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Review Commentary

Chemical messengers: mediated oxidations with the enzyme laccase†

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ABSTRACT: The use of low molecular-weight compounds (viz., mediators) in combination with fungal laccase makes the enzyme suitable for the oxidation of 'non-natural' non-phenolic substrates. Benzyl alcohols are thus oxidised to carbonylic products by laccase/mediator systems in the presence of oxygen, although laccase cannot oxidise these substrates directly. The reaction is carried out by the oxidised form of the mediator (Med_{ox}), generated on its interaction with laccase, and the structure of the Med_{ox} species is crucial for the mechanism of the ensuing non-enzymatic oxidation of the substrate. 1-Hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI), violuric acid (VLA) and TEMPO have been investigated as mediators, and experimental evidence is provided that enables the radical hydrogen atom transfer route with the laccase/HBT, laccase/HPI and laccase/VLA systems to be assessed unambiguously, although the laccase/TEMPO system follows a different and ionic oxidation route. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: enzymes; laccase; radicals; radical ions; delignification; mechanisms; bond dissociation energies; Hammett treatments

INTRODUCTION

Hermes (or Mercury, in Latin) was the Greek god who acted as a 'messenger' between the kingdom of the Gods, on top of Mount Olympus, and the humans on Earth, enabling the two worlds to communicate. Moving to the realm of chemistry, it is essential to find a counterpart for the RNA-messenger, being responsible for communication between the world of genetic information stored in DNA and the 'protein factory' embodied by the ribosomes. There is, however, another important class of chemical messengers, which is perhaps less known but will represent the focus of this contribution. This is the class of chemical messengers that in Nature enable the oxidative degradation of lignin to take place in rotten wood by specialised enzymes, produced by basidiomycete fungi. Wood is an amazing and complex material, made of cellulose and lignin, interwoven with hemicelluloses. The fungi focus on cellulose as the source of energy for their metabolism. To fulfil this task they first need to get rid of lignin, and therefore excrete enzymes capable of performing an oxygen-dependent degradation of the lignin network (Fig. 1).[1,2] Lignin is a three-dimensional polymer and, as such, it would not fit into the active site of an enzyme, owing to the size difference. To solve this 'communication' problem between enzyme and substrate, and to make reactivity with lignin possible, the enzymes resort to messengers or, better, to mediators (as they are known in this context).[3,4] Lignin peroxidase, a heme-enzyme endowed with a redox potential as high as 1.4 V, employs the natural metabolite veratryl alcohol (i.e. 3,4-dimethoxybenzyl alcohol; VA) as a mediator.[1,5] The O_2-activated form of Lignin peroxidase oxidises VA by electron abstraction, and the resulting $VA^{\cdot+}$ (Med_{ox}) diffuses away from the active site, reaches lignin and carries out a mono-electronic oxidation of the polymer. In this way the oxidised mediator behaves as a 'messenger' (or, more precisely, as an electron shuttle) between the enzyme and the substrate, solving the communication problem between the two partners.

Subtler and less well known is the mediation phenomenon with the other ligninolytic enzyme, i.e. laccase, a 'blue copper' oxidase.[3,6] It contains four copper ions in the active site, enabling the mono-electronic oxidation of four molecules of substrate at the expenses of oxygen,

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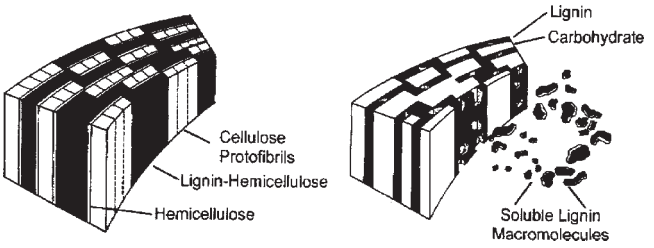
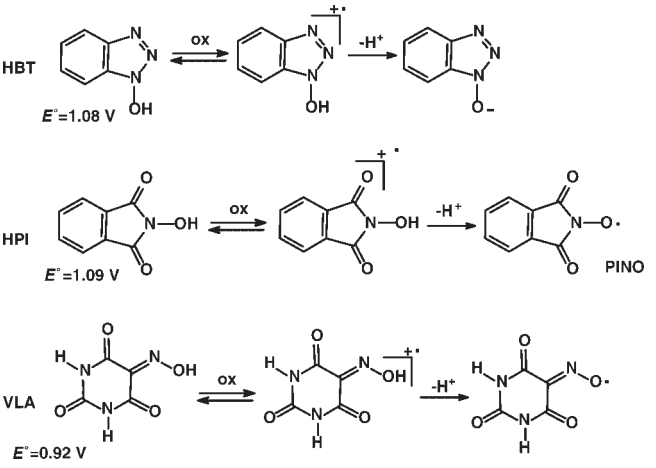


