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Comparing the catalytic efficiency of some mediators of laccase

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Abstract

The mechanism of oxidation of non-phenolic substrates by laccase/mediators systems has been investigated. Oxidation of 4-methoxybenzyl alcohol (**1**), taken as a benchmark reaction, enabled us to compare and to rank the relative ability of twelve mediators: TEMPO proved most effective, and a ionic mechanism is suggested for its action. Data on intermolecular selectivity of substrate oxidation are in favour of an electron transfer (ET) mechanism in the case of ABTS-mediated oxidations, and of a radical mechanism in HBT- and HPI-mediated reactions. Investigation by cyclic voltammetry (CV) of some of the mediators revealed that an important role in determining the mechanism of substrate oxidation may be played by the stability of the oxidised form of the mediator, as well as by its redox potential.

Keywords: Laccase; Mediators; Electron transfer; H-atom abstraction; Lignin degradation; TEMPO

1. Introduction

White-rot fungi are able to break down the structure of the natural polymer lignin by resorting to specific enzymes [1,2]. These enzymes, and the reaction mechanism they use, have attracted both academic and applied interest, for example in the paper industry [2,3]. Lignin peroxidase (LiP) [4] and manganese peroxidase (MnP) [5] are two enzymes secreted by fungi for the oxidative depolymerisation of lignin, and can oxidise even its non-phenolic constituents [6]. Laccase is another enzyme expressed under lignin-olytic conditions [7]; its X-ray structure has been recently made available [8]. Because of its lower redox potential with respect to LiP and MnP [9], laccase can oxidise only phenolic fragments of lignin. This is unfortunate, because the ready availability of laccase and its convenient manipulation would make it attractive for an environmentally benign bleaching of kraft pulps, if only it were able to oxidise the non-phenolic residues from the oxygen delignification [3]. The latter compounds make it difficult to efficiently bleach the pulp for paper making. Recent reports, however, have disclosed that appropriate substances can 'mediate' the oxidation of non-phenolic substrates by laccase [10–13], thereby, extending the enzymatic reactivity towards 'uncommon' substrates. This is consonant with the catalytic role that some metabolites, produced by enzymes, have in Nature in similar circumstances [14,15].

The role of mediators in an enzymatic oxidation is outlined in Fig. 1 for the case of laccase [16].

A mediator could be a small molecule that acts as a sort of 'electron shuttle': once it is oxidised by the enzyme, it diffuses away from the enzymatic pocket and in turn oxidises any substrate that, due to its size, could not directly enter the enzymatic pocket [17]. Alternatively, the oxidised mediator could rely on an oxidation mechanism not available to the enzyme, thereby extending the range of substrates accessible

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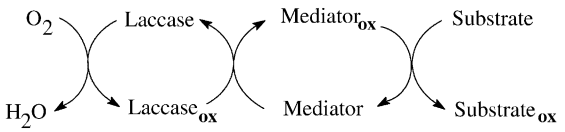

Fig. 1. The role of a mediator of the enzymatic activity.

to it [6]. As an example, veratryl alcohol (**4**) appears to be the natural mediator of the LiP activity of the fungus *Phanerochaete chrysosporium* [14], while 3-hydroxyanthranilic acid is reported to mediate the laccase activity of the fungus *Pycnoporus cinnabarinus* towards non-phenolic lignin structures [15]. In keeping with this idea, the discovery of new and efficient mediators of laccase could enable the attempt of an enzymatic bleaching of kraft pulps. We have, therefore, decided to study the mediation phenomenon with laccase. Although this topic has been already addressed in [10–13], we deemed that a more systematic investigation was needed. The efficiency of a number of mediators has been, therefore, compared with respect to a model reaction, and inferences about the operating mechanism have been made in the most promising mediation cases.

2. Experimental procedures

2.1. Enzyme preparation

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 [18], and an activity of 10,000 U/ml determined spectrophotometrically by the standard reaction with ABTS [19].

2.2. Materials

All the substrates and solvents were commercially available (Aldrich). Acetonitrile was purified by distillation over P_2O_5 for the electrochemical experiments.

