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Research Article

Reactivity of *p*-Coumaroyl Groups in Lignin upon Laccase and Laccase/HBT Treatments

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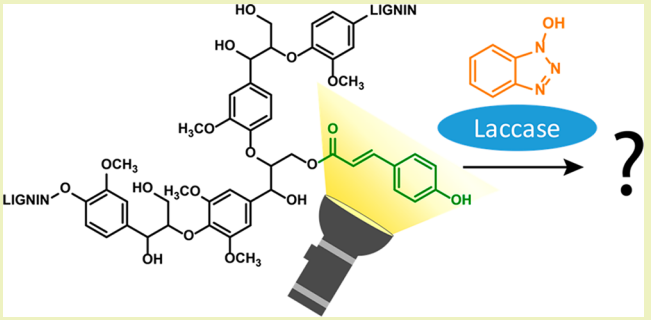
ABSTRACT: Laccase−mediator systems (LMS) are potential green tools for oxidative degradation and modification of lignin. Although LMS convert both phenolic and nonphenolic lignin structures, phenolic structures are more prone to react. Remarkably, in a previous study on laccase/HBT treatment of grasses, we observed the accumulation of *p*-coumaroyl moieties in residual lignin, even though such groups are free phenolic structures. To provide more insights into this apparent paradox, here, we studied the reactivity of *p*-coumaroyl groups in lignin and model compounds using HSQC NMR and RP-UHPLC-PDA-MS^n, respectively. It was found that a *p*-coumaroylated model compound (VBG-*p*CA), in contrast to its nonacylated analogue, was rapidly converted by laccase and laccase/HBT, resulting in oxidative coupling and HBT-mediated degradation, respectively. The high reactivity of VBG-*p*CA was related to the phenolic character of the *p*-coumaroyl group. Upon laccase/HBT treatment of two grass lignin isolates, *p*-coumaroyl groups accumulated in residual lignin, indicating that *p*-coumaroyl groups in polymeric lignin display different reactivity than those in model compounds. On the basis of additional experiments, we propose that *p*-coumaroyl groups in lignin polymers can be oxidized by laccase/HBT but undergo HSQC-undetectable radical coupling or redox reactions rather than degradation.



KEYWORDS: *Lignocellulosic biomass, Corn stover, Wheat straw, Enzyme catalysis, Mediator, Laccase, Degradation, p-Coumarate*

INTRODUCTION

Lignocellulosic biomass is a promising resource for the sustainable production of biobased fuels and chemicals. Efficient conversion of lignocellulosic biomass, however, is challenging due to its recalcitrant structure. A major factor limiting efficient conversion is the presence of lignin, a complex aromatic heteropolymer.[1] Over recent years, laccase (EC 1.10.3.2) and laccase−mediator systems have gained extensive attention as potential green tools for lignin degradation. However, despite the insights from various mechanistic studies,[2−5] the reactivity of lignin in laccase and LMS incubations is still not fully understood.

Lignin consists, depending on its botanical origin, of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) subunits. These subunits form a polymer through various C−O and C−C linkages, of which the *β-O*-4′ linkage is the most abundant one.[6,7] The botanical origin of lignin affects not only the S/G/H ratio but also the extent and type of C_γ-acylation. Whereas no C_γ-acylation occurs in softwood lignin, several hardwood lignins (i.e., those from willow, aspen, poplar, and palms) are C_γ-acylated by *p*-hydroxybenzoyl and acetyl groups,[7−9] and grass lignins can be extensively *p*-coumaroylated and acetylated at the C_γ−OH group.[7,10,11]

Laccases are oxidases that catalyze one-electron oxidation of aromatic substrates. Their redox potential (<800 mV vs NHE)[2] is, however, too low to oxidize the nonphenolic substructures of lignin, which account for up to 90% of the lignin polymer.[12] To overcome this recalcitrance, a mediator, such as 1-hydroxybenzotriazole (HBT), can be added to form a laccase−mediator system (LMS). In such a LMS, laccase oxidizes the mediator, which, in turn, oxidizes the nonphenolic lignin substructure. In the case of a laccase/HBT system, the latter step is generally suggested to occur via hydrogen atom transfer (HAT), resulting in benzylic radicals.[5] These radicals can, subsequently, react further via multiple pathways. The final outcome of these radical reactions is dependent on the local structures neighboring the benzylic radical.[4] In various biomass substrates, LMS treatments have been shown to induce oxidation and degradation of lignin interunit linkages, resulting in delignification (up to 50%) of the biomass.[13−15]

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Recently, we observed that laccase/HBT treatment of milled corn stover and wheat straw resulted in extensive loss (>40%) of interunit linkages, which are largely part of nonphenolic lignin structures. In contrast, *p*-coumaroylated substructures accumulated in the residue upon laccase/HBT treatment, suggesting that these substructures are poor targets for the laccase/HBT system.[15] A similar observation was reported by Rencoret et al. upon laccase/HBT treatment of sugar cane bagasse.[14] These observations are remarkable, as *p*-coumaroyl moieties in lignin are generally suggested to occur as free phenolic groups (i.e., unetherified at the phenolic OH group).[10,16,17] Admittedly, compared to other phenolic structures, *p*-coumaric acid is poorly oxidized by laccase and by ABTS radical cations (and possibly also for laccase/HBT).[18] Nonetheless, based on its phenolic structure, it should still be more easily oxidized than the nonphenolic substructures of lignin.[19−21] Thus far, it remains unknown how this apparent recalcitrance of *p*-coumaroylated lignin substructures could be explained. In fact, very little is known at all on the reactivity of *p*-coumaroyl moieties in lignin upon oxidative treatments.

