Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 2161–2169

BIOORGANIC & MEDICINAL CHEMISTRY

The Early Oxidative Biodegradation Steps of Residual Kraft Lignin Models with Laccase

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Received 9 March 1998; accepted 20 July 1998

Abstract—A number of model compounds resembling the fundamental bonding patterns of residual kraft lignin, including a series of stilbenes, were incubated with laccase from *Trametes versicolor* in the presence and absence of delignification 'mediators' ABTS and HBT. The condensed kraft lignin model compounds seem to undergo initial degradation by laccase mainly via benzylic oxidation, demethylation and hydroxylation reactions. Phenolic 5-5′, diphenylmethane and α-5 lignin models were found to be degraded mainly via side-chain oxidation reactions. Among the models studied, a phenolic stilbene was found to be the most reactive, yielding several products showing side-chain oxidation/transposition, demethoxylation and hydroxylation reactions. Non-phenolic 5-5′, diphenylmethane and stilbene model compounds were found unreactive even in the presence of the laccase-mediator system.

Introduction

Residual lignin from kraft pulping processes is a three dimensional polymer linked mainly by condensed (i.e., carbon–carbon) bonds and some ether linkages between dihydrophenyl propionic, diphenylmethane and stilbene units, most of which are not readily hydrolyzable. In view of the renewed interest in alternative bleaching processes, the use of enzymes as mild and environmentally benign bleaching agents is being considered by the pulp and paper industry.[1] Among these laccase (benzenediol: oxygen oxidoreductase; E.C. 1.10.3.10), a multicopper oxidase which performs one-electron oxidations of a variety of aromatic substrates, holds a pre-eminent position. Laccase alone, however, has little effect on pulps, but when used in the presence of mediators such as 2,2′-azinobis-(3-ethyl-benzthiazoline-6-sulfonate) (ABTS) or 1-hydroxybenzotriazole (HBT) laccase has been shown to effectively demethylate kraft pulps.[2] In order to elucidate the delignification mechanism catalyzed by laccase, several studies have been carried out on both phenolic and non-phenolic monomeric, arylglycerol β-O-4 and β-1 ether lignin model compounds.[3–5] The use of radical mediators has become of interest since the early report that non-phenolic lignin model compounds, unreactive towards laccase alone, could be oxidized in the presence of laccase and ABTS,[3] and that kraft pulp could be delignified. Since then, 1-hydroxybenzotriazole was found to mediate pulp delignification in the presence of laccase.[6] It is also likely that the relatively small radical mediators can act as diffusible lignin oxidizing agents since they can access inner lignin structures in the cell wall as opposed to the relatively large enzyme.[7]

Kraft lignin contains significant amounts of condensed structures such as 5-5′, α-5, diphenylmethane, and stilbene units.[8–13] To date there are no literature studies dealing with the behaviour of laccase toward such lignin moieties, despite the fact that their presence is very significant within the kraft lignin backbone. The aim of this work is to elucidate the reactivity of laccase toward condensed lignin model compounds in the presence or

Key words: laccase; oxidation; lignin models.
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0968-0896/98/$ - see front matter
PII: S0968-0896(98)00173-4

absence of ABTS and HBT. A series of model compounds resembling the fundamental bonding patterns of residual kraft lignin were thus incubated with laccase from *Trametes versicolor*. In order to elucidate the initial steps in the oxidation degradation products, the reactions were conducted under conditions of limited oxygen supply and low enzyme activity. This experimental design allowed for the early metabolites of the reaction to be detected, characterized and quantified. Our efforts were also supplemented with experiments where oxygen was bubbled in the reactor accompanied by extensive degradation of the examined models.

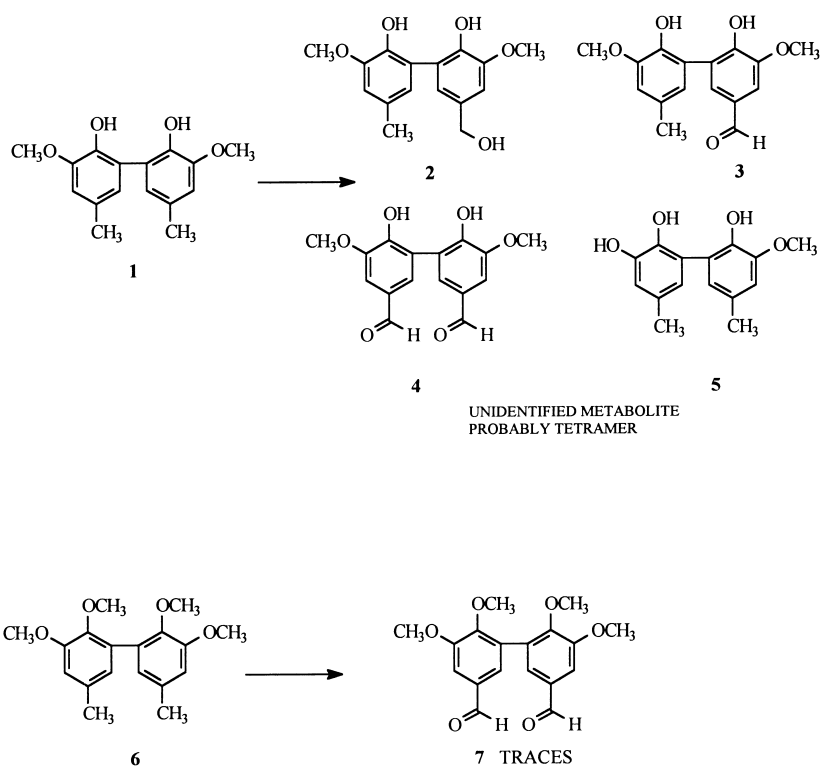
Results

Biodegradation of 5-5′ condensed models dihydrocreosol 1 and dihydromethyl veratrole 6

