich moieties in hardwood lignins by the spectral analysis, since the inherent errors in the determination of wave length of maximum absorbance are unavoidable. The methoxyl content is a most important indication of structural principle in lignin.

These chemical data in the present investigation has evidently shown the existence of syringyl unit-predominant lignin in hardwoods.

**Experimental**

Following woods; beech (Fagus crenata Blume), Yamamomo (Myrica rubra Sieb. et Zucc.), and Kiri (Paulownia tomentosa Steud.) were used in the present experiment. The wood powder (40-80 mesh) was extracted with warm water for 72 hr, boiling water for 25 min, and ethanol/benzene (1:1) for 24 hr, successively. MWL of the extractive-free powder was prepared as described previously. Dioxane lignin of beech residual wood powder, from which MWL was preextracted, was prepared as described by Browning.

**Mercuration of lignin preparation**

To a suspension of 8 g of MWL/ or dioxane lignin in 50 ml of absolute ethanol was added a solution of 18 g of mercuric acetate in a mixture of 500 ml of absolute ethanol and 33 ml of anhydrous acetic acid, and the mixture was refluxed for 4 hr. The progress of the reaction was accompanied with production of insoluble material.

**Fractionation of mercurated lignin preparation**

The hot reaction solution was immediately separated from the brown material by decantation and evaporated in vacuo to dryness. The residue was dissolved in 20 ml of 95% dioxane, and then the insoluble material (mercuric acetate) was filtered off. The clear solution was added dropwise to 500 ml of 2% acetic acid with vigorous stirring. Precipitates formed were washed with 2% acetic acid (3000 rpm, 5 times) and water (11000 rpm, 2 times) by using a centrifuge, and dried in vacuo over phosphorous pentoxide and potassium hydroxide. The milky powder thus obtained was suspended in 15 ml of a 2:1 mixture
of dichloroethane and ethanol and stirred for 1 hr, and then the soluble fraction was added dropwise to ether with stirring. Milky powder (Fraction I) was obtained. The insoluble residue in the mixture of dichloroethane and ethanol was fractionated and purified successively according to the scheme summarized in Fig. 11. On the other hand the brown material described above was purified as described previously, and used as Fraction IV (beech and Kiri MWLs) and Fraction VII (Yamamomo MWL). The insoluble residue of the mercurated beech dioxane lignin in 95% acetic acid, which contains more or less amounts of mercury (I) ion, was taken off, and the soluble fraction was used as Fraction V for analyses.

Demercuration of each fraction

Each fraction was demercurated with 15% hydrochloric acid and the total content of mercury was determined by the procedure as described previously.

Nitrobenzene oxidation of demercurated lignin preparation

Ten mg of each demercurated fraction was subjected to alkaline nitrobenzene oxidation,
and TMS derivatives of the aromatic aldehydes obtained were analyzed by gas-liquid chromatography as described previously.

IV. Concluding remarks

Enzymic dehydrogenation polymers (DHPs) of lignin monomers were subjected to alkaline nitrobenzene oxidation, acidolysis, and alkaline potassium permanganate and hydrogen peroxide oxidation. Degradation products of the DHPs were analyzed by gas-liquid chromatography. Chromatograms for DHP of p-coumaryl alcohol (p-DHP) showed the presence of p-hydroxybenzaldehyde and p-hydroxyphenylpropanones on nitrobenzene oxidation and acidolysis, which suggest the presence of p-hydroxyphenylglycerol-β-p-hydroxyphenyl ether moiety, and also the presence of anisic, 4-methoxyisophthalic, 4-methoxy-o-phthalic, 3,3'-dehydrodianisic, and 2-methoxydiphenyl ether-5,4'-dicarboxylic acids with a trace of methoxytrimesic acid on permanganate-hydrogen peroxide oxidation. Molar ratios of dimethyl 3,3'-dehydrodianisate to methyl anisate and dimethyl 4-methoxyisophthalate to methyl anisate were very similar to those of dimethyl 5,5'-dehydrodiveratrate to methyl veratrate and dimethyl isohemipinate to methyl veratrate, respectively. These results indicate that the degree of condensation at C-3 and C-5 between the DHP of p-coumaryl alcohol and DHP of coniferyl alcohol differs little, and that the possibility of double condensations at C-3 and C-5 in the p-hydroxyphenyl unit of the p-DHP is quite small.

Methylated Grass and bamboo MWLs were hydrolyzed, remethylated, and then subjected to alkaline potassium permanganate and hydrogen peroxide oxidation successively. Methylesters of carboxylic acids produced were analyzed by gas-liquid chromatography. Chromatograms for grass and bamboo MWLs showed the presence of 2,3,2'-trimethoxydiphenyl-5,5'-dicarboxylic, 2,3- and 2,2'-dimethoxydiphenyl ether-5,4'-dicarboxylic acids which could not be detected for softwood and hardwood lignins, indicating a peculiar feature of grass lignins. The chromatograms also showed the presence of anisic, 4-methoxyisophthalic, and a trace of methoxytrimesic acids as well as acids of veratrole and trimethoxybenzene series. No 3,3'-dehydrodianisic and 2-methoxydiphenyl ether-5,4'-dicarboxylic acids were found. From these results, it was concluded that the grass lignin is qualitatively but not quantitatively similar to hardwood lignin in the occurrence of the structural elements composed of p-hydroxyphenyl and guaiacyl, two guaiacyl, and guaiacyl and syringyl units through diphenyl ether and biphenyl, and that the structural elements composed of two p-hydroxyphenyl units through diphenyl ether and biphenyl may not participate.

From the results obtained by the methylation-permanganate oxidation of p-DHP and grass lignin, it was confirmed that the occurrence of diphenyl ether moiety is characteristic of the structure of mature lignin, and the occurrence of diphenyl ether moiety in lignin was discussed in relation to Sphagnum polyphenol.

