Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates

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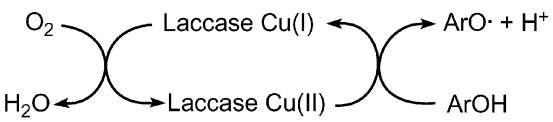
Steric and redox issues of phenolic and non-phenolic substrates are investigated for a better insight of the reactivity features of the phenoloxidase laccase. Whenever a substrate is endowed with a redox potential too high for direct monoelectronic oxidation by the enzyme, or else is too much encumbered to access the enzymatic pocket, redox mediators overcome the problem, behaving as an interface between enzyme and substrate. For example, the small-sized mediator ABTS, once oxidised by laccase, fruitfully interacts with bulky substrates, 2,4,6-tri(Bu^t)-phenol providing a significant case. Other mediators, for example HBT, resort to a radical oxidation mechanism precluded to laccase, and can react with non-phenolic substrates which are impossible for the enzyme. The advantages provided by the mediators are discussed, and suitable phenolic compounds, as precursors of phenoxyl radical intermediates, emerge as a new proficient class. They could be the true natural mediators of laccase in the oxidative delignification. In fact, phenoxyl radical fragments generated by laccase from lignin, or from phenolic monomer residuals from the building up of lignin polymer or else deriving from lignin by oxidation with other ligninolytic enzymes, could oxidise non-phenolic residues of lignin thereby causing the breakdown of its alkyl network. The novel mechanistic probe 3,5-di(Bu^t)-4-OH-benzyl alcohol enables the decoupling of the reactivity channels of a phenolic *vs.* a benzylic alcohol moiety in the enzymatic oxidation of bifunctional substrates having structural features comparable to portions of lignin. Experimental support is thereby attained for the central role of laccase in biodelignification, in spite of the seemingly lower oxidation power of this enzyme with respect to other and stronger oxidising enzymes excreted by ligninolytic fungi.

Introduction

Laccase, a class of 'blue copper' oxidase excreted by basidiomycete fungi,[1] contains four copper ions (one T1 copper and a T2/T3 trinuclear copper cluster)[1,2] and cooperates with other enzymes in the biodelignification process.[1–4] Four molecules of a reducing substrate are oxidised by this enzyme coupled to the four-electron reduction of oxygen to water.[2,5] In view of the low redox potential of the T1 Cu(II) site, which is in the range of 0.5–0.8 V *vs.* NHE depending on the fungal source,[1,2,6] laccase typically oxidises phenols (phenoloxidase activity; Scheme 1) or phenolic lignin units, due to matching redox features.[1,7] Electron abstraction and subsequent deprotonation gives rise to phenoxyl radicals that undergo oligomerisation, or cleavage of the aromatic ring upon dioxygen attack.[8,9]

Non-phenolic substrates, having redox potentials above 1.3 V,[7a,10] are more resistant to monoelectronic oxidation and are not oxidised by laccase directly. Consistently, lignin is a recalcitrant phenolic polymer with respect to oxidation,[1,11] because non-phenolic groups represent more than 70% of its total residues.[12] This would seem to limit the relevance of laccase in the biodelignification. However, suitable compounds, often referred to as *redox mediators*,[13] enable laccase to oxidise indirectly non-phenolic lignin units or substrates.[14] Following monoelectronic oxidation of the mediator by the enzyme, the oxidised mediator (Med_{ox} in Scheme 2) reacts with non-phenolics, such as benzylic alcohols and ethers, according to mechanisms unattainable to laccase, as for example by H-atom abstraction. Several laccase/mediator systems have been investigated, and found capable of oxidizing benzylic lignin models or lignin itself at the expense of oxygen.[7,10,14–16]

Among the mediators, those presenting the >N–OH moiety (1-hydroxybenzotriazole, HBT; *N*-hydroxyphthalimide, HPI; violuric acid, VLA; Scheme 3) proved very efficient towards benzylic substrates, through a radical H-abstraction route of oxidation involving the aminoxyl radical (>N–O$^\bullet$) intermediate as the Med_{ox} species.[7,14,15] TEMPO, a stable >N–O$^\bullet$ species, proved the most efficient mediator towards benzyl



Scheme 1 The redox cycle of the phenoloxidase laccase.

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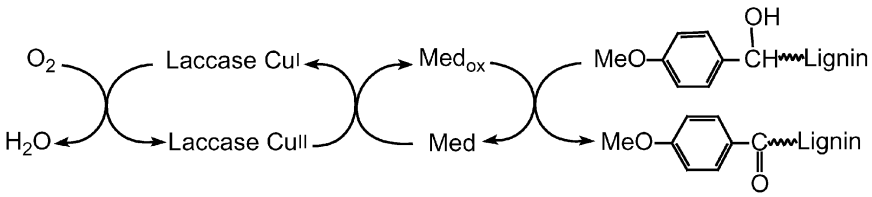
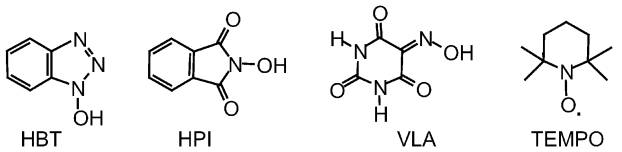


Scheme 2 The redox cycle of a laccase-mediator system.



Scheme 3 Common non-natural mediators of laccase.