Compound **1** was incubated with laccase both in the presence and in the absence of the radical mediators ABTS and HBT to give compounds **2**, **3**, **4**, and **5** in variable yields (Scheme 1). When the enzyme load was 0.5 U/mL the extent of degradation ranged from 9.4 to 10.3% (Table 1). In the presence of a lower enzyme load the conversion of **1** was 4.6–6.5%.

The presence of radical mediators did not significantly affect the degree of conversion and product distribution (Table 2, Entry 1). Compounds **2**, **3**, and **4** are all products of side-chain oxidation of the benzyl group to hydroxymethyl, aldehyde and double oxidation to dialdehyde, respectively; the major component being, under all experimental conditions examined, the benzyl alcohol derivative **2**. Along with side-chain oxidation products, compound **5**, a metabolite produced upon demethylation of one of the methyl-aryl ether moieties, was also identified. In the presence of higher enzyme activity another metabolite with a high molecular weight was detected, and tentatively assigned as being a compound possibly emerging via a radical coupling pathway.[4,14,15] Table 2 (Entry 1) shows the quantitative amounts of the metabolites obtained.

When the fully methylated model **6** was incubated under the same experimental conditions, no reaction occurred, with the exception that traces of the dialdehyde **7** were detected in the presence of ABTS when high enzyme levels were used (Scheme 1). When the reaction was performed in the presence of bubbled oxygen, the extent of degradation was found to be 61.7% for model compound **1** in the presence of laccase alone, while the non-phenolic compound **6** was essentially unreacted even in the presence of ABTS or HBT.



Scheme 1. Laccase biodegradation products of 5-5′ condensed models **1** and **6**. **2** is the major product recovered.

Table 1. Extent of biodegradation of Kraft lignin models after treatment with laccase

	Reaction Conditions[a]					
Compound	A	B	C	D	E	F
	(%) of conversion of the model compounds[b]					
1	6.5	4.6	9.4	10.3	9.8	61.7
6	—	—	—	TRACE	—	—
8	14.9	8.6	41.8	40.4	36.5	100
12	—	—	—	—	—	—
13	9.7	12.8	32.9	29.5	38.8	100

[a] A: Laccase 0.05 U/ml; B: Laccase 0.05 U/mL, ABTS 1 mM; C: Laccase 0.5 U/mL; D: Laccase 0.5 U/mL, ABTS 1 mM; E: Laccase 0.5 /mL, HBT 1 m; F: Laccase 0.5 U/mL, bubbled oxygen. Model compounds **6** and **12** were also treated with laccase and ABTS or HBT 1 mM. In both cases (experiments carried out in triplicate), the non-phenolic models were found to be completely unreactive.
[b] Measured by quantitative GC analyses.

Table 2. Product profiles for the biodegradation of condensed models after treatement with laccase

		Reaction Conditions[a]				
Entry	Products	A	B	C	D	E
		Yield (%)[b]				
1	**2**	1.8	1.3	6.0	5.6	2.8
	3	0.6	0.3	0.0	0.1	1.4
	4	—	0.1	0.1	0.2	0.0
	5	0.3	0.2	0.2	0.1	0.0
2	**9**	8.0	6.1	29.3	31.9	14.4
	10	0.3	0.1	0.7	0.8	0.8
	11	0.4	0.2	0.7	0.5	0.6
3	**14**	5.0	3.6	20.8	22.0	14.2
	15	1.4	1.4	0.6	0.6	0.4
	16	0.4	0.2	0.8	0.8	0.5
	17	—	—	0.6	0.7	—
	18 + 19[c]	1.8	0.7	5.1	3.4	2.3

Entry 1: Product profiles for the biodegradation of 5-5′ condensed model **1**; Entry 2: product profiles for the biodegradation of diphenylmethane model **8**; Entry 3: Product profiles for the biodegradation of α-5 condensed model **13**. =
[a] A: Laccase 0.05 U/mL; B: Laccase 0.05 U/mL, ABTS 1 mM; C: Laccase 0.5 U/mL; D: Laccase 0.5 U/mL, ABTS 1 mM; E: Laccase 0.5 U/mL, HBT 1 mM.
[b] Evaluated by quantitative GC analyses.
[c] Due to the partial overlaping of the CG peaks of these isomers, they were integrated together.

