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Degradation of biphenyl lignin model compounds by laccase of *Trametes versicolor* in the presence of 1-hydroxybenzotriazole and heteropolyanion $[SiW_{11}VO_{40}]^{5-}$

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Abstract

A series of phenolic/non-phenolic biphenyl model compounds mimicking 5-5′ type "condensed" lignin substructures were subjected to the oxidation with laccase of *Trametes versicolor* in the presence of 1-hydroxybenzotriazole (HBT) or $[SiW_{11}VO_{40}]^{5-}$ ($SiW_{11}V$) as mediators. Phenolic models suffered a significant degradation in both the laccase-mediator systems (LMS), which was more pronounced, however, in the case of $SiW_{11}V$-mediated oxidation. This result was explained, at least partially, by HBT decomposition and by the increased extent of competing radical coupling reactions of phenolic models in the HBT–laccase reaction system. The non-phenolic biphenyl model was non-reactive in the presence of $SiW_{11}V$ and degraded substantially in the presence of HBT. The main degradation pathways of lignin model compounds were deduced based on the analysis of the detected oxidation products.

Keywords: Lignin; Lignin model compounds; Heteropolyanion; Laccase; 1-Hydroxybenzotriazole; Oxidation

1. Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are a group of enzymes of the multi-copper oxidases family possessing broad substrate specificity with respect to the electron donor [1]. Laccases are widely distributed in white-rot fungi (*Trametes versicolor*, *Phanerochaete chrysosporium* and *Phlebia radiata* among others), which are involved in the natural biodegradation of lignin [2]. In the catalytic cycle, laccase reduces dioxygen to water and simultaneously performs one-electron oxidation of polyphenolic substrates [3]. The initial reaction products are oxygen-centred radicals (or cation radicals), which react further through non-enzymatic routes. Laccase-assisted reactions strongly depend on the enzymes redox potential, temperature and pH of the reaction medium [4].

In the last two decades, a strong effort was made in order to understand the mechanisms of the lignin biodegradation by laccase [5,6] and to find the feasible industrial enzymatic applications, mostly for the delignification/bleaching of kraft pulps—a raw material for the papermaking [6]. However, the catalytic efficiency of laccase is insufficient for many industrial processes (effluent treatment, delignification of lignocellulosic materials, etc.) and the so-called redox mediation is required [6]. The acceptable biodelignification of unbleached pulps is possible using a laccase-mediator

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system (LMS), which constitutes a promising alternative to conventional polluting bleaching technologies [6]. The catalytic mechanisms of LMS can be presented in a simplified mode as a sequence of redox cycles where electrons, withdrawn from the substrate (lignin) by oxidized form of the mediator, are transferred by laccase to oxygen, which plays a role of the terminal electron acceptor. The use of mediators in practice is hindered by their costs, regeneration problems and degradation in oxidation reactions.

In the literature, a series of mediators from the category of phenolic and non-phenolic types, consumable/degradable during their redox turnover, have been described [6,7]. Alternatively, robust transition metal complexes [8] and heteropolyanions [9] as mediators for laccase-assisted reactions were proposed. 1-Hydroxybenzotriazole (HBT) was suggested as one of the most effective organic mediators [7] and undecatungstovanadosilicate heteropolyanion ($[SiW_{11}VO_{40}]^{5-}$ or simply $SiW_{11}V$) as one of the perspective inorganic [9] mediators for LMS. The comparison of the laccase-catalyzed delignification efficiency in the presence of HBT showed better performance than in the presence of $[SiW_{11}VO_{40}]^{5-}$ at the same reaction conditions [9]. This fact was explained tentatively by poor reactivity of lignin non-phenolic and "condensed" (biphenyl, α-aryl, etc.) structures in the $SiW_{11}V$–laccase reaction system.