As an enhanced understanding of the reactivity of *p*-coumaroyl groups in lignin is expected to be helpful in further optimization of LMS-based lignin conversion, we investigated the reactivity of *p*-coumaroyl groups in detail. Hereto, we studied the reactions of a *p*-coumaroylated lignin model compound (VBG-*p*CA, Figure 1) in laccase and laccase/HBT

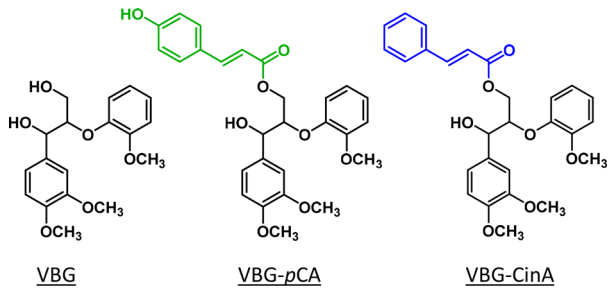

Figure 1. Molecular structures of the model compounds VBG, VBG-*p*CA, and VBG-CinA used in this study.

treatments and compared this to the reactivity of a cinnamoylated model compound (lacking the phenolic hydroxyl group) and a nonacylated model compound (VBG-CinA and VBG, respectively, Figure 1) using RP-UHPLC-PDA-MS^n. In addition, to investigate whether the reactivity of *p*-coumaroyl moieties in polymeric lignin was comparable to that in the model compound, we used 2D HSQC NMR to study the structure of lignin isolates from corn stover and wheat straw before and after laccase/HBT treatment.

MATERIALS AND METHODS

Materials. Acylated lignin model compounds were enzymatically synthesized and purified using a previously reported method.[22] The used VBG-*p*CA and VBG-CinA (Figure 1) consisted for approximately 95% and 100% of trans isomers, respectively.[22] Veratryglycerol-*β*-guaiacyl ether (VBG) was purchased from ABCR Chemicals (Karlsruhe, Germany), and guaiacylglycerol-*β*-guaiacyl ether (GBG) was obtained from TCI Chemicals (Tokyo, Japan). Laccase from *Trametes versicolor* was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was partially purified as described earlier.[23] The activity of both enzymes was determined spectrophotometrically by oxidation of ABTS (1 U = 1 *μ*mol of ABTS oxidized per minute at pH 5). Wheat straw lignin isolate (WSL) and corn stover lignin isolate (CSL) were obtained from previous studies at our laboratory. Briefly, CSL was obtained by aqueous dioxane extraction of ball-milled corn stover followed by lyophilization and was further purified by incubation with a cellulolytic enzyme cocktail.[24] WSL was obtained similarly but with cellulolytic enzyme treatment before dioxane extraction.[25] WSL and CSL had lignin contents of 85% and 90% (w/w), respectively.[24,25] All other chemicals were obtained from Sigma-Aldrich and were of analytical grade. Water was prepared using a Milli-Q water purification system (Merck Millipore, Billerica, MA).

Incubation of Lignin Models with Laccase and Laccase/HBT. Stock solutions were prepared by dissolving model compounds at a concentration of 1 mM in citrate/phosphate buffer (pH 4, 20/40 mM) containing 25% (v/v) methanol. Addition of methanol was necessary to ensure complete solubilization of the acylated model compounds. No spontaneous hydrolysis or transesterification was observed, indicating that the ester bonds of the model compounds were stable under these solvent conditions. Stock solutions of HBT (10 mM) and laccase (25 U mL^{-1}) were prepared in the same buffer. The stock solutions were then mixed to obtain final substrate and HBT concentrations of 80 and 400 *μ*M, respectively, and a laccase activity of 2 U mL^{-1}. The relatively high HBT:mediator ratio was used to ensure (i) a clear difference between incubations with and without HBT and (ii) a reasonable conversion of nonphenolic lignin models under the nonoptimal conditions used (i.e., 25% methanol). After 1 and 24 h, 40 *μ*L aliquots of the samples were transferred to a clean tube and immediately cooled in ice−water to stop the enzymatic activity. Control incubations containing only model compounds or only model compounds with HBT were performed similarly. In these cases, only a sample incubated for 24 h was analyzed. After cooling for 10 min in ice water, samples were centrifuged (10 000 × *g*, 5 min, 4 °C) and immediately transferred to the autosampler of the UHPLC system (at 4 °C) prior to RP-UHPLC-PDA-MS analysis.

Incubation of Lignin Models with Laccase-Free Oxidized VBG-*p*CA. Nonacylated lignin model compounds were dissolved at 1 mM in citrate/phosphate buffer (pH 4, 20/40 mM) containing 25% (v/v) methanol, and 60 *μ*L of these solutions was transferred to 2 mL reaction tubes. Amicon Ultra-0.5 centrifugal filters (Merck Millipore) with a normalized molecular weight limit of 10 kDa were placed on top of these solutions and were loaded with 130 *μ*L of buffer and 20 *μ*L of VBG-*p*CA solution (1 mM). Subsequently, 20 *μ*L of laccase solution was added to obtain a final activity of 6 U mL^{-1}. The tubes were then immediately centrifuged (10 000 × *g*, 40 °C) until >90% of the liquid had passed through the filter (approximately 30 min). As controls, identical incubations were performed in which laccase was replaced with buffer. The filtrates were collected and analyzed using RP-UHPLC-PDA-MS^n. The retentates were mixed with 200 *μ*L of MeOH and centrifuged (10 000 × *g*, 5 min, 20 °C) prior to RP-UHPLC-PDA-MS^n analysis.

Incubation of Lignin Isolates with Laccase/HBT. Laccase/HBT treatment of CSL and WSL was performed based on a previously described protocol.[15] Hereto, approximately 12 mg of WSL and CSL were suspended in 240 *μ*L of a 200 mM citrate buffer at pH 4 containing 100 mM HBT. To this suspension, 60 *μ*L of a laccase stock solution (10 U mL^{-1}) was added to obtain a final HBT concentration of 80 mM and a laccase activity of 50 U g^{-1} lignin isolate. In addition, control samples were prepared by suspending 12 mg of WSL and CSL in 300 *μ*L of buffer. In contrast to the model compound incubations, no methanol was added, as methanol addition did not solubilize WSL and CSL. All samples were incubated at 40 °C in a thermomixer at 650 rpm shaking. After 24 h, the samples were removed from the thermomixer and washed 5 times with 1 mL of MQ water. The residues were frozen in liquid nitrogen and lyophilized prior to NMR spectroscopy.

Saponification of Lignin Isolates. For mild saponification of the laccase/HBT-treated and untreated CSL and WSL, approximately 10 mg of sample was incubated with 0.5 mL of 1 M NaOH at 30 °C for 24 h under 300 rpm shaking.[26] Subsequently, the samples were acidified by adding 6 M acetic acid to reach a pH of approximately 6 (as indicated by pH paper). After centrifugation (10 000 × *g*, 5 min, 20 °C), the supernatants were collected and stored at 4 °C. Prior to

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RP-UHPLC-PDA-MS analysis, the supernatants were diluted 20 times in MQ water. The residue was washed 4 times with 1 mL of MQ water, frozen in liquid nitrogen, and lyophilized prior to NMR spectroscopy.

