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Oxidation of the *erythro* and *threo* forms of the phenolic lignin model compound 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol by laccases and model oxidants

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ABSTRACT

Mixtures of equal amounts of the *erythro* and *threo* forms of the phenolic arylglycerol β-aryl ether 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol were oxidized (i) with laccases from *Trametes versicolor*, *Agaricus bisporus*, *Myceliophthora thermophila* and *Rhus vernicifera*, (ii) with laccase-mediator systems consisting of *T. versicolor* laccase and ABTS or HBT, and (iii) with various model oxidants including cerium(IV) ammonium nitrate (CAN), lignin peroxidase, Fenton's reagent, and lead(IV) tetraacetate (LTA). All the laccases exhibited a similar preferential degradation of the *threo* form. The mediator ABTS counteracted the *threo* preference of laccase, but the mediator HBT did not affect it. The outer-sphere model oxidants CAN and lignin peroxidase showed a preferential degradation of the *threo* form. LTA and Fenton's reagent did not exhibit any stereo-preference. The results suggest that laccases of different origin, primary structure, and redox potential behave as typical outer-sphere oxidants in their interaction with the diastereomers of the arylglycerol β-aryl ether.

1. Introduction

Lignins are complex aromatic biopolymers found in the cell walls of vascular plants [1]. The most common substructure in lignin is the arylglycerol β-aryl ether (Fig. 1). Most of the arylglycerol β-aryl ethers in lignin are non-phenolic (**1**, Fig. 1), but some of them are phenolic (**2**, Fig. 1) [2]. Phenolic arylglycerol β-aryl ethers and related non-phenolic arylglycerol β-aryl ethers (examined in a previous study [3]) react differently on oxidation. Phenol oxidation in general results in so called oxidative phenol coupling leading to biphenyls [4]. It is therefore important to study both phenolic and non-phenolic arylglycerol β-aryl ethers. Furthermore, the laccases examined in this study are not capable of direct oxidation of non-phenolic arylglycerol β-aryl ethers. These fundamental differences between phenolic and non-phenolic arylglycerol β-aryl ethers justify the investigation of phenolic substrates as a complement to previous investigations of non-phenolic substrates [3].

Arylglycerol β-aryl ethers exist in two diastereomeric forms: *erythro* and *threo* (see compound **3** in Fig. 1). The proportions of the *erythro* and the *threo* forms differ in hardwood lignin (the *erythro* form is predominant) and softwood lignin (equal amounts of the *erythro* and *threo* forms are present) [5,6].

In nature, lignin is primarily degraded by white-rot fungi. The fungi secrete enzymes, such as peroxidases and laccases, which are capable of oxidizing lignin substructures. Various mediators, organic compounds or metal ions, may take part in the process. Several aspects of the initial phase of lignin biodegradation warrant further attention: the susceptibility of the different substructures in lignin and their stereo-isomeric forms to biologically relevant oxidants, the possible involvement of reactive oxygen species like hydroxyl radicals, and the identification of the substrates of peroxidases and laccases in vivo.

It has previously been shown that many biologically relevant oxidants, such as lignin peroxidase and some laccase-mediator systems, preferentially oxidize the *threo* isomer of non-phenolic arylglycerol β-aryl ethers [3,7]. A strong unspecific oxidant such as Fenton's reagent exhibits no stereo-preference [3]. Laccases typically oxidize phenolic compounds directly and assisted with mediators also non-phenolic compounds [8]. This contribution is focused on a phenolic lignin model compound (**3**, Fig. 1), which, unlike a related non-phenolic compound (**3** with methylated phenol group) [3], is susceptible to direct oxidation by laccases without the assistance of a mediator.

