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On the Reactions of Two Fungal Laccases Differing in Their Redox Potential with Lignin Model Compounds: Products and Their Rate of Formation

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Laccases (EC 1.10.3.2) are multicopper oxidases able to oxidize phenolic compounds such as lignin-related polyphenols. Since the discovery that so-called mediators effectively extend the family of laccase substrates, direct interactions between lignin-like materials and laccase have gained much less attention. In this work, the aim was to characterize oxidation products formed in direct laccase-catalyzed oxidation of different guaiacylic and syringylic lignin model compounds with two different laccases: a low redox potential *Melanocarpus albomyces* laccase and a high redox potential *Trametes hirsuta* laccase. By following the formation of different, mainly biphenylic (5-5) and benzylic oxidation products, it was found that although both of these enzymes generated practically the same pattern of products with particular types of syringyl and guaiacyl compounds, in some cases a clear difference in the rates of their formation was observed. The results also confirm further to the suggestions that syringylic compounds are able to act as mediators in their own oxidation reactions and also that in some instances acetylation of phenolic material may produce altered, unexpected structures.

KEYWORDS: Laccase; lignin; model compound; guaiacyl; syringyl; oxidation; product structure; nuclear magnetic resonance spectroscopy; liquid chromatography−mass spectrometry; electrospray ionization mass spectrometry; redox potential; *Trametes hirsuta*; *Melanocarpus albomyces*

INTRODUCTION

Laccases (EC 1.10.3.2) are multicopper oxidases able to catalyze one-electron oxidation, with concomitant reduction of O_2 to H_2O, of various substrates such as mono-, di-, and polyphenols, aminophenols, diamines, and some inorganic compounds (*1*−*3*). Lignin, an amorphous polyphenol accounting for approximately 30% of the organic carbon in the biosphere, is composed of three different types of phenylpropane units and contains *p*-hydroxyphenyl, guaiacyl, and syringyl types as aromatic moieties (none, one or two methoxyls *ortho* to the phenolic hydroxyl) (*4*, *5*). Softwood lignin contains mainly guaiacyl units, hardwood lignin contains guaiacyl and syringyl units, and both lignins also have low amounts of *p*-hydroxyphenyl units. Grass lignin contains guaiacyl and syringyl units, and more *p*-hydroxyphenyl units than wood lignin. The phenylpropane units are linked together to form a seemingly randomly organized polymeric network, with several types of linkages, the most abundant being the *β*-*O*-4 type (45−60%) (*5*). Other types of linkages are *β*-5, *β*-*β*, 5-5, 5-5/*β*-*O*-4 (dibenzodioxocin), 5-*O*-4, and *β*-1.

Laccase is able to oxidize directly only phenolic subunits of lignin, but the substrate range can be widened to nonphenolic units by the use of so-called mediators (*6*). Since the discovery of laccase-mediator system and its potential in industrial processes utilizing lignocellulosic material, laccase−synthetic mediator reactions have gained much more attention compared to reactions without such mediators. Obviously, laccase always reacts directly with lignin to some extent. For example, laccases polymerize softwood lignin and oxidize lignin precursors to synthetic lignin (*7*). Recently, there have also arisen questions about the importance of so-called natural mediators (*8*). Therefore, a thorough knowledge about the types of products formed and reaction mechanisms involved in laccase-induced reactions without synthetic mediators is of crucial importance in developing new applications for laccase with lignin.

Reactions of laccase and lignin have been studied earlier with some individual model compounds. These studies have mainly focused on lignin model compounds containing a syringyl unit as the phenolic moiety. Dimeric *β*-*O*-4 model compound syringylglycol *β*-guaiacyl ether (**7**) was oxidized by *Polyporus versicolor* laccase from benzylic position and also cleaved between Cα and phenyl ring to guaiacoxyacetaldehyde and 2,6-dimethoxy-*p*-benzoquinone (*9*). Syringylglycerol *β*-guaiacyl ether (**8**) gave different products in two different studies. Wariishi et al. (*10*) observed the formation of guaiacoxy propionic acid and 2,6-dimethoxy-*p*-benzoquinone (Cα−aryl cleavage), syringaldehyde and guaiacoxyethanol (Cα−C*β* cleavage), and also guaiacol (*O*−C*β* cleavage) by *Coriolus versicolor* laccase, when Kawai et al. (*11*) detected a product of benzylic hydroxyl oxidation, 2,6-dimethoxy-*p*-hydroquinone and glyceraldehyde-2-guaiacyl ether (Cα−aryl cleavage)

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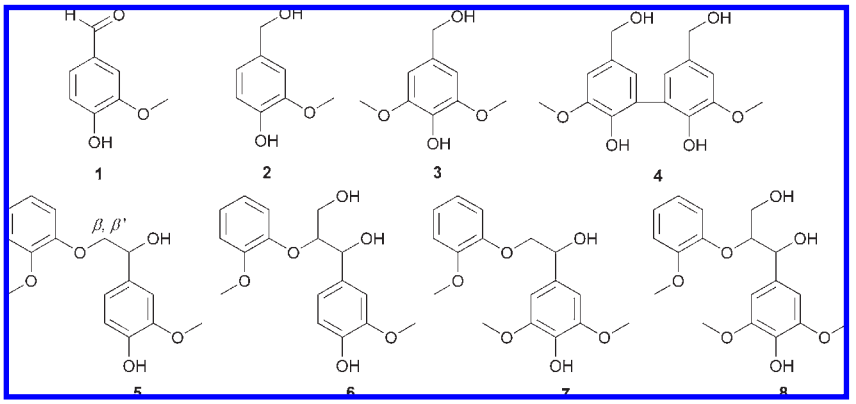

Figure 1. Lignin model compounds used in the study.

and guaiacol (*O*−C*β* cleavage) by the same *C. versicolor* laccase. Oxidation of syringylic *β*-1 dimers by *C. versicolor* laccase yielded products resulting from benzylic hydroxyl oxidation, Cα−C*β* cleavage, and alkyl−aryl cleavage (*12*, *13*).

There are even fewer studies about oxidation of model compounds containing guaiacyl units as the phenolic moiety (*4*, *5*, *7*). These types of lignin substructures are, however, abundant in softwoods. Vanillyl alcohol (**2**) has been oxidized by *Trametes versicolor* laccase to 4,4′-dihydroxy-3,3′-dimethoxybenzophenone and vanillin (**1**) (*14*). Oxidation of methyl to hydroxymethyl and demethylation by *T. versicolor* laccase have also been observed with diphenylmethane, α-5, and stilbene type models (*15*).

To understand more clearly the reactions of laccase and lignocellulosic material, we investigated in this study how the redox potential of the laccase and reaction time affect the product pattern. Direct reactions between two types of laccases, one from an ascomycete *Melanocarpus albomyces* (low redox potential laccase) and another from a white rot fungus *Trametes hirsuta* (high redox potential laccase), with eight different lignin model compounds representative for softwood and hardwood lignins (**1**−**8**, **Figure 1**) were elucidated. The formation of oxidation products was analyzed as a function of time with high-performance liquid chromatography (HPLC) and liquid chromatography−mass spectrometry (LC-MS). By using these methods, the order, by which the products were formed, and the formation rates of the products were followed, as well as the differences between low and high redox potential laccases. The structures of the products, deduced on the basis of their mass values from LC-MS, were further verified by fractionating the acetylated products and analyzing them with nuclear magnetic resonance (NMR) spectroscopy and high-resolution electrospray ionization mass spectrometry (ESI-MS).

MATERIALS AND METHODS

General. All commercial reagents and solvents were used as received unless otherwise mentioned. For preparative HPLC methanol−water was used as the eluent (isocratic flow) with the following equipment: ISCO 2350 HPLC pump, Shimadzu SPD-6A UV spectrophotometric detector, Shimadzu C-R6A Chromatopac data processor, reverse phase column (Waters SymmetryPrep C_{18}, 19 × 150 mm, 7 μm). Flash chromatography purifications and fractionations were done with silica gel 60 (Merck) and ethyl acetate−toluene as the eluent. For thin-layer chromatography silica gel 60 F_{254} TLC aluminum sheets (Merck) were used.