RESULTS AND DISCUSSION

Reactivity of Acylated and Nonacylated Model Compounds with Laccase and Laccase/HBT. VBG, VBG-*p*CA, and VBG-CinA were incubated with laccase in the presence or absence of HBT to investigate whether and how acylation of VBG influences its reactivity.

When unacylated VBG was incubated with laccase alone, no conversion was observed, as expected based on its nonphenolic structure (Figures 2A and 3, Tables 1 and 2).[21,27] In the case

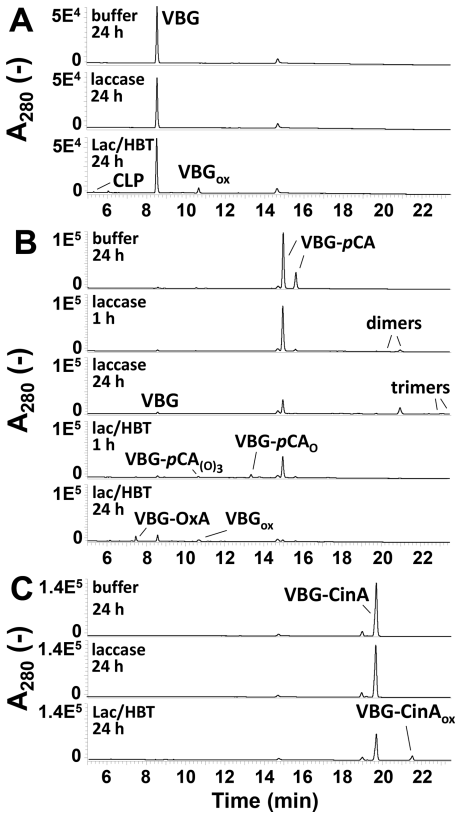

Figure 2. RP-UHPLC-UV$_{280}$ chromatograms of VBG (A), VBG-*p*CA (B), and VBG-CinA (C) after incubation with buffer, laccase, or laccase/HBT for 1 or 24 h. HBT and BT (see Table 1) eluted within 5 min and are therefore not visible in the figure. Second VBG-*p*CA peak corresponds to the cis isomer of VBG-*p*CA and was formed spontaneously upon exposure to light.

where the laccase/HBT system was used, a slight conversion of 4% was observed after 24 h. This conversion is low in comparison to a previous study and is likely caused by the presence of MeOH as cosolvent in the current study, which decreases laccase activity.[21,28] The conversion mainly resulted in formation of its C$_\alpha$-oxidized analogue (VBG$_{ox}$) and trace amounts of a cleavage product (CLP), as also found in previous studies (Figures 2A and 3, Tables 1 and 2).[21,29]

In the case of VBG-*p*CA, several conversions were observed. First, trans−cis isomerization was shown to occur (Figure 2B, Table 1). Isomerization also occurred in the absence of laccase and has been reported more often upon exposure of *p*CA derivatives to light.[30,31] Moreover, in contrast to VBG, VBG-*p*CA was readily oxidized by laccase alone with 39% and 82% substrate conversion after 1 and 24 h, respectively (Figure 2B, Tables 1 and 2). This conversion resulted in formation of multiple products with molecular formula $C_{54}H_{54}O_{16}$, which were annotated as VBG-*p*CA dimers. In addition, after 24 h, a small amount of VBG-*p*CA trimers and (unacylated) VBG was formed (Figures 2B and 3, Tables 1 and 2). Hence, laccase is able to oxidize VBG-*p*CA in the absence of a mediator, and this mainly results in oxidative coupling, as previously also observed with other phenolic lignin substructures.[23,32] The fact that multiple VBG-*p*CA dimers were formed is most likely related to trans−cis isomerization but possibly also due to formation of various new C−C or C−O linkages. The exact structures of the formed bonds (e.g., 5-5′, *β*-5′, and 4-*O*-5′) were not further investigated.

When VBG-*p*CA was incubated with the laccase/HBT system, faster conversion was observed than in the incubation with laccase alone, as indicated by substrate conversions of 71% and 97% after 1 and 24 h, respectively (Table 2). In addition, a completely different product profile was obtained. VBG-*p*CA dimers were absent at all incubation times. Instead, after 1 h, reaction products corresponding to $C_{27}H_{28}O_9$ (VBG-*p*CA$_O$) and $C_{27}H_{28}O_{11}$ (VBG-*p*CA$_{O3}$) were detected as well as trace amounts of $C_{27}H_{28}O_{10}$ (VBG-*p*CA$_{O2}$), indicating that hydroxylation of VBG-*p*CA occurred (Figure 2B and Table 1). After 24 h, the abundance of hydroxylated VBG-*p*CA products had decreased, which coincided with formation of relatively large amounts of VBG and VBG$_{ox}$ (Figures 2B and 3, Tables 1 and 2). In addition, a compound corresponding to $C_{20}H_{22}O_9$ and small amounts of $C_{20}H_{20}O_9$ were detected, which were tentatively annotated as a VBG-oxalate ester (VBG-OxA) and its C$_\alpha$-oxidized analogue (VBG-OxA$_{ox}$). Thus, overall, addition of HBT to VBG-*p*CA incubations shifted the reaction outcome from polymerization to degradation. The (hydroxylated) *p*-coumaroyl moieties that were cleaved off were not detected in the RP-UHPLC-PDA-MS analysis, most likely due to further reactions (e.g., polymerization) of these moieties.