The abundance of biphenyl type structures in lignin ranges from 2 to 5 mol % in angiosperms to about 20 mol % in gymnosperms and may reach up to 30% in the residual lignin of unbleached pulps [10]. These structures are considered as essential intermediates in the depolymerization of lignin by laccase [11] and one of the most difficult to degrade with LMS [12]. The knowledge about biphenyl lignin structures behaviour in HBT–laccase system is rather incomplete being, sometimes, in contradiction to known literature data, for example, as far as the reactivity of non-phenolic units is concerned [12]. There is no information available about the behaviour of biphenyl lignin structures in the $SiW_{11}V$–laccase reaction system.

In this report, effort was made to examine the degradation patterns of phenolic and non-phenolic biphenyl (5-5′) type lignin substructures using dimeric model compounds in the reaction systems containing HBT or $[SiW_{11}VO_{40}]^{5-}$ and laccase produced by fungi *T. versicolor*.

2. Results and discussion

2.1. Biodegradation of phenolic 5-5′ type lignin models

The phenolic lignin model compounds of 5-5′ type were (2,2′-dioxy-3,3′-dimethoxy-5,5′-dioxymethyl)-biphenyl (**I**) and (2-oxy-2′,3,3′-trimethoxy-5,5′-dioxymethyl)biphenyl (**II**). These models have been involved in biodegradation with laccase in the presence of HBT or $[SiW_{11}VO_{40}]^{5-}$ as mediators. The incubation conditions (40 °C; pH 4.5; 5 h; laccase activity of 0.30 U/ml) were selected based on the results of optimisation experiments discussed previously [6,9]. The mediators did not completely suppress the activity of laccase as revealed from the corresponding enzymatic essays after the treatments.

In both the reaction systems, HBT–laccase (HBT–L) and $[SiW_{11}VO_{40}]^{5-}$–laccase ($SiW_{11}V$–L), model **I** was degraded completely yielding a series of non-aromatic products (mostly aliphatic/olefinic dicarboxylic acids) resulting from the cleavage of aromatic ring. Only a small amount of dehydrodivanillin was detected. In the experiment with HBT–L, a notable proportion of yellow coloured polymeric product was formed possibly via a known radical coupling reaction of biphenyl type structures in the presence of laccase [11].

The model compound **II** showed a conversion of 52% in the HBT–L reaction system and 72% in the $SiW_{11}V$–L reaction system (Table 1). The main identified aromatic reaction products were compounds **IV**–**VII** reflecting the oxidative cleavage of the phenolic aromatic group in model **II** (Fig. 1). The major metabolite **V** (Table 1) evidences that in both tested LMS exactly the phenolic group of **II** was degraded first followed by the oxidation of non-phenolic group. The non-phenolic moiety of dimeric model **II** was easier to oxidise with HBT–L than with $SiW_{11}V$–L as witnessed by the ratio of products **V** and **VII** (Table 1). The formation of notable amounts of **V** can also be explained as the result of direct oxidation of **II** by laccase, because in the absence of mediators, about 18% of **II** was converted giving **V** (3.4% of yield) as a major low molecular aromatic product together with a small amount of **VI** (0.2% of yield).

The oxidation product **III** (Fig. 1) was detected only in the experiment with $SiW_{11}V$–L (Table 1). The

Table 1
Results of the oxidation of phenolic model **II** by HBT–L and $SiW_{11}V$–L

Compound	MS (*m*/*z*) data as TMS derivatives (rel. int., %)	Yield (%)	
		HBT–L	$SiW_{11}V$–L
II	536 ($M^{+\bullet}$, 29); 447 (13); 343 (26); 285 (12); 207 (19), 147 (65); 73 (100)	48.0	28.0
III	522 ($M^{+\bullet}$, 35); 492 (24); 403 (14); 329 (20); 287 (12), 147 (45); 73 (100)	–	24.1
IV	356 ($M^{+\bullet}$, 20); 267 (29); 251 (40); 237 (23); 207 (12) 163 (64); 73 (100)	12.3	19.0
V	370 ($M^{+\bullet}$, 13); 355 (27); 325 (22); 281 (45); 237 (34) 207 (38); 73 (100)	4.8	1.1
VI	268 ($M^{+\bullet}$, 26); 237 (30); 223 (16); 207 (9); 179 (28); 136 (44); 73 (100)	0.9	6.6
VII	282 ($M^{+\bullet}$, 22); 267 (31); 251 (14); 223 (29); 207 (5) 193 (34); 73 (100)	–	0.8