Biodegradation of model compounds (3,3′-dihydroxy-4,4′-dimethoxy-6,6′-dimethyl) diphenylmethane 8 and (3,3′4,4′-tetramethoxy-6,6′-dimethyl) diphenylmethane 12

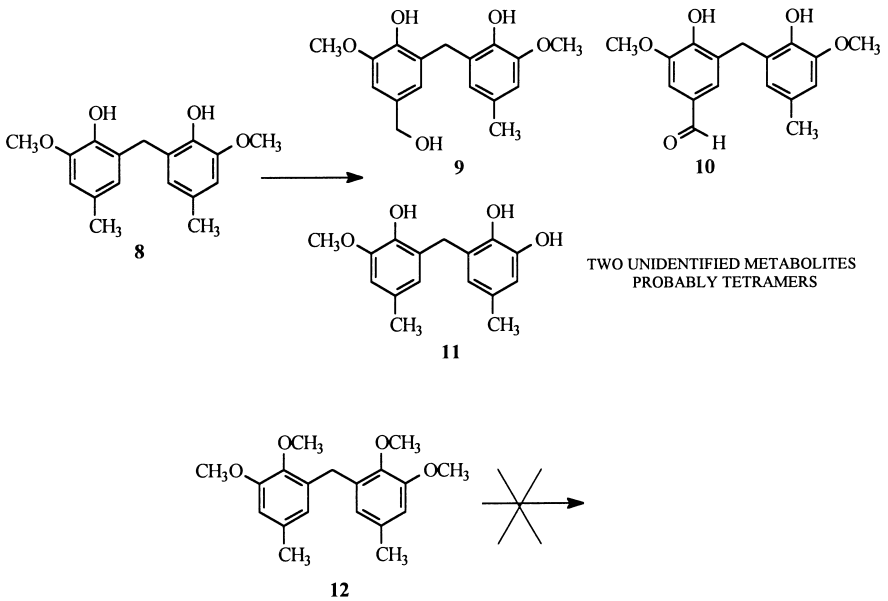
Compound **8** was treated with laccase from *Trametes versicolor* to give **9**, **10**, and **11** in variable yields (Scheme 2). The extent of conversion ranged from 41.8–36.5 % in incubations performed with 0.5 U/mL and 14.9–8.6% in the presence of 0.05 U/mL of laccase (Table 1).

The presence of radical mediators did not significantly affect the reaction pathway and the extent of degradation. In this case two products witnessing side-chain oxidations, **9** and **10** arising from the oxidation at the benzylic position to hydroxymethyl and aldehyde, respectively, were detected. A demethylated product, **11**, was also identified (Scheme 2). In the presence of higher amounts of laccase two more metabolites were detected, both of high molecular weight. In any case, the major metabolite detected was compound **9**, that is, the product of side-chain oxidation to benzyl alcohol (Table 2, Entry 2). The lack of further oxidation products to carboxylic derivatives was expected. In fact the laccase catalyzed oxidation of aromatic methyl and hydroxymethyl groups with oxygen usually is very selective and stops to benzaldehyde step. In the presence of excess of oxygen, autooxidation processes may yield to carboxylic derivatives.

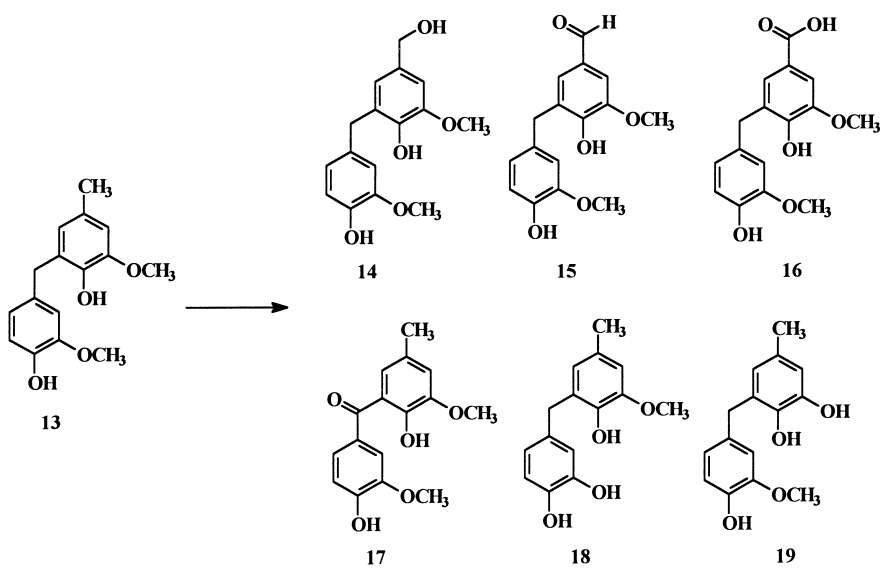
When the etherified diphenylmethane model **12** was incubated with the enzyme no reaction was detected under any of the experimental conditions used in this effort (Scheme 2). When the reaction was carried out in the presence of bubbled oxygen, the phenolic model compound **2** was completely degraded, while the non-phenolic model **12** was found completely inert, even in the presence of ABTS or HBT (Table 1).