Model Compounds and Enzymes. *Model Compounds*. Of the used compounds vanillin was commercial (**1**, Merck). Other compounds were prepared according to well-known methods. Vanillyl (**2**) and syringyl (**3**) alcohols were reduced from the corresponding aldehydes with $NaBH_4$ in ethanol. Dehydrodivanillyl alcohol (*16*) (**4**), guaiacylglycol *β*-guaiacyl ether (*17*) (**5**), guaiacylglycerol *β*-guaiacyl ether (*17*) (**6**), syringylglycol *β*-guaiacyl ether (*9*) (**7**), and syringylglycerol *β*-guaiacyl ether (*18*) (**8**) were synthesized according to the previously reported methods. Synthesis of the guaiacylic *β*-*O*-4 trimer used in LC-MS mass calibration will be published elsewhere (*19*). The synthesized products were purified by crystallization, with preparative HPLC or flash chromatography.

Enzymes. The *M. albomyces* laccase was overproduced in *Trichoderma reesei* and purified as described earlier (*20*). The *T. hirsuta* laccase was produced in its native host and purified (*21*).

HPLC and LC-MS Analysis of Products as a Function of Time. *Analysis Equipment, Reagents, and Procedure*. HPLC was performed using reverse-phase columns (Agilent ZORBAX Eclipse XDB-C8, 4.6 mm × 15 cm, 5 μm, or XDB-C18, 2.1 mm × 100 mm, 3.5 μm), methanol−water as the eluent, and Agilent 1100 series HPLC. The C_{18} column was used for dimeric *β*-*O*-4 model compounds and the C_8 column for the other compounds. Gradient elution was applied starting from 50% methanol (v/v). The flow rate was 0.1 mL min^{-1} at the gradient elution phase (15 min) and 0.2 mL min^{-1} with 100% methanol.

LC-MS in negative-ion mode was acquired using the same HPLC followed by Mariner ESI-TOF (PerkinElmer Biosystems). Ionization was enhanced by spraying a 2.5% NH_3 solution from a separate line with syringe pump at 10 μL min^{-1} rate to the ionization chamber along with the analyte. Mass calibration was done with the starting material present in the sample and guaiacylic *β*-*O*-4 trimer injected at the beginning of the analysis.

Reaction Procedure for HPLC Analysis. Experiments were carried out with both laccases, and 12.5 mM model compound solutions were used. Solvent was dioxane/sodium succinate buffer (25 mM, pH 4.5) 2:8 with dimeric *β*-*O*-4 compounds and 1:9 with other compounds. Dioxane was distilled over sodium before use. Enzyme dosages of 2 nkat mL^{-1} for dehydrodivanillyl alcohol (**4**) and 1 nkat mL^{-1} for other compounds were used. Laccase activity was determined using ABTS as described by Niku-Paavola et al. (*22*). Enzyme was added to 10 mL of the model compound solution. Samples (0.5 mL) were taken at 1, 5, 10, and 30 min and 1, 2, 4, and 24 h to vials containing 1−2 mg of NaN_3, a known inhibitor of laccase (*23*). A control sample (0 min) was taken before the enzyme was added.

HPLC and LC-MS Samples. From each 0.5 mL sample two samples were prepared for HPLC. Of the original sample, 0.4 mL was diluted by adding 1.6 mL of water and 2.0 mL of methanol. The diluted sample was divided into two 2 mL samples and to one was added 2 μL of 50 mg mL^{-1} guaiacol solution (guaiacol from Merck) to reveal any material loss. All samples were analyzed with HPLC and some of them with LC-MS to identify oxidation products. The mass spectral data are in **Table 1**.

NMR Analysis and High-Resolution ESI-MS of Acetylated Oxidation Products. *Analysis Equipment, Reagents, and Procedure*. A Varian Inova 500 MHz spectrometer was used for NMR measurements, which were performed at 27 °C. Deuterated chloroform ($CDCl_3$) was used as a solvent and internal reference for all samples. High-resolution mass spectra were acquired with Bruker Daltonics microTOF ESI-TOF. Methanol/water (80:20) was used as a solvent, and the concentration of all samples was 1 μg mL^{-1}. Trifluoroacetic acid (0.1%) was added to the sample to enhance ionization when necessary. For internal mass calibration, Agilent ES Tuning Mix (G2421A; 1/50 dilution

Table 1. Mass Spectral Data for Oxidation Products Studied with LC-MS

compound (m/z [M − 1]$^-$)	m/z [M − 1]$^-$ for oxidation products
1 (151)	301
2 (153)	151, 301, 303, 305, 423, 575
3 (−[a])	181
4 (305)	301, 303, 575, 577
5 (289)	577
6 (319)	637
7 (319)	317
8 (349)	347

[a] Syringyl alcohol was not ionized at the applied conditions.

in acetonitrile/water, 1:1) or sodium formiate (0.1% formic acid and 2.5 mM NaOH in water/isopropanol, 1:1) was added to the sample (1:1).

Reaction Procedure for NMR Studies. Reaction was carried out with that laccase which gave highest yield of all oxidation products at 24 h so that they all could be identified. Thus, *M. albomyces* laccase was used for compounds **2**, **3**, **6**, **7**, and **8** and *T. hirsuta* laccase for **1**, **4**, and **5**. Solvents and concentrations of the model compounds were the same as in the procedure for HPLC analysis. The amount of the model compound solution (50−150 mL) was dependent on the number of oxidation products expected on the basis of HPLC and LC-MS analyses. Enzyme dosage was 1 nkat mL^{-1} to final volume in all preparative oxidations. After enzyme addition, solution was stirred for 24 h. Products were then extracted twice with ethyl acetate. The organic phase was washed with water and brine and dried with Na_2SO_4, and the solvent was evaporated. In the case of vanillin (**1**), a considerable amount of solid material was formed as could be expected when **9** is formed, and it was separated by suction filtration after ethyl acetate was added. Solids were washed with ethyl acetate and water, and the filtrate was then handled as in all other cases. Yields of the mixtures (m-%) were as follows: **1**, 0.25 g (86%); **2**, 0.16 g (55%); **3**, 0.28 g (81%); **4**, 0.07 g (30%); **5**, 0.29 g (100%); **6**, 0.10 g (50%); **7**, 0.28 g (100%); **8**, 0.26 g (85%). The mixture of the products was then acetylated in pyridine/acetic anhydride (1:1) for 24 h at room temperature. Yields after acetylation were as follows: **1**, 0.36 g; **2**, 0.21 g; 3, 0.34 g; **4**, 0.09 g; **5**, 0.39 g; **6**, 0.17 g; **7**, 0.30 g; and **8**, 0.33 g.

Fractionation of Oxidation Product Mixtures. Product mixtures were fractionated with flash chromatography or preparative HPLC (**2**). Total yields (m-% to acetylated mixtures) of material recovered after fractionation were as follows: **1**, 0.31 g (86%); **2**, 0.14 g (67%); **3**, 0.05 g (15%); **4**, 0.08 g (89%); **5**, 0.27 g (69%); **6**, 0.13 g (76%); **7**, 0.22 g (0.73%); **8**, 0.25 g (76%). Fractionated oxidation products were analyzed with NMR [^{1}H, ^{13}C, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments] and high-resolution ESI-MS. Nearly all of the fractions contained only one product.

Spectral Data for Selected Acetylated Oxidation Products. *Tetraacetate of 5,5′-bis(hydroxymethyl)-3,3′-dimethoxy-2,2′-biphenyldiol* (***4***): ESI-MS (positive), m/z 492.1856 [M + NH$_4$]$^+$ ($C_{24}H_{30}NO_{10}$ requires 492.1864), 497.1403 [M + Na]$^+$ ($C_{24}H_{26}NaO_{10}$ requires 497.1418), 513.1145 [M + K]$^+$ ($C_{24}H_{26}KO_{10}$ requires 513.1158).