On the basis of the fact that the abundance of hydroxylated products decreased along with formation of degradation products, it seems likely that VBG-*p*CA degradation occurred (mainly) after initial hydroxylation. As no hydroxylation was observed in incubations with laccase alone, it is clear that HBT is, somehow, involved in the hydroxylation reactions. A plausible route for hydroxylation of VBG-*p*CA is via formation of covalent HBT adducts as intermediates, as suggested earlier for hydroxylation of other phenolic molecules.[33] The HBT adducts could then decompose into hydroxylated VBG-*p*CA and either HBT or its degradation product benzotriazole (BT) (see Figure 4). Corroborating evidence for the latter pathway is the remarkably high conversion of HBT to BT in VBG-*p*CA incubations (Figure S2). Although reduction of HBT to BT is common in laccase/HBT incubations[21,23,34] and was also observed in incubations with VBG or VBG-CinA, this occurred to a substantially higher extent in incubations of VBG-*p*CA (see Figure S2). On the basis of the above discussion, we propose that the laccase/HBT system first hydroxylates VBG-*p*CA, after which subsequent degradation takes place, yielding VBG and VBG-OxA (Figures 3 and 4).

The latter two species may then undergo C$_\alpha$-oxidation, as generally observed in laccase/HBT treatments,[21,27] to form VBG$_{ox}$ and VBG-OxA$_{ox}$. Laccase-catalyzed conversion of VBG-CinA, a nonphenolic analogue of VBG-*p*CA, was only observed in the presence of HBT with VBG-CinA$_{ox}$ as the only reaction

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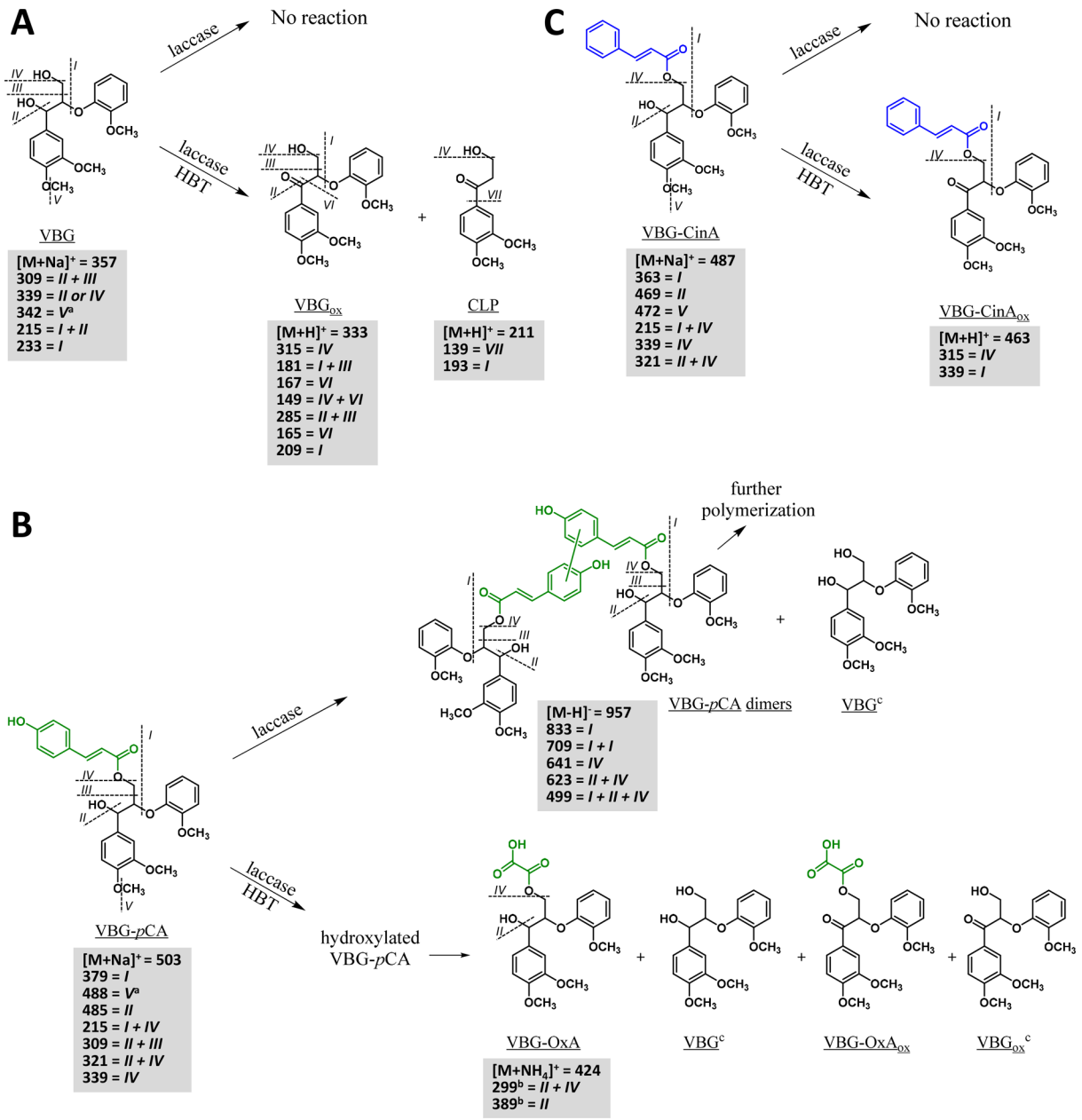

Figure 3. Schematic overview of the final reaction outcomes of VBG (A), VBG-*p*CA (B), and VBG-CinA (C) incubations with laccase and laccase/HBT. Proposed fragmentation patterns of substrates and products resulting in the MS^2 fragments reported in Table 1 are shown in the gray boxes. Since multiple isomers of the VBG-*p*CA dimers are formed, the linkage type between the two VBG-*p*CA molecules is not specified in this figure. Hydroxylation of VBG-*p*CA (to VBG-*p*CA_O and VBG-*p*CA_{O3}) is suggested to occur prior to eventual degradation to VBG-OxA, VBG, and VBG_{ox} but is not shown in the figure. [a]This fragmentation is suggested to be a radical fragmentation. [b]The NH_4^+ ion is lost during this fragmentation. [c]For fragmentation pattern, see Figure 3A.

product detected (Figures 2C and 3, Tables 1 and 2). Neither degradation of the ester bond nor hydroxylation was observed, as in the case of VBG-*p*CA, indicating that the phenolic character of the *p*-coumaroylated model plays a key role in its reactivity. Although VBG-CinA was converted slower than VBG-*p*CA, remarkably, its conversion was faster than that of VBG (Table 2).