Laccases (EC 1.10.3.2) are a group of multi-copper enzymes that reduce molecular oxygen to water but show relatively low

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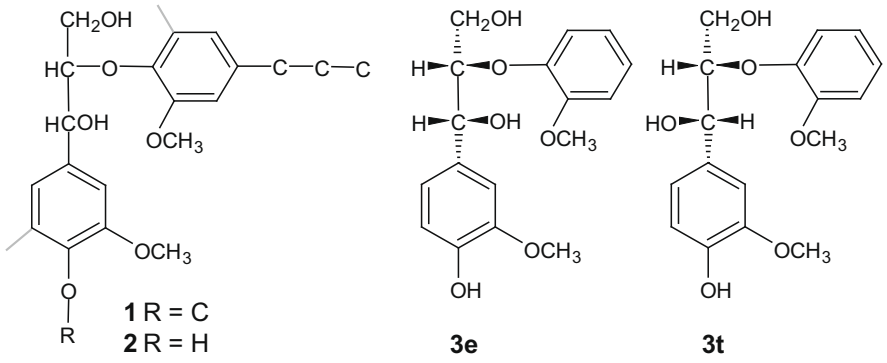

Fig. 1. Non-phenolic (**1**) and phenolic (**2**) lignin structural elements of the arylglycerol β-aryl ether type and the *erythro* (**3e**) and *threo* (**3t**) isomers of the phenolic arylglycerol β-aryl ether 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**3**). The conformations of the diastereomers of **3** are available through X-ray diffraction studies (see [28] and [29]).

specificity with regard to the substrate they oxidize, which is typically a phenolic compound. Laccases have been thoroughly studied with biophysical methods, but in general rather little is known about their physiological roles, the identity of their substrates in vivo, and the way they interact with their substrates. Four laccases of widely different origin and with different properties were included in this investigation (Table 1). The laccase from the Japanese lacquer tree (*Rhus vernicifera*) (Table 1) is involved in wound healing, while laccases from white-rot fungi, such as *Trametes versicolor* (Table 1), are generally believed to be involved in lignin degradation.

Equal amounts of the *erythro* and *threo* forms of the phenolic lignin model compound 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**3**, Fig. 1) were oxidized with the four different laccases (Table 1) and the reaction mixtures were analyzed with high-performance liquid chromatography–diode array detector (HPLC–DAD). For comparison, laccase-mediator systems, lignin peroxidase, and the non-enzymic oxidants cerium(IV) ammonium nitrate (CAN), Fenton's reagent and lead(IV) tetraacetate (LTA) were included in the study.

2. Materials and methods

2.1. Chemicals

Reagent grade chemicals were used unless otherwise stated. The *erythro* and *threo* forms of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**3** in Fig. 1) were synthesized according to Li et al. [9]. Mixtures of diastereomers were prepared by weighing in desired amounts of **3e** and **3t**, which were available as separate preparations.

2.2. Enzymes

Laccases from *Trametes versicolor, Agaricus bisporus, Myceliophthora thermophila* and *Rhus vernicifera* were used. Laccase from *T. versicolor* was prepared as described previously [10]. Laccase from *A. bisporus* was purchased from Jülich Fine Chemicals (Jülich, Germany), dissolved in 20 mM phosphate buffer (pH 6.0), and filtered before use. Laccase from *M. thermophila* was obtained from Novozymes (Bagsvaerd, Denmark). It was purified on a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) and dialyzed against 10 mM imidazole buffer (pH 6.0) before use. Laccase from *R. vernicifera* was kindly provided by Dr. Bengt Reinhammar. Lignin peroxidase was prepared from *T. versicolor*, as previously described [11].

2.3. Enzyme assays

A UV-1601PC instrument from Shimadzu (Kyoto, Japan) was used to spectrophotometrically determine the laccase and lignin peroxidase activities. The reaction mixture of the laccase activity assay contained 2,2′-azinobis(3-ethylbenzothiazolone-6-sulfonic acid) (ABTS) (0.4 mM), acetate buffer (50 mM, pH 5.0), and laccase. The absorbance change was monitored at 414 nm for 5 min (temperature 21 °C). The amount of laccase that formed 1 μmol of ABTS radical cation ($\varepsilon = 3.6 \times 10^4$ [12]) per min was defined as one unit.

The reaction mixture of the lignin peroxidase activity assay contained tartrate buffer (100 mM, pH 3.0), veratryl alcohol (3,4-dimethoxybenzyl alcohol) (2 mM), hydrogen peroxide (0.2 mM), and lignin peroxidase. The reaction was monitored at 310 nm for 5 min (temperature 21 °C). The amount of enzyme that formed 1 μmol of veratraldehyde ($\varepsilon = 9.3 \times 10^3$ [13]) per min was defined as one unit.