Diacetate of 1-(4-hydroxy-3-methoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethanol (***5***): ^{1}H NMR (499.82 MHz, CDCl$_3$, 27 °C) δ 2.11 (3H, s, aliph −OCOCH$_3$), 2.31 (3H, s, arom −OCOCH$_3$), 3.83 (3H, s, 2′-OCH$_3$), 3.85 (3H, s, 3-OCH$_3$), 4.23 (1H, dd, 11.0 Hz, 3.9 Hz, β′H), 4.30 (1H, dd, 11.0 Hz, 8.0 Hz, βH), 6.15 (1H, dd, 8.0 Hz, 3.9 Hz, αH), 6.89−7.03 (6H, m, ArH), 7.04 (1H, d, 1.6 Hz, 2-H); ^{13}C NMR (125.69 MHz, CDCl$_3$, 27 °C) δ 20.62 (arom −OCOCH$_3$), 21.12 (aliph −OCOCH$_3$), 55.89, 56.00 (3-OCH$_3$, 2′-OCH$_3$), 72.21 (βC), 73.76 (αC), 111.36 (2-C), 112.61 (3′-C), 115.60 (arom C−H), 119.06 (6-C), 120.95, 122.36, 122.82 (each arom C−H), 135.99 (1-C), 139.77 (4-C), 148.08 (1′-C), 150.18 (2′-C), 151.10 (3-C), 168.90 (arom −OCOCH$_3$), 170.01 (aliph −OCOCH$_3$); ESI-MS (positive), m/z 392.1700 [M + NH$_4$]$^+$ ($C_{20}H_{26}NO_7$ requires 392.1704), 397.1238 [M + Na]$^+$ ($C_{20}H_{22}NaO_7$ requires 397.1258), 413.0998 [M + K]$^+$ ($C_{20}H_{22}KO_7$ requires 413.0997).

Diacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethanol (***7***): In the same fraction with compound **18**; ^{1}H NMR (499.82 MHz, CDCl$_3$, 27 °C) δ 2.12 (3H, s, aliph −OCOCH$_3$), 2.32 (3H, s, arom −OCOCH$_3$), 3.82 (6H, s, 3-OCH$_3$, 5-OCH$_3$), 3.83 (3H, s, 2′-OCH$_3$), 4.23 (1H, dd, 11.0 Hz, 3.9 Hz, β′H), 4.29 (1H, dd, 11.0 Hz, 7.8 Hz, βH), 6.12 (1H, dd, 7.8 Hz, 3.9 Hz, αH), 6.67 (2H, s, 2-H, 6-H), 6.90−6.96 (4H, m, ArH); ^{13}C NMR (125.69 MHz, CDCl$_3$, 27 °C) δ 20.41 (arom −OCOCH$_3$), 21.12 (aliph −OCOCH$_3$), 55.94 (2′-OCH$_3$), 56.15 (3-OCH$_3$, 6-OCH$_3$), 72.23 (βC), 74.07 (αC), 103.67 (2-C, 6-C), 112.57 (3′-C), 115.59, 120.94, 122.36 (4′-6′-C), 128.66 (4-C), 135.48 (1-C), 148.07 (1′-C), 150.17 (2′-C), 152.18 (5-C, 3-C), 168.62 (arom −OCOCH$_3$), 169.98 (aliph −OCOCH$_3$); ESI-MS (positive), m/z 422.1807 [M + NH$_4$]$^+$ ($C_{21}H_{28}NO_8$ requires 422.1809), 427.1350 [M + Na]$^+$ ($C_{21}H_{24}NaO_8$ requires 427.1363), 443.1100 [M + K]$^+$, ($C_{21}H_{24}KO_8$ requires 443.1103).

Triacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1,3-propanediol (***8***): ESI-MS (positive), m/z 494.2008 [M + NH$_4$]$^+$ ($C_{24}H_{32}NO_{10}$ requires 494.2021), 499.1563 [M + Na]$^+$ ($C_{24}H_{28}NaO_{10}$ requires 499.1575), 515.1304 [M + K]$^+$ ($C_{24}H_{28}KO_{10}$ requires 515.1314).

Tetraacetate of 3,3′-bis[2-(2-methoxyphenoxy)-1-ethanol]-5,5′-dimethoxy-6,6′-biphenyldiol (***10***): ^{1}H NMR (499.82 MHz, CDCl$_3$, 27 °C) δ 2.07 (6H, br s, arom −OCOCH$_3$), 2.12 (6H, br s, aliph −OCOCH$_3$), 3.815, 3.817 (6H, s + s, 2B-OCH$_3$, 2′B-OCH$_3$), 3.87 (6H, br s, 5A-OCH$_3$, 5′A-OCH$_3$), 4.22 (2H, dd, 11.0 Hz, 3.9 Hz, β2H, β2′H), 4.30 (2H, dd, 11.0 Hz, 7.8 Hz, β1H, β1′H), 6.12 (1H, dd, 7.8 Hz, 3.9 Hz, α′H), 6.13 (1H, dd, 7.8 Hz, 3.9 Hz, αH), 6.88−6.96 (10H, m, ArH), 7.050, 7.046 (2H, d + d, each 2.1 Hz, 4A-H, 4′A-H); ^{13}C NMR (125.69 MHz, CDCl$_3$, 27 °C) δ 20.23 (6A-OCOCH$_3$, 6′A-OCOCH$_3$), 21.01, 21.03 (α-OCOCH$_3$, α′-OCOCH$_3$), 55.89, 56.02 (5A-OCH$_3$, 5′A-OCH$_3$, 2B-OCH$_3$, 2′B-OCH$_3$), 72.20, 72.24 (βC, β′C), 73.69, 73.75 (αC, α′C), 110.65, 110.68 (4A-C, 4′A-C), 112.53 (3B-C, 3′B-C), 120.26 or 120.92 (2A-C, 2′A-C), 115.59, 120.26 or 120.92, 122.35 (4B-6B-C, 4′B-6′B-C), 131.13 (1A-C, 1′A-C), 135.21, 135.24 (3A-C, 3′A-C), 137.42, 137.45 (6A-C, 6′A-C), 148.00 (1B-C, 1′B-C), 150.11 (2B-C, 2′B-C), 151.44, 151.45 (5A-C, 5′A-C), 168.67 (6A-OCOCH$_3$, 6′A-OCOCH$_3$), 170.00 (α-OCOCH$_3$, α′-OCOCH$_3$); ESI-MS (positive), m/z 764.2931 [M + NH$_4$]$^+$ ($C_{40}H_{46}NO_{14}$ requires 764.2913), 769.2452 [M + Na]$^+$ ($C_{40}H_{42}NaO_{14}$ requires 769.2467), 785.2206 [M + K]$^+$ ($C_{40}H_{42}KO_{14}$ requires 785.2206).

Triacetate of 5-carboxaldehyde-5′-hydroxymethyl-3,3′-dimethoxy-2,2′-biphenyldiol (***12***): ^{1}H NMR (499.82 MHz, CDCl$_3$, 27 °C) δ 2.08 (3H, s, 2′-OCOCH$_3$), 2.12 (3H, s, −CH$_2$OCOCH$_3$), 2.13 (3H, s, 2-OCOCH$_3$), 3.88 (3H, s, 3′-OCH$_3$), 3.93 (3H, s, 3-OCH$_3$), 5.10 (2H, s, −CH$_2$−), 6.86 (1H, d, 1.8 Hz, 6′-H), 7.00 (1H, d, 1.8 Hz, 4′-H), 7.38 (1H, d, 1.7 Hz, 6-H), 7.51 (1H, d, 1.7 Hz, 4-H), 9.93 (1H, s, −COH); ^{13}C NMR (125.69 MHz, CDCl$_3$, 27 °C) δ 20.04 (2-OCOCH$_3$), 20.41 (2′-OCOCH$_3$), 21.03 (−CH$_2$OCOCH$_3$), 56.17 (3′-OCH$_3$), 56.33 (3-OCH$_3$), 65.67 (−CH$_2$−), 109.70 (4-C), 112.06 (4′-C), 121.85 (6′-C), 126.83 (6-C), 130.32 (1-C), 131.96 (1′-C), 134.37, 134.39 (5-C, 5-C′), 137.35 (2′-C), 142.74 (2-C), 151.37 (3′-C), 152.21 (3-C), 167.76 (2-OCOCH$_3$), 168.29 (2′-OCOCH$_3$), 170.54 (−CH$_2$OCOCH$_3$), 190.68 (−COH); ESI-MS (positive), m/z 448.1596 [M + NH$_4$]$^+$ ($C_{22}H_{26}NO_9$ requires 448.1602), 453.1160 [M + Na]$^+$ ($C_{22}H_{22}NaO_9$ requires 453.1156), 469.0902 [M + K]$^+$ ($C_{22}H_{22}KO_9$ requires 469.0895).