2.3. Enzymatic reactions

The oxidation reactions were performed at room temperature in stirred water solution (3 ml), buffered at pH 5 (0.1 M in citrate) and purged with O_2 for 30 min prior to the addition of the reagents. The concentration of the reagents was: [substrate], 20 mM; [mediator], 6 mM, with 10 units of laccase. The reaction time was 24 h, in general. The yields of oxidation (Table 1) were determined by GC analysis with respect to an internal standard (acetophenone or *para*-methoxyacetophenone), suitable response factors being determined from authentic products. A Varian 3400 Star instrument, fitted with a 20 m × 0.25 mm methyl silicone gum capillary column, was employed in the GC analyses. The identity of the products was also confirmed by GC–MS analyses, run on a HP 5892 GC, equipped with a 12 m × 0.2 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV. The competition experiments of **1** and **3** were similarly run on a 40 mmol amount of each of the substrates

Table 1
Oxidation reactions of **1** by laccase/mediator systems: a comparison of efficiency[a]

Mediator	Yield of **2** (%)[b]
–	0
ABTS	22
ABTS without O_2	18
ABTS without laccase	0
ABTS at pH 3.5	51
HAA	2
MV	1
TPA	1
PT	3
VLA	65
HPI	70
HBT	76
HBT at pH 3.5	95
HBT without O_2	70
TEMPO	99
TEMPO without laccase	1
TEMPO without O_2	99
TEMPO at pH 3.5	99
4-Hydroxy-TEMPO	98
4-Acetamido-TEMPO	98
3-Carbamoyl-PROXYL-ene	28

[a] Conditions: [**1**] = 20 mM, [Mediator] = 6 mM, [laccase] = 3 U/ml, reaction time: 24 h at room temperature, pH = 5. The solution (3 ml) had been purged with oxygen for 30 min before the beginning of the reaction.

[b] GC yields were determined by the internal standard method, with respect to the molar amount of **1**.

Table 2
Reactivity of substrate with selected laccase/mediator systems[a]

Substrate, E^o (V; in H_2O)	Oxidised product	Yield (%) with		
		ABTS	HBT	TEMPO
3 (2.4)	**5**	2	30	92
1 (1.7)	**2**	22	76	99
4 (1.4)	**6**	37	92	99
1 + **3**[b]	**2** + **5**	30[c]	4.1[c]	1.9[c]
7	**8**	52	–	–
9	**10**	2	41	–
11 (ca. 1.7)	**2**	0	–	–
11 (ca. 1.7)[d]	**2**	0	–	–

[a] Experimental conditions as in Table 1; reaction time, 24 h.
[b] Competition experiment of a 40 mmol amount for each substrate.
[c] Reported as k_1/k_3 relative reactivity ratios (see Reaction (1)).
[d] For a double amount of the enzyme, and under an oxygen over-pressure; 72 h reaction time.

(Table 2) and the yields of products **2** and **5** determined after a suitable reaction time.

2.4. Electrochemical determinations

Cyclic voltammetry (CV) experiments were carried out with an Amel 5000 potentiostat, in a 0.01 M sodium acetate buffer at pH 4.7, containing 0.1 M $LiClO_4$ and 4% (v/v) of acetonitrile, with a glassy-carbon working electrode (planar disk, ∅ 3 mm), a platinum counterelectrode (surface, 1 cm^2) and a saturated calomel reference electrode (SCE). The concentration of the substrate was in the range of 0.5–2 mM.

3. Results

3.1. Mediation efficiency

Some mediators of laccase have been already described by several authors [10–13,20–27], but a satisfactory comparison of mediation efficiency is hampered by the fact that different reaction conditions were adopted and diverse substrates used. In order to enable a more systematic evaluation of the relative efficiency of the mediators, we have, therefore, selected the oxidation of 4-methoxybenzyl alcohol (**1**) as a benchmark reaction. This compound is a non-phenolic substrate and, as such, is not a natural target of laccase [15]. Consequently, our evaluation of the mediation activity is not masked by the spontaneous reactivity of the enzyme with **1**. Additionally, the structure of **1** mimics typical structural moieties of lignin, such as the cumaryl or the veratryl building blocks [28], or also some of the oligomers resulting from the oxidative degradation of wood pulp [13]. Therefore, any good mediator of the oxidation of **1** could be considered a promising candidate to explore a laccase-based bleaching procedure.

The oxidation reactions of **1** were performed at room temperature in a buffered (pH 5) water solution, previously purged with O_2 (Fig. 2).

The yield of 4-methoxybenzaldehyde (**2**) from **1** was determined by GC analysis. The results are reported in Table 1, while the structure and abbreviated names of the mediators are given in Fig. 3.