2.4. Oxidation experiments with the erythro and threo forms of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (3)

All oxidations were performed at room temperature (21 °C) and the total volume of the reaction mixture was 1 ml. The mixture contained **3** (concentration 1 mM, **3e**:**3t** ratio = 1), acetate buffer (20 mM, pH 5.0), and the oxidant. Both enzymic and non-enzymic oxidants were studied. The enzymic oxidants were the laccases

Table 1
Origins of laccases studied, redox potentials, and percent identity with *Trametes versicolor* laccase.

Organism	Kingdom/division	$E^{0'}$ (mV vs. NHE[a])	Identity (%)[b]	References[c]
Trametes versicolor (a white-rot fungus)	Basidiomycota	785	100	[36] CAA77015
Agaricus bisporus (button mushroom)	Basidiomycota	–	48	-Q12542
Myceliophthora thermophila (a thermophilic fungus)	Ascomycota	480	33	[37] AAE35046
Rhus vernicifera (Japanese lacquer tree)	Plantae	440	29	[37] BAC20342

[a] Normal hydrogen electrode.
[b] Amino-acid sequence identity with *T. versicolor* laccase A (lcc2 gene product) [38].
[c] References to redox potentials and accession number of sequences.

from *T. versicolor*, *A. bisporus*, *M. thermophila* and *R. vernicifera*, two laccase-mediator systems, and lignin peroxidase from *T. versicolor*. The laccase-mediator systems consisted of laccase from *T. versicolor* and the mediators ABTS or 1-hydroxybenzotriazole (HBT). The non-enzymic oxidants were cerium(IV) ammonium nitrate (CAN), Fenton's reagent and lead(IV) tetraacetate (LTA). All samples for analysis were taken in duplicates.

Laccase: The reaction mixtures with the laccases from *T. versicolor*, *A. bisporus*, *M. thermophila* and *R. vernicifera* contained 0.01, 0.25, 0.02, and 1.0 units of laccase, respectively. In the oxidation experiments with the laccases from *T. versicolor* and *M. thermophila*, samples were taken after 15, 30, 60, and 120 min. In the experiments with the laccases from *A. bisporus* and *R. vernicifera*, samples were taken after 1, 2, 4, and 8 h. *Laccase-mediator systems:* The reaction mixture contained 0.01 units of laccase from *T. versicolor* and was 1 mM with respect to the mediator (ABTS or HBT). Samples were taken after 15, 30, 60, and 120 min. *Lignin peroxidase:* The reaction mixture contained 0.5 units of *T. versicolor* lignin peroxidase and hydrogen peroxide (0.5 mM). Samples were taken after 15, 30, 60, and 120 min. *CAN*: The reaction mixture was 2 mM with respect to CAN. Samples were taken after 1 h. *LTA*: The reaction mixture was 1 mM with respect to LTA. Samples were taken after 1 h. *Fenton's reagent*: The reaction mixture contained iron(II) chloride (4 mM) and hydrogen peroxide (4 mM). Samples were taken after 1 h. *Negative controls:* No oxidant was added to mixtures containing substrate and buffer. Samples were taken after 8 h.

The samples were flash freezed in liquid nitrogen and were then stored at −20 °C. Before analysis with HPLC (see below), the samples were thawed and filtered through 0.45 μm membrane filters.

2.5. High-performance liquid chromatography (HPLC)

The *erythro* and *threo* forms of **3** were separated and quantified using a VP series HPLC system (Shimadzu). The system was equipped with an XTerra Phenyl analytical column (5 μm, 2.1 × 150 mm) (Waters, Milford, MA, USA) and an XTerra MS C18 (5 μm, 2.1 × 10 mm) guard column. The injection volume was 20 μl. The eluent consisted of a mixture of (A) Milli Q water (Millipore, Bedford, MA, USA) and (B) acetonitrile, and was 2 mM with respect to formic acid. The flow rate was 0.2 ml/min. An SPD-M10Avp diode array UV–Vis detector (λ = 280 nm) and the LCsolution 1.0 software (Shimadzu) were used for detection and quantification. The gradient started with a linear gradient from 5% to 10% B over 5 min, continued with a linear gradient from 10% to 20% B over 30 min, and a linear gradient from 20% to 90% B over 15 min. The column was thereafter reconditioned isocratically with 5% B for 15 min. A chromatogram of the separation of **3e** and **3t** is shown in Fig. 2.