Biodegradation of the α-5 model compound (2,4′-dihydroxy-3,3′-dimethoxy-5-methyl)-diphenylmethane 13

The extent of biodegradation of the α-5 model compound **13** with laccase ranged from 29.5–38.7% in the presence of 0.5 U/mL of enzyme to 9.6–20.4% in the presence of 0.05 U/mL of enzyme (Table 1). The main metabolite detected, **14**, is a product of side-chain oxidation to benzyl alcohol. Besides **14**, two products, **15** and **16**, of oxidation to aldehyde and carboxylic moieties respectively were identified, albeit in low amounts (Scheme 3). The formation of **16** can be rationalized by autooxidation of **14** or **18** in the presence of oxygen. A product indicating side-chain oxidation in the benzylic position, compound **17**, was also identified. Besides side-chain oxidation, two demethylation metabolites, **18**, **19**, were detected (Table 2, Entry 3). High molecular weight metabolites were not detected. In general the radical mediators ABTS and HBT had no effect on the extent of degradation and the distribution of metabolites.



Scheme 2. Laccase biodegradation products of diphenylmethane models **8** and **12**. **9** is the major product recovered.



Scheme 3. Laccase biodegradation products of α-5 model compound **13**. **14** is the major product recovered.

When the reaction was performed in the presence of bubbled oxygen, the model compound **13** was completely degraded.

Biodegradation of 4,4′-dihydroxy-3,3′-dimethoxystilbene 20 and 3,3′,4,4′-tetramethoxystilbene 25

Stilbenoids, which are formed during pulping, constitute a significative structural feature of the residual kraft lignin.[8] Stilbene units are known to be fairly oxygen sensitive,[16,17] and under our reaction conditions they reacted even in the absence of laccase. It is noteworthy that the nature of the products obtained from laccase incubation and direct oxygen degradation are the same. In the absence of enzyme the degradation reached 24% of the starting material, while in the presence of 0.5 U/mL of laccase the extent of degradation ranged from 77.6 to 90.0% (Table 3).

Table 3. Extent of begradation of Stilbene models

Compound	Reaction Conditions[a]			
	A	B	C	D
	Conversion of the model compounds 20 and 25 (%)[b]			
20	24.0	77.6	80.6	90.0
25	9.0	9.3	9.1	9.3

[a] A: Blank; B: Laccase 0.5 U/mL; C: Laccase 0.5 U/mL, ABTS 1 mM; D: Laccase 0.5 U/mL, HBT 1 mM.
[b] Evaluated by quantitative GC analyses.

From the incubation of 4,4′-dihydroxy-3,3′-dimethoxy-stilbene **20**, four main metabolites were detected in comparable amounts (Scheme 4). Two of them, compounds **21** and **23** were products of olefinic oxidation and rearrangement,[17] while **22** and **24** were characterized as products of aromatic hydroxylation and demethoxylation, respectively (Scheme 4). In Table 4 the amounts of the detected metabolites obtained both in the presence and absence of radical mediators are reported. For these models ABTS and HBT showed an activating effect on the oxidation of the stilbene moiety compared to laccase alone.

The non-phenolic stilbene model **25** showed a somewhat reduced reactivity toward laccase as witnessed by the fact that its oxidation products were obtained in amounts roughly equivalent to those obtained in the absence of enzyme (Table 3). When the reaction was carried out in the presence of ABTS and HBT, no differences in the reactivity of the system were apparent, the only metabolites detected being the hydroxylation product **27** and traces of **28**, a product of olefinic oxidation and rearrangement.[17]

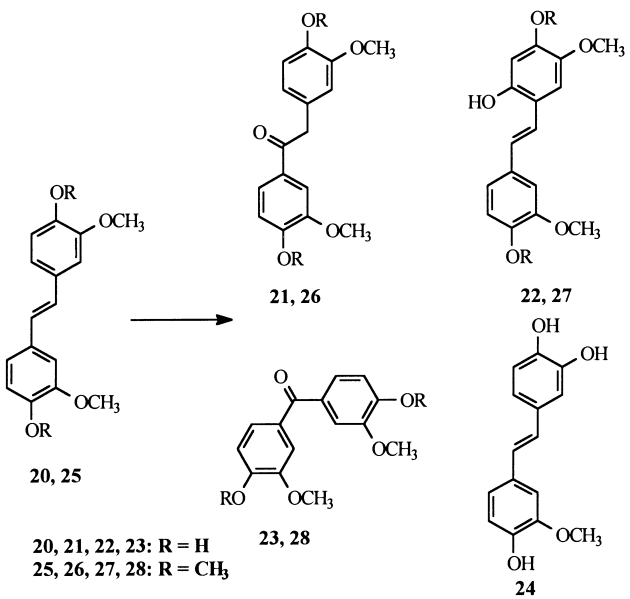
Table 4. Product profiles for the degradation of Stilbene models by laccase

Product	Reaction Conditions[a]			
	A	B	C	D
	Yield (%)[b]			
21	3.4	17.0	26.8	21.6
22	3.4	13.3	28.0	22.4
23	7.0	32.5	1.0	17.1
24	8.1	12.6	26.8	27.0
26	1.6	1.6	1.6	1.6
27	0.9	0.9	0.9	0.9
28	TRACE	TRACE	TRACE	TRACE

[a] A: Blank; B: Laccase 0.5 U/mL; C: Laccase 0.5 U/mL, ABTS 1 mM; D: Laccase 0.5 U/mL, HBT 1 mM.
[b] Evaluated by quantitative GC analyses.