Triacetate of 5′-(5″-carboxaldehyde-2″-hydroxy-3″-methoxyphenyl)-6,9-bis(hydroxymethyl)-3′,4,11-trimethoxydibenzo[d,f][1,3]dioxepin-2-spiro-4′-cyclohexa-2′,5′-dienone (***13***): ^{1}H NMR (499.82 MHz, CDCl$_3$, 27 °C) δ 2.14 (6H, s, aliph −OCOCH$_3$), 2.21 (3H, s, arom −OCOCH$_3$), 3.70 (3H, s, 3′-OCH$_3$), 3.90 (6H, s, 4-OCH$_3$, 11-OCH$_3$), 3.91 (3H, s, 3″-OCH$_3$), 5.16 (4H, s, −CH$_2$−), 5.90 (1H, d, 3.0 Hz, 2′-H), 6.86 (1H, d, 3.0 Hz, 6′-H), 7.02 (2H, d, 1.6 Hz, 5-H, 10-H), 7.16 (2H, d, 1.6 Hz, 7-H, 8-H), 7.45 (1H, d, 1.7 Hz, 6″-H), 7.51 (1H, d, 1.7 Hz, 4″-H), 9.92 (1H, s, −CHO); ^{13}C NMR (125.69 MHz, CDCl$_3$, 27 °C) δ 20.50 (arom −OCOCH$_3$), 21.03 (two aliph −OCOCH$_3$), 55.43 (3′-OCH$_3$), 56.05 (4-OCH$_3$, 11-OCH$_3$), 56.32 (3″-OCH$_3$), 66.01 (two −CH$_2$−), 109.23 (2-C = 1′-C), 109.58 (2′-C), 111.88 (4″-C), 112.05 (5-C, 10-C), 120.30 (7-C, 8-C), 125.66 (6″-C), 129.82 (1″-C), 134.06 (1O−C-C-C−C−3O−), 134.40 (6-C, 9-C), 134.54, 134.57 (5′-C, 5″-C), 138.97 (1O−C−, 3O−C), 142.18 (6′-C), 143.44 (2″-C), 150.67 (3′-OCH$_3$), 152.26 (3″-C), 153.04 (4-C, 11-C), 167.52 (arom −OCOCH$_3$), 170.79 (two aliph −OCOCH$_3$), 178.03 (4′—C═O), 190.55 (−CHO); ESI-MS (positive), m/z 703.1987 [M + H]$^+$ ($C_{37}H_{35}O_{14}$ requires 703.2021), 725.1819 [M + Na]$^+$ ($C_{37}H_{34}NaO_{14}$ requires 725.1841), 741.1566 [M + K]$^+$ ($C_{37}H_{34}KO_{14}$ requires 741.1580).

Diacetate of 9-carboxyaldehyde-5′-(5″-carboxyaldehyde-2″-hydroxy-3″-methoxyphenyl)-6-hydroxymethyl-3′,4,11-trimethoxydibenzo[d,f]-[1,3]dioxepin-2-spiro-4′-cyclohexa-2′,5′-dienone (***14***) *or diacetate of 6-carboxyaldehyde-5′-(5″-carboxyaldehyde-2″-hydroxy-3″-methoxyphenyl)-9-hydroxymethyl-3′,4,11-trimethoxydibenzo[d,f][1,3]dioxepin-2-spiro-4′-cyclohexa-2′,5′-dienone* (***15***)*:* Numbering of atoms in NMR assignations according to compound **14**; ^{1}H NMR (499.82 MHz, $CDCl_3$, 27 °C) δ 2.15 (3H, s, −OCOCH$_3$), 2.22 (3H, s, arom −OCOCH$_3$), 3.71 (3H, s, 3′-OCH$_3$), 3.92 (6H, br s, 3″-OCH$_3$, 4-OCH$_3$), 3.97 (3H, s, 11-OCH$_3$), 5.18 (2H, s, −CH$_2$−), 5.87 (1H, d, 3.0 Hz, 2′-H), 6.85 (1H, d, 3.0 Hz, 6′-H), 7.05 (1H, d, 1.6 Hz, 5-H), 7.22 (1H, d, 1.6 Hz, 7-H), 7.44 (1H, d, 1.7 Hz, 6″-H), 7.52 (1H, d, 1.7 Hz, 4″-H), 7.55 (1H, d, 1.6 Hz, 10-H), 7.70 (1H, d, 1.6 Hz, 8-H), 9.93 (1H, s, 5″-CHO), 10.05 (1H, s, 9-CHO); ^{13}C NMR (125.69 MHz, $CDCl_3$, 27 °C) δ 20.48 (arom −OCOCH$_3$), 21.04 (aliph −OCOCH$_3$), 55.49 (3′-OCH$_3$), 56.11, 56.27 (3″-OCH$_3$, 4-OCH$_3$), 56.35 (11-OCH$_3$), 65.89 (−CH$_2$−), 109.14 (2′-C), 110.02 (2-C = 1′-C), 110.49 (10-C), 112.04 (4″-C), 112.47 (5-C), 120.23 (7-C), 123.89 (8-C), 125.44 (6″-C), 129.66 (1″-C), 133.25 (either of 1O−C-C−C−C−3O−), 134.58, 134.63, 134.67, 134.78, 134.87 (either of 1O−C−C−C−C−3O−, 5′-C, 5″-C, 6-C, 9-C), 138.99 (3O−C), 141.60 (6′-C), 143.39 (2″-C), 144.38 (1O−C−), 150.86 (3′-C), 152.29 (3″-C), 153.08 (4-C), 153.97 (11-C), 167.58 (arom −OCOCH$_3$), 170.77 (aliph −OCOCH$_3$), 177.91 (4′-C=O), 190.50 (5′-CHO), 190.84 (9-CHO); ESI-MS (positive), *m*/*z* 659.1759 [M + H]$^+$ ($C_{35}H_{31}O_{13}$ requires 659.1759), 681.1576 [M + Na]$^+$ ($C_{35}H_{30}NaO_{13}$ requires 681.1579), 697.1340 [M + K]$^+$ ($C_{35}H_{30}KO_{13}$ requires 697.1318).

Monoacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethanone (***18***)*:* In the same fraction with compound **7**; ^{1}H NMR (499.82 MHz, $CDCl_3$, 27 °C) δ 2.35 (3H, s, −OCOCH$_3$), 3.87 (3H, s, 2′-OCH$_3$), 3.88 (6H, s, 3-OCH$_3$, 5-OCH$_3$), 5.27 (2H, s, βH), 6.86−6.98 (4H, m, ArH) 7.34 (2H, s, 2-H, 6-H); ^{13}C NMR (125.69 MHz, $CDCl_3$, 27 °C) δ 20.37 (−OCOCH$_3$), 55.81 (2′-OCH$_3$), 56.31 (3-OCH$_3$, 5-OCH$_3$), 72.49 (βC), 105.21 (2-C, 6-C), 112.21 (3′-C), 114.90, 120.86, 122.62 (4′-6′-C), 132.54 (1-C), 133.37 (4-C), 147.32 (1′-C), 149.75 (2′-C), 152.38 (3-C, 5-C), 168.06 (−OCOCH$_3$), 193.78 (αC); ESI-MS (positive), *m*/*z* 361.1267 [M + H]$^+$, ($C_{19}H_{21}O_7$ requires 361.1282), 383.1091 [M + Na]$^+$ ($C_{19}H_{20}NaO_7$ requires 383.1101), 399.0823 [M + K]$^+$ ($C_{19}H_{20}KO_7$ requires 399.0841).