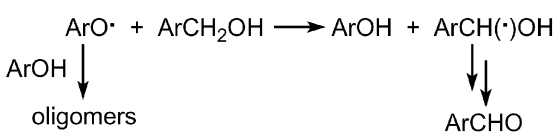
alcohols, but the oxidation mechanism is ionic and not radical in this case.[14,15,17]

The quest for possible natural mediators of laccase has been undertaken, and phenoxyl radicals have been recently suggested for this role.[7] Once generated from phenolic groups through the phenoloxidase activity of the enzyme (Scheme 1), the phenoxyl radicals could remove benzylic hydrogens from non-phenolic residues (as much as the above >N–O• intermediates do), thereby mediating a radical oxidation of lignin polymer. Experimental evidence supports this hypothesis, since phenoxyl radicals from phenolphthalein or its derivatives have been found able to oxidise benzylic model compounds by H-abstraction,[7a,18] before being depleted in competing phenol-coupling oligomerisation (Scheme 4).

In this paper we have addressed the key issue of mediation of laccase by phenoxyl radicals once more, and have expanded the study to the oxidation of bifunctional substrates having *both* a phenol *and* a benzyl alcohol moiety, this being a recurring structural feature in lignin.[12] One of the experimental problems we met was how to sort out unambiguously the contribution of solubility and steric issues upon the direct reactivity of laccase toward the bifunctional substrates, with or without mediators. A central role of laccase in biodelignification emerges from the present study.

Results and discussion

Laccase from the fungus *Trametes villosa* (*viz*. *Poliporus pinsitus*) was employed. The oxidation reactions were performed at room temperature in buffered (0.1 M sodium citrate, pH 5) water solution, initially purged with O_2 before the addition of the enzyme. In many cases a mixed solvent (water –dioxane) needed to be used to increase the solubility of partially hydrophobic substrates, at the expense of a moderate reduction of the enzyme activity.[19] It was first necessary to evaluate unambiguously any specific effect affecting the direct reactivity of laccase with either a phenol or a benzyl alcohol group. To assess the extent of oxidation in these experiments, we monitor the consumption of the substrate, because it is easier and less ambiguous than any quantification of oligomeric mixtures of products.



Scheme 4 Partition of a phenoxyl radical between H-abstraction from a non-phenolic substrate and coupling with another phenolic precursor.

Oxidation of non-phenolics

Laccase does not react with benzyl alcohol directly,[1] nor with the mono-methoxy (*e.g*., *p*-anisyl alcohol) or 3,4-dimethoxy (*i.e*., veratryl alcohol, VA) or 2,4,5-trimethoxy (only 3% of the corresponding aldehyde being obtained) derivatives, nor with 4-MeO-ethylbenzene,[20] all being model compounds of recurring structural motifs in lignin.[12] Basically, the half-wave potential of these substrates (given in Table 1),[10,21] which is above 1.2 V and beyond the electron-abstraction reach of laccase (0.78 V *vs*. NHE, in the specific case of *Trametes villosa* laccase),[6a] prevents monoelectronic oxidation to occur. Accordingly, neither anisole, *i.e*., the methyl ether derivative of phenol, is oxidised by laccase due to the unsuited anodic potential ($E^{1/2}$ 2.0 V).[10] However, the half-wave potential of 1,2,4,5-tetramethoxybenzene ($E^{1/2}$ 1.05 V *vs*. NHE) is accessible to laccase, and indeed this non-phenolic is quantitatively oxidised, as also hinted in the literature,[22] whereas the positional isomer 1,2,3,5-tetramethoxybenzene is not, being the removal of electrons more difficult in view of the higher half-wave potential ($E^{1/2}$ 1.44 V *vs*. NHE). In conclusion any substrate, regardless its phenolic or non-phenolic nature, is a sound candidate for the monoelectronic oxidation by laccase, provided that the electrochemical potential is suitable.

As anticipated in the Introduction, recalcitrant non-phenolic substrates can be oxidised if laccase is used together with >N–OH mediators (Scheme 3 and Table 1) which, in view of their pK_a values,[7a] are extensively deprotonated (>N–O$^-$) in solution at pH 5. The radical route delineated in Scheme 5 operates through the intervention of the aminoxyl radical (>N–O•) as the active Med_{ox} species.[14]

Redox features of the substrate have no major importance upon a radical H-atom transfer (HAT) route, where only the enthalpic balance between the breaking and forming of bonds is relevant.[7a] For many >N–O• species the energy of the NO–H bond being formed on H-abstraction (BDE_{OH} values in the 78–88 kcal mol^{-1} range)[7a] matches that of the scissile benzylic C–H bond in the substrate.[23] Mediator TEMPO, instead, undertakes an ionic route of oxidation of benzylic

Table 1 Recovery of substrate, or extent of oxidation, in reaction of phenolic or non-phenolic substrates with laccase or laccase/mediator systems, for 24 h at 25 °C[a]

Substrate	$E_{sub}^{1/2}$, in V *vs.* NHE (in H_2O) [b]	Recovered substrate (%) with laccase in water	Recovered substrate (%) with laccase in a mixed solvent[c]	Oxidized product (%) with laccase/TEMPO	Oxidized product (%) with laccase/HBT
Benzyl alcohol	2.68 (in MeCN)	99[d]	—	92[e]	30[e]
p-Anisyl alcohol	1.98 (in MeCN)	99[d]	—	98[e]	76[e]
VA	1.36	98[d]	—	99[e]	92[e]
2,4,5-Trimethoxy-benzyl alcohol	*ca.* 1.2	95[d]	60[g]	85[e]	—
4-MeO-ethylbenzene	*ca.* 1.7 (in MeCN)	—	99[d]	<1[cd]	28[ch]
1,2,4,5-Tetra-methoxybenzene	1.05 (in MeCN)	—	<1[fi]	—	—
1,2,3,5-Tetra-methoxybenzene	1.44 (in MeCN)	—	90	—	—
Anisole	2.0 (in MeCN)	99[d]	—	—	—
Phenol	0.87	<1[f]	—	—	—
4-Bu^t-phenol	0.82	<1[f]	—	—	—
β-Naphthol	0.87	98[d]	<1[f]	—	—
2,4,6-Trichlorophenol	1.01	95[d]	<1[fi]	—	—
2,4,6-Tri(Bu^t)-phenol	0.71	99[d]	99[d]	<1[cd]	40[cj]

