Methoxyl-deficient Structural Elements in Lignin

of Sweetgum Decayed by a Brown-rot Fungus

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Methoxyl-deficient units in lignin isolated from wood of sweetgum
(*Liquidambar styraciflua* L.) decayed by the brown-rot fungus
Lenzites trabea Pers. ex Fries were investigated by identifying and
quantitatively estimating some ethoxybenzoic acids formed on
oxidative degradation of ethylated samples. Major products formed
were 4-ethoxy-3-methoxybenzoic acid (V), 3,5-dimethoxy-4-ethoxy-
benzoic acid (VII), 3,4-diethoxybenzoic acid (VI), and 3,4-diethoxy-5-
methoxybenzoic acid (VIII). V and VII arose from normal lignin
units, but VI and VIII were formed from methoxyl-deficient units,
disclosing that the fungus had demethylated phenolic units of the
guaiacyl and syringyl types. Much larger yields of VI and VIII, and
of V and VII, were obtained from a sample refluxed with 0.2 M HCl
in dioxane/water (9:1) ("acidolysis") before ethylation-oxidation,
showing that the fungus also had demethylated etherified (non-
phenolic) units of the guaiacyl and syringyl types within the lignin
molecule. However, such non-phenolic units had been demethylated
less extensively than phenolic units. Both phenolic and non-phenolic
structures of the syringyl type had been demethylated more ex-
tensively than corresponding structures of the guaiacyl type.

Similar investigations of Björkman lignin prepared from sound
wood of sweetgum indicated that small amounts of 3,4-dihydroxy-
phenyl and 3,4-dihydroxy-5-methoxyphenyl units (III and IV) were
already present.

In the attack of wood by the Basidiomycetes known as brown-rot fungi,
carbohydrates are decomposed and removed, leaving a residue almost
equal in weight to the entire lignin in the original wood. Lignin freed in this
way from its normal intimate association with wood carbohydrates is known
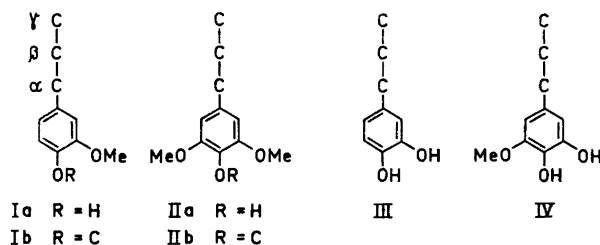as "enzymatically liberated lignin".[1]

This lignin has qualities which make it attractive for comparison with
other isolated lignins in chemical and physical studies.[2] However, it has not
been determined to what extent the fungi alter the chemical and physical

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properties of the original lignin. An enzymatically liberated lignin was therefore compared with lignin from sound wood to ascertain the nature and extent of changes brought about by the fungus. The first studies of lignin isolated from brown-rotted wood indicated that the lignin was essentially unaltered,[1] but subsequent studies [2],[3a],[4],[5] have confirmed an early report [6] that the fungi cause a decrease in the methoxyl content. It also has been shown that the fungi cause an increased IR absorption in the carbonyl region [2] and certain less defined changes.[3b] Researches described here were aimed at determining the structural significance of the decreased methoxyl content of the lignin.

A lignin of sweetgum (*Liquidambar styraciflua* L.), prepared in connection with an earlier study [7] by use of the brown-rot fungus *Lenzites trabea* Pers. ex Fries, was separated by preparative gel permeation chromatography into four fractions of different molecular weight; these were used in the present investigation. Björkman lignin ("milled wood lignin") [8] from sound sweetgum wood was used for comparison.

Preliminary studies [9] with one fraction disclosed the presence of catechol (3,4-dihydroxyphenyl) moieties (III and IV) in the lignin; these structures

Iα R = H   IIα R = H   III   IV
Ib R = C   IIb R = C

were formed by fungal demethylation of lignin units of the guaiacyl (Iα) and syringyl (IIα) types.* Enzymatically liberated lignin of spruce (*Picea sitchensis* Carr.) prepared with the brown-rot fungus *Poria monticola* Murr. also contained catechol moieties of type III.[9]

The presence of *o*-diphenol structures was demonstrated in the following way. Samples of the lignins were ethylated to convert free phenolic hydroxyl groups to ethoxyl groups. Subsequent oxidative degradation produced ethoxybenzoic acids. The carboxyl group of these acids was derived primarily from C-α of the propyl side chains; a few units have an aryl substituent on C-β.[10–12] The methyl esters of the ethoxybenzoic acids were identified by gas chromatography-mass spectrometry using procedures developed for similar investigation of methylated lignins.[13],[14] The occurrence of *o*-diethoxybenzoic acids VI and VIII among the products reflected the presence of catechol moieties in the lignins (*cf.* Ref. 15).