formation of *p*-hydroquinone type structures in the oxidation of lignin model compounds with $SiW_{11}V$ was suggested previously as the result of two consecutive one-electron oxidations of the phenolic unit leading to the formation of intermediate cyclohexadienyl cation followed by the hydrolytic cleavage of phenyl–alkyl linkage [13]. The same mechanism of the nucleophilic substitution of methylol group in model **II** may be proposed in the $SiW_{11}V$–L reaction system. Compound **III** is an eventual intermediate for the metabolites **IV**–**VII**.

The absence of product **III** after the reaction of dimer **II** with HBT–L may signify its low stationary concentration in the solution or the preferable formation of other oxidation intermediates. For example, two one-electron oxidation steps could result in, instead of *p*-hydroquinone type product **III**, the vanillin type derivative **IIIa** (Fig. 1). Though the product **IIIa** was detected only in negligible amounts (lower than 0.1% of yield), the oxidation of benzyl alcohol group to the aldehyde moiety in the dehydrodivanillyl alcohol type structures with HBT–L may be an important

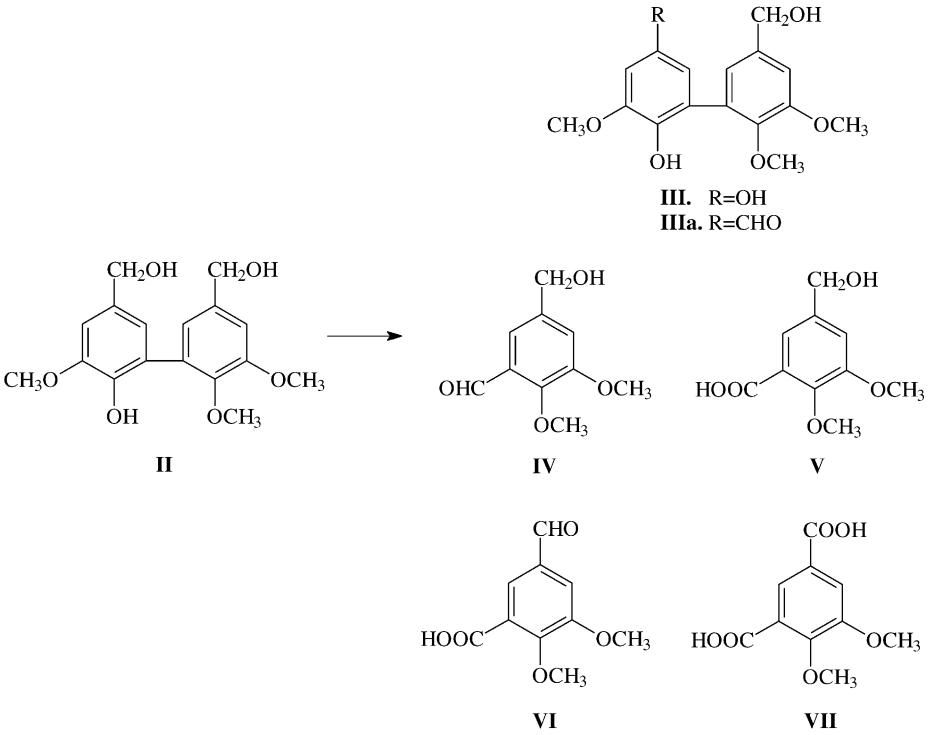

Fig. 1. Biodegradation products released from the oxidation of phenolic lignin model **II**.