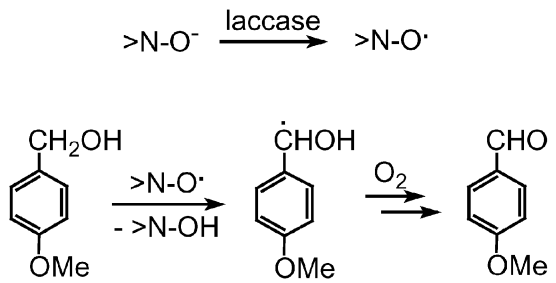
[a] Conditions: [substrate] 20 mM, 10 U of laccase; [mediator] 6 mM, if employed. [b] From ref. 10 and 21; in general, run at 25 °C, acid pH and 0.1 V s^{-1}. [c] In H_2O : dioxane 1 : 1. [d] Quantitative recovery of the substrate. [e] The product is the corresponding aldehyde. [f] No substrate is recovered. [g] 2,4,5-Trimethoxybenzaldehyde is detected (3%), in addition to a dimeric derivative formed by acid catalysis (see Experimental). [h] As a mixture of 4-MeO-acetophenone and 1-(4-MeO-phenyl)ethanol (from ref. 20). [i] Minor amounts of quinone are detected by GC-MS. [j] 10% of di(Bu^t)quinone is also detected by GC-MS (see Experimental).

alcohols to aldehydes through the oxoammonium ion (>N=O^+) acting as the Med_{ox} species (Scheme 6).[7a,14,15,17]

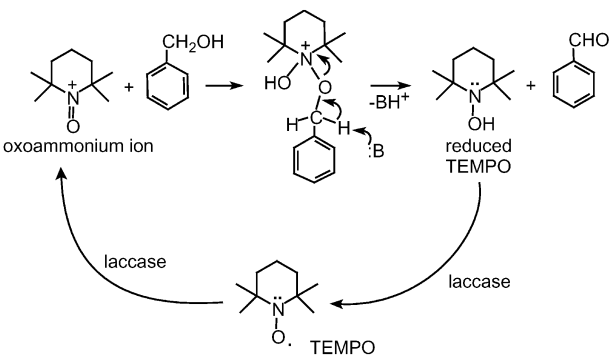
Table 1 shows that the functional group of benzyl alcohols, which is unreactive with laccase, is susceptible to oxidation by the specific Med_{ox} form (either >N–O• or >N=O^+) of suitable mediators through mechanisms other than the monoelectronic one of the enzyme. Finally, oxidation of an alkylarene, such as 4-MeO-ethylbenzene, takes place appreciably through the HAT route with laccase/HBT, and solubility problems can be overcome by the use of mixed solvents.[20] The ionic route of TEMPO, in contrast, does not operate with the alkylarenes, the necessary requirement of a nucleophilic attack onto TEMPO-oxoammonium being unfeasible for this kind of substrate, while possible for the lone-pair of benzyl alcohols.[17,20]

Oxidation of phenols

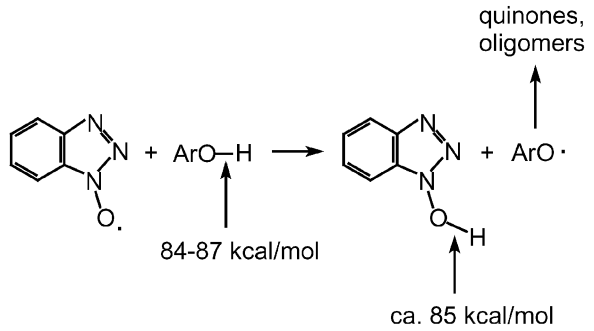
Laccase promotes the monoelectronic oxidation of phenols because the redox potential of phenolic substrates (0.5–0.9 V, at acid pH)[21] and enzyme (0.6–0.8 V)[1,2,6] is well matched; this allows the transfer of electrons and ensuing quantitative depletion of the substrate, as reported in Table 1 for phenol itself. Phenoxyl-to-phenol oligomers are produced *via* oxygen-to-nucleus or nucleus-to-nucleus couplings.[24] The same occurs with a more hydrophobic phenol such as 4-*tert*-butyl-phenol; however β-naphthol, 2,4,6-trichlorophenol and 2,4,6-tri(Bu^t)-phenol, in spite of the appropriate redox potential value, are not oxidised by laccase. There is certainly an effect of a reduced (or lack of) solubility in water, because β-naphthol and 2,4,6-trichlorophenol are fully consumed whenever the reaction is run in a water : dioxane (1 : 1) mixed solvent, but this is insufficient for 2,4,6-tri(Bu^t)phenol which continues to be unreactive. Here steric factors may hinder the approach of the substrate to the enzyme active site, and consequently preclude the oxidation. Not all the phenols are therefore fitting substrates for the phenoloxidase laccase! This steric problem is solved by suitable mediators. The >N–O• species generated from HBT by laccase, in view of its small size and matching value of BDE (Scheme 7),[7a] abstracts H-atom from the O–H bond of 2,4,6-tri(Bu^t)phenol and affords the corresponding phenoxyl radical. The latter, having the 2,4,6-positions hindered, undergoes preferential loss of *t*-Bu• to give di-*t*-butyl-*p*-benzoquinone (10%), besides oligomeric products from



Scheme 5 The radical H-atom transfer (HAT) route of oxidation of a non-phenolic substrate by laccase and >N–OH mediators.