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* In referring to the aromatic portions of the substituted phenylpropane units that comprise lignin, we have, for convenience, used the terms guaiacyl and syringyl to refer to 4-hydroxy-3-methoxyphenyl and 3,5-dimethoxy-4-hydroxyphenyl moieties. Units of the guaiacyl and syringyl types, but with no free 4-hydroxyl group, *i.e.* linked through the 4-oxygen atom to a carbon atom of an adjacent phenylpropane unit (see Ref. 10), are referred to as "etherified" or "non-phenolic".

These ethylation-oxidation studies have now been extended to include a quantitative comparison of the four fractions of enzymatically liberated sweetgum lignin and the sweetgum Björkman lignin, and to investigate whether the fungus had demethylated etherified (non-phenolic) units of the guaiacyl (Ib) and syringyl (IIb) types.

RESULTS

Some properties of the enzymatically liberated lignin and the Björkman lignin are given in Table 1. Gel chromatography of the former [7] had provided four fractions of decreasing molecular weight, which together amounted to 80 % of the lignin in the original non-decayed wood. The estimated contribution of each individual fraction to the total lignin in the original wood is given in Table 1 (see Ref. 7). The Björkman lignin represented 20–25 % of the total

Table 1. Properties of four fractions of enzymatically liberated lignin and Björkman lignin from sweetgum.

Lignin sample	$\overline{M}_n$ [a]	Proportion of lignin in wood %[b]	Methoxyl %	Decrease in methoxyl %[c]
Enzymatically liberated lignin				
Fraction 1	5000	30	16.5	23.8
Fraction 2	2600	15	15.3	28.5
Fraction 3	1100	21	14.9	30.4
Fraction 4	400	14	11.8	44.9
Björkman lignin	4300	20–25	21.4	—

[a] From Ref. 7 (enzymatically liberated lignin) and Ref. 16 (Björkman lignin).
[b] Calculated on basis of original wood from data of Ref. 7 (enzymatically liberated lignin).
[c] By comparison with the methoxyl content of the Björkman lignin.

lignin. The methoxyl contents of the four fractions increased with molecular weight, but all had methoxyl contents substantially lower than that of the Björkman lignin. All four fractions were free of carbohydrates, as judged by the absence of sugars in acid hydrolysates.* Other characteristics of the fractions have been given earlier.[7]

The four fractions and the Björkman lignin were ethylated and then oxidized with $KMnO_4$ at pH 12 and 80°, then with 5 % H_2O_2 at pH 9–10 and 50°.[13,14] The methyl esters of the resultant acids V–VIII were separated and estimated by gas chromatography, after identification by comparison of their retention times and mass spectra with those of authentic samples. The major degradation acids from all the lignin samples were 4-ethoxy-3-methoxy-benzoic acid (V) and 3,5-dimethoxy-4-ethoxybenzoic acid (VII) (Table 2).

* These determinations were kindly conducted by Dr. Knut Kringstad, North Carolina State University at Raleigh, U.S.A.

Table 2. Yields of products formed on oxidative degradation of ethylated lignin samples.

Lignin sample	Products; yields [a] in μmoles/100 mg original lignin					
	V	VI	VII	VIII	Total V−VIII	IX
Enzymatically liberated lignin						
Fraction 1	7.7	2.4	8.2	3.9	22.2	0.6
Fraction 2	7.3	2.7	9.5	4.6	24.1	1.0
Fraction 3	7.3	2.5	10.5	4.8	25.1	0.8
Fraction 4	9.0	3.6	15.7	7.6	35.9	3.4
Björkman lignin	17.8	<0.4	11.0	<0.8	∼29	− [b]

[a] Determined by gas chromatography of methyl esters.
[b] Not detected.