Diacetate of 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1-propanone (***19***)*:* ^{1}H NMR (499.82 MHz, $CDCl_3$, 27 °C) δ 2.05 (3H, s, aliph −OCOCH$_3$), 2.33 (3H, s, arom −OCOCH$_3$), 3.75 (3H, s, 2′-OCH$_3$), 3.85 (6H, s, 2-OCH$_3$, 6-OCH$_3$), 4.47 (1H, dd, 12.1 Hz, 7.5 Hz, γH), 4.72 (1H, dd, 12.1 Hz, 3.6 Hz, γ'H), 5.63 (1H, dd, 7.5 Hz, 3.6 Hz, βH), 6.83 (1H, td, 7.9 Hz, 1.5 Hz, 4′-H or 5′-H), 6.87 (1H, dd, 7.9 Hz, 1.5 Hz, 3′-H), 6.93 (1H, dd, 7.9 Hz, 1.5 Hz, 6′-H), 7.00 (1H, td, 7.9 Hz, 1.5 Hz, 4′-H or 5′-H), 7.48 (2H, s, 2-H, 6-H); ^{13}C NMR (125.69 MHz, $CDCl_3$, 27 °C) δ 20.28 (arom −OCOCH$_3$), 20.65 (aliph −OCOCH$_3$), 55.63 (2′-OCH$_3$), 56.22 (3-OCH$_3$, 5-OCH$_3$), 64.37 (γC), 80.40 (βC), 105.76 (2-C, 6-C), 112.58 (3′-C), 117.97 (6′-C), 120.95, 123.44 (4′-C, 5′-C), 132.54 (1-C), 133.32 (4-C), 146.67 (1′-C), 150.19 (2′-C), 152.28 (3-C, 5-C), 167.93 (arom −OCOCH$_3$), 170.85 (aliph −OCOCH$_3$), 194.24 (αC); ESI-MS (positive), *m*/*z* 455.1284 [M + Na]$^+$ ($C_{22}H_{24}NaO_9$ requires 455.1313), 471.1020 [M + K]$^+$ ($C_{22}H_{24}NaO_9$ requires 471.1052).

Triacetate of 10,15-dihydro-1,3,6,8,11,13-hexamethoxy-2,7,12-triol-5H-tribenzo[a,d,g]cyclononene (***20***)*:* ^{1}H NMR (499.82 MHz, $CDCl_3$, 27 °C) δ 2.31 (9H, s, −OCOCH$_3$), 3.76 (9H, s, 3-OCH$_3$, 8-OCH$_3$, 13-OCH$_3$), 3.88 (9H, s, 1-OCH$_3$, 6-OCH$_3$, 11-OCH$_3$), 4.05 (3H, d, 13.8 Hz, 5′-H, 10′-H, 15′-H), 4.45 (3H, d, 13.8 Hz, 5-H, 10-H, 15-H), 7.20 (3H, s, 4-H, 9-H, 14-H); ^{13}C NMR (125.69 MHz, $CDCl_3$, 27 °C) δ 20.61 (−OCOCH$_3$), 30.10 (−CH$_2$−), 56.06 (3-OCH$_3$, 8-OCH$_3$, 13-OCH$_3$), 60.85 (1-OCH$_3$, 6-OCH$_3$, 11-OCH$_3$), 110.53 (4-C, 9-C, 14-C), 131.50 (2-C, 7-C, 12-C), 125.21, 138.65 (C=C in cyclononatriene ring), 150.24 (3-C, 8-C, 13-C), 150.61 (1-C, 6-C, 11-C), 168.74 (−OCOCH$_3$); ESI-MS (positive), *m*/*z* 642.2554 [M + NH$_4$]$^+$ ($C_{33}H_{40}NO_{12}$ requires 642.2545), 647.2097 [M + Na]$^+$ ($C_{33}H_{36}NaO_{12}$ requires 647.2099), 663.1843 [M + K]$^+$ ($C_{33}H_{36}KO_{12}$ requires 663.1838).

Diacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethen-1-ol (***21***)*:* ^{1}H NMR (499.82 MHz, $CDCl_3$, 27 °C) δ 2.32 (3H, s, α-OCOCH$_3$), 2.33 (3H, s, arom −OCOCH$_3$), 3.82 (6H, s, 3-OCH$_3$, 5-OCH$_3$), 3.88 (3H, s, 2′-OCH$_3$), 6.61 (2H, s, 2-H, 6-H), 6.82 (1H, s, βH), 6.93 (1H, td, 7.9 Hz, 1.3 Hz, 5′-H), 6.96 (1H, dd, 7.9 Hz, 1.3 Hz, 3′-H), 7.10 (1H, td, 7.9 Hz, 1.6 Hz, 4′-H), 7.14 (1H, dd, 7.9 Hz, 1.6 Hz, 6′-H); ^{13}C NMR (125.69 MHz, $CDCl_3$, 27 °C) δ 20.41 (arom −OCOCH$_3$), 20.61 (α-OCOCH$_3$), 56.12 (2′-OCH$_3$), 56.16 (3-OCH$_3$, 5-OCH$_3$), 101.06 (2-C, 6-C), 112.91 (3′-C), 118.88 (6′-C), 121.01, 124.78 (4′-C, 5′-C), 128.65 (4-C), 131.63 (1-C), 133.81 (αC), 134.07 (βC), 146.37 (1′-C), 150.14 (2′-C), 152.27 (3-C, 5-C), 168.24 (α-OCOCH$_3$), 168.65 (arom −OCOCH$_3$); ESI-MS (positive), *m*/*z* 420.1652 [M + NH$_4$]$^+$ ($C_{21}H_{26}NO_8$ requires 420.1653), 425.1202 [M + Na]$^+$ ($C_{21}H_{22}NaO_8$ requires 425.1207), 441.0948 [M + K]$^+$ ($C_{21}H_{22}KO_8$ requires 441.0946).

Monoacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-2-propen-1-one (***22***)*:* ^{1}H NMR (499.82 MHz, $CDCl_3$, 27 °C) δ 2.35 (3H, s, −OCOCH$_3$), 3.85 (3H, s, 2′-OCH3), 3.88 (6H, s, 2-OCH$_3$, 6-OCH$_3$), 4.73 (1H, d, 2.5 Hz, γH), 5.29 (1H, d, 2.5 Hz, γ'H), 6.95 (1H, td, 7.9 Hz, 1.3 Hz, 5′-H), 6.98 (1H, dd, 7.9 Hz, 1.3 Hz, 3′-H), 7.06 (1H, dd, 7.9 Hz, 1.6 Hz, 6′-H), 7.16 (1H, td, 7.9 Hz, 1.6 Hz, 4′-H), 7.40 (2H, s, 2-H, 6-H); ^{13}C NMR (125.69 MHz, $CDCl_3$, 27 °C) δ 20.43 (−OCOCH$_3$), 55.77 (2′-OCH$_3$), 56.28 (3-OCH$_3$, 5-OCH$_3$), 100.55 (γC), 106.98 (2-C, 6-C), 112.83 (3′-C), 121.29 (5′-C), 121.63 (6′-C), 125.87 (4′-C), 132.76 (4-C), 133.96 (1-C), 143.12 (1′-C), 150.85 (2′-C), 151.91 (3-C, 5-C), 157.66 (βC), 168.14 (−OCOCH$_3$), 189.32 (αC); ESI-MS (positive), *m*/*z* 762.2765 [2M + NH$_4$]$^+$ ($C_{40}H_{44}NO_{14}$ requires 762.2756), 767.2327 [2M + Na]$^+$ ($C_{40}H_{40}NaO_{14}$ requires 767.2310), 783.2101 [2M + K]$^+$ ($C_{40}H_{40}KO_{14}$ requires 783.2050).

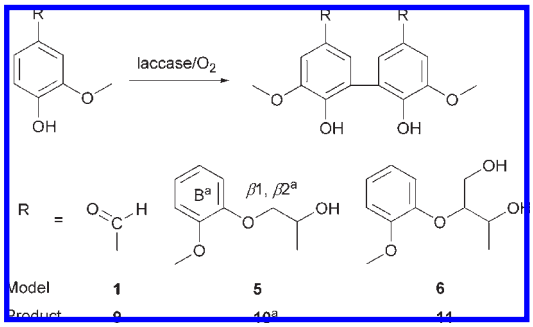

Figure 2. Oxidation of vanillin (**1**) and guaiacylic β-*O*-4 dimers (**5** and **6**) in laccase-catalyzed reactions.