3. Results and discussion

Equal amounts of the *erythro* and *threo* forms of the phenolic lignin model compound 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**3**) (Fig. 1) were oxidized with laccases from *T. versicolor, A. bisporus, M. thermophila* and *R. vernicifera* to investigate their diastereomer selectivity. Laccase-mediator systems and some model oxidants, which unlike laccases are also capable of oxidizing non-phenolic arylglycerol β-aryl ethers [3], were included in the investigation for comparison. The

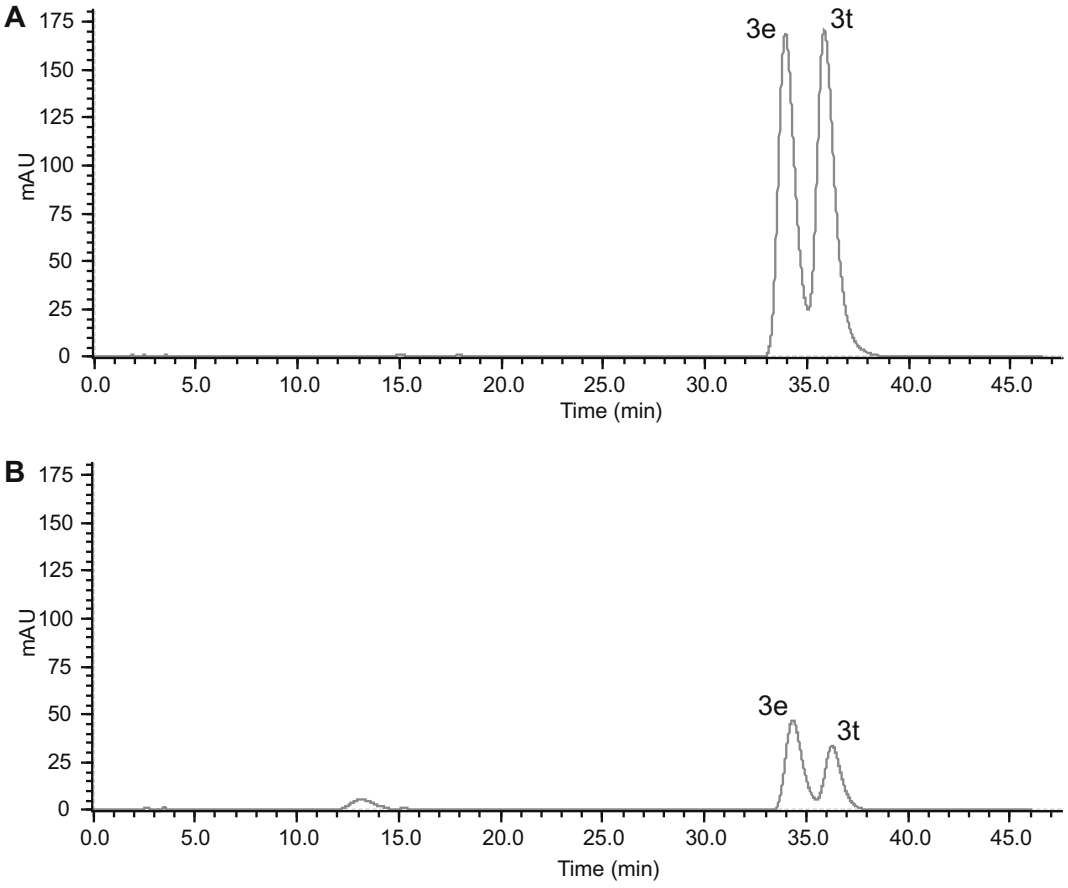

Fig. 2. HPLC chromatograms of the *erythro* (**3e**) and the *threo* (**3t**) isomers of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol: (A) negative control, (B) sample after 120 min oxidation with *T. versicolor* laccase.

Table 2
Percentage of initial amounts and isomer ratios of the *erythro* (**3e**) and *threo* (**3t**) forms of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**3**)[a].