Scheme 6 The ionic route of oxidation of benzyl alcohols by laccase/TEMPO.



Scheme 7 Thermochemical balance for the radical HAT mechanism between a >N–O• intermediate and a phenol acting as a H-donor substrate.

oxygen-to-nucleus couplings,[24d] followed by loss of *t*-Bu•. No oxidation of 2,4,6-tri(Bu^t)phenol takes place instead with laccase/TEMPO, because the ionic route through TEMPO-oxoammonium is structurally precluded to a phenol.

Depletion of 2,4,6-tri(Bu^t)phenol with laccase occurs more significantly (*i.e.*, 55%) in the presence of Phenol Red (Table 2). This particular phenol, once monoelectronically oxidised by laccase to a relatively stable phenoxyl radical,[7a,18] performs as a mediator by efficiently abstracting the H-atom from the O–H bond of 2,4,6-tri(Bu^t)phenol. The subsequent reactivity channels of the 2,4,6-tri(Bu^t)phenoxyl radical described above with the N–OH mediator (*cf.* Scheme 7) then become accessible. Finally, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (*viz.* ABTS)[7a,10,14,15] mediates laccase in an even more extensive (90%) oxidative degradation of 2,4,6-tri(Bu^t)phenol. The Med_{ox} form of ABTS (*i.e.*, ABTS$^{\bullet+}$) is in fact endowed with a redox potential (0.69 V)[10] suitable to monoelectronic oxidation of the phenolic substrate (0.71 V), the subsequent depletion of which occurs by loss of *t*-Bu• to give di-*t*-butyl-*p*-benzoquinone (30%) besides oligomeric products. Therefore, the oxidative degradation of the recalcitrant 2,4,6-tri(Bu^t)phenol takes place appreciably either by the intervention of radical mediators according to the HAT route or, and more extensively, by electron transfer to ABTS$^{\bullet+}$. In both ways the steric incompatibility of the encumbered phenol towards the active site of laccase, which hampered a direct oxidation, is overcome.

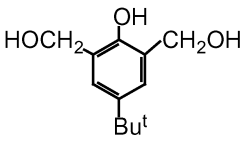
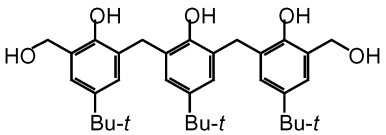
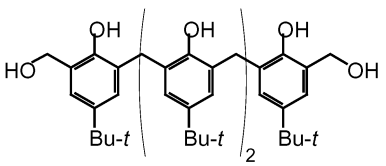
Comparable is the steric bulk of 2,6-di(Bu^t)-4-Me-phenol and 2,6-di(Bu^t)-4-MeO-phenol, or that of the disubstituted 2,6-di(Bu^t)phenol, which are largely recovered unreacted on exposure to laccase (Table 2). One concludes that sterically demanding groups *ortho* to the phenolic OH prevent the approach of the substrate to the enzyme active site, and any ensuing oxidation. In keeping with this explanation 2,4-di(Bu^t)phenol, having only one encumbered *ortho* position, is *partially* oxidised by laccase (25%) and a dimeric coupling product (*ca.* 10%) detected.

The size of the 2,6 substituents must be large enough to hinder the approach to the active site, as indicated by the quantitative oxidation of the smaller 2,4,6-trimethylphenol by laccase (only 1% recovered; *cf.* also 2,4,6-trichlorophenol in Table 1), to give oligomeric products plus 4-hydroxy-3,5-dimethylbenzaldehyde (20%). Significant also is another trend of reactivity reported in Table 2. The structurally complex phenol **1** is soluble in water and less encumbered than 2,4,6-tri(Bu^t)phenol, in view of the smaller size of the CH_2OH groups in the *ortho* position. Consistently, it can approach the enzyme and is quantitatively oxidised monoelectronically to oligomeric and ring-cleavage products. Mediation of laccase would consequently appear unnecessary but, whenever TEMPO is added, an important change in the product mixture is found. The intermediate phenoxyl radical of **1**, resulting from interaction with laccase, is trapped by TEMPO (a radical scavenger),[25] and both the phenol-coupling and ring-cleavage pathways are accordingly put off. Only the oxidation of the hydroxymethyl groups of **1** into formyl-groups is observed,[26] in keeping with similar transformations of benzyl alcohol groups by TEMPO-oxoammonium (Scheme 6).[17] Therefore, TEMPO intercepts and stops the normal reactivity pattern of laccase towards the 'phenolic part' of **1**, while the enzyme-produced TEMPO-oxoammonium oxidises the non-phenolic side-chain of **1** regioselectively. With the trimeric derivative of **1** (*i.e.*, **2**), lack of solubility in water hampers the *direct* oxidation by laccase, this being even more pronounced with tetrameric derivative **3**. Use of the mixed solvent improves the situation with **2**, but not with **3**. Oxidation of the latter takes place extensively with the laccase/TEMPO system but, once again, it is specifically directed towards the CH_2OH side-chain.[26] Besides, monoelectronic oxidation of **3** takes place quantitatively with laccase/ABTS through the ABTS$^{\bullet+}$ intermediate, as also observed for the encumbered 2,4,6-tri(Bu^t)-phenol. This stresses the importance of small-sized diffusible mediators for an efficient oxidation of bulky substrates, including phenolic ones.