The fractions of enzymatically liberated lignin gave rise to substantial amounts of 3,4-diethoxybenzoic acid (VI) and 3,4-diethoxy-5-methoxybenzoic acid (VIII). The total yield of products V − VIII increased with decreasing molecular weight of the fractions (Table 2), reflecting an increasing content of free 4-hydroxyl groups (in structures Ia, IIa, III, and IV) with decreasing molecular weight.
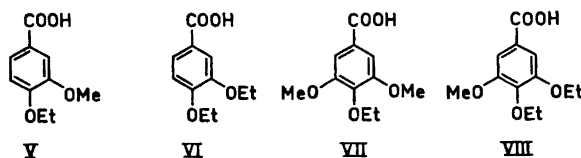
Small amounts of the two diethoxybenzoic acids VI and VIII also were formed from Björkman lignin (Table 2), indicating minor amounts of *o*-diphenol structures in the sound material.

In addition to compounds V − VIII, lesser amounts of 3,4,5-triethoxybenzoic acid (IX) were formed from the four fractions of enzymatically liberated lignin. The yields of this product were similar for fractions 1 − 3, but greater for fraction 4 (Table 2). Small amounts of several other aromatic acids (*cf.* Refs. 13, 14) were formed on oxidation of the ethylated lignins, but were not identified.

The results described above concern products derived from phenolic units in the lignin, *i.e.* units with a free hydroxyl substituent *para* to the propyl side chain. However, in most of the phenylpropane units in lignin this *para*-oxygen atom is in an ether bond. In spruce Björkman lignin, for example, about three-fourths of the phenylpropane units are etherified in this way.[17] The question therefore arose whether etherified (non-phenolic) units inside the enzymatically liberated lignin also had been demethylated by the fungus. Assuming that half or more of the phenylpropane units in the lignin fractions did not contain a free *para*-hydroxyl group, it can be calculated from the methoxyl contents (Table 1) that the percentages of demethylation probably

could not be accounted for on the basis of demethylation of phenolic units alone. Thus it appeared likely that non-phenolic units also had been demethylated. This was confirmed by investigations described below.

It has been shown that acidolysis of lignin by refluxing with 0.2 M HCl in dioxane/water (9:1) results in depolymerization accompanied by a substantial increase in the phenolic hydroxyl content.[18,19] This is due to cleavage of the arylglycerol-β-aryl ether bond in structures of type X, and to a lesser extent to cleavage of other types of aryl ether bonds (see Refs. 17 – 19). It has been demonstrated with a large number of model compounds that demethylation of structures of types Ia and IIa does not occur during acidolysis.[20]

Samples of fraction 2 were therefore ethylated, subjected to 8 h acidolysis, re-ethylated, and oxidatively degraded. Determination of the yields of products V – VIII revealed large increases in each over the yields from the non-acidolyzed fraction (Table 3; cf. Table 2). This provides clear evidence that fungal demethylation was not limited to structural units bearing free phenolic hydroxyl groups. More definitively, the result is consistent with the presence of structures of types XI and XII in the enzymatically liberated lignin. A slight increase in the yield of IX was also obtained from fraction 2 after acidolysis (cf. Tables 2 and 3), a result which is consistent with the presence of a small proportion of structures of type XIII (see Discussion).

Table 3. Yields of ethoxybenzoic acids formed on oxidative degradation of lignin samples following ethylation, acidolysis, and re-ethylation.

	Products; yields[a] in μmoles/100 mg original lignin					
Lignin sample	V	VI	VII	VIII	Total V – VIII	IX
Enzymatically liberated lignin						
Fraction 2	17.3	4.3	30.1	9.3	61.0	1.4
Björkman lignin	34.6	<0.4	58.0	<1.2	~93	–[b]

[a] Determined by gas chromatography of methyl esters.
[b] Not detected.

Ethylated Björkman lignin was also acidolyzed, re-ethylated and then oxidatively degraded in the same way as fraction 2; determination of the principal products (V and VII) revealed large increases in yields over the non-acidolyzed material (cf. Tables 2 and 3).