RESULTS

Eight different lignin model compounds were used to study reactions of laccases with lignin (**Figure 1**): vanillin (**1**), vanillyl alcohol (**2**), syringyl alcohol (**3**), dehydrodivanillyl alcohol (**4**), guaiacylglycol β-guaiacyl ether (**5**), guaiacylglycerol β-guaiacyl ether (**6**), syringylglycol β-guaiacyl ether (**7**), and syringylglycerol β-guaiacyl ether (**8**). Two different laccases were used: *Melanocarpus albomyces* and *Trametes hirsuta* laccases. The T1 copper redox potentials are 470 mV for *M. albomyces* laccase (*20*) and 780 mV for *T. hirsuta* laccase (*24*).

Structures of Oxidation Products. The oxidation products were first deduced on the basis of the data from LC-MS and later confirmed with NMR and high-resolution ESI-MS. The same oxidation products were obtained with both laccases and, thus, the fractionation and detailed analysis were performed with only one of the laccases.

With vanillin (**1**) and guaiacylic β-*O*-4 dimers **5** and **6**, only one type of oxidation product, a 5-5 coupling product, was observed (**Figure 2**). These kinds of products were highly expected as typical and well-known oxidation products in guaiacylic lignin model compound oxidations.

Vanillyl alcohol (**2**) gave the most complicated distribution of oxidation products (**Figure 3**). Three different kinds of 5-5 coupling products were formed: one formed straightforward from two radicals of vanillyl alcohol (**4**), and the other two had one (**12**) or two (**9**) benzylic hydroxyl groups oxidized. Vanillin (**1**), a product formed when the benzylic hydroxyl of vanillyl alcohol is oxidized, was also formed. The structures of trimeric and tetrameric (based on mass values in MS) oxidation products remained unfortunately unresolved, because they were in the same fraction in nearly equimolar amounts.

Dehydrodivanillyl alcohol (**4**, **Figure 4**) gave the same benzylic hydroxyl oxidized products **9** and **12** observed with vanillyl alcohol (**2**). In addition, dibenzodioxepin-type structures, with one or two oxidized benzylic hydroxyls, were observed (**13**−**15**). It was impossible to determine whether **14** or **15** or both were present in the product mixture. The tetramer found in the oxidation of vanillyl alcohol (**2**) is probably like structure **14** or **15**, because it has the same mass value and dehydrodivanillyl alcohol is an oxidation product of vanillyl alcohol (**2**).

Oxidation products detected for syringyl alcohol (**3**, **Figure 5**) were syringaldehyde (**16**) and 2,6-dimethoxy-*p*-benzoquinone (**17**). The latter of these was observed by absorption spectrum from HPLC, and the structure was confirmed with NMR, because it was not ionized at the applied LC-MS conditions.

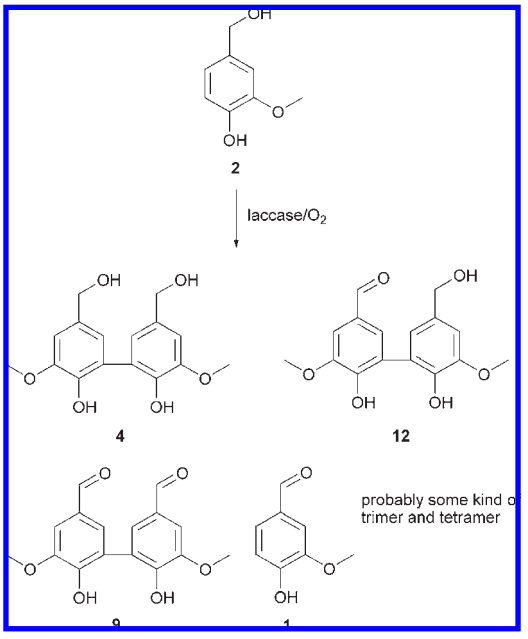

Figure 3. Oxidation products of vanillyl alcohol (**2**) in laccase-catalyzed reactions.

The only oxidation products observed for syringylic *β*-*O*-4 dimers **7** and **8** were **18** and **19** (**Figure 6**), resulting from benzylic hydroxyl oxidation.

Products Resulting from Acetylation of Syringylic Compounds. In the case of syringyl compounds a few products were observed only after acetylation. These were formed from product mixtures of syringyl alcohol (**3**) and of *β*-*O*-4 dimers **7** and **8**.

The product from acetylation of oxidized syringyl alcohol (**3**) was cyclotrisyringylene (**20**) (**Figure 7**). This product was also formed in small amounts when **3** was acetylated without oxidation by laccase. It is also highly probable that syringyl alcohol was

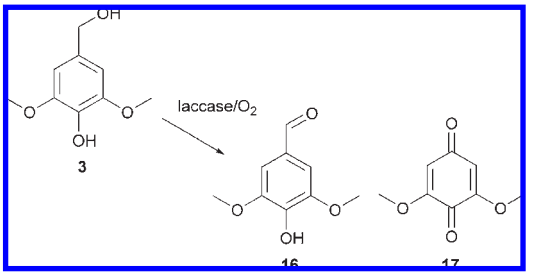

Figure 5. Oxidation products of syringyl alcohol (**3**) in laccase-catalyzed reactions.

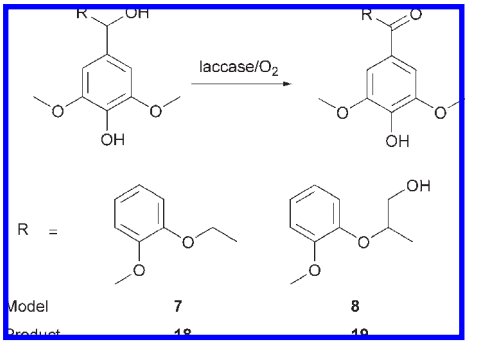

Figure 6. Oxidation products of dimeric *β*-*O*-4 compounds containing phenolic syringyl unit (**7** and **8**) in laccase-catalyzed reactions.

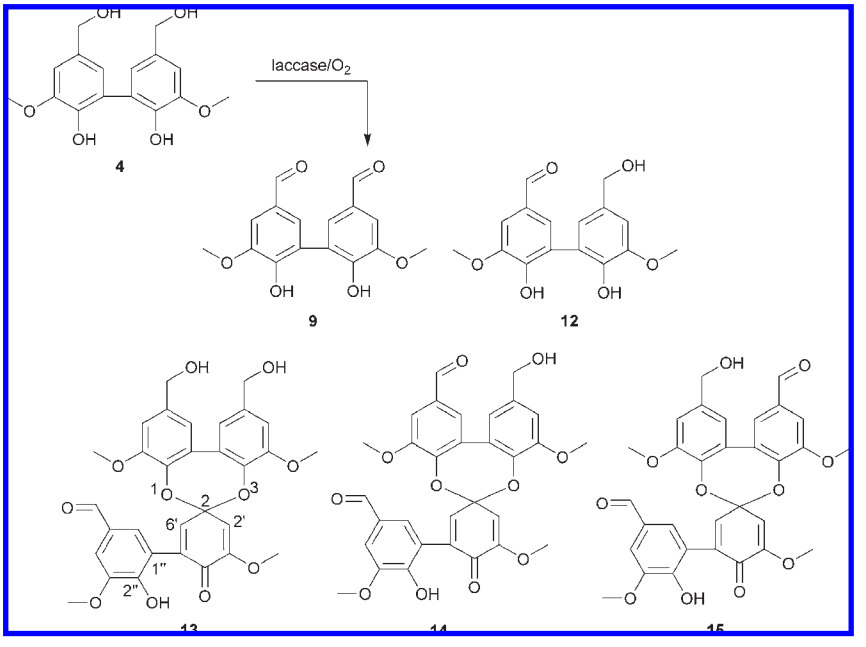

Figure 4. Oxidation products of dehydrodivanillyl alcohol (**4**) in laccase-catalyzed reactions.

even further polymerized during acetylation because of the low yield of material recovered after fractionation. It was actually clearly seen that dark material remained above the silica gel and did not dissolve during fractionation.