Phenols as radical mediators of laccase towards non-phenolics

Structural features make the phenoxyl radical from Phenol Red sufficiently stable and 'slow' in self-coupling routes,[18] so to enable H-abstraction (*cf.* Scheme 4). In fact Phenol Red mediates laccase efficiently in the radical oxidation of the encumbered 2,4,6-tri(Bu^t)phenol (in Table 2). Taking advantage from this long-living phenoxyl radical, the laccase/Phenol Red system has been exploited for the benchmark oxidation of *p*-anisyl alcohol, the most simple lignin model compound, into the corresponding aldehyde in reasonable yield (Table 3).[7a] Inspired by this result, we searched for any ability of the encumbered phenoxyl radical from 2,4,6-tri(Bu^t)phenol to mediate similarly, but no significant oxidation of *p*-anisyl alcohol with laccase and 2,4,6-tri(Bu^t)phenol was obtained. This may simply confirm the inability of laccase to convert this phenol into the phenoxyl radical for steric reasons. Conversely, catechol (1,2-dihydroxybenzene) compares well with Phenol Red because it mediates laccase in the oxidation of *p*-anisyl alcohol appreciably. *Ortho*-diphenols are recurring structural motifs in lignin and, whenever sufficiently stable as phenoxyl radicals, they could perform as natural mediators of laccase towards non-phenolic and phenolic units. Similarly effective is the 1,2,3-trihydroxy-analogue pyrogallol, and even more so hydroquinone, but not 2,6-$(MeO)_2$-phenol, which sometimes is used to assay the activity of laccase.[27] Neither barbituric nor uric acids, which are natural compounds

Table 2 Consumption of encumbered phenols, and evidence of product(s), in the aerobic oxidations with laccase or laccase/mediator systems, at 25 °C[a]

Substrate	Recovered substrate (%) with laccase	Recovered substrate (%) with laccase/HBT	Recovered substrate (%) with laccase/ Phenol Red	Recovered substrate (%) with laccase/ ABTS	Recovered substrate (%) with laccase/ TEMPO
2,4,6-Tri(Bu^t)phenol	99	60; 10% quinone	45	10; 30% quinone	99
2,6-Di(Bu^t)-4-Me-phenol	99	90; 2% quinone, tr. 4-CH_2OH	47; 1% 4-CH_2OH, 9% quinone	2; 2% 4-CHO, 13% quinone,	—
2,6-Di(Bu^t)-4-MeO-phenol	93; 4% quinone	82; 13% quinone	—	1; 75% quinone	—
2,6-Di(Bu^t)phenol	94	—	—	—	—
2,4-Di(Bu^t)phenol	75; 10% dimer	—	—	—	—
2,4,6-Tri(Me)phenol	1; 20% aldehyde	—	—	—	99
 **1**	<1[b]	—	—	—	<1 (but different products)
 **2**	82[bc]	40	—	—	5
 **3**	99[d]	—	—	<1	13

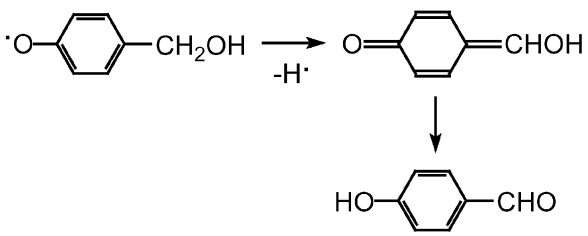
[a] Conditions as in Table 1, in H_2O : dioxane 1 : 1. [b] In buffered water. [c] Becomes 45% in the mixed solvent. [d] Both in buffered water and in the mixed solvent.

structurally related to the proficient mediator VLA,[7a] appear to mediate laccase toward *p*-anisyl alcohol.

Additional examples of aryloxyl radicals, which are sufficiently stable to play an essential role for the hosting protein, are known and found in the ribonucleotide reductase, galactose oxidase and prostaglandine H synthase enzymes, besides photosystem II.[28]

Oxidation of bifunctional substrates

Anodic oxidation of phenols bearing suitable substituents in *para*, such as methyl groups, is known to give rise to quinone-methide and dimerisation products.[29] Because the presence of *both* a phenol *and* a benzyl alcohol (or other benzylic groups) on the same aromatic ring is widespread in lignin,[12] by the same token any laccase-induced monoelectronic oxidation of the phenolic group in a bifunctional substrate could 'shift' intramolecularly from the phenoxyl radical to the benzylic center (Scheme 8).[30] This would enable subsequent transformation/oxidation of the latter, as much as any mediated



Scheme 8 Conceivable functionalisation of a side-chain in *para* to a phenoxyl radical.

Table 3 Yield of *p*-anisaldehyde from the aerobic benchmark oxidation of *p*-anisyl alcohol with laccase and phenolic or phenolic-like mediators

Substrate	% aldehyde with laccase/ Phenol Red	% aldehyde with laccase/ 2,4,6-tri(Bu^t) phenol	% aldehyde with laccase/ catechol	% aldehyde with laccase/ hydroquinone	% aldehyde with laccase/ pyrogallol	% aldehyde with laccase/2,6-$(MeO)_2C_6H_3OH$	% aldehyde with laccase/ barbituric acid
p-Anisyl alcohol	20 (35, after 48 h)	<2	19	32	18	≤1	≤1

Table 4 Recovery of bifunctional substrates (having both phenol and benzyl alcohol moieties) under aerobic oxidation with laccase or laccase/mediator systems, in H_2O : dioxane 1 : 1 at 25 °C