Inspection of Table 1 confirms the expected lack of reactivity of laccase alone with benzylic alcohol **1**. However, addition of mediator ABTS to the enzyme resulted in a significant conversion of **1** into aldehyde **2**. ABTS has been already described as an efficient mediator of laccase activity towards non-phenolic

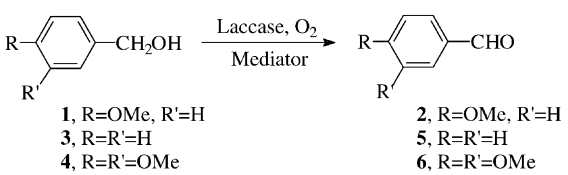

Fig. 2. Oxidations by the laccase/mediator systems.

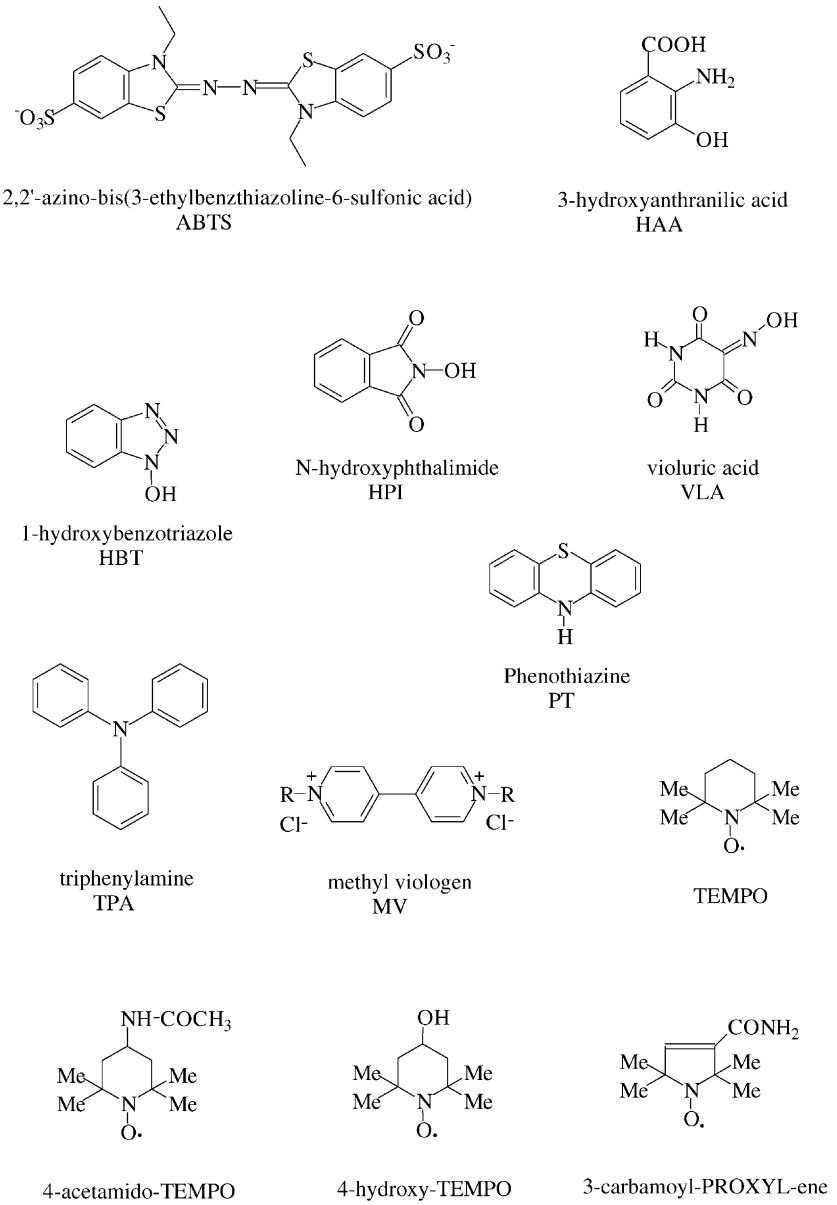

Fig. 3. Structure and shortened name of the mediators.

substrates [12,21,22,24,25]: Table 1 shows that it is not 'the best' mediator, though.