Overall, the above-described incubations show that *p*-coumaroylation increases rather than decreases the reactivity of a nonphenolic lignin model in laccase and laccase/HBT treatments. Thus, the previously found accumulation of *p*CA groups in the residual fraction of laccase/HBT-treated wheat straw and corn stover cannot be attributed to an intrinsic recalcitrance of the *p*CA moieties.[15]

Reactivity of *p*CA Moieties upon Laccase/HBT Treatment of Wheat Straw and Corn Stover Lignin Isolates. As the relatively high reactivity of VBG-*p*CA conflicts with the apparent low reactivity of *p*CA groups upon laccase/HBT treatments of wheat straw and corn stover, we investigated whether, somehow, the cell wall matrices of wheat straw and corn stover could have selectively protected the *p*CA groups from laccase/HBT activity in our previous study.[15] Therefore, here, the reactivity of *p*CA groups in purified lignin isolates from corn stover (CSL) and wheat straw (WSL), lacking an intact cell wall matrix, was studied upon treatment with

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Table 1. Compounds Detected with RP-UHPLC-PDA-ESI-FTMS and RP-UHPLC-PDA-ESI-ITMS after Incubation of VBG, VBG-*p*CA, or VBG-CinA with Laccase in the Presence or Absence of HBT[a]

RT (min)	annotation	molecular formula	ion	obs/calc mass (Da)	mass error (ppm)	MS^2 fragments
			substrates and mediator			
3.63	HBT	$C_6H_5N_3O$	$[M+H]^+$	135.04329/135.04326	0.23	119 (100), 80 (86), 91 (75), 53 (39), 116 (37)
6.64	BT	$C_6H_5N_3$	$[M+H]^+$	119.04842/119.04835	0.30	92 (100), 102 (43), 65 (25)
15.00	VBG-*p*CA (trans)	$C_{27}H_{28}O_8$	$[M+Na]^+$	480.17841/470.17842	−0.02	379 (100), 488 (27), 485 (25), 215 (26), 309 (24),, 321 (15), 339 (11)
15.66	VBG-*p*CA (cis)	$C_{27}H_{28}O_8$	$[M+Na]^+$	480.17841/480.17842	−0.02	379 (100), 488 (27), 485 (25), 215 (26), 309 (24), 321 (15), 339 (11)
19.71	VBG-CinA	$C_{27}H_{28}O_7$	$[M+Na]^+$	464.18308/464.18350	−0.87	363 (100), 469 (40), 472 (38), 215 (28), 321 (23), 339 (18), 197 (16)
			reaction products			
5.40	CLP	$C_{11}H_{14}O_4$	$[M+H]^+$	210.08961/210.08921	1.92	139 (100), 193 (18)
7.42	VBG-OxA	$C_{20}H_{22}O_9$	$[M+NH_4]^+$	406.12656/406.12639	0.41	299 (100), 389 (43)
8.61	VBG	$C_{18}H_{22}O_6$	$[M + Na]^+$	334.14155/344.14164	−0.25	309 (100), 339 (22), 342 (14),, 215 (6), 233 (6)
9.30	VBG-OxA_{ox}	$C_{20}H_{20}O_9$	$[M+H]^+$	404.11146/404.11074	1.80	N.D.
10.68	VBG-pCA_{O3}	$C_{27}H_{28}O_{11}$	$[M-H]^-$	528.16410/528.16316	1.7	509 (100)
10.75	VBG_{ox}	$C_{18}H_{20}O_6$	$[M + H]^+$	332.12586/332.12599	−0.37	315 (100), 181 (69), 167 (68), 149 (66), 285 (42), 165 (43), 209 (40)
11.20	VBG-pCA_{O2}	$C_{27}H_{28}O_{10}$	$[M+Na]^+$	512.16904/512.16825	1.48	N.D.
13.36	VBG-pCA_O	$C_{27}H_{28}O_9$	$[M-H]^-$	496.17380/496.17334	−0.89	371 (100), 161 (14)
19.80−21.50	VBG-*p*CA dimers (multiple)	$C_{54}H_{54}O_{16}$	$[M-H]^-$	958.34141/958.34119	1.42	833, 709, 641, 623, 499[b]
22.10−23.30	VBG-*p*CA trimers (multiple)	$C_{81}H_{80}O_{24}$	$[M+NH_4]^+$	1436.5061/1436.5040	1.47	N.D.
21.57	VBG-$CinA_{ox}$	$C_{27}H_{26}O_7$	$[M +H]^+$	462.16774/462.16786	−0.24	315 (100), 339 (8)

[a]MS^2 fragments were obtained using RP-UHPLC-PDA-ESI-ITMS. All other values were obtained using RP-UHPLC-PDA-ESI-FTMS. Proposed fragmentation patterns are shown in Figure 3. MS^2 spectra of substrates and products can be found in Figure S1. N.D. = Not determined. [b]Abundance varies between dimer isomers.

Table 2. Substrate Recoveries and (Estimated) Product Yields of VBG, VBG-*p*CA, and VBG-CinA after 1 and 24 h Incubations with Laccase or Laccase/HBT[a]

incubation	time	substrate recovery (%)	product yields (%)
VBG + laccase	1 h	100	-
	24 h	100	-
VBG + laccase/HBT	1 h	∼100	VBG_{ox} (0.9)
	24 h	96	CLP (0.3); VBG_{ox} (3.7)
VBG-pCA + laccase	1 h	61	VBG (4.1); VBG-pCA dimers and trimers (N.D.)
	24 h	18	VBG (5.6); VBG-pCA dimers and trimers (N.D.)
VBG-pCA + laccase/HBT	1 h	29	VBG (5.3); VBG-OxA[b] (1.4); VBG-pCA_O (N.D.); VBG-pCA_{O2}(N.D.);VBG-pCA_{O3}(N.D.)
	24 h	3	VBG (24.7); VBG-OxA[b] (16.9); VBG_{ox} (N.D.[c]); VBG-OxA_{ox}(N.D.);VBG-pCA_{O3}(N.D.)
VBG-CinA + laccase	1 h	100	-
	24 h	100	-
VBG-CinA + laccase/HBT	1 h	81	VBG-$CinA_{ox}$ (1.1)
	24 h	63	VBG-$CinA_{ox}$ (11.1)

[a]In control incubations without laccase, no conversion was observed in all cases. N.D. = Not determined. [b]Yields of VBG-OxA were estimated by assuming a molar extinction coefficient equal to VBG. [c]In this incubation, VBG_{ox} could not accurately be quantified due to coelution with VBG-pCA_O

laccase/HBT. 2D HSQC NMR analysis revealed that the laccase/HBT treatment substantially altered the structure of lignin isolates, as shown by a 3- and 20-fold increased abundance of C_α-oxidized structures for CSL and WSL, respectively, and a ∼37% decrease in interunit linkages (Figure 5, Table 3). The relative abundance of *p*CA groups was shown to increase in both CSL and WSL (Table 3). These findings are in good agreement with those previously obtained in incubations of (unfractionated) CS and WS,[15] indicating that the cell wall matrix does not play a major role in the reactivity of lignin *p*CA moieties in laccase/HBT treatments.