oxidation route [12]. The fact of the different reaction behaviour of model **II** with two LMS indicates the different oxidation mechanisms with $SiW_{11}V$ and HBT. Indeed, $SiW_{11}V^{V}$ oxidises the lignin substructures via the electron transfer mechanism [14], whereas in the presence of laccase, HBT oxidises the former presumably via the radical routes [15,16]. For the reasons of radical reaction statistics, the formation of **IIIa** in the HBT–L reaction system may be more pronounced than with $SiW_{11}V$–L, where **IIIa** was not detected. Considering the analysis of degradation products, the general scheme reflecting the main biodegradation pathways in $SiW_{11}V$–L and HBT–L systems may be proposed (Fig. 2).

The fact of the higher conversion of dimeric model **II** in the oxidation with $SiW_{11}V$–L, when compared

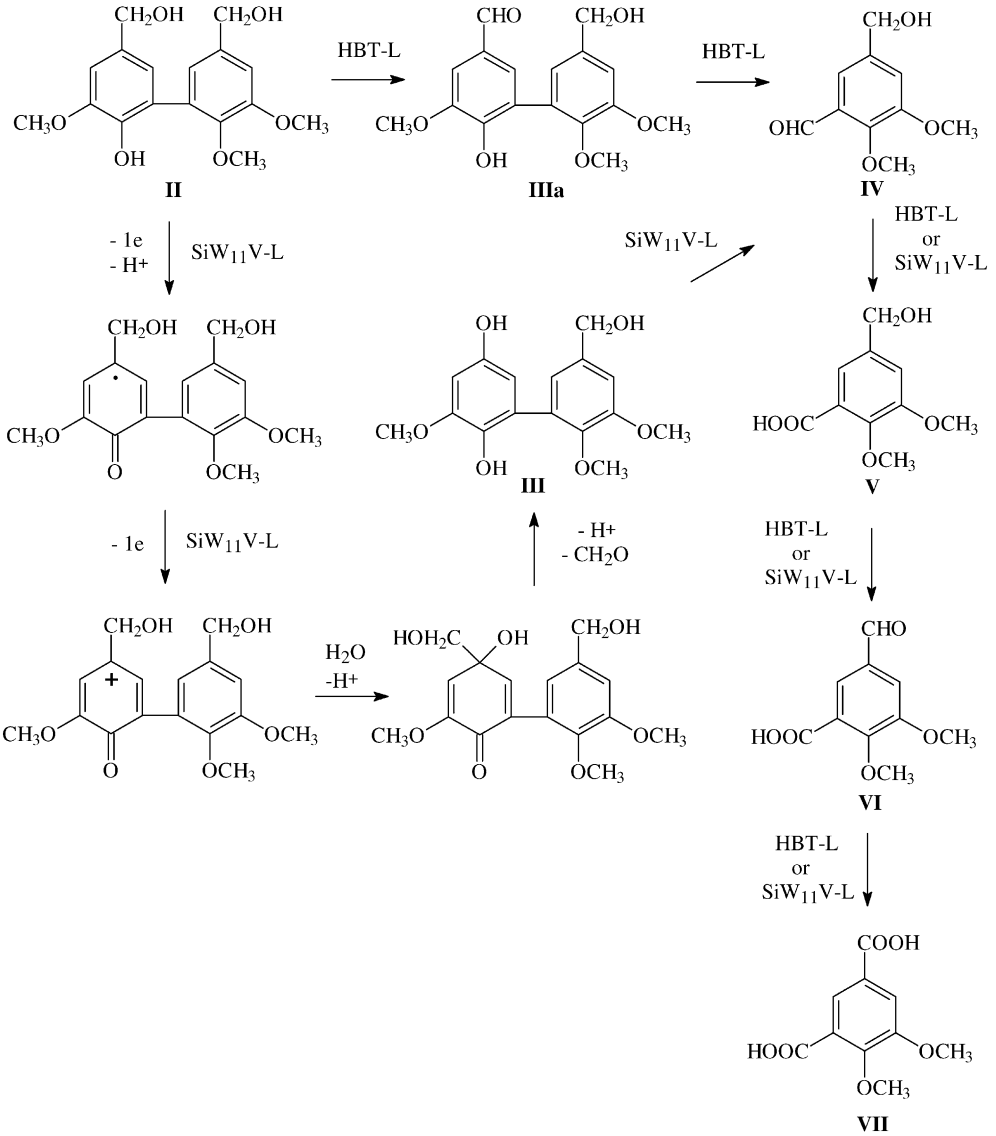

Fig. 2. Biodegradation pathways of phenolic lignin model **II** in the oxidation with HBT–L and $SiW_{11}V$–L. Reduced/oxidised forms of mediators are as follows: HBT/HBT$^{\bullet}$ in the HBT–L system and $SiW_{11}V^{IV}$/ $SiW_{11}V^{V}$ in $SiW_{11}V$–L system.

to that obtained with HBT–L (Table 1), contradicts with the formal thermodynamic rules of the redox reactions. More specifically, HBT$^{\bullet}$ (benzotriazolyl-1-oxide radical), an active mediator form in the lignin oxidation with HBT–L, possesses much higher E_{pa} (about +1.1 V versus NHE at pH 6 [17]) than E_{pa} of $SiW_{11}V^{V}$ (+0.74 V versus NHE at pH 5 [18]) and should react faster with phenolic substrate. Several explanations for the reaction behaviour observed may be deduced. Typically, HBT suffers a partial degradation in the LMS catalytic cycle and produces catalytically inactive