The lack of reactivity of ABTS in the absence of the enzyme confirms that laccase first oxidises ABTS, and then the oxidised mediator converts **1** to **2**, in keeping with the scheme of Fig. 1. The nature of the oxidised state(s) accessible to ABTS (i.e. the radical cation or the dication form) will be commented on later. However, the higher yield of **2**, obtained upon decreasing the pH of the solution from 5 to 3.5, in spite of the fact that the natural pH of laccase is around 5 [7], might indicate that the dication of ABTS is the form responsible for the oxidation of the substrate; in fact, $ABTS^{++}$ is more stable at acidic pH [16]. An

increase of mediation efficiency with ABTS at lower pH values had been already reported [12]. Finally, the effect of pre-purging with O_2 appears marginal upon the yield of **2**: this could be due to the modest amount of laccase we employed, that would be more than enough for carrying out the catalytic cycle at the expenses of the low amount of oxygen already present in the solvent.

In contrast to a literature claim [15], the 'natural mediator' HAA resulted ineffective in the oxidation of **1**. Equally not effective were triphenyl amine (TPA), methyl viologen (MV) and phenothiazine (PT), in spite of their capability to enter redox cycles. Another known mediator of laccase activity is HBT [12,13,23,26,27]: from Table 1 it results more efficient than ABTS in the oxidation of **1**. Similarly, effective is HPI, another *N*-hydroxy compound, that is known to be a good catalyst of the alkane oxidation with molecular oxygen, in combination with a Co(II) species [29]. Violuric acid (VLA) is at present under scrutiny as a redox catalyst [27,30]; it is an *N*-hydroxy compound as well, and did mediate the conversion of **1** to **2** to the same extent as HBT and HPI. Consequently, the *N*-hydroxy moiety proves an important structural feature for mediating the 'non natural' activity of laccase [27]. It seems likely that an *N*-oxyl radical, generated from this *N*-hydroxy moiety by laccase, is the active species in the oxidation cycle. It must be finally stressed out that the yields in Table 1 are calculated with respect to the molar amount of **1**, the molar amount of the mediator being only one-third than that. Accordingly, some yields of **2** become greater than 100%, if evaluated with respect to the mediator, then implying an oxidation process with turnover.

An obvious extrapolation of the simple hypothesis of an *N*-oxyl radical, as the reactive intermediate in the oxidations mediated by HBT, HPI (and VLA), led us to test TEMPO as a mediator of laccase. TEMPO is a stable *N*-oxyl radical, and is responsible for radical processes of wide synthetic interest [31–36]. More specifically, it has been described as a highly selective catalyst in the oxidation of alcohols with bis-acetoxyiodobenzene (BAIB) [37]. Table 1 shows that TEMPO gave indeed the best performance towards **1**; it does require laccase, thus, confirming its true role of mediator, as delineated in Fig. 1. Further elaboration on the structure of TEMPO led us to try also two of its derivatives, i.e. the 4-acetamido- and the 4-hydroxy-TEMPO, as well as a derivative of the five-membered *N*-oxyl radical PROXYL. In all these cases there was conversion of **1** to **2**, but the yields were at most comparable with that of TEMPO.

The results reported in Table 1 allow the assessment of the relative efficiency of twelve mediators of laccase: this is the first time that such a number of mediators is compared under the same conditions, and TEMPO emerges as the most efficient one. Clearly, a better understanding of this spread of mediation efficiency would require the knowledge of the oxidation mechanism accessible to the reactive species generated from each mediator (i.e. Med_{ox} in Fig. 1) upon its interaction with laccase. To this aim, we investigated additional substrates that could be informative on this respect.

3.2. Substrate reactivity

Mediation by ABTS is suggested to involve the oxidation of the substrate by an electron transfer (ET) step [10,16]. In this event, the reactivity of mediation should correlate with the oxidation potentials within a series of structurally homogeneous substrates, the easier to oxidise substrate owing to react more easily [18]. Benzyl alcohol (**3**) is less oxidisable (E^o 2.68 V/NHE in acetonitrile, and ca. 2.4 in H_2O) [38] than **1** (E^o 1.98 V/NHE in acetonitrile, and ca. 1.7 in H_2O) [39], due to the lack of methoxy group, whereas **4**, bearing two methoxy groups, is easier to oxidise (E^o 1.36 V/NHE in H_2O; in acetonitrile it should be ca. 1.6 V) [40]. Consistently, oxidation of **3** with the laccase/ABTS system (Table 2) under the same conditions described in Table 1, gave a negligible formation of benzaldehyde (**5**), while the yield of veratryl aldehyde (**6**) from **4** was even higher than that of **2** from **1**. This consistency between reactivity and redox potential, within a series of structurally homogeneous substrates, provides an experimental support to the hypothesis of an ET mechanism with the laccase/ABTS system. In contrast, with mediation by TEMPO, both the conversion of **3** to **5**, as well as that of **4** to **6**, occurred in very high yields, thereby suggesting the operation of an oxidation mechanism that does not respond to the redox features of the substrate. Mediation by HBT gave a somewhat intermediate result, because conversion to **5** was not