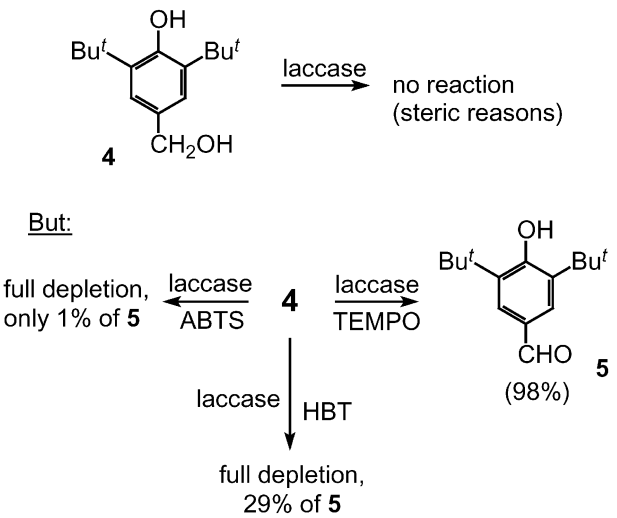
Substrate	% recovery with laccase	% recovery with laccase/ABTS	% recovery with laccase/TEMPO	% recovery with laccase/HBT	% recovery with laccase/HPI
3,5-Di(Bu^t)-4-OH-benzyl alcohol (**4**)	95	0; <1% of **5**	0; 100% of **5**	1; 29% of **5**	
3,5-Di(Bu^t)-4-OMe-benzyl alcohol (**6**)	99	95	—	83; 17% of **7**	93; 7% of **7**
2,4,6-Tri(Me)phenol	1; 20% aldehyde	—	99	—	—
2,4,6-Tri(Me)-anisole	97	—	—	—	—

radical oxidation regiospecifically directed towards the benzylic group would do.

Bulky substituents in *ortho* to free phenolic groups in lignin, while blocking (or retarding) the normal coupling channels of the intermediate phenoxyl radical (*cf.* Table 2),[2a] could partially drive the reactivity toward the conjugated benzylic center; this would induce a C_α–C_β cleavage of the alkyl chains in lignin, as already documented,[31] even on suitable arylalkyl models which yield aromatic aldehydes as chain-cleaved products.[15a,32] Therefore, a depolymerisation of lignin without the need for external/added mediators would ensue. This endorses the function of phenols not only as 'natural' substrates but also as natural mediators of laccase, supporting a central role of the latter in biodelignification. Consistently, the monoelectronic oxidation of hindered phenols, such as 2,6-di(Bu^t)-4-methylphenol (and others), by DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) has been already described.[33] The formation of dimers *via* C–C or C–O coupling, subsequent dissociation into radicals and further disproportionation to a quinone–methide, evolving in the formation of 3,5-di(Bu^t)-4-hydroxybenzaldehyde as a benzylic oxidation product, are documented.

A problem of clear interpretation of the results in bifunctional substrates arises, however. In fact, the reactivity of the phenolic group that reacts with laccase directly, as well as the reactivity of the benzylic group that *does not* react in the absence of mediators, are somewhat 'geared' (see Experimental). For example, it is quite obvious that a simple bifunctional substrate such as 4-hydroxy-benzyl alcohol reacts with laccase quantitatively, being a phenol. However, this is not sufficient indication for any participation of the benzylic moiety to overall reactivity through the operation of the delocalisation/dehydrogenation (or disproportionation)[33] pathway depending on the phenoxyl radical and quinone-methide, as delineated in Scheme 8. In order to unambiguously decouple the reactivity channels of the two functional groups, appropriate substrates ought to be used.

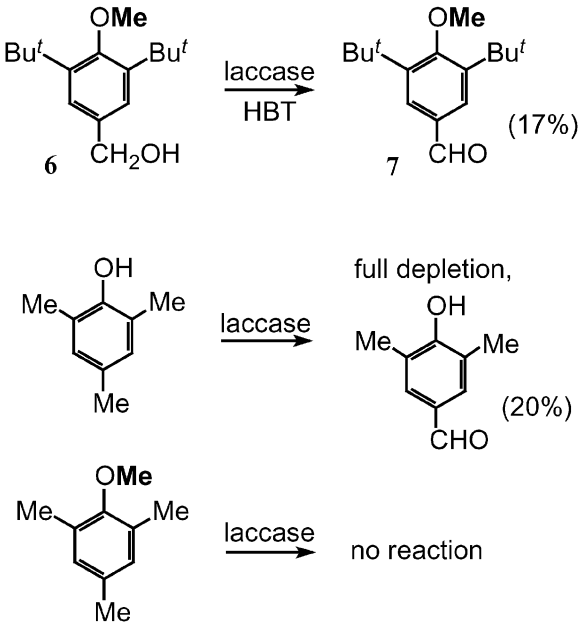
In this context, 3,5-di(Bu^t)-4-hydroxy-benzyl alcohol (**4**) becomes a significant mechanistic probe (Scheme 9). It is recovered 95% unchanged by exposure to laccase (Table 4), in keeping with the lack of reactivity of 2,4,6-encumbered phenols (Table 2). However, phenol **4** substantially gains in reactivity under the presence of mediators. Monoelectronic oxidation *via* $ABTS^{\bullet+}$, generated from ABTS by laccase, takes place as it did in the case of 2,4,6-tri(Bu^t)phenol (Table 2), leading to full consumption of **4** through the typical reactivity channels of phenolic oxidations (*i.e.*, oligomers and ring-



Scheme 9 Reaction pathways with probe substrate **4**.

opening products, which escaped our GC detection being either high-boiling or water-soluble, respectively), made available by this small-sized Med_{ox} species. No evidence for participation from the 'spectator' CH_2OH group in *para* to the overall reactivity is obtained, since less than 1% of 3,5-di(Bu^t)-4-hydroxy-benzaldehyde (**5**) is detected, *i.e.* the specific oxidation product of the benzylic moiety. The oxidation of **4** with laccase/TEMPO gives an almost opposite outcome (Table 4). Full consumption of **4** is now accompanied by quantitative and regioselective conversion into aldehyde **5**, consistent with the operation of the ionic mechanism of oxidation of the benzyl alcohol moiety by TEMPO-oxoammonium. Finally, the intervention of the radical Med_{ox} species $>N–O^{\bullet}$ from the use of laccase/HBT promotes the H-abstraction route, and leads to the consumption of **4** with a significant formation (29%) of **5**. Does the latter derive from *direct* H-abstraction from the benzylic C–H bond of the CH_2OH group, or from initial H-abstraction from the phenolic O–H *followed* by the resonance/dehydrogenation route outlined in Scheme 8?