derivatives [7]. Actually, about 30% of HBT was degraded in the assay with model **II** as revealed by GC analysis of the reaction products. At the same time, no significant degradation of $SiW_{11}V$ was detected (UV-Vis control). Hence, the deactivation of the mediator is one of the reasons for the poorer oxidation of model **II** with HBT–L than with $SiW_{11}V$–L. Another point could be the insufficient oxidation rate of HBT by laccase resulting in the active mediator form HBT$^{\bullet}$. In spite of the absence of reliable data on the oxidation rates of HBT and $SiW_{11}V^{IV}$ with laccase, there are evidences in the literature indicating the apparent difficulties on HBT oxidation with laccase, relatively to other organic mediators [7]. In contrast to HBT, $SiW_{11}V^{IV}$ is oxidised very quickly by laccase even at room temperature [9]. In other words, in the LMS catalytic cycle, the oxidation of the reduced form of the mediator (HBT) may slow the effective rate of the lignin oxidation. One more reason for the differences in the oxidation of model **II** in two tested LMS could be the difference in the extent of coupling reactions of one-electron oxidised phenolic substrates hindering the biodegradation. Though coupling reactions of phenolic substrates take place both in the presence of HBT$^{\bullet}$ [19] and $SiW_{11}V^{V}$ [13], these should be more pronounced with HBT–L than with $SiW_{11}V$–L. Such a proposition is confirmed by the significant polymerisation of phenolic model **I** in the oxidation experiment with HBT–L unlike with $SiW_{11}V$–L. The relatively low yield of the oxidation products obtained in the reaction of **II** with HBT–L (Table 1) can indicate the formation of oligomeric products as well.