	Percentage of initial amount		Isomer ratio[b] (**3e:3t**)
	3e	**3t**	
Negative control	100	100	50:50
Laccases			
A. bisporus laccase	26	18	59:41
M. thermophila laccase	35	26	58:42
R. vernicifera laccase	42	34	55:45
T. versicolor laccase	27	19	58:42
Laccase-mediator systems			
T. versicolor laccase/ABTS	21	20	51:49
T. versicolor laccase/HBT	30	21	59:41
Model oxidants			
CAN	28	20	58:42
Fenton's reagent	38	38	50:50
Lignin peroxidase	32	25	56:44
LTA	20	20	50:50

[a] The data shown are from reaction mixtures in which 20–40% of the initial amount of **3** remained after oxidation.
[b] Relative standard deviations: ⩽2% (negative control samples) and ⩽6% (oxidation reactions).

erythro/*threo* ratio in remaining substrate when approximately the same amount of substrate had been consumed (70 ± 10%) was taken as a measure of the relative diastereoselectivity of the enzymes and model oxidants. This ratio also reflects the relative rate of reaction of the stereoisomers with the particular enzyme or model oxidant examined.

The use of lignin model compounds offers the advantage of studying a homogeneous substrate in the oxidation reactions. Although experiments with lignin would be of interest, it has to be taken into account that the heterogeneity of the polymer, with respect to the presence of both non-phenolic and phenolic arylglycerol β-aryl ether substructures and the presence of other types of lignin substructures, would make the results more complex and difficult to interpret.

Nakatsubo et al. [14] studied the acid-catalyzed equilibration of **3** and found that the equilibrium mixture consisted of similar amounts of the *erythro* and *threo* forms. This suggests that the diastereomers of **3** are equally stable. No degradation of **3** could be detected in negative controls (no oxidant added).

The laccases used in this study have widely different origins, primary structures, and redox potentials (Table 1). The physiological role of many laccases is not very well understood, but the laccases in Table 1 represent enzymes that are engaged in different kinds of activities and they can be assumed to act on different reducing substrates. Despite the enzymic diversity (Table 1), the substrate, **3**, was oxidized by all the laccases (Table 2). The *erythro*:*threo* ratio (**3e**/**3t**) was 1.0 at the beginning of the experiments. When a substantial portion of the substrate was consumed (60–80%), all laccases showed a clear preferential oxidation of the *threo* form and *erythro*:*threo* ratios in remaining **3** ranged from 1.2 to 1.4 (Table 2). Fig. 3A shows the dependence of the *erythro:threo* ratio on the amount of substrate oxidized in the laccase experiments. This figure indicates that the slightly different *erythro:threo* ratios observed (Table 2) are merely a consequence of how far the reactions have proceeded: the ratios are very similar for different laccases when the same amount of **3** has been degraded.

There were only a few minor product peaks present in the chromatograms of reaction mixtures of **3** and the different laccases. Oxidation of **3** (e.g. by peroxidase [15]) generates primarily a biphenyl as a result of oxidative phenol coupling. Oxidation of lignin-related phenols in general results in formation of biphenyls (e.g. laccase-catalyzed oxidation of 3-(4-hydroxy-3-methoxy)-1,2-propanediol [16]). However, the biphenyls formed from **3** are too hydrophobic to show up in the chromatograms of samples from the laccase-catalyzed reactions.