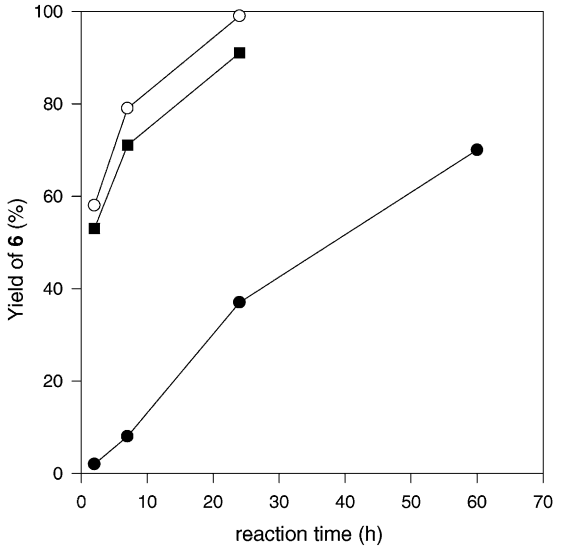

Fig. 4. Yields of aldehyde **6** from the reactions of three laccase/mediator systems: (○) TEMP; (■) HBT; (●) ABTS.

negligible, and that to **6** was high. This could be due to the operation of a third type of mechanism.

Fig. 4 very qualitatively shows that the profiles of oxidation yield versus reaction time for the three laccase/mediator systems towards substrate **4** are indeed rather different, and confirms the more efficient reaction with TEMPO.

A more quantitative evaluation of the reactivity with these laccase/mediator systems was attempted in competition experiments towards equimolar amounts of **1** and **3**. The relative reactivity (viz. intermolecular selectivity) of these two substrates was determined (as k_1/k_3) (Reaction (1)) by measuring the molar amounts of the corresponding oxidation products **2** and **5** from GC analyses (Table 2).

$$\begin{aligned} \mathbf{1} &\xrightarrow{\mathbf{k_1}} \mathbf{2} \\ \mathbf{3} &\xrightarrow{\mathbf{k_3}} \mathbf{5} \end{aligned} \qquad (1)$$

$$\frac{k_1}{k_3} = \frac{\ln[\mathbf{1}]_o/[\mathbf{2}]_t}{\ln[\mathbf{3}]_o/[\mathbf{5}]_t}$$

In the above equation, that is derived from the known kinetic scheme for competing processes [41] and holds true whenever the kinetic order of the two competing reactions is the same, $[\mathbf{1}]_o$ and $[\mathbf{3}]_o$ are the initial concentrations of the competing substrates, and $[\mathbf{2}]_t$ and $[\mathbf{5}]_t$ are the concentrations of the corresponding products after 24 h reaction time. A k_1/k_3 substrate relative reactivity equal to 4.1 was obtained with the laccase/HBT system. When this competition experiment was similarly run with the laccase/ABTS or laccase/TEMPO systems, k_1/k_3 reactivity ratios of 30 and 1.9, respectively, were obtained. This variety of results confirmed the dominant role of the Med_{ox} species (rather than that of laccase; see Fig. 1) in the interaction with the substrate, and hinted at an intermediate ability of mediator HBT to discriminate among substrates of different electronic activation.