2.2. Biodegradation of non-phenolic 5-5′ type lignin model

The non-phenolic lignin model **VIII** ((2,2′,3,3′-tetramethoxy-5,5′-dioxymethyl)biphenyl) was subjected to the oxidation with HBT–L, $SiW_{11}V$–L and laccase under the same conditions as phenolic model compounds **I** and **II**. As could be expected, compound **VIII** was inactive in the oxidation with laccase without mediators. At the same time, former non-phenolic model showed the distinct reaction behaviour with two LMS tested. Almost the same conversion of **VIII**, as with phenolic model II, was reached when HBT was applied as a mediator (Table 2). In contrast, model **VIII** was inactive in the $SiW_{11}V$-mediated oxidation. These reaction features are completely in agreement with known high reactivity of HBT$^{\bullet}$ [20] and the inertness of $SiW_{11}V$ [21] in the oxidation of non-phenolic lignin structures. The reactivity of lignin substructures with radical mediator HBT$^{\bullet}$ should not be affected very strongly by their E_{pa} [16], whereas the oxidation with $SiW_{11}V$ via the electron transfer mechanism is sensitive to the electron donating properties of the substrate [14,18]. Hence, rather high E_{pa} (about +1.0 V versus NHE at pH 2 [22]) of non-phenolic lignin substructures explains the inertness of **VIII** in the reaction with $SiW_{11}V$ (E_{pa} = 0.74 V versus NHE at pH 5 [18]).

The main oxidation products of **VIII** are presented in Fig. 3. Eventually, HBT$^{\bullet}$ abstracts firstly the benzylic hydrogen from **VIII** resulting in the product **IX**, after the second electron abstraction and deprotonation. The next oxidation steps with HBT$^{\bullet}$ give product **X**. All detected metabolites (**IX** and **X**) reflect

Table 2
Results of the oxidation of non-phenolic model **VIII** by HBT–L

Compound	MS (*m/z*) data as TMS derivatives (rel. Int., %)	Yield (%)
VIII	478 ($M^{+\bullet}$, 46); 389 (22); 373 (21); 255 (12); 150 (40); 73 (100)	45.0
IX	404 ($M^{+\bullet}$, 38); 373 (13); 315 (44); 282 (23); 207 (63); 157 (11); 73 (100)	17
X	492 ($M^{+\bullet}$, 40); 403 (27); 299 (11); 239 (9); 207 (22); 157 (28); 73 (100)	19

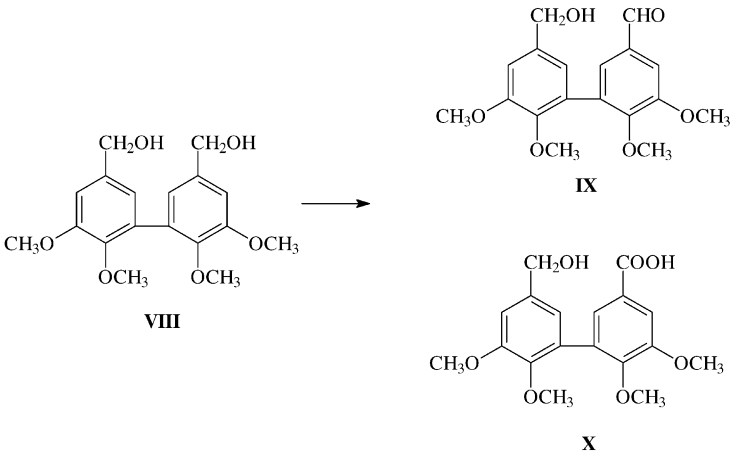

Fig. 3. Biodegradation products released from the oxidation of non-phenolic lignin model **VIII**.

the oxidation of the side-chain of the dimeric model (methylol group in this particular case). This degradation pathway usually leads to the C_α–C_β oxidative cleavage in the propane chain of non-phenolic structures and is one of the principal pathways in the lignin biodegradation by laccase [5,20].

The experiment with **VIII** did not confirm the inactivity of non-phenolic biphenyl type structures in the HBT–L reaction system suggested previously [12]. Though the same source of laccase (*T. versicolor*) was used in the present study, as in the work [12], the particular incubation conditions (pH of inoculation solution, laccase purity, the mediator charge, organic solvent used for the dissolution of the model, etc.) could influence the results of the oxidation.