Discussion

Laccases are oxygen-dependent enzymes that reduce oxygen to water. The reaction mechanism of laccase on lignin model compounds has been widely studied to understand the mechanisms of the biological degradation



Scheme 4. Laccase biodegradation products of stilbene models **20** and **25**.

of wood and evaluate the possible use of this oxidative enzyme in delignification processes.[4,14,15] The phenoxy radical intermediates formed during this process can further disproportionate and consequently initiate lignin degradation.[4,14,15]

The main early metabolites detected were, for all the 5-5′ diphenylmethane and α-5 models, products of oxidation at the benzylic positions. The oxidation of methyl groups on aromatic rings to benzyl alcohols or benzaldehydes is usually accomplished in organic synthesis using transition metal oxidants, but it is rather difficult to stop the reaction at the aldehyde stage, since the oxidation products are more easily oxidized than the starting materials. The occurrence of this selective oxidation on benzylic groups to aldehyde on non-phenolic systems in the presence of the laccase-ABTS system, has recently been reported.[18]

Besides the products of side-chain oxidation, demethoxylation and perhaps oxidative coupling products were also detected. Such reactivity patterns have already been observed for laccase oxidations of lignins and lignin model compounds.[2,15,19] This behaviour is analogous to the overall reactivity found between hydroxy radicals and lignin model compounds. This analogy is reasonable since the main intermediates involved in such reactions are believed to be phenoxy radicals.[20]

The phenolic diphenylmethane and α-5 structures, **8** and **13**, respectively, were found to be remarkably more reactive than the 5-5′ dicreosol model compound **1**, that was degraded by the laccase treatments only in low amounts. The diphenylmethane model **8**, which showed the highest reactivity, also showed the highest amount of high molecular weight metabolites, probably arising from radical coupling reactions.[4,14,15] The strongly enhanced reactivity of 5-5′, diphenylmethane and α-5 models in the presence of bubbled oxygen (which caused their near complete degradation), constitutes experimental evidence supporting our contention that the other experiments were performed under limited oxygen supply. This experimental design effectively allowed the isolation and characterization of the early metabolites occurring during the laccase and laccase-mediator oxidative processes.

The degradation pathway of the phenolic stilbene model **20**, besides showing demethylation and hydroxylation reactions, was shown to yield a product of benzylic oxidation, **21**, and a product arising from complex oxidation-transposition reactions, **23**. These transformations are characteristic of stilbenes in the presence of hydroxy radicals.[4,14] Once again this similarity can be due to the presence of common phenoxy radical intermediates. The high reactivity of the stilbene unit suggests that during the laccase delignification of kraft pulp the process of degradation of stilbenes could play an important role.

The condensed kraft lignin model compounds seem to initially be degraded by laccase, mainly via side chain oxidation, demethylation and hydroxylation reactions. These transformations can be considered as aids to delignification since they increase the hydrophilic nature of the lignin moieties. Most significantly, however, such reactions introduce activated sites on lignin structures. Oxidative coupling reactions that inhibit delignification were found to occur only in minor amounts under the acidic conditions examined, and are therefore considered irrelevant.

The inertness of the non-phenolic 5-5′, diphenylmethane and stilbene models toward laccase is in accord with data previously reported for methoxy and dimethoxy benzenes.[21,22] However, their inertness in the presence of radical mediators was unexpected. In fact, it has been reported that the radical mediator ABTS is able to bring about the oxidation of non-phenolic systems characterized by a redox potential higher than laccase such as veratryl alcohol or non-phenolic β-O-4 model compounds.[3] In contrast, recent findings have also shown that the reactivity of benzylic alcohols in the presence of the laccase-mediator system depends on the substitution pattern on the aromatic ring.[18,23] In particular, 2,6-disubstituted benzylic alcohols were found to be unreactive with the laccase-mediator system.

Most of the work on laccase and laccase/mediator systems has been carried out in aqueous buffers. In principle one could argue that the presence of dioxane in our experiments could affect the functionality of laccase and the laccase-mediator reaction rate and/or redox potential. However there are several literature examples of the use of laccases in the presence of considerable amounts of organic solvents.[18,23–25]

In an effort to address this concern, the activity of laccase was assayed under these conditions. The presence of 50% dioxane reduced the enzyme oxidation rate by about 70–80%, but the enzyme was still functional. In fact assays of enzymatic activity of laccase kept 24 h in 50% dioxane solution showed residual 80% activity. With respect to the redox potential of the laccase-mediator system in the presence of dioxane, laccase-mediator treatments in mixed solvents have been reported to occur successfully under analogous reaction conditions.[18,23,24]

According to our data, the radical mediators ABTS and HBT do not play an important role in the degradation of non-phenolic 5-5′, diphenylmethane and stilbene

models. It has been recently reported that laccase, in the presence of either ABTS or HBT can effectively delignify kraft pulps.[5] On the basis of our data one may suggest that the role of such mediators, in kraft pulps, is to act as diffusible lignin oxidizing agents of phenolic systems, since these relatively small compounds can access the inner lignin structure with greater facility than the relatively large enzyme.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Varian XL 300 spectrometer. Mass Spectroscopy (MS) was performed with Hewlett-Packard 5971 mass-selective detector on a Hewlett-Packard 5890 gas chromatograph.