Potential Explanations for the Accumulation of *p*CA Groups in Residual Lignin upon Laccase/HBT Treatment. From the above it is clear that upon laccase/HBT treatment, *p*CA groups of *p*-coumaroylated model compounds were rapidly hydroxylated and degraded whereas *p*CA groups in polymeric lignin isolates accumulated in the residue. Thus, the reactivity of *p*CA groups in polymeric lignin is not accurately reflected by the reactivity of VBG-*p*CA. In order to

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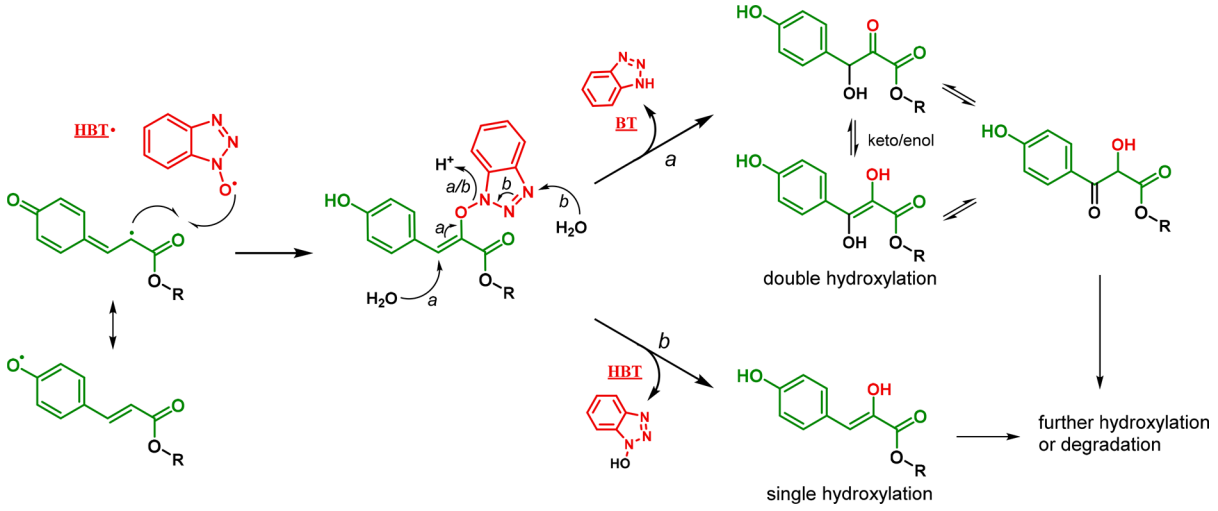

Figure 4. Proposed reaction mechanism for the hydroxylation of VBG-*p*CA observed upon laccase/HBT treatment. R corresponds to VBG (without its primary hydroxyl group). It should be noted that the exact position of the hydroxyl groups is unknown and that VBG-pCA_{O3} is expected to be hydroxylated at the aromatic ring. Putative mechanisms for eventual ring hydroxylation and further degradation to VBG-OxA are depicted in Figures S3 and S4. It should be noted that the HBT adduct could also be attacked by methanol (instead of H_2O). Products of such methanol addition were found in trace amounts (see Table S1 for RP-UHPLC-MS data). Instead of a simultaneous water addition and BT loss to the HBT adduct, the upper reaction could proceed via an initial formation of a quinone methide (with concurrent loss of BT) followed by water addition to the benzylic position.