A claim of the literature attracted our attention [24]. The laccase/ABTS system was reported to be unreactive towards 2,6-disubstituted benzyl alcohols, and the inference was made that at least one aromatic hydrogen atom ought to be present at the *ortho*-positions of the substrate for making the enzymatic reaction possible. This conclusion was based on the lack of oxidation of 2,6-difluorobenzyl alcohol. Since the laccase/ABTS system appears to respond to the redox potential of the substrate (see earlier sections), the choice of 2,6-difluorobenzyl alcohol did not appear sound to us, because the strong electron-withdrawing effect of two fluorine atoms would make this particular alcohol very resistant towards a one-electron oxidation [18]. We have instead taken 2,4,6-trimethoxybenzyl alcohol (**7**), where the *ortho*-positions are substituted by groups that are electron-donors, and exposed it to the laccase/ABTS system. Formation of 2,4,6-trimethoxybenzaldehyde (**8**) occurred in 52% yield. In contrast, reaction of 4-chlorobenzyl alcohol (**9**), that has free *ortho*-positions but an electron-withdrawing substituent in *para*, gave a negligible conversion to the corresponding aldehyde **10**. It is concluded that the laccase/ABTS system does accept 2,6-disubtituted benzyl alcohols, provided that the effect of the substituents does not depress the redox potential of the substrate, in agreement with the operation of the ET mechanism of oxidation. In sharp contrast, reaction of **9** with the laccase/HBT system gave oxidation to **10** in 41% yield, confirming the operation of a mechanism (possibly radical) that neglects the redox potential of the substrate.

Another report described an efficient oxidation of alkylbenzenes to aldehyde derivatives by the laccase/ABTS system [42]. Such a reaction did not occur, instead, under our conditions with

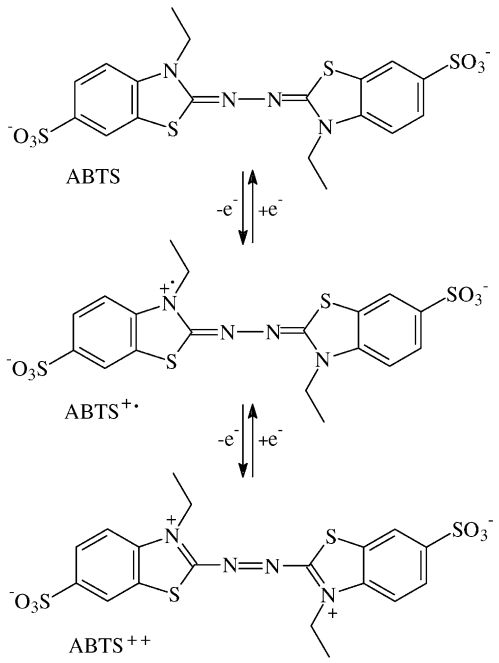

Fig. 5. The oxidised states of ABTS.

4-methoxyethylbenzene (**11**), even though the redox potential of alkylbenzene **11** [43] is comparable to that of **1**. This could be in part due to the lower amount of laccase present in our experiment. However, doubling the amount of laccase, and keeping the reaction flask under oxygen for 72 h [42], did not result in any conversion to **2**.

3.3. Electrochemical survey

Recently, a careful electrochemical characterisation of ABTS (Fig. 5) was carried out in aqueous solution (0.05 M citrate buffer, pH 4.0) with a glassy-carbon electrode ($\varnothing = 3$ mm) [16]. CV at 0.2 V/s showed a reversible one-electron oxidation of ABTS to $ABTS^{\bullet+}$, with $E^{o} = 0.472$ V versus Ag/AgCl; it was followed by a second reversible one-electron oxidation of $ABTS^{\bullet+}$ to $ABTS^{++}$, occurring at $E^{o} = 0.885$ V versus Ag/AgCl.

We have repeated these determinations, and analogously found $E^{o} = 0.69$ V for $ABTS^{\bullet+}$/ABTS, and 1.1 V for $ABTS^{++}$/$ABTS^{\bullet+}$, both data being versus NHE at a glassy-carbon electrode, in a 96/4 (v/v) water/acetonitrile mixed solvent. In the case of mediator HBT, instead, the Authors could not obtain a reversible potential, and reported only an E^{p} of 0.87 V versus Ag/AgCl for the oxidation of this compound at 0.5 V/s [16]. When repeating their experiment at a faster scan (2 V/s), we obtained a reversible oxidation, with $E^{o} = 1.08$ V/NHE. This suggests the occurrence of an ensuing chemical reaction from $HBT^{\bullet+}$, probably its deprotonation. The latter reaction could be prevented by reverting the sweep at a faster rate. CV of the two *N*-hydroxy compounds HPI and VLA, that also mediated the laccase activity towards **1** (Table 1), was analogously carried out. At 1 V/s, we could obtain a reversible oxidation potential ($E^{o} = 1.09$ V) for HPI; oxidation of VLA was instead reversible (i.e. 0.916 V) even at rather slow scans (e.g. 5 mV/s) [30]. Further electrochemical investigations are in progress, in order to better assess the electrochemical properties of the mediators and to hopefully obtain kinetic data for the ensuing chemical events (i.e. deprotonation steps) in the case of unstable Med_{ox} forms.