All solvents were ACS reagent grade and were redistilled and dried according to standard procedures. Chromatographic purifications were performed on columns packed with Merck silica gel 60, 230–400 mesh for flash technique. Thin-layer chromatography was carried out using Merck platten Kieselgel 60 F254.

Characterization of metabolites. Gas chromatography and gas chromatography-mass spectrometry of the reaction products were performed using a DB1 column (30 m×0.25 mm and 0.25 mm film thickness), and an isothermal temperature profile of 100 °C for the first two min, followed by a 20 °C/min temperature gradient to 300 C and finally an isothermal period at 300 °C for 10 min. The injector temperature was 280 °C. Chromatography grade helium was used as the carrier gas. The fragmentation patterns were compared to those of authentic samples.[17,26–31]

Kraft lignin model compounds. The model compounds **1**, **8**, **13**, **20**, and **25** were synthesized by known methods.[26,27] Non-phenolic models dihydromethyl veratrole **6** and (3,3′4,4′-tetramethoxy-6,6′-dimethyl) diphenylmethane **12** were synthesized by methylating the corresponding phenols **1** and **8** using dimethylsulfate (Me_2SO_4) under alkaline conditions. Phenols **1** and **8** (0.1 mol) were dissolved in 10 mL of NaOH 30% (aq solution) under magnetic stirring, and Me_2SO_4 (0.2 mole) were added at 60 °C during 1 h. After 2 h the reaction mixtures were cooled, neutralized with ammonium chloride, and extracted with ethyl acetate. The organic solvent was evaporated under reduced pressure, and the crude products were crystallized from hexane-ethyl acetate to give **6** and **12** in quantitative yield.

Dihydromethyl veratrole 6. ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 6H, CH_3), 3.64 (s, 6H, OCH_3), 3.88 (s, 6H, OCH_3), 6.64–6.74 (m, 4H, CH); ^{13}C NMR (CDCl3) δ 21.26 (CH_3), 55.79 (OCH_3), 60.64 (OCH_3), 112.58 (CH), 123.53 (CH), 132.59 (C) 132.81 (C), 144.60 (C), 152.30 (C). Mass Spectrometry data are reported in Table 5.

(3,3′4,4′-Tetramethoxy-6,6′-dimethyl) diphenylmethane 12. ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 6H, CH_3), 3.76 (s, 6H, OCH_3), 3.85 (s, 6H, OCH_3), 3.96 (s, 2H, CH_2), 6.52–6.60 (m, 4H, CH); ^{13}C NMR ($CDCl^3$) δ 21.30 (CH_3), 29.07 (CH_2), 55.62 (OCH_3), 60.37 (OCH_3), 111.21 (CH), 122.87 (CH), 133.29 (C) 134.46 (C), 144.88 (C), 152.66 (C). Mass Spectrometry data are reported in Table 5.

Oxidation of residual kraft lignin model compounds. Due to the low solubility of kraft lignin model compounds in water, their oxidation was performed in a 1/1 (v/v) dioxane:water solvent mixture. The substrate (2 mM) was dissolved in dioxane:acetate buffer pH 5 0.05 M (10 mL, 1/1 v/v), in the presence or absence of either ABTS or HBT (1 mM), and treated with purified laccase from *Trametes versicolor* supplied by Dr. R. Bourbonnais of PAPRICAN with either 0.05 U/mL or 0.5 U/mL at 40 °C. The reaction mixtures were kept in open vials under vigorous stirring in order to ensure constant oxygen saturation of the solutions throughout the experiment.[5] The reactions were conducted in the dark in order to avoid possible photochemical oxidation.

After 24 h the reactions were halted by heating the mixtures in boiling water. A suitable amount of benzoic acid as internal standard (2 mM), dissolved in dioxane, was then added to the reaction mixtures. The solvent was evaporated under reduced pressure, the residues were solubilized in ethyl acetate and pyridine. The solutions were dryed over anhydrous $MgSO_4$ and filtered. The organic solvent was evaporated under reduced pressure. In order to analyze the reaction products the residues were dissolved in 1 mL of pyridine and submitted to silylation by addition of N,O-bis(trimethylsilyl)-acetamide. After 30 min the mixtures were subjected to gas chromatography (GC) and gas chromatography-mass spectrometric (GC-MS) analyses.

Enzyme assays. Laccase activity was determined by oxidation of ABTS.[32] The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate, pH 5.0, and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm (e_{420} = $3.6\times104\ M^{-1}cm^{-1}$). Enzyme activity was expressed in units (U = mmol of ABTS oxidized per minute).[4,7] The determination of the laccase activity was also performed in a dioxane/Na-acetate buffer 1/1 (v/v) solvent system.