In the initial experiments [9] diethyl sulfate was used as ethylating agent, and the yield of VII from non-acidolyzed Björkman lignin of sweetgum was higher than was found in subsequent studies (Table 2). It is suspected that the higher yield in the first studies was the result of partial hydrolysis of the sample during ethylation. In the present studies diazoethane was used to ethylate the non-acidolyzed samples, so that this problem was avoided.

DISCUSSION

These investigations show that *Lenzites trabea* demethylates both free and etherified units of the guaiacyl and syringyl types in sweetgum lignin. A hydroxyl group *ortho* to the methoxyl group is not required for demethylation. Recent studies have shown that demethylation of vanillic acid by the bacterium *Pseudomonas fluorescens* also does not require the *ortho*-hydroxyl group, because both veratric acid and 3-methoxybenzoic acid are demethylated almost as readily.[21] Demethylation by *P. fluorescens* involves direct oxidation of the methyl group, which is then eliminated as formaldehyde, leaving a free phenolic hydroxyl group.[21] Demethylation of lignin by brown-rot fungi may involve a similar process.

Table 4. Ratios A and B (see text) for products formed on ethylation-oxidation of four fractions of enzymatically liberated lignin of sweetgum.

Lignin sample	Ratio A	Ratio B
Fraction 1	0.24	0.32
Fraction 2	0.27	0.33
Fraction 3	0.26	0.31
Fraction 4	0.29	0.33

The approximate extent to which the fungus had demethylated uncondensed structural units in the lignin can be estimated from the quantitative results given in Tables 2 and 3. Ratios A and B reflect the relative proportion of methoxyl-deficient units of the guaiacyl and syringyl types (Table 4).

$$A = \frac{\text{yield of VI}}{\text{yield of V} + \text{yield of VI}}$$

$$B = \frac{\text{yield of VIII}}{\text{yield of VII} + \text{yield of VIII}}$$

"Condensed" units in lignin, *i.e.* units linked through their aromatic nuclei to adjacent units (see Refs. 10, 22, 23), are irrelevant to this consideration, since they do not give rise to products V – VIII.

Comparison of the ratios A and B for the four (non-acidolyzed) fractions (Table 4) indicates that the extent of demethylation of phenolic units of the guaiacyl or syringyl types (Ia or IIa) was similar for each fraction.
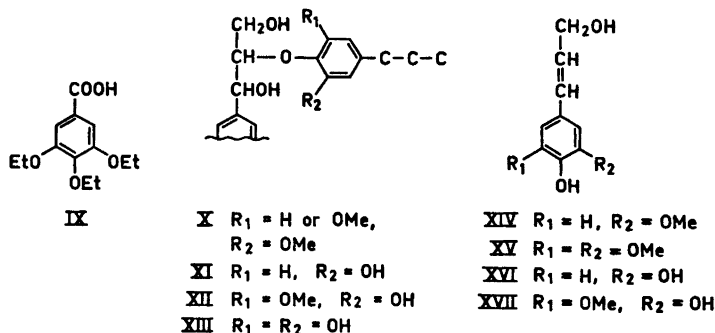
Phenolic units of both the guaiacyl (Ia) and syringyl (IIa) types had been demethylated to a greater extent than the corresponding non-phenolic units Ib and IIb. Subtraction of the yields of V – VIII formed from non-acidolyzed fraction 2 (Table 2) from the yields for acidolyzed fraction 2 (Table 3) gives the yields of products formed from uncondensed, etherified (non-phenolic) units released on acidolysis. Calculation of ratios A and B using these difference values gives 0.14 for units of the guaiacyl type (ratio A) and 0.19 for units of

the syringyl type (ratio B). Much higher ratios (0.27 and 0.33) were obtained for the non-acidolyzed fraction (Table 4). This difference in demethylation between non-phenolic and phenolic units possibly reflects differences in accessibility of the units in the polymer to the demethylating enzyme system of the fungus.

For both phenolic and non-phenolic units it is apparent that units of the syringyl type had been more extensively demethylated than those of the guaiacyl type. This preferential demethylation of units of the syringyl type may merely reflect the fact that these units have two methoxyl groups, so that there is a greater probability that the demethylating enzyme system would come in contact with them.