In order to solve this mechanistic conundrum, conversion of the phenolic group of **4** into a methoxy derivative, *i.e.* 3,5-di(Bu^t)-4-OMe-benzyl alcohol (**6**), was devised. In this case, any HAT route (*cf.* Scheme 5) could occur only on the CH_2OH group, the expected product being 3,5-di(Bu^t)-4-OMe-benzaldehyde (**7**) (Scheme 10). This strategy was inspired by finding that, whereas 2,4,6-trimethylphenol reacts



Scheme 10 In search of a genuine phenol-dependent benzylic functionalisation.

quantitatively with laccase, 2,4,6-trimethylanisole does not do so and is recovered *quantitatively* (Table 4). Obviously, conversion of the phenolic into a methyl ether group, in 2,4,6-trimethylanisole, by enhancing the redox potential of the substrate (compare anisole *vs.* phenol in Table 1), hampered the 'normal' phenoloxidase route (to oligomeric coupling and ring-cleavage products) and substrate depletion. But it prevented also any oxidation of the methyl group(s) of 2,4,6-trimethylanisole into formyl moiety, at variance with the partial (20%) oxidation of the 4-methyl group that is observed for 2,4,6-trimethylphenol. It is therefore likely that the latter oxidation is a phenol-dependent side-chain functionalisation, in keeping with Scheme 8. Consistent with this observation and with our expectation, oxidation of the ether **6** by laccase/HBT gave 17% of aldehyde **7**, more than 80% of **6** being recovered unreacted. If such an extent of formation of **7** may be taken as the intrinsic reactivity of the CH_2OH group of **6** in the HAT route, it supports our claim that the more abundant formation (29%, in Scheme 9) of aldehyde **5** in the corresponding HAT route of oxidation of **4** by laccase/HBT truly testifies for the phenoxyl-dependent side-chain functionalisation in *para* outlined in Scheme 8.

Conclusions

Assessment of product yields or extent of consumption of the substrate provide a way to monitor reactivity in the monoelectronic oxidation reaction by laccase. The importance of the redox potential of the substrate is stressed. Phenols, having electrochemical potential comparable to that of the enzyme, are typical natural substrates, whereas structurally different compounds, endowed with substantially higher electrochemical potential, are not. However, laccase can directly oxidase a non-phenolic substrate whenever its electrochemical potential is sufficiently low, 1,2,4,5-tetramethoxybenzene being a fitting example. Quite the opposite, solubility or steric issues make the oxidation of specific phenolic substrates unattainable to laccase, 2,4,6-tri(Bu^t)-phenol providing a significant case. In this event, redox mediators enable the enzyme to solve the problem, because the oxidised mediator acts as the interface between laccase and the substrate. Small-sized mediators can fruitfully interact with encumbered substrates that cannot access the enzymatic pocket directly. On the other hand, substrates endowed with high electrochemical potential can be oxidised by radical mediators through the operation of a H-abstraction mechanism inaccessible to the enzyme itself. On this topic we stress the importance of phenoxyl radicals. They could be the true natural mediators of laccase for the oxidative degradation of the aromatic polymer lignin, where phenolic but mostly non-phenolic (benzylic) residues are present. Phenoxyl radicals produced by the phenoloxidase activity of laccase either from lignin, from phenolic monomers residuals from the building up of lignin polymer,[34] or deriving from lignin by oxidation with other ligninolytic enzymes,[1] provided that they are sufficiently long-living, could mediate laccase towards the oxidation of non-phenolic lignin units. Steric effects could grant this long-living feature to appropriate phenoxyl radicals, enabling them to perform as radical mediators before being depleted in competing oligomerisation pathways.[7a,18,35] In keeping with this point, the structural peculiarity of the aromatic groups of lignin, where both phenolic and benzylic moieties are often present together as substituents, is remarked upon and 3,5-di(Bu^t)-4-OH-benzyl alcohol exploited as a significant mechanistic probe. The experimental information acquired reveals that initial formation of a phenoxyl radical on the one side of the aromatic bifunctional molecule might activate the benzylic substituent on the other side. By extrapolation, the geared reactivity of these two kinds of functional groups could induce the breakdown of the alkyl network of the polymer lignin under oxygen-assisted radical routes, enabling its degradation. In conclusion, the experimental evidence presented here supports the relevant role of laccase in the biodelignification, despite the seemingly lower power of this enzyme with respect to other and stronger oxidising enzymes produced by ligninolytic fungi, such as Lignin peroxidase or manganese peroxidase.[1]

Experimental

General remarks

NMR spectra were taken on an AC 300 Bruker instrument. A VARIAN CP 3800 GC, fitted with a 30 m × 0.25 mm methyl silicone gum capillary column (CPSil5CB), was employed in the analyses. The identity of the products was confirmed by GC-MS analyses, run on a HP 5892 GC, equipped with a 30 m × 0.2 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV. An Agilent HPLC system (pump, degasser, UV-Vis detector, and solvent delivery system), equipped with a Supelcosil LC-18-DB 25 cm × 4.6 mm column, was used, as well as a Perkin Elmer Sciex API 365 liquid-mass spectrometer.

Materials

Most of the mediators and substrates were commercially available (Aldrich), or already present in the laboratory.[7a,10,15,17] Phenols **1**, **2** and **3** were available from a previous investigation.[26] Buffers were prepared using ultrapure water obtained from a MilliQ apparatus.