3. Experimental

3.1. Enzyme production

A culture of white-rot fungus *T. versicolor* obtained from the National Institute of Industrial Engineering and Technology (INETI, Portugal) was employed. The growth of mycelium was held on Petrie plates at 28 °C using a medium described by Tien and Kirk [23]. The liquid cultures were prepared by sterile transference of mycelia fungus to the *T. versicolor* defined medium (TDM) described by Roy and Archibald [24] with 0.1% of Tween 80. After 8 days of the fermentation (maximal concentration of laccase) at 28 °C, cultures were filtered and concentrated by ultrafiltration. The concentrated solution was saturated with ammonium sulphate and cooled to 4 °C for 1 h. The precipitate was separated by centrifugation (15,000 × *g*, 30 min, 4 °C), re-dissolved in a small amount of citrate/phosphate (0.05 mM/0.1 mM) buffer (pH 4.5), dialysed overnight against the same buffer and used as a crude laccase solution. No peroxidase activity was detected (test with veratryl alcohol in the presence of H_2O_2).

3.2. Laccase activity analysis

Laccase activities were measured at 40 °C using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The reaction mixture contained 0.4 mM ABTS in citrate/phosphate (0.05 mM/0.1 mM) buffer at pH 4.5 [25] in a total volume of 2000 μl. Oxidation of ABTS was monitored through absorbance increase in 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). One unit of enzyme activity is defined as the amount of enzyme required to oxide 1 μM of ABTS per minute.

3.3. Mediators

1-Hydroxybenzotriazole was a commercial product supplied by Aldrich Chem. Comp. (Madrid). Sodium salt of $[SiW_{11}VO_{40}]^{5-}$ (0.2 M solution) was synthesised according to known protocol [26].

3.4. Lignin model compounds

Phenolic and non-phenolic biphenyl lignin model compounds were synthesised by oxidative coupling of vanillin in the presence of $FeSO_4$ followed by methylation/reduction of dehydrodivanillin obtained according to established protocols [27]. ^{1}H NMR spectra were recorded on a Bruker AMX 300 spectrometer operating at 300.13 MHz (25 °C). TMS was used as an internal standard (δ 0.00).

3.4.1. (2,2′-Dioxy-3,3′-dimethoxy-5,5′-dioxymethyl)biphenyl (I)

^{1}H NMR (DMSO-d_6): 3.81 (s, 6H, 3,3′-OC$\underline{H}_3$); 4.39 (d, 4H, C$\underline{H}_2$OH, J = 5.6 Hz); 5.04 (t, 2H, CH$_2$O$\underline{H}$, J = 5.6 Hz); 6.66 (d, 2H, H-4,4′, J = 1.7 Hz); 6.87 (d, 2H, H-6,6′, J = 1.7 Hz); 8.25 (large peak, 2,2′-O$\underline{H}$,). EI/MS (*m*/*z*) as TMS derivative (rel. int.): 594 ($M^{+\bullet}$, 29); 505 (5); 417 (63); 343 (9); 299 (6); 147 (10); 73 (100).

3.4.2. (2-Oxy-2′,3,3′-trimethoxy-5,5′-dioxymethyl)biphenyl (II)

^{1}H NMR (DMSO-d_6): 3.49 (s, 3H, 2′-OC$\underline{H}_3$); 3.81 (s, 3H, 3-OC$\underline{H}_3$); 3.82 (s, 3H, 3′-OC$\underline{H}_3$); 4.40 (d, 2H, C$\underline{H}_2$OH, J = 5.3 Hz); 4.44 (d, 2H, C$\underline{H}_2$′OH, J = 5.6 Hz); 5.04 (t, 2H, CH$_2$O$\underline{H}$, J = 5.3 Hz); 5.16 (t, 2H, CH$_2$O$\underline{H}$, J = 5.6 Hz); 6.59 (s, 1H, H-4′); 6.65 (s, 1H, H-4); 6.89 (s, 1H, H-6); 6.96 (s, 1H, H-6′); 8.26 (large peak, 2-O$\underline{H}$). EI/MS (*m*/*z*) as TMS derivative (rel. int.): 536 ($M^{+\bullet}$, 29); 447 (13); 343 (26); 285 (12); 207 (19), 147 (65); 73 (100).