As mentioned above the ethylation-oxidation studies showed that the fractions were similar with respect to ratios A and B (Table 4). These ratios therefore provide no direct indication of why the fractions differed in extents of demethylation as evidenced by their differing methoxyl contents (Table 1). However, since the total yields of V — VIII increased with decreasing molecular weight (*i.e.* the amount of phenolic units of the types giving rise to products V — VIII increased with decreasing molecular weight), and since phenolic units were demethylated to a greater extent than non-phenolic units, the total amount of demethylation should increase with increasing content of phenolic units, *i.e.* with decreasing molecular weight. This can account for the differences between fractions 1, 2, and 3 in methoxyl contents. However, fraction 4, which had the lowest molecular weight, had a methoxyl content which indicated that it apparently had lost 45 % of its initial methoxyl groups in comparison to the Björkman lignin (Table 1); this difference in methoxyl content was too great to be explained by the extents of demethylation indicated by the values of ratios A and B given in Table 4.

In part the low methoxyl content of fraction 4 apparently was due to the presence of a polyphenolic contaminant. It has been found recently that sound sapwood of sweetgum contains small amounts of a polyphenolic component or "tannin".[24] This tannin has not been studied extensively, but ethylation-oxidation yields substantial amounts of 3,4-diethoxybenzoic acid (VI) and especially 3,4,5-triethoxybenzoic acid (IX), and acid hydrolysis yields substantial amounts of gallic acid.[24] It is therefore evident that the sweetgum tannin contains galloyl residues and 3,4-dihydroxyphenyl moieties. Acid hydrolysis [24] of fraction 4 yielded 1.6 μmole of gallic acid per 100 mg of sample, an amount equivalent to about half the IX formed on ethylation-oxidation. Fraction 4 thus contained some tannin material, which lowered its methoxyl content. This tannin probably accounted also for part of the VI formed on ethylation-oxidation of fraction 4. Acid hydrolysis of fractions 1 — 3 yielded no gallic acid, indicating that galloyl residues were not present in these fractions. Nevertheless, it is possible that part of the 3,4-diethoxybenzoic acid (VI) formed on ethylation-oxidation of fractions 1 — 3 was derived from a polyphenolic component. Although the sweetgum tannin contains galloyl residues and 3,4-dihydroxyphenyl moieties, it is not known whether both structures occur in the same molecule. It is possible therefore that in the present study fractions 1 — 3 contained some polyphenolic material which had 3,4-dihydroxyphenyl moieties but not galloyl residues.

CH₂OH R₁
CH—O—⟨ ⟩—C–C–C
CHOH R₂

COOH

EtO   OEt
    OEt

CH₂OH
CH
CH
R₁   R₂
OH

IX

X   R₁ = H or OMe,
R₂ = OMe

XI   R₁ = H, R₂ = OH

XII   R₁ = OMe, R₂ = OH

XIII   R₁ = R₂ = OH

XIV   R₁ = H, R₂ = OMe

XV   R₁ = R₂ = OMe

XVI   R₁ = H, R₂ = OH

XVII   R₁ = OMe, R₂ = OH

Some of the 3,4,5-trihydroxyphenyl moieties that gave rise to IX on ethylation-oxidation of fractions 1 – 3 (and, in part, fraction 4) were formed *via* a "double-demethylation" of structures of the syringyl type. Since acidolysis followed by ethylation-oxidation of the enzymatically liberated lignin led to a slightly increased yield of IX (*cf*. Tables 2 and 3), the presence of "double-demethylated" non-phenolic units of the syringyl type (structures of type XIII) is indicated.

Table 5. Ratio *C* for products formed on ethylation-oxidation of Björkman lignin and four fractions of enzymatically liberated lignin of sweetgum.