Synthesis of 3,5-di(Bu^t)-4-OMe-benzyl alcohol (6). A direct conversion of the commercially available 3,5-di(Bu^t)-4-OH-benzyl alcohol (Aldrich) into the title compound, by methylation of the phenolic hydroxyl group with MeI and KOH in MeCN, proved unfeasible because it led to polymeric material. This confirms the mutual interaction of the OH and CH_2OH groups in the *para* hypothesised in Scheme 8. Commercially available 3,5-di(Bu^t)-4-OH-benzaldehyde (Aldrich) was instead successfully methylated with a fourfold molar amount of MeI in MeCN solution at 80 °C for 36 h, using *t*-BuOK as the base. The obtained 3,5-di(Bu^t)-4-OMe-benzaldehyde (*m*/*z* 248) was quickly purified by short-path column chromatography on silica gel with hexane : diethyl ether 30 : 1 as the eluent, and reduced with $NaBH_4$ in 2-propanol containing NaOH. Product **6** was obtained in a 47% overall yield, and analogously purified by column chromatography, to a solid (*m*/*z* 250) melting at 94–96 °C. ^{1}H-NMR δ ($CDCl_3$): 7.26 and 7.24 (s, 2H, ArH), 4.62 (s, 2H, CH_2OH), 3.69 (s, 3H, OCH_3), 1.75 (s, 1H, OH), 1.43 (s, 18H, 2 × CMe_3); ^{13}C-NMR δ ($CDCl_3$): 159.3 (C_{Ar}–OMe), 144.0 (C_{Ar}–CH_2OH), 134.9 (C_{Ar}–CMe_3), 127–125 (ArH), 66.0 (ArOCH_3), 64.3 (CH_2OH), 35.9 (CMe_3), 32.2 (Me).

Enzyme purification. Laccase from a strain of *Trametes villosa* (*viz*. *Poliporus pinsitus*) (Novo Nordisk Biotech) was employed. It was purified by ion-exchange chromatography on Q-Sepharose by elution with phosphate buffer, and laccase fractions having an absorption ratio A_{280}/A_{610} of 20–30 were considered sufficiently pure.[7a,26] The collected fractions were concentrated in cellulose membrane tubing (Sigma) by dialysis against poly(ethylene glycol), a final activity of 5000 U mL^{-1} being determined spectrophotometrically by the standard assay with ABTS.[36]

Enzymatic reactions

The aerobic oxidation reactions were performed at room temperature in stirred water solution (3 mL), buffered at pH 5 (0.1 M in sodium citrate) and purged with O_2 for 30 min prior to the addition of the reagents.[13,14] In general, the initial concentration of the reagents was: [substrate] 20 mM, 3 U mL^{-1} of laccase, besides [mediator] 6 mM, when employed. The reaction time was 24 h at room temperature, an atmosphere of oxygen being kept in the reaction vessel by means of a hemi-inflated latex balloon. In many cases, a 1 : 1 buffered water : dioxane mixed solvent (3 mL) was employed to ensure full solubility of the substrates. The reaction mixture was extracted with ethyl acetate, dried over Na_2SO_4 and characterised by GC and GC-MS analyses. The recovery of substrate or the yield of oxidation product was reckoned by GC analysis with respect to an internal standard (acetophenone or 4-methoxyacetophenone or 4-methylbenzophenone), using suitable response factors. With simple phenolic substrates, oligomeric coupling products are expected;[8,9] in a few instances, evidence for these was qualitatively obtained through LC-MS analyses.[26] In the oxidation of 2,4,6-trichlorophenol with laccase, minor amounts of dichloroquinone were detected from GC-MS (*m*/*z* 176). In the oxidation of 2,4,6-tri(Bu^t)phenol, the formation of di(Bu^t)quinone was inferred from GC-MS analysis (*m*/*z* 220); it was possible to isolate it by a short-path column of silica gel (hexane : diethyl ether 30 : 1 as the eluent) from the crude material of the oxidation with laccase/HBT; it has a mp 66–68 °C. ^{1}H-NMR δ ($CDCl_3$): 6.49 (s, 2H, C=C–H), 1.27 (s, 18H, C(CH_3)$_3$); ^{13}C-NMR δ ($CDCl_3$): 189.7 (*C*=O), 188.3 (*C*=O), 158.5 (Me_3CC=CH), 130.7 (Me_3CC=*C*H), 36.2 (CMe_3), 29.9 (CH_3)$_3$. In the oxidation of 2,4,5-trimethoxybenzyl alcohol with laccase, a dimeric compound (*m*/*z* 348) was detected by GC-MS. It possibly originates from the benzyl alcohol precursor by proton-induced loss of water, followed by attack of the resulting carbocation onto a second molecule of precursor, and loss of formaldehyde.

HPLC analyses

With substrates **1–3**, consumption after 24 h reaction time was determined by HPLC. The analyses were carried out with gradients of water–methanol–isopropanol mixtures, containing 0.03% trifluoroacetic acid, at 0.5–1 mL min^{-1} flow rate. Quantitation of unreacted substrate was achieved by using 2-bromonaphthalene (Aldrich) as the internal standard. The standard was added to the reaction crude, which was then diluted in the mobile phase and filtered through 0.2 μm Teflon syringe filters (Superchrom Varisep) prior to analysis. Liquid mass spectrometry (LC-MS) analyses were carried out using a triple quadrupole Perkin Elmer Sciex API 365 spectrometer with a turbo-ion spray interface. Samples were diluted in HPLC-grade methanol, filtered through 0.2 μm Teflon syringe filters, and directly injected into the ion spray chamber without chromatographic separation.

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