Oxidation of kraft lignin model compounds in the presence of bubbled oxygen. The model substrate (2 mM) was dissolved in dioxane:acetate buffer pH 5 0.05 M (10 mL, 1/1 v/v) and treated with laccase 0.5 U/mL at 40°C in the presence or absence of either ABTS

Table 5. Mass spectrometric data

Product	Derivative[a]	MS (m/z) data (%)
1	—	274 (M, 100), 241 (24), 227 (21), 199 (19)
1	-Si $(CH_3)_3$	418 (M, 38), 403 (M-15, 18), 388 (M-30, 26), 287 (14), 179 (11), 73 (100)
2	-Si $(CH_3)_3$	506 (M, 28), 329 (100), 147 (8), 73 (87)
3	-Si $(CH_3)_3$	432 (M, 21), 417 (M-15, 26), 301 (8), 73 (100)
4	-Si $(CH_3)_3$	446 (M, 12), 431 (M-15, 18), 357 (4), 315 (8), 73 (100)
5	-Si $(CH_3)_3$	476 (M, 16), 373 (7), 358 (10), 147 (14), 73 (100)
6	—	302 (M, 100), 287 (M-15, 16), 272 (M-30, 21) 256 (93), 241 (25), 213 (23), 128 (28)
7	—	330 (M, 100), 315 (M-15, 12), 300 (M-30, 18), 284 (96), 269 (27), 241 (22)
8	—	288 (M, 84), 255 (21), 241 (8), 151 (33), 138 (100), 123 (7)
8	-Si $(CH_3)_3$	432 (M, 56), 417 (M-15, 12), 402 (M-30, 24), 73 (100)
9	-Si $(CH_3)_3$	520 (M, 9) 505 (M-15, 5), 417 (7), 343 (22), 73 (100)
10	-Si $(CH_3)_3$	446 (M, 28), 431 (M-15, 62), 357 (6), 269 (8), 179 (8), 73 (100)
11	-Si $(CH_3)_3$	490 (M, 13), 431(8), 342 (66), 269 (6), 193 (8), 117 (12), 73 (100)
12	—	316 (M, 100), 301 (M-15, 11), 269 (23), 255 (21), 165 (26), 151 (99), 135 (78)
13	—	274 (M, 95), 241 (12), 151 (28), 150 (100), 149 (22), 124 (18), 121 (13)
13	-Si $(CH_3)_3$	419 (M + 1, 26), 418 (M, 100), 403 (M-15, 26), 388 (M-30, 91), 358 (23), 343 (18), 179 (20), 73 (81)
14	-Si $(CH_3)_3$	506 (M, 14), 491 (M-15, 4), 476 (M-30, 6), 387 (8), 297 (9), 209 (11), 147 (12), 73 (100)
15	-Si $(CH_3)_3$	432 (M, 22), 414 (M-15, 13), 402 (M-30, 16), 193 (16), 73 (100)
16	-Si $(CH_3)_3$	520 (M, 18), 505 (M-15, 11), 430 (7), 417 (11), 343 (28), 193 (11), 147 (13), 73 (100)
17	-Si $(CH_3)_3$	432 (M, 24), 417 (M-15, 22), 372 (8), 314 (11), 223 (14), 179 (21), 121 (16), 73 (100)
18	-Si $(CH_3)_3$	476 (M, 22), 446 (M-30, 9), 417 (14), 387 (19), 343 (12), 314 (13), 179 (17), 73 (100)
19	-Si $(CH_3)_3$	476 (M, 26), 446 (M-30, 13), 417 (18), 387 (12), 343 (9), 314 (15), 179 (19), 73 (100)
20	-Si $(CH_3)_3$	417 (M + 1, 31), 416 (M, 100), 401 (M-15, 4), 386 (M-30, 28), 356 (19), 281 (5), 253 (14), 178 (11), 73 (96)
21	-Si $(CH_3)_3$	432 (M, 6), 417 (M-15, 4), 223 (M-209, 100), 209 (M-223, 12), 179 (21), 165 (9), 73 (32)
22	-Si $(CH_3)_3$	504 (M, 71), 489 (M-15, 6), 474 (M-30, 11), 444 (M-60, 4), 342 (8), 73 (100)
23	-Si $(CH_3)_3$	418 (M, 63), 403 (M-15, 28), 388 (M-30, 25), 373 (M-45, 12), 358 (M-60, 26), 223 (58), 193 (62), 165 (60), 73 (100)
24	-Si $(CH_3)_3$	475 (M + 1, 16), 474 (M, 46), 444 (M-30, 8), 356 (18), 253 (6), 73 (100)
25	—	301 (M + 1, 21), 300 (M, 100), 285 (M-15, 39), 225 (16), 210 (14), 210 (9), 181 (14), 152 (15), 139 (14), 128 (16)
26	—	316 (M, 5), 302 (36), 271 (24), 227 (12), 207 (16), 165 (100), 137 (22)
27	-Si $(CH_3)_3$	389 (M + 1, 11), 388 (M, 69), 373 (M-15, 6), 358 (M-30, 5), 345 (12), 315 (14), 287 (18), 73 (100)
28	—	302 (32), 271 (M-31, 24), 227 (14), 207 (16), 165 (100) 137 (19)

[a]-: underivatized; -Si $(CH_3)_3$: trimethylsilylated with N,O-bis(trimethylsilyl)-acetamide.

or HBT (1 mM). Oxygen was continously bubbled in the reaction mixtures. All reactions were conducted in the dark in order to avoid photochemical oxidation. After 4 h the reactions were halted by heating the mixtures in boiling water.