Lignin sample	Ratio *C*
Enzymatically liberated lignin	
Fraction 1	1.2
Fraction 2	1.4
Fraction 3	1.6
Fraction 4	1.8
Fraction 2, acidolyzed	1.8
Björkman lignin	0.6
Björkman lignin, acidolyzed	1.7

The ratio (*C*) of products derived from uncondensed units of the syringyl type to those derived from uncondensed units of the guaiacyl type allows further comparison of the lignin samples to be made (Table 5).

$$C = \frac{\text{yield of VII} + \text{yield of VIII}}{\text{yield of V} + \text{yield of VI}}$$

This ratio for the four non-acidolyzed fractions was found to increase with decreasing molecular weight, and to be significantly lower for the Björkman

lignin. Products derived from the two acidolyzed samples (Björkman lignin and fraction 2) gave ratios similar to each other.*

For both the Björkman lignin and fraction 2 ratio C was much larger for acidolyzed than non-acidolyzed material (Table 5). Larsson and Miksche [14] recently observed a similar large increase in the "syringyl/guaiacyl" ratio of the major products formed on methylation-oxidation of Björkman lignin of birch (*Betula verrucosa* Ehrh.) following acidolysis or "sulfate cooking". The probable reasons for this large increase are discussed by Larsson and Miksche.[14]

The ratios C for the products formed on ethylation-oxidation of all of the fractions were much larger than that for the Björkman lignin *before* acidolysis (Table 5). It is possible that the fungus caused a partial depolymerization of the lignin with release of phenolic hydroxyl groups. Such depolymerization would be expected to result in larger values for ratio C for products formed on ethylation-oxidation; the observed increase in ratio C with decreasing molecular weight of the fractions is consistent with this.

The total yield of V − VIII after acidolysis of fraction 2 was lower than the yields from the Björkman lignin after the same treatment (Table 3). The reason for this difference is not clear; it is possible that some structures that otherwise would give rise to V − VIII were altered by the fungus.

Small amounts of VI and VIII were among the products formed on ethylation-oxidation of the Björkman lignin of sweetgum (Table 2). These were obtained also with a second preparation of Björkman lignin of sweetgum [24] and with a Björkman lignin of birch (*B. verrucosa* Ehrh.),[25] indicating small amounts of catechol structures in unattacked lignins of these two woods. The origin of these groups is not known, but their presence raises the question whether, in addition to the major precursors of hardwood lignin, *i.e.* coniferyl alcohol (XIV) and sinapyl alcohol (XV),[22,23] small amounts of the corresponding dihydroxy compounds, *viz.* caffeyl alcohol (XVI) and 5-methoxycaffeyl alcohol (XVII), may have been incorporated into the lignins. Freudenberg and Heel [26] showed that caffeyl alcohol readily forms a polymer on dehydrogenation by a phenol-oxidizing enzyme; they considered the possibility that XVI may be incorporated into lignin.

EXPERIMENTAL

Dioxane was purified according to Vogel.[27]

Ultraviolet spectra of lignins were measured with a Beckman DK-2 instrument, using dioxane/water 1:1 or 7:3 as solvent. Alkaline solutions (in connection with measurements of the $\Delta\varepsilon$-curves for ionization [28]) were prepared by adding 60 μl of 5 M aqueous NaOH directly to 3.0 ml solutions of lignins in 1 cm cuvettes.

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* *Note added in proof.* It was shown recently that the "guaiacyl" compound 4-hydroxy-3-methoxyphenylethylamine is enzymatically hydroxylated to 3,4-dihydroxy-5-methoxyphenylethylamine on incubation with mammalian liver homogenates (Bennington, F. and Morin, D. *Experientia* **24** (1968) 33). In the present study, such a hydroxylation of guaiacyl structures in the lignin by the fungus would have led to the 3-methoxycatechol structure which gave rise to acid VIII on ethylation followed by oxidative degradation. However, hydroxylation of guaiacyl structures apparently does not play an important role in our case since the "syringyl/guaiacyl" ratios for products V − VIII (*i.e.*, ratios C) *after acidolysis* were very similar for the Björkman lignin and the fungus-degraded lignin (Table 5). Any substantial hydroxylation of guaiacyl units to 3-methoxycatechol moieties would have affected this ratio by decreasing the amount of guaiacyl structures as well as by increasing the apparent amount of syringyl structures.

The following reference compounds (V—IX) were prepared.

4-Ethoxy-3-methoxybenzoic acid (V), *3,4-diethoxybenzoic acid (VI)*, and *3,5-dimethoxy-4-ethoxybenzoic acid (VII)* were prepared by ethylation of vanillic acid, protocatechuic acid, and syringic acid with diethyl sulfate. M.p.: V, 195—196° (lit.[29] 193—194°); VI, 164—167° (lit.[30] 165—166°); VII, 122—123° (lit.[31] 123—124°).