Figure 1. Degradation of lignin in wood

which is reduced to water. For its low redox potential of 0.5–0.8 V, laccase attacks the easily oxidisable phenolic residues of lignin, but these represent less than 20% of all the functional groups of the polymer. The benzylic alcohol and ether groups, which add up to about 70% of the residues of lignin, and that are more difficult to oxidise by electron transfer (redox potentials above 1.5 V), cannot be oxidised by laccase. Yet, many 'white-rot' fungi do not excrete the stronger oxidant Lignin peroxidase but produce laccase, and nevertheless can degrade lignin in wood efficiently.[6] To explain this paradox, the hypothesis has been advanced that low molecular weight, easily oxidisable natural metabolites can act as mediators (Med, in Scheme 1) of laccase.[4,6] So far, no natural mediator of laccase has been identified unambiguously, but non-natural mediators have been found and tested,[3,4,6–8] confirming the feasibility of the laccase/mediated oxidation of non-phenolic compounds, as delineated in Scheme 1.

The mediator is oxidised by the O_2-activated enzyme, and in turn oxidises the non-phenolic residues of lignin by resorting to oxidation mechanisms not available to laccase. We have gradually discovered that many oxidised mediators follow radical oxidation routes. However, when we entered this area of research the mechanistic details of the mediated oxidations of laccase were not known precisely, or had only been inferred from qualitative evidence. Because some of the non-natural mediators, in combination with laccase, were finding interesting applications, for example in synthetic procedures,[9] or in bioremediation of water effluents,[10] or also in the paper industry,[11] it seemed appropriate to try to understand the features of the mediation phenomenon more deeply. Our studies with laccase from the fungus *Poliporus pinsitus*,[8,12] combined with previous literature evidence, have shown that some valuable non-natural mediators share the N—OH structural feature (*N*-hydroxyphthalimide, HPI; violuric acid, VLA; 1-hydroxybenzotriazole, HBT). Another efficient mediator is 2,2′,6,6′-tetramethylpiperidine-*N*-oxyl (TEMPO), a well-known and stable aminoxyl radical. How and why do these species mediate the oxidation activity of laccase?



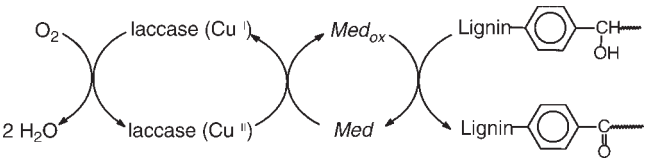
Scheme 2

In a preliminary electrochemical study,[8] we have provided independent evidence that the N—OH mediators can be oxidised mono-electronically at the electrode to give radical cations; the latter species deprotonate to aminoxyl radicals (> N—O˙, in Scheme 2).

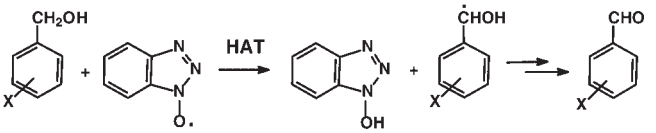
Among the aminoxyl radicals, Scheme 2 shows phthalimide *N*-oxyl (PINO), which is already known to synthetic chemists for its involvement in a radical oxidation procedure, the so-called Ishii reaction, being generated from HPI by chemical oxidants.[13] Thus, it could be suggested that *Poliporus pinsitus* laccase, in view of its redox