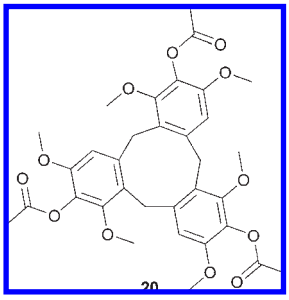

Figure 7. Product detected after acetylation of laccase-oxidized syringyl alcohol (**3**).

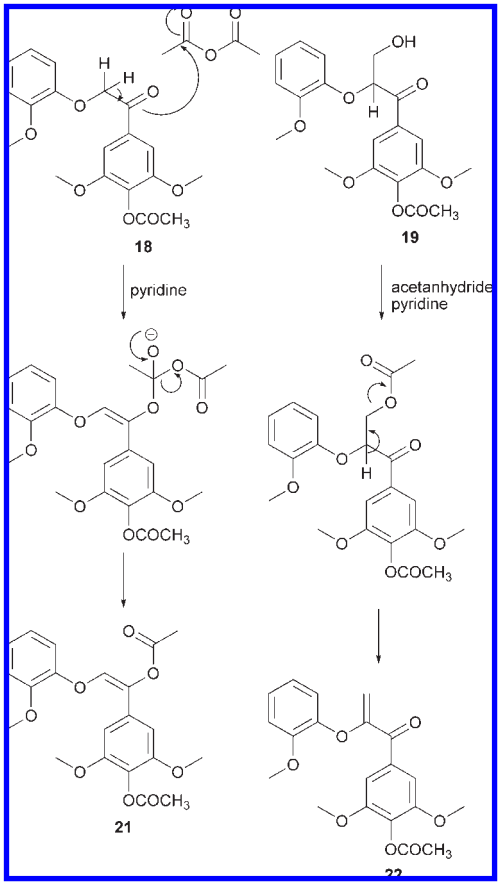

Figure 8. Tentative mechanism for products formed from oxidation products **18** and **19** as a result of acetylation.

Products **21** and **22** were most probably formed from oxidation products **18** and **19** and can be explained by the acidic proton next to a carbonyl group formed in oxidation and the presence of basic pyridine (**Figure 8**).

Oxidation Product Amounts Recovered from Fractionation. Yields of oxidation products and starting material recovered from oxidations after fractionation to the original starting material are shown in **Table 2**. In cases when there was more than one product in a fraction, amounts were estimated on the basis of ^{1}H NMR integrals (structures were first assured from HSQC and HMBC spectra).

Formation of Oxidation Products as a Function of Time. Formation of products was analyzed as a function of time with HPLC and LC-MS analyses. Analyses were performed with both laccases to reveal possible differences between the enzymes.

First detection times of oxidation products for *M. albomyces* laccase are in **Table 3**, and those for *T. hirsuta* laccase are in **Table 4**.

DISCUSSION

On the basis of our results, the main reactions of lignin model compounds oxidized by laccase are formation of 5-5 dimers and oxidation of a benzylic hydroxyl group. Only one product was formed by these reactions with vanillin (**1**) and β-*O*-4 dimers **5**−**8**. From these compounds, the guaiacylic model compounds gave 5-5 coupling products and syringylic models were oxidized from the benzylic position.

From syringyl alcohol (**3**), two oxidation products were formed: the benzylic hydroxyl oxidation product syringaldehyde (**16**) and 2,6-dimethoxy-*p*-benzoquinone (**17**). The latter has been previously shown to be formed from syringaldehyde through 2,6-dimethoxy-*p*-hydroquinone (*25*), which we did not, however, detect.

Vanillyl alcohol (**2**) and dehydrodivanillyl alcohol (**4**) gave very similar products, which is well understood by the fact that **4** is the main product from oxidation of **2**. Actually, one of the reasons why **4** was also taken to the study was, that it revealed how some products of **2** were formed. Identified products of vanillyl alcohol (**2**) were, besides dehydrodivanillyl alcohol (**4**), dehydrodivanillin (**9**), biphenylic compound of vanillin and vanillyl alcohol (**12**), and vanillin (**1**). Formation of **9** and **12** from vanillyl alcohol (**2**) could in principle take place in two ways: (1) two molecules of **2** are coupled to form **4**, which is then further oxidized from benzylic positions or (2) **2** is first oxidized to form **1**, followed by 5-5 coupling. The first reaction is probably dominating, because vanillin (**1**) and dehydrodivanillin (**9**) were formed after **12** and dehydrodivanillyl alcohol (**4**) gave also products **9** and **12**.

The other products that were formed from dehydrodivanillyl alcohol (**4**) were of dibenzodioxepin type (**13**−**15**), which had one

Table 2. Yield of Material Recovered from Oxidations (Reaction Time = 24 h) after Fractionation

model	yield (%)	product	yield (%)	product	yield (%)	product	yield (%)	product	yield (%)	total yield (%)
1	35.1	**9**	43.5							78.6
2	0.7	**1**	2.7	**4**	<13.9[a]	**9**	4.7	**12**	2.5	<24.5[b]
3	c	**16**	7.5	**17**	2.1	**20**	3.0			12.6
4	7.6	**9**	3.4	**12**	3.1	**13**	8.6	**14,15**	2.9	25.6
5	8.0	**10**	18.7							26.7
6	35.9	**11**	3.6							39.5
7	29.5	**18**	30.4	**21**	5.7[d]					65.6
8	12.0	**19**	47.5	**22**	3.1[d]					62.6

[a] Low amount of unidentified material was present; yield calculated from the total weight of the fraction. [b] The true yield might be slightly higher, because structures of trimeric and tetrameric compounds could not be resolved (yield of their fraction was 7% in m-%). [c] Starting material was not recovered, probably because it reacted during acetylation. [d] A product resulting from a reaction of the oxidation product during acetylation.

or two oxidized benzylic hydroxyls. The formation of dibenzodioxepins as lignin dehydrogenation products is a known reaction (*26*, *27*), but as far as we know, oxidation of their side chains as well has not been reported before. As was mentioned before, we could not confirm whether the structure with two benzylic hydroxyls oxidized was **14** or **15**. The "parent" dibenzodioxepin, in which no benzylic hydroxyls are oxidized, was absent from the product mixture. This is understandable in a way that benzylic hydroxyl oxidation of dehydrodivanillyl alcohol (**4**) starts well before any dibenzodioxepins were detected and the benzylic oxidation may also be the most preferred reaction.

Interestingly, we were not able to find any degradation products with syringylic β-*O*-4 dimers **7** and **8** as others have done, and as was mentioned before, the two previous studies for syringylglycerol β-guaiacyl ether (**8**) gave two sets of products (*9*−*11*). Also, the products that we found for vanillyl alcohol (**2**) were different from those that others found, except for vanillin (**1**) (*14*). The reasons for different results are not very clear, but a few that might affect the outcome are different enzyme activities, reaction temperatures, substrate concentrations, etc. The differences are not most probably due to different laccases, because we observed the same products with both laccases used. On the other hand, Wariishi et al. and Kawai et al. had different products in the case of syringylglycerol β-guaiacyl ether using the same laccase from *C. versicolor* (*10*, *11*).

Table 3. First Detection Times of Oxidation Products in Reaction Catalyzed by *M. albomyces* Laccase

model	1 min	5 min	10 min	30 min	1 h	2 h	4 h	24 h
1						**9**		
2		**4**					**12**	**1**, **9**, trimer, tetramer
3			**16**					**17**
4[a]			**12**				**13**	**9**, **14**, **15**
5		**10**						
6				**11**				
7		**18**						
8			**19**					

[a] Enzyme dosage twice as high (based on activity) as in other experiments.

Table 4. First Detection Times of Oxidation Products in Reactions Catalyzed by *T. hirsuta* Laccase

model	1 min	5 min	10 min	30 min	1 h	2 h	4 h	24 h
1			**9**					
2		**4**				**12**	**1**	**9**, trimer, tetramer
3			**16**					**17**
4[a]			**12**	**13**				**9**, **14**, **15**
5		**10**						
6			**11**					
7		**18**						
8				**19**				

[a] Enzyme dosage twice as high (based on activity) as in other experiments.