3,4-Diethoxy-5-methoxybenzoic acid (VIII). 3-Methoxy-5-methyl-*o*-benzoquinone (1.14 g, 7.5 mmoles), prepared according to Teuber and Staiger,[32] was suspended in 25 ml of cold ethanol, and solid NaBH₄ added slowly with stirring to the cooled ethanolic mixture until the red color had disappeared, leaving a clear pale yellow solution. After addition of 15 ml of water and acidification with dilute H_2SO_4, the solution was extracted with chloroform, and the extract washed with water, dried over Na_2SO_4, and evaporated, leaving a yellow oil (0.90 g=80 %) which crystallized on standing (m.p. 65°, from ether-hexane; 3,4-dihydroxy-5-methoxytoluene, 65° [33]). Half a gram was ethylated in dioxane water (2:1, 16 ml containing 400 mg of Na_2SO_3 to inhibit oxidation) by alternate, dropwise addition of diethyl sulfate and 10 M NaOH under nitrogen. The pH was maintained above 8, and the temperature at 80—85°. After 1 h the alkaline solution was extracted with chloroform, and the chloroform layer washed with 1 M HCl, water, and dried over Na_2SO_4. Evaporation of solvent left a brown oil. This was dissolved in 17 ml of *t*-butanol, and 34 ml of 1.5 % Na_2CO_3 solution was added. The solution was stirred vigorously at 80—85° as 5% $KMnO_4$ was added during 6 h at a rate sufficient to maintain a purple color. Excess $KMnO_4$ was destroyed by addition of ethanol, and the MnO_2 removed by filtration. The cooled alkaline filtrate was washed with chloroform, then acidified and extracted with chloroform. The extract was washed, dried over Na_2SO_4, and evaporated, leaving an oil which crystallized. Recrystallization from chloroform-petroleum ether yielded 181 mg of VIII; fine white needles, m.p. 113—114° (lit.[34] 112—113°). Yield: 23 % on the basis of 3,4-dihydroxy-5-methoxytoluene.

3,4,5-Trimethoxybenzoic acid and *3,4,5-triethoxybenzoic acid (IX)* were purchased from Fluka A.G., and recrystallized from chloroform/hexane.

To prepare the methyl esters of reference compounds for gas chromatography, small amounts of acids V—IX were dissolved in methanol and methylated with diazomethane in ether.

Diazoethane in ether/dimethylformamide. Diazoethane in ether was prepared by modification of a procedure for the preparation of diazopropane.[35] *N*-Nitroso-*N*-ethylurea (3.0 g) was added during 1 min to a vigorously stirred mixture of 10 ml of 10 M NaOH and 30 ml of ether in an ice bath. Stirring was continued for 30 min and the dark yellow ether solution decanted onto 3—4 g of KOH pellets and left standing at 0° for 2 h. The solution was filtered off and added to 30—40 ml of dimethylformamide (DMF) in an open vessel. The ether was allowed to evaporate for 30 min, and the resulting solution of diazoethane in ether/DMF (0.4 g $C_2H_4N_2$=35 % yield) was used immediately.

Ethylation of lignins. Lignins (40—100 mg) were dissolved in 5 ml of DMF and 5 ml diazoethane solution added at once and again after 1, 3, 5, and 7 days (5 additions); the reaction mixture was kept in a loosely corked flask in the dark at room temperature. Two days after the final addition of diazoethane, solvents were removed by film evaporation (<50°) to give pale yellow to brown glassy solids. UV spectra of the ethylated lignins were not altered by addition of base, indicating that the lignins contained no phenolic hydroxyl groups (see Ref. 28). Treatment of a representative ethylated fraction or ethylated Björkman lignin with periodate yielded only traces of methanol (or ethanol), confirming the absence of phenolic hydroxyl groups (see Ref. 17).

Oxidation of ethylated lignins (cf. Refs. 13, 14). Lignins (40—100 mg) ethylated as above were dissolved in ~10 ml *t*-butanol/water (1:1) and added dropwise to a vigorously stirred 1% aqueous solution (75 ml) of Na_2CO_3, followed by 5% $KMnO_4$ during 3 h at a rate sufficient to maintain a purple color. During the first 30 min the reaction mixture was heated to 80°, at which temperature it was maintained. The reaction was stopped by adding a few ml of ethanol; the MnO_2 then was removed by filtration, and washed with a few ml of hot 1 % Na_2CO_3.