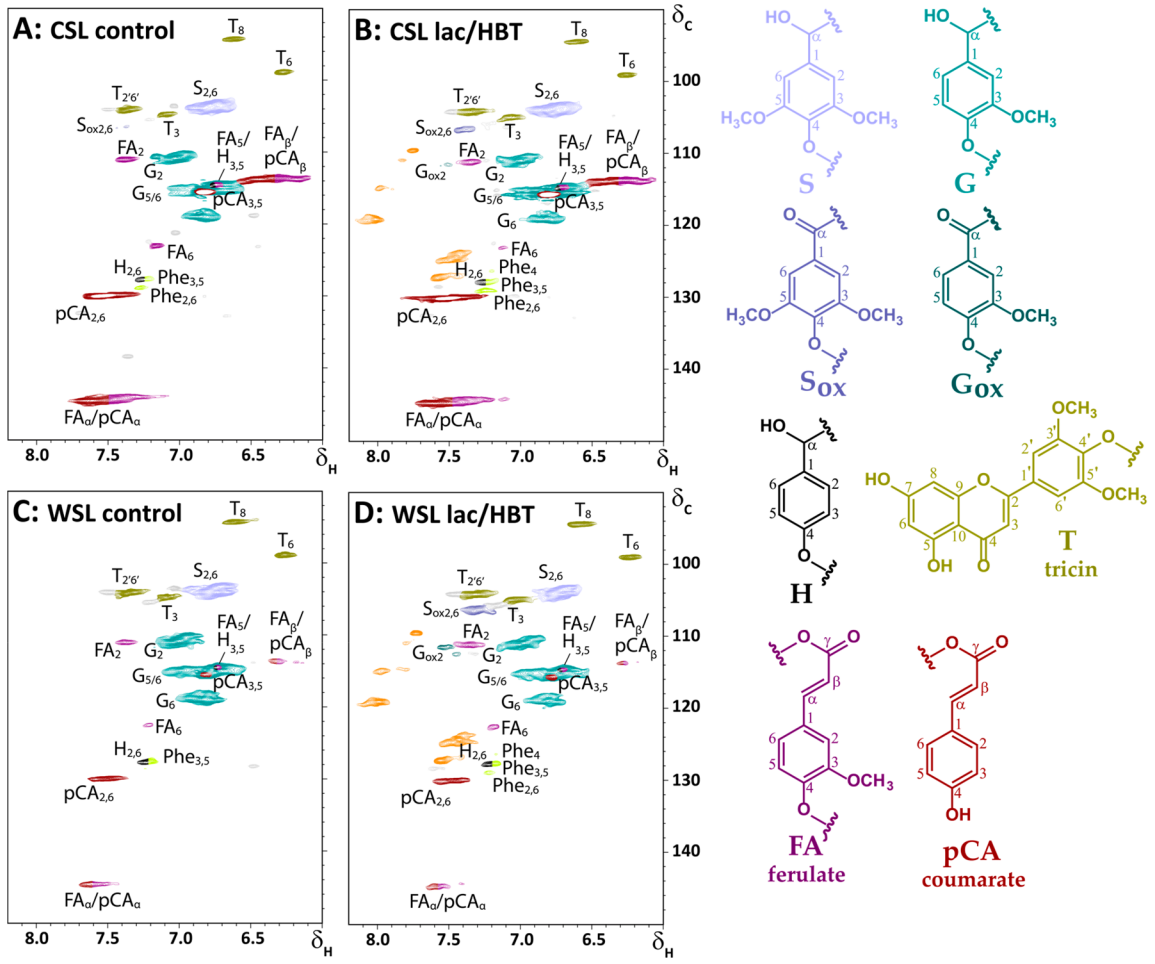

Figure 5. Aromatic regions of HSQC spectra of residues (i.e., insoluble and washed material) of CSL (A and B) and WSL (C and D) after incubation with buffer (A and C) or laccase/HBT (B and D). Lignin substructures corresponding to the colored annotations are shown on the right. Phe = phenylalanine. Correlations in gray are unannotated; those in orange correspond to signals from HBT and its degradation products.[15] Annotation was performed based on the literature.[10,35,36] Aliphatic regions of the spectra can be found in Figure S5.

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Table 3. Relative Abundances of Lignin Subunits, Hydroxycinnamates and Interunit Linkages in CSL and WSL after Incubation with Buffer (Control) Or Laccase/HBT

	CSL control	CSL lac/HBT	WSL control	WSL lac/HBT
lignin subunits (%)				
H	2	2	3	3
G	55	49	63	48
G_{ox}	1	4	1	10
S	39	39	33	30
S_{ox}	2	7	0	10
S/G	0.73	0.85	0.53	0.68
hydroxycinnamates[a]				
*p*CA	60	78	8	9
FA	11	9	5	10
interunit linkages[a]				
β-O-4′	32	23	43	28
β-5′	2	0	6	3
β-*β*′	1	0	0	0
β-*β*′ (tetrahydrofuran)	5	2	0	0
total	40	25	49	31

[a]Amount per 100 lignin subunits (i.e., S + S_{ox} + G + G_{ox} + H).

shine more light on this discrepancy and on the fate of *p*CA groups in polymeric lignin, two scenarios were investigated, which are discussed in detail below.

HSQC-Undetectable Radical Coupling of pCA Groups in Lignin upon Laccase/HBT Treatment. Although, based on the accumulation of *p*CA groups in residual lignin, it seems that *p*CA groups in polymeric lignin are relatively unreactive upon laccase/HBT treatments, it could be speculated that *p*CA moieties underwent radical coupling to other *p*CA moieties or other types of lignin substructures and that the result of this was undetectable in the HSQC NMR spectra. On the basis of reported chemical shifts of model compounds, it seems indeed possible that covalent coupling of *p*CA results in very small changes in chemical shifts (<1 ppm in the C dimension and <0.1 ppm in the H dimension), especially when coupling occurs via 5-5′ bond formation or etherification of the *p*-OH group.[37]

In order to investigate whether new ether or C−C bonds were formed upon laccase/HBT treatment, the residues of laccase/HBT-treated CSL and WSL and control incubations were saponified (1 M NaOH, 30 °C, 24 h). The saponification residues were analyzed using HSQC NMR to determine the residual *p*CA content, and the supernatant was analyzed using RP-UHPLC-PDA-MS to quantify the released *p*CA. If radical coupling between *p*CA and other lignin substructures occurred, saponification is expected to result in a lower removal of *p*CA from laccase/HBT-treated lignin than from control-treated lignin. In the case of radical coupling between *p*CA groups, it can be expected that dicoumarates are released upon saponification. In the case of CSL, HSQC analysis showed an almost equal removal of *p*CA groups from the laccase/HBT-treated and control-treated lignin upon saponification (Table 4, Figure S6), suggesting that laccase/HBT treatment did not induce coupling between *p*CA groups and the lignin backbone to a substantial extent. The fact that, even for untreated CSL, a small fraction of *p*CA was still present after complete saponification could be due to the following reasons: (i) despite extensive washing, a small amount of free

Table 4. Relative Abundance of pCA in CSL and WSL (Control and Laccase/HBT Treated) Before and after Saponification, and Absolute Amounts of pCA Released upon Saponification[a]

	pCA/100 Ar unsaponified[b]	pCA/100 Ar saponified[b]	pCA released (μg/mg)[c]
CSL control	60	15	74.2
CSL + laccase/HBT	78	16	60.5
WSL control	8.1	0	12.0
WSL + laccase/HBT	9.4	1.7	9.9

[a]Saponification of pCA groups was complete in all samples (see Figure S3). [b]Determined using HSQC NMR (see Figure S5 for spectra). [c]Determined using RP-UHPLC-PDA-MS

*p*CA was not removed, or (ii) a minor fraction of *p*CA does not occur as free phenolic groups in native corn stover lignin but is etherified at the *p*-OH group, as recently proposed for sugar cane bagasse.[38]

Surprisingly, the results obtained from HSQC experiments did not match with the RP-UHPLC-PDA-MS analysis. Whereas HSQC analysis of the residues suggested a similar removal of pCA upon saponification, RP-UHPLC-PDA-MS analysis showed an 18% lower release of *p*CA from the laccase/HBT-treated CSL than from the control sample (Table 4).