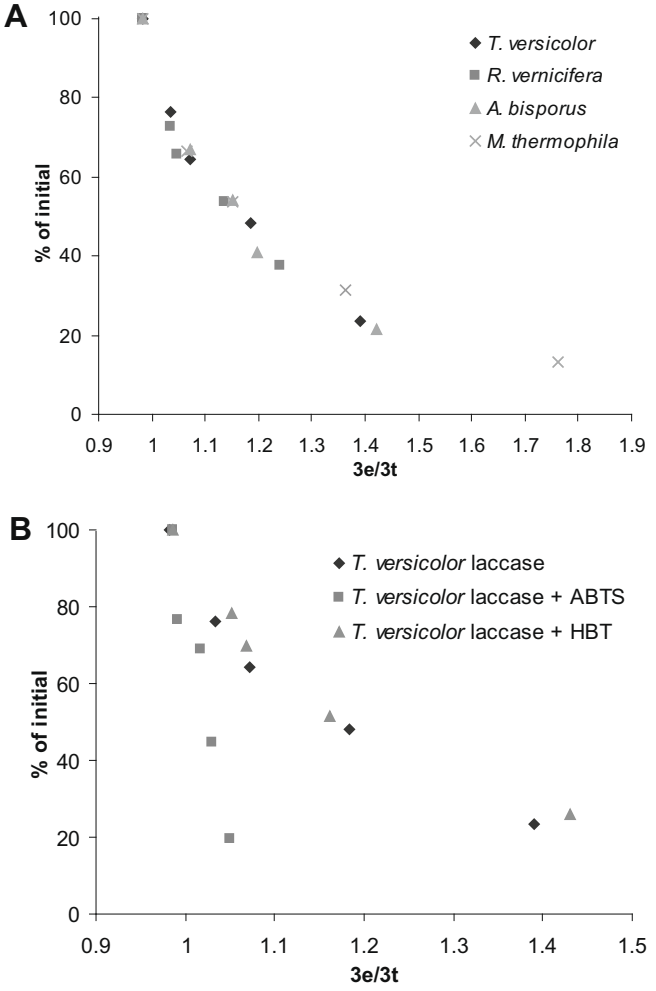

Fig. 3. The relation between the ratio of the *erythro* (**3e**) and the *threo* (**3t**) isomers of 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**3**) and the fraction of **3** remaining after oxidation with laccases from *T. versicolor*, *R. vernicifera*, *A. bisporus* and *M. thermophila* (A) and laccase from *T. versicolor* with and without the mediators ABTS and HBT (B). The relative standard deviations of the measurements of remaining amounts and ratios were ⩽10% and ⩽4%, respectively.

Mediators are typically used to extend the substrate range of laccases from being mainly phenolic compounds to encompass also non-phenolic compounds that are normally not oxidized even by laccases with high redox potentials [8]. In oxidation experiments with a non-phenolic lignin model compound (**3** with methylated phenol group) the stereo-preference is dependent on the nature of the mediator [3]. Most mediators, including HBT, give a moderate but significant preferential degradation of the *threo* isomer, while the ABTS-mediated reaction preferentially degrades the *erythro* isomer [3]. Experimental series with the laccase-mediators ABTS and HBT are included in the present study, despite the fact that **3** is a phenolic compound that is oxidized directly by laccase without the presence of a mediator. In these experiments, **3** may be oxidized both directly by the enzyme and indirectly via the mediator.

The laccase–HBT system showed a preferential degradation of the *threo* form of **3** (Table 2). When about one fourth of the initial amount of substrate remained, the *erythro:threo* ratio was 1.4 regardless of the presence or absence of HBT (Table 2, see also Fig. 3B). Any pronounced stereo-preference was not observed in

oxidation experiments with the laccase/ABTS system (Table 2, Fig. 3B). Considering that direct oxidation by laccases results in *threo* preference (Table 2), it is possible that mediation by ABTS gives *erythro* preference and that the result obtained is a consequence of a combination of direct and mediated oxidation of **3**. Alternatively, the mediated oxidation is predominant and there is no clear stereo-preference.

Arylglycerol β-aryl ether **3** was also oxidized with the model oxidants CAN, lignin peroxidase, LTA, and Fenton's reagent. CAN and lignin peroxidase gave results that were very similar to the results obtained with the laccases. When 20–40% of the substrate remained, CAN, lignin peroxidase and the laccases gave *erythro*:*threo* ratios between 1.2 and 1.4 (Table 2). LTA and Fenton's reagent did not exhibit any stereo-preference (Table 2).

Oxidation of a non-phenolic model compound (**3** with methylated phenol group) with CAN or lignin peroxidase resulted in a clear *threo* preference [3]. CAN and lignin peroxidase also resemble each other with respect to product formation [17]. LTA preferentially oxidized the *threo* form of the non-phenolic lignin model compound, while Fenton's reagent did not exhibit any stereo-preference [3]. In general, the stereo-preference is more pronounced in oxidation experiments with non-phenolic arylglycerol β-aryl ethers [3] than it is in oxidation experiments with phenolic arylglycerol β-aryl ethers (Table 2). Such a difference is not unexpected considering the distinctiveness of oxidation reactions of phenolic compounds [4,15].