3.4.3. (2,2′,3,3′-Tetramethoxy-5,5′-dioxymethyl)biphenyl (VIII)

^{1}H NMR (DMSO-d_6): 3.49 (s, 6H, 2,2′-OCH$_3$); 3.82 (s, 6H, 3,3′-OC$\underline{H}_3$); 4.45 (d, 4H, C$\underline{H}_2$OH, J = 5.5 Hz); 5.17 (t, 2H, CH$_2$O$\underline{H}$, J = 5.5 Hz); 6.66 (d, 2H, H-4,4′, J = 1.6 Hz); 6.99 (d, 2H, H-6,6′, J = 1.6 Hz). EI/MS (*m*/*z*) as TMS derivative (rel. int.): 478 ($M^{+\bullet}$, 46); 389 (22); 373 (21); 255 (12); 150 (40); 73 (100).

3.5. Oxidation of lignin model compounds

The substrate, dimeric lignin model compound (40 μmol), was dissolved in 600 μl of dimethylformamide (DMF) and added to 7.0 ml of citrate/phosphate (0.05 mM/0.1 mM) buffer (pH 4.5) together with HBT (20 μmol) dissolved in 50 μl DMF under stirring. The solution was flushed with oxygen at 30 °C during 30 min. The laccase solution (2.0 ml) was added to reach the final enzyme activity in the mixture 0.30 U/ml and the incubation was carried out on air (in dark) at 40 °C during 5 h under constant stirring. In the case of $SiW_{11}V$-mediated treatment, 100 μl of its 0.2 M solution (20 μmol) was added together with laccase solution to the oxygen-flushed sodium citrate/phosphate buffer solution containing the model compound as described above.

The reaction mixture was quickly acidified to pH 2 and extracted 3× 10 ml by chloroform (partition coefficients were determined for compounds **I**, **II**, **VIII** and extrapolated for the oxidation products). The combined organic layer was dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Oxidation products were dissolved in 1 ml of pyridine and submitted to silylation by addition of 400 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Chem. Co., Madrid) and of 100 μl trimethylclorosilane (TMC, Sigma Chem. Co., Madrid).

3.6. Analysis of oxidation products

Oxidation products (as TMS derivatives) were analysed by GC-FID (Varian model 3350) using dimethylphthalate as an internal standard and identified by GC-MS (Trace GC 2000 series coupled with Finnigan Trace MS mass spectrometer (EI, 80 eV)) using a DB-1 capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: initial temperature 100 °C; temperature rate 5 °C/min; final temperature 280 °C; injector temperature 270 °C; detector temperature 290 °C. The identification of oxidation products was made using MS spectral library or based on the comparison of their retention times and the mass spectra with those of pure compounds synthesised in the laboratory.

4. Conclusions

The phenolic 5-5′ type lignin model compounds **I** and **II** showed a significant degradation with laccase in the presence of both HBT and $SiW_{11}V$. The reactivity

of the phenolic model **II** in the oxidation mediated by $SiW_{11}V$ was higher than in the experiment with HBT. The last fact is related, at least partially, with HBT degradation and with the extent of competing radical coupling reactions of the dimeric model in HBT–L system. Both examined phenolic models suffered extensive aromatic ring cleavage.

The non-phenolic 5-5′ type lignin model compound **VIII** was inactive in the oxidation with laccase mediated by $SiW_{11}V$, whereas its reactivity was rather high when HBT was used as a mediator. The degradation of the non-phenolic model **VIII** in the HBT–L reaction system occurred predominantly via the oxidation of side chain.

Considering the results of model experiments, more pronounced kraft pulp delignification observed with HBT–L, than with $SiW_{11}V$–L [9], may be explained by well-balanced degradation of both phenolic and non-phenolic lignin substructures in the presence of HBT.

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