4. Discussion

If we refer to the role of the mediator outlined in Fig. 1, the following conclusions can be drawn. The redox potential of laccase is quite independent of the fungi it originates from, and is typically around 0.7–0.8 V [9]. Our determinations of E^{o} for ABTS (0.69 and 1.1 V), HBT (1.08 V), HPI (1.09 V), and VLA (0.916 V), indicate that the redox potential of the enzyme is high enough to convert all these mediators into an oxidised state (Med_{ox}) by one-electron abstraction. The occurrence of the catalytic cycle of Fig. 1 through the action of Med_{ox} is indeed corroborated by the observed oxidation of substrate **1** to **2** under mediation by ABTS, HBT, HPI or VLA. However, what is the structure of the Med_{ox} form for each of these mediators, and what kind of mechanism does it follow in the oxidation of the substrate? Taking ABTS as an example, its Med_{ox} form is found to oxidise benzyl alcohol **1** much better than the structurally similar alcohol **3** ($k_{\mathbf{1}}/k_{\mathbf{3}}$ of 30, Table 2), that differs only for a higher redox potential; this supports an ET mechanism of oxidation (see Fig. 6).

With this laccase/mediator system, an upper limit around 1.8 V does emerge for the redox potential of the substrate. In this scenario, if $ABTS^{++}$ ($E^{o} = 1.1$ V)

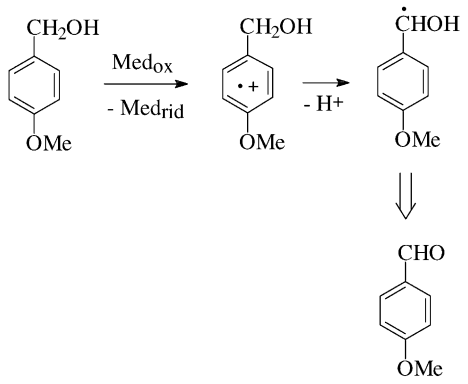

Fig. 6. The ET route.

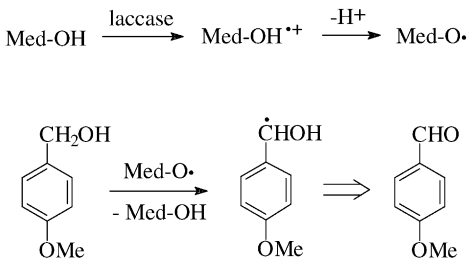

Fig. 7. The radical HAT route.

were the Med_{ox} form, a moderately endoergonic ET step would be required for the oxidation of substrates approaching the 1.8 V threshold value (such as **1**), while a much more endoergonic ET would be required if the Med_{ox} form were the weaker $ABTS^{\bullet+}$ (E^{o} = 0.69 V). It seems, therefore, likely that the Med_{ox} form of ABTS is the dication, but this statement will require additional experimental support from future investigations. We can only add, at present, that the stability of $ABTS^{++}$ is known to increase at lower pH values [16], at which we indeed find that the laccase/ABTS system gives a higher yield of **2** from **1** (Table 1). A similar ET mechanism (Fig. 6) could even be accessible to mediator VLA, that is electrochemically stable in its Med_{ox} form, whose redox potential (0.916 V) is close to that of $ABTS^{++}$. Finally, the lack of mediation activity with PT and MV is not surprising, because the Med_{ox} form ($E^{p} = 0.3$ V) of the former compound is too weak an oxidant [44], and the redox potential of MV ($E^{o} = -0.44$ V) is unsuited with respect to the redox potential of the substrates here investigated.

As for HBT, the efficiency this mediator shows with a substrate as difficult to oxidise as **3** would rather support a radical H-atom transfer pathway (HAT, in Fig. 7).