Acknowledgements

The authors would like to acknowledge Mr. R. Bourbonnais for supplying the purified enzyme used in this effort, and Dr. F. Archibald for providing constructive criticism during the writing of this paper. This work was supported by the Paprican Maintaining Member Companies, and forms a part of a research program under the auspice of the Protein Engineering Network Centre of Excellence (PENCE).

References and Notes

1. Reid, I. D.; Paice, M. G. *FEMS Microb. Rev.*, **1994**, *13*, 369.

2. Bourbonnais, R.; Paice, M. G. *Appl. Microbiol. Biotechnol.* **1992**, *36*, 823.

3. Bourbonnais, R.; Paice, M. G. *FEBS Lett.* **1990**, *267*, 99.

4. Kawai, S.; Umezawa, T.; Higuchi, T. *Archiv. Biochem. Biophys.* **1988**, *262*, 99.

5. Bourbonnais, R.; Paice, M. G.; Freiermuth, B.; Bodie, E.; Borneman, S. *Appl. Environ. Microbiol.* **1997**, in press.

6. Call, H. P. *World Patent Application* **1994**, WO 94/29510.

7. Bourbonnais, R.; Paice, M. G.; Reid, I. D.; Lanthier, P.; Yaguchi M. *Appl. Env. Microb.* **1995**, *61*, 1876.

8. Eriksson, T.; Gierer, J. J. *Wood Chem. Technol.* **1985**, *5*, 53.

9. Gellerstedt,G.; Lindfors, E. L. *Holzforshung* **1984**, *38*, 151.

10. Gierer, J. *Wood Sci. Technol.* **1980**, *14*, 241.

11. Gierer, J. *Holzforshung* **1982**, *36*, 43.

12. Gierer, J. *Wood Sci. Technol.* **1985**, *19*, 289.

13. Chiang, V. L.; Kolppo, K.; Stokke, D. D. *Wood and Pulp Chem.* **1989**, 593.

14. Caldwell, E. S.; Steelink, C. *Bioch. Bioph. Acta* **1969**, *189*, 420.

15. Lundquist, K.; Kristersson, P. *Biochem. J.* **1985**, *229*, 277.

16. Gierer, J.; Yang, E.; Reitberger, T. *Holzforschung* **1996**, *50*, 342.

17. Gierer, J.; Yang, E.; Reitberger, T. *Holzforschung* **1996**, *50*, 353.

18. Potthast, A.; Rosenau, T.; Chen, C. L.; Gratzl, J. S. *J. Mol. Catal. A: Chem*. **1996**, *108*, 5.

19. Higuchi, T. *Wood Sci. Technol*. **1990**, *24*, 23.

20. Gierer, J.; Yang, E.; Reitberger, T. *Holzforshung*, **1992**, *46*, 495.

21. Kersten, P. J.; Kalianaraman, B.; Hammel, K. E.; Reinhammar, B.; Kirk, T. K. *Biochem. J*. **1990**, *268*, 475.

22. Muheim, A.; Fiechter, A.; Harvey, P. J.; Shoemaker, E. *Holzforschung*, **1992**, *46*, 121.

23. Chen, C. L.; Gratzl, J.; Kirkman, A. G.; Miyakosky, T. *Abstracts of Papers*, Fifth Brazilian Symposium on the Chemistry of Lignins and Other Wood Components: Curitiba, Brazil, 1997; 547–556.

24. Potthast, A.; Rosenau, T.; Chen, C. L.; Gratzl, J. S. *J. Org. Chem*. **1995**, *60*, 4320.

25. Ikeda, R.; Sugihara, J.; Uyama, H.; Kobayashi, S. *Macromol*. **1996**, *29*, 8702.

26. Douglas, F. X.; Reeve, W.; McKague, A. B. *J. Wood Chem. Technol*., **1996**, *16*, 35.

27. Wong, D. F.; Leary, G.; Arct, G. *Res. Chem. Intermed*. **1995**, *21*, 329.

28. Gierer, J.; Lindberg, O. *Acta Chem. Scand. Ser. B*, **1980**, *B34*, 161.

29. Falke, J. *Sven. Paappersttidn* **1984**, *87*, R133.

30. Falke, J.; Lindgaard, J. P. *Toxical Environ. Chem*. **1985**, *10*, 1.

31. Gierer, J.; Nilvebrant, N. O. *J. Wood Chem. Technol*. **1991**, *11*, 171.

32. Wolfenden, B. S.; Wilson, R. L. *J. Chem. Soc. Perkin Trans. 2* **1982**, 805.