Compared to other reaction conditions, the greatest difference seems to be in the amounts of the used enzyme activities. These are, however, difficult to compare because of the different activity determination methods. For example, Kawai et al. (*13*) used an activity of 2400−4800 nkat mL^{-1} (activity determined with syringaldazine) and Crestini et al. (*14*) used 10 U mL^{-1} [equal to 167 nkat mL^{-1}, activity measured with ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate)]. In our study, the activity used was 1−2 nkat mL^{-1} (activity determined with ABTS). Thus, it seems that the most important factor, when reaction product distributions are concerned, is the amount of the used activity. The amount is probably proportional to the concentration of radicals formed, which most likely has an impact to the course of the reaction.

The products that resulted from acetylation were observed only in the case of syringyl compounds. This is also an indication of their higher reactivity in general compared to guaiacyl compounds and can be seen also in practice, for example, in model compound synthesis. Compound **22** was also detected by Kawai et al. (*11*) with GC-MS after acetylation, but they thought it was formed during GC analysis. Compounds similar to cyclotrisyringylene (**20**), for example, cyclotriveratrylene and its derivatives, are usually synthesized in acidic conditions [for example, Vériot et al. (*28*)], but are apparently also formed in basic conditions. Because there might be quite complicated reactions, as in the case of syringyl alcohol (**3**) that can oligomerize or polymerize, the high reactivity of syringylic compounds should be taken into account when they are modified prior to analysis.

By examining the total material yields at different stages, after laccase oxidation and after fractionation of acetylated products, it can be seen that the greatest yield loss for syringyl alcohol (**3**) occurred during fractionation. It shows also that polymerization occurred indeed during acetylation and not at the oxidation stage. With vanillyl alcohol (**2**), dehydrodivanillyl alcohol (**4**), and guaiacylglycerol β-guaiacyl ether (**6**), greatest yield loss occurred during enzymatic oxidation, or most probably at the isolation stage, indicating that they were polymerized already during laccase oxidation.

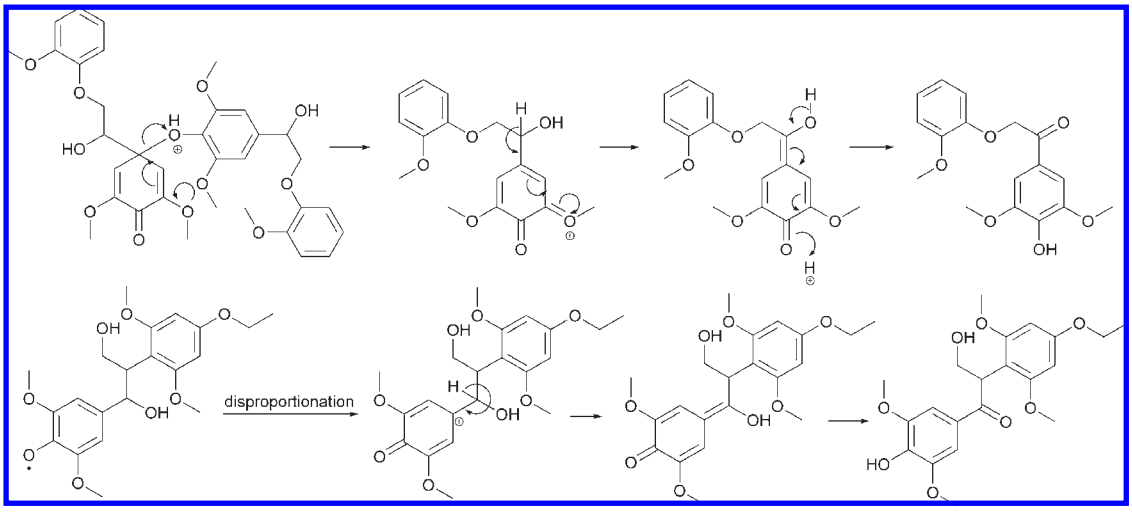

Figure 9. Previously suggested mechanisms for benzylic hydroxyl oxidation: upper mechanism taken from Kirk et al. (*9*) and lower from Kawai et al. (*13*).

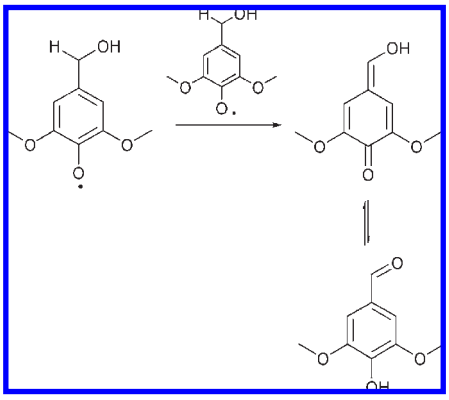

Figure 10. Mechanism suggestion for benzylic hydroxyl oxidation catalyzed by laccase.

As was mentioned earlier, coupling to 5-5 dimers seems to be faster compared to benzylic hydroxyl oxidation. The other possible products, dibenzodioxepins and *p*-benzoquinone, are formed even later. Results also show that for guaiacylic compounds benzylic hydroxyl oxidation is not preferred. Guaiacylic *β-O*-4 dimers were not oxidized to ketones at all, and side-chain oxidation of dehydrodivanillyl alcohol (**4**) took place more rapidly than that of vanillyl alcohol (**2**). This result is also consistent with a previous finding that in the early reaction stages laccase polymerizes phenolic lignin model compounds even when there is a mediator present, and a mediator then degrades the formed polymer (*29*).

There were also some differences in the reaction rates between the two laccases used. *T. hirsuta* laccase formed more rapidly dehydrodivanillin (**9**) from vanillin (**1**) and products that were formed after primary products from vanillyl alcohol (**2**) and dehydrodivanillyl alcohol (**4**). Tetrameric **11** from guaiacylglycerol *β*-guaiacyl ether (**6**) was also formed more rapidly by *T. hirsuta* laccase. *M. albomyces* laccase was faster in forming **19** from syringylglycol *β*-guaiacyl ether (**8**).

Coupling to biphenylic products is a well-known reaction in the oxidation of guaiacylic lignin model compounds. For the benzylic hydroxyl oxidation by laccase, two different mechanisms have been proposed (**Figure 9**). Kirk et al. suggested that the first step of the reaction, after formation of phenoxyl radical, is combination of two molecules (*9*). Kawai et al., on the other hand, proposed a reaction mechanism based on disproportionation, enabled by the formation of a highly resonance stabilized syringyl-type cyclohexadienone cation (*13*). We now suggest also an alternative, simpler mechanism, wherein a hydrogen atom from the benzylic position would be transferred to another phenoxyl radical (**Figure 10**). The only ionic step in this mechanism would be tautomerization to the final product. In other words, one substrate molecule would act as a mediator in the oxidation of another molecule and no high-energy intermediates are generated. Recently, a very similar mechanism was proposed for a laccase-catalyzed reaction, wherein a phenol acts as a mediator that oxidizes a nonphenolic compound (*30*). Of course, the mechanism proposed is, as are the other ones, only a hypothetical one.

As was mentioned above, the product distributions in different studies seem to correlate with the amount of enzyme activity used. This could also have an effect on the mechanisms by which the products are formed. Thus, when the amount of the enzyme activity used is high, it becomes more probable that two radicals couple in a way that degradation products are formed.

In conclusion, on the basis of studies performed with different lignin model compounds, it seems that the main reactions of laccase with these compounds are coupling to biphenylic (5-5) products and benzylic hydroxyl oxidation. If there is a choice between reactions in the same system, as is the case with guaiacylic compounds, formation of biphenylic compounds dominates and this reaction also occurs at a faster rate. Also, high and low redox potential laccases, *T. hirsuta* and *M. albomyces*, form in practice the same reaction products, but there are some clear differences in the formation rates. Interestingly, *T. hirsuta* laccase forms products more quickly from guaiacylic models, whereas *M. albomyces* laccase seems to be slightly faster with syringylic models. This may be a sign of difference in substrate specificity between the laccases, supporting our earlier findings (*31*). On the basis of comparison to other studies, it seems that the product distributions and the reaction routes are dependent on the dosages of the used enzyme activities.

NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP August 25, 2009, a correction was made to ^{13}C NMR data for compound **20**; the corrected version was reposted August 26, 2009.

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