It has been considered that no polymer is given by enzymic dehydrogenation of sinapyl alcohol. In the present investigation, however, a considerable amount of lignin-like polymer
(s-DHP) was found to be formed from sinapyl alcohol alone by the Zutropf method in the presence of peroxidase and hydrogen peroxide.

A constitutional model of the s-DHP was established on the basis of analyses of UV, IR, PMR and CMR spectra of the s-DHP, the determination of contents of methoxyl, free phenolic hydroxyl, α-carbonyl, p-hydroxybenzyl and p-alkoxybenzyl alcohol groups, the estimation of amounts of α- and β-O-4 linkages, the determination of molecular weight, the yields of syringaldehyde and trimethylgallic acid on alkaline nitrobenzene and permanganate-hydrogen peroxide oxidation, and the yields of Hibbert's monomers and syringaresinol on acidolysis.

The formation of s-DHP gives a foundation on a possible occurrence of syringyl lignin in hardwood tissues.

A view which is positive in denying the occurrence of syringyl lignin in nature has hitherto been influential. However it has been known that the relative amounts of syringyl units in angiosperm lignins differ from species to species, and tissues to tissues. A possibility of the occurrence of syringyl lignin should not be overlooked. To reveal the existence of syringyl lignin, very effective methods have been required. Spectral analyses of ultrathin sections of woods with a UV microscope have offered potential information.

The present application of mercuration of hardwood lignin is also of advantage for the selective isolation of syringyl lignin. The mercuration is a cationid substitution for lignin not involving loss of methoxyl group. Generally acetoxymercuri derivatives of phenols are easily dissolved in acetic acid but hardly in neutral organic solvent. Therefore acetic acid was used for specific fractionation of mercurated hardwood lignins. The distinct difference of the solubility in acetic acid between mercurated s-DHP and c-DHP was found, and s-DHP and c-DHP were separated from a mercurated 1:1 mixture of s-DHP and c-DHP by stepwise fractionation. Syringyl unit-predominant lignin were finally isolated and characterized from woods of beech and Yamamomo (Myrica rubra Sieb. et Zucc.) by the application of mercuration. Methoxyl content was determined to be 24.5% for a lignin fraction obtained from beech MWL, to be 27.8% for a lignin fraction from beech dioxane lignin, and to be 25.2% for a lignin fraction from Yamamomo MWL, respectively.
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The author thanks Associate Professor Shigeyuki Yoshida of Laboratory of Landscape and Architecture, Kagawa University for his courtesy in gathering vascular plants.
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p-ヒドロキシフェニル並びにシリンギルリグニンに関する研究

山崎 徹

イネ科植物リグニンは著量のp-ヒドロキシフェニルプロパン体を構成単位として含んでいる。この論文において先ずp-クマールアルコールの脱水素重合物（p-DHP）の化学的性質との関連でイネ科植物リグニン中のp-ヒドロキシフェニル核の存在様式について研究を行った。p-DHPの酸化分解生成物中から芳香族アルデヒドを、アシドリシス生成物中から多量のヒーバートのケトン類を得た。これらの結果はp-DHP中にβ-O-4構造が存在していることを強く示唆している。メチル化p-DHPのKmno₄・H₂O₂分解生成物中から主生成物として5種の芳香族酸を、微量生成物としてメトキシトリメチレン酸を得た。一方、フェノールアルコールの脱水素重合物（c-DHP）をメチル化後、Kmno₄・H₂O₂で分解してp-DHPから生成するアミノソール系分解物に対応するペラトリール系分解物をすべて同じモル比で得た。以上の結果からp-クマールアルコールの脱水素重合の機構は本質的にフェノールアルコールのそれと同一であることが明らかとなった。イネ科植物リグニンのメチル化物をKmno₄・H₂O₂で分解し、生成物中から得られたもとで発見リグニンから得られなかったp-ヒドロキシフェニル核とグワヤシル核から構成されているジフェニル酸を一種、ジフェニルエーテル酸を二種得たが、p-ヒドロキシフェニル核が2種類の構成をもっていることを明らかにした。

天然にメトキシシリンギル高含量のリグニンは見出されていないことおよびシリンギルアルコール単独ではラッカーゼ・O₂の作用で高分子を与えないことの理由から、従来シリンギルプロパン単位から構成されているシリンギルリグニンは自然界に存在しないと考えられていた。しかし、この論文において着目下法によりシリンギルアルコールがベレオキシダーゼ・H₂O₂の作用で多量のリグニン亜重合物（s-DHP）を与えることを明らかにした。s-DHPのメトキシシリンギルあるいはそのメチル化物のKmno₄・H₂O₂による分解並びにアシドリシスによる生成物の同定、種々の官能基分析、UV、IR、¹H-NMR並びに¹³C-NMRスペクトル解析および分子量測定結果に基づき、シリンギルプロパン七重体から成るs-DHPのモデル構造を明らかにした。s-DHPの形成は天然にシリンギルリグニンが生成している可能性を強く示唆している。さらにこの論文において中性有機溶媒中に懸濁したs-DHPとc-DHPのアセットキシシリンギル亜酸に対する溶解度に非常に大きな差があることを見出し、s-DHPとc-DHPの混合物を無水エタノール中酢酸第二水酸で水銀化し、酢酸で分画することによってs-DHPとc-DHPをそれぞれ分離する手法を確立した。この水銀化法を適用し、ブナ属（Fagus crenata）およびヤマモモ属（Myrica rubra）からそれぞれメトキシシリンギル含有率27.8％、25.2％のほとんどシリンギルプロパン単位から構成されているリグニンを単離した。
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