Combined filtrate and washings were cooled, extracted with ether and hexane, neutralized with 2 M H_2SO_4, and evaporated to 30 ml. To this solution were added 0.8 g of Na_2CO_3 and 5 ml of 30 % H_2O_2. The mixture was kept at 50° for 10 min. Excess H_2O_2 was then destroyed with $KMnO_4$, and excess $KMnO_4$ with $Na_2S_2O_5$. One ml of 3,4,5-trimethoxybenzoic acid solution (1.53 mg in 1 % Na_2CO_3) was added as internal standard

for gas chromatography, and the solution acidified with 2 M H_2SO_4. This mixture was extracted with four portions of chloroform/acetone (1:1, 45 ml/portion), then with chloroform. Extraction of compounds V—IX and internal standard was complete with this procedure. The extract was washed with ~30 ml of H_2O, and solvent removed.

The residue was dissolved in 2—3 ml of methanol (4°) and methylated by addition of diazomethane in ether. Excess diazomethane was destroyed with glacial acetic acid, ether was added, and the solution was washed with $NaHCO_3$ solution and water. Evaporation of solvent left a small amount of yellow oil. This was dissolved in 100—300 μl of acetone for gas chromatography.

Gas chromatography. Column dimensions: 200×0.3 cm o.d. stainless steel tubing. Solid support: Chromosorb G, acid-washed and treated with dimethyldichlorosilane, 80—100 mesh. Stationary phases: A) Silicone elastomer OV-1 (Applied Sciences Laboratories), 5 % by weight of solid support; or B) Apiezon L, 5 % by weight of solid support. Temperature: column A 187°, column B 210°. Injection: 300°. Detector: 230°. Carrier gas: N_2, 25 ml/min. Detector: flame ionization. The instrument was used with dual matched columns.

Quantitative determinations of compounds VI—IX (as methyl esters) were made with standard curves comparing peak areas with that of the internal standard, 3,4,5-trimethoxybenzoic acid methyl ester. The methyl ester of V was determined with column B, since in column A it was not separated well from an unidentified component which appeared to result from the treatment with diazoethane. With column B, however, it was necessary to use the methyl ester of VII (determined previously on column A) as internal standard, since 3,4,5-trimethoxybenzoic acid methyl ester was not separated from the methyl ester of VI with column B.

Gas chromatography-mass spectrometry. Compounds V—IX were identified by the retention times of their methyl esters relative to that of the internal standard and by mass spectrometric comparison of their methyl esters with synthetic samples using an LKB 9000 gas chromatograph-mass spectrometer unit. a) *Gas chromatography.* Column: as above, except 100 cm long, and with General Electric silicone elastomer SE-30 (5 % by weight of solid support) as stationary phase. Temperature: 180°. Carrier gas: He, 25 ml/min. b) *Mass spectrometry.* Molecule separators: 230°. Ion source: 270°. Electron energy: 70 eV.

Acidolysis [18,19] *of ethylated lignin and subsequent re-ethylation and oxidative degradation.* Lignin (~60 mg) ethylated with diazoethane was dissolved in 30 ml of 9:1 dioxane/2 M HCl and refluxed under pre-purified nitrogen for 8 h. The pH was then adjusted to 3—4 with 0.4 M $NaHCO_3$, and the solvents removed to about 2 ml. Methyl cellosolve and water were added to give 10—15 ml of a solution in approximately 2 parts methyl cellosolve to 1 part water. Alternate, dropwise addition of 10 M NaOH and diethyl sulfate (85—90°, with stirring, N_2 atmosphere) effected ethylation. After 1 h, excess diethyl sulfate was hydrolyzed by stirring the alkaline solution for 30 min at 85—90°. The pH of the cooled reaction mixture was adjusted to about 7, and solvents were removed by film evaporation. The residue was dissolved in *t*-butanol/water (1:1, about 10 ml) and added to 75 ml of vigorously stirred 1 % Na_2CO_3. Oxidations and analyses were accomplished as described above.

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