There is a clear difference between results obtained with Fenton's reagent and results obtained with ligninolytic enzymes, such as lignin peroxidase and laccase, with respect to (i) product formation [17,18], (ii) stereo-preference in the oxidation of a non-phenolic arylglycerol β-aryl ether [3], and (iii) stereo-preference in the oxidation of a phenolic arylglycerol β-aryl ether (this work). The possible involvement of hydroxyl radicals generated by Fenton's reagent in the biodegradation of lignin has been extensively discussed. As judged from studies of fungal ligninolytic cultures [18,19] the Fenton's reagent type of reactions is not involved during the initial phase of lignin biodegradation.

The different redox potentials of the laccases studied (Table 1) did not affect the stereo-preference (Table 2, Fig. 3A). The following redox potentials (versus NHE) have been reported for the other oxidants: $ABTS^{\bullet+}$/ABTS 0.69 V [20], $ABTS^{++}/ABTS^{\bullet+}$ 1.10 V [20], HBT 1.08 V [20], Ce^{4+}/Ce^{3+} 1.76 V [21], Pb^{4+}/Pb^{2+} 1.69 V [21], and hydroxyl radicals 2.80 V [22,23]. The lack of stereo-preference of Fenton's reagent can be attributed to the very high redox potential of hydroxyl radicals leading to unspecific interaction with the substrate.

CAN and lignin peroxidase react very similarly with regard to both stereo-preference [3,7] (this work) and product pattern [17]. Ce(IV) represents a typical outer-sphere oxidant [24], and therefore it is reasonable to assume that lignin peroxidase acts like that as well. Pb(IV) is a typical inner-sphere oxidant, although its involvement in outer-sphere electron transfer is also conceivable [24]. Concerning laccase-mediator systems, coupling products between phenolic substrates and ABTS have been proposed to form [25–27]. These observations include experiments with the phenolic lignin model compound used in this study [27]. The occurrence of coupling products suggests a close interaction between oxidized ABTS and the phenolic substrate and is consistent with an inner-sphere type of mechanism. It is noteworthy that neither LTA nor laccase/ABTS show the marked *threo* preference observed for the other oxidants (Table 2). As the laccases (Table 1) give the same result as CAN and lignin peroxidase, *i.e.* preferential degradation of the *threo* form, it is reasonable to assume that they interact with **3** in a way that resembles the outer-sphere electron transfer mechanism.

Although the divergence of the laccases studied is large (Table 1) and although the conformations of the diastereomers of **3** are quite different [28,29], all laccases investigated show the same stereo-preference. The interactions between laccases and their substrates are not well known, but structural and electrochemical studies have provided some knowledge about the transfer of electrons in laccases (reviewed in [30]). Laccases can be involved in direct electron transfer (DET) and enzymes that have been investigated in this respect include laccase A from *T. versicolor* [31] and the laccase from *R. vernicifera* [32]. In DET, electrons are transferred from the electrode and further through the enzyme to the T1 copper site, which is situated about 7 Å from the surface [30]. From the T1 site, the electrons are then transferred to the trinuclear T2/T3 copper cluster, where molecular oxygen is reduced to water. DET has also been observed in experiments with lignin peroxidase [33]. Electron transfer through DET permits oxidation of large substrates by interaction between the enzyme surface and the reductant. The widespread occurrence of this type of electron transfer among blue multi-copper oxidases [34] appears consistent with our finding that widely different laccases oxidize the diastereomers of the phenolic arylglycerol β-aryl ether **3** in a similar way.

In conclusion, this investigation demonstrates that certain oxidizing agents, including a set of laccases from different sources, exhibit stereo-preference in the oxidation of a phenolic lignin model compound, while others, including Fenton's reagent, do not. The results serve as a complement to previous investigations of stereo-preference of oxidation of non-phenolic lignin model compounds, which are not oxidized directly by oxidants such as laccases. Studies of the stereo-preference and of other chemical fingerprints generated by enzymes and other biologically relevant oxidants offer a possibility to explore the nature of the oxidants employed by fungi during the decay of lignocellulose [35].

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