Although the initial step is the oxidation of HBT to $HBT^{\bullet+}$ by laccase, deprotonation of $HBT^{\bullet+}$ would follow, as the moderately irreversible cyclic voltammogram indicates, to give the *N*-oxyl radical. The latter eventually abstracts the benzylic hydrogen from the substrate, thereby giving rise to the aldehyde and producing HBT back. In this radical route, the redox potential of the substrate will have a very modest relevance on reactivity (see the experiment with **9** and the k_1/k_3 value, Table 2) The efficiency of this mediator does depend on the pH, as in the case of ABTS (Table 1), and we are presently investigating this point. The conclusion in favour of the radical mechanism with HBT is likely to hold also for the other *N*-hydroxy compound HPI, whose radical cation deprotonates as well as the one of HBT. The case of the other *N*-hydroxy compound, i.e. VLA, is instead uncertain yet. The electrochemical evidence suggests that it may give a stable radical cation on interaction with laccase, and therefore, it could oxidise substrates endowed with a suitable redox potential according to the ET route (Fig. 6). On the other hand, being VLA a *N*-hydroxy compound, it could alternatively follow the HAT route (Fig. 7) with difficult to oxidise substrates.

The mediators from the TEMPO family, that are 'stable' *N*-oxyl radicals, represent a more complex case. Their intervention in the oxidation of substrate **1** does require laccase (see Table 1), so that the oxidation is not simply due to the interaction of the substrate with the *N*-oxyl radical. One could suggest that the 'active' form of TEMPO is the oxoammonium ion [31,33], formed by oxidation of the *N*-oxyl radical form, as it is proposed in the TEMPO-catalysed oxidation of alcohols [36,37,45]. The oxoammonium ion would be responsible for the oxidation of the alcohol to aldehyde in a process that requires no ET, as the low k_1/k_3 selectivity (i.e. 1.9, Table 2) between substrates of different electronic activation indicates. Laccase would then regenerate TEMPO from the hydroxylamine form (Fig. 8), so that the oxoammonium ion can be restored either through acid-induced disproportionation of TEMPO, or through oxidation by laccase. Since laccase is re-oxidised by oxygen, this promising synthetic process represents a 'green' oxidation

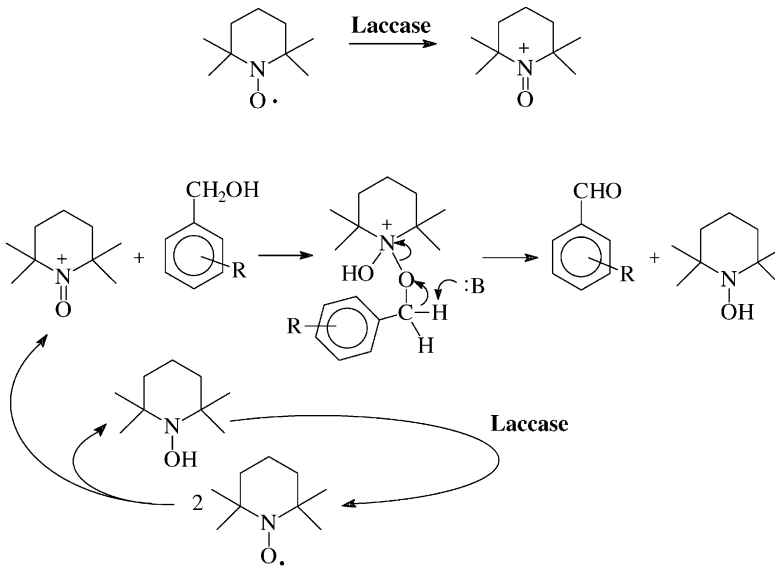

Fig. 8. Suggested mechanism of oxidation by TEMPO.

of alcohols by air, catalysed by the laccase/TEMPO system [45].

More in general, the present study allows to compare the efficiency of twelve mediators of laccase under the same experimental conditions, thereby enabling a more systematic knowledge of this topic; it also provides mechanistic evidence in favour of the possible operation of at least three oxidation routes with these mediators, depending on subtle characteristics of the substrate and of the mediator; it finally gives a more thorough electrochemical insight into the redox features of some of these mediators, that supports the mechanistic rationalisation. The results show that some laccase/mediator systems, both for their reactivity and for the oxidation mechanism they employ, are worth investigating in view of the development of new and environment respectful procedures for the bleaching of kraft pulps, in combination with the modern oxygen delignification techniques.

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