In the case of WSL, both HSQC and RP-UHPLC-PDA-MS analysis showed an approximately 18% lower removal of *p*CA from laccase/HBT-treated lignin than from the control (Table 4). Although it remains unclear why the HSQC and RP-UHPLC-PDA-MS data showed nonmatching results in the case of CSL, the above suggests that part of the *p*CA groups may undergo coupling to other lignin substructures. In none of the samples, dicoumarates were detected using RP-UHPLC-PDA-MS, suggesting that radical coupling between *p*CA moieties did not occur (data not shown).

pCA Moieties As Redox Shuttles in Lignin Polymers. Another potential explanation for the apparent low reactivity of *p*CA groups in polymeric lignin could be that *p*CA groups are oxidized by laccase/HBT but act as redox shuttles (or mediators) rather than undergoing coupling or degradation reactions. It has been suggested that during lignin biosynthesis, lignin-*p*CA conjugates function as redox shuttles to assist in formation of other lignin radicals and that *p*CA radicals do not undergo oxidative coupling.[39] Possibly, *p*CA groups in polymeric lignin display similar reactivity when incubated with laccase/HBT. As it is challenging to prove such mediator activity in polymeric lignin or biomass, we tested whether VBG-*p*CA could act as a mediator between laccase and other lignin model compounds. Hereto, incubations were performed in a centrifugal filter with a 10 kDa cutoff value. VBG-*p*CA solutions were loaded on top of the filter. Laccase was then added, and the samples were immediately filtered onto a solution of VBG or its phenolic analogue GBG (guaiacylglycerol-*β*-guaiacyl ether). The filtrates were then analyzed using RP-UHPLC-PDA-MS to investigate whether VBG or GBG was oxidized by laccase-free oxidized VBG-*p*CA. In a control incubation, in which laccase (without VBG-*p*CA) was filtered onto an ABTS solution (1 mM), no color formation was observed, confirming that the filtrate was free of laccase (data not shown). Upon filtration of laccase-treated VBG-*p*CA onto GBG, a new peak corresponding to $C_{34}H_{38}O_{12}$ was formed, which was annotated as a dimer of GBG (Figure 6C and 6D), indicating that the produced VBG-*p*CA radicals were able to

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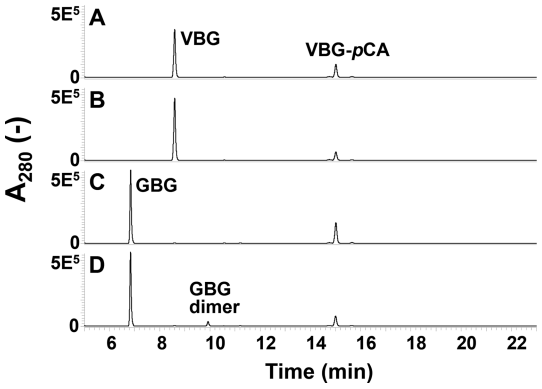

Figure 6. RP-UHPLC-UV_{280} chromatograms of VBG incubated with VBG-*p*CA (A), VBG incubated with oxidized laccase-free VBG-*p*CA (B), GBG incubated with VBG-*p*CA (C), and GBG incubated with oxidized laccase-free VBG-*p*CA (D). GBG dimer was identified based on its molecular formula ($C_{34}H_{38}O_{12}$; mass error 0.06 ppm). VBG-*p*CA dimers were formed in the presence of laccase but accumulated above the filter membrane and were therefore not detected in the filtrates.

act as redox shuttles and thereby oxidize GBG. Nevertheless, the newly formed GBG dimer accounted for only 7% of the UV_{280} peak area, indicating that GBG was only converted to a limited extent. Upon incubation, also dimerization of VBG-*p*CA occurred but the resulting products accumulated above the filter membrane (data not shown) and are therefore not visible in Figure 6. Upon incubation of VBG with oxidized VBG-*p*CA, no conversion of VBG was observed (Figure 6A and 6B). In an additional experiment, equimolar amounts of VBG and VBG-*p*CA were incubated with laccase (i.e., without filter membrane). After 24 h, extensive dimerization of VBG-*p*CA was observed, although also a minor conversion of VBG to VBG_{ox} (i.e., <1%) occurred (data not shown). Overall, these results indicate that VBG-*p*CA could act as a redox shuttle toward other lignin structures but preferentially undergoes radical coupling.

Thus, based on these experiments, it seems not very likely that *p*CA groups in lignin act as *efficient* redox shuttles. Nevertheless, there is an important difference between VBG-*p*CA and the *p*CA groups in polymeric lignin that is difficult to compensate for in model systems: whereas VBG-*p*CA molecules are soluble and can rapidly diffuse through the incubation buffer, the *p*CA groups in polymeric lignin are covalently bound to the insoluble polymer, resulting in low mobility. Since oxidative coupling of *p*CA groups and possibly also hydroxylation and degradation (see Figure 4) require covalent bond formation to the *p*CA groups, it is not inconceivable that these reactions are very slow in the case where the *p*CA groups are immobile (i.e., in polymeric lignin). Possibly, these *p*CA groups undergo electron transfer instead, as this can occur rapidly over relatively long distances (>15 Å).[40] On the basis of the available data, this can, however, not be proven.

CONCLUSIONS

We studied the reactivity of *p*-coumaroyl groups in laccase and laccase/HBT treatments of lignin and lignin model compounds in detail. *p*-Coumaroyl moieties of lignin model compounds were shown to rapidly react in both laccase and laccase/HBT incubations, resulting in oxidative coupling (with laccase) and various degradation products (with laccase/HBT). The phenolic character of the *p*-coumaroyl moiety was found to be essential for these rapid conversions. Upon laccase/HBT treatment of polymeric lignin isolates, *p*CA groups accumulated in the residual lignin, implying that these groups do not undergo degradation and thus display different reactivity than *p*CA groups of model compounds. Although this discrepancy is still not fully resolved, we suggest that *p*CA groups in lignin polymers may be oxidized by laccase/HBT but undergo HSQC-undetectable radical coupling or may act as redox shuttles instead of undergoing degradation reactions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c02376.

Detailed experimental information (RP-UHPLC-PDA-MS^n and 2D NMR spectroscopy), additional UHPLC-UV_{280} chromatograms, MS^2 spectra and HSQC NMR spectra, and putative reaction mechanisms for aromatic ring hydroxylation of *p*CA moieties and VBG-OxA formation (PDF)

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Notes

The authors declare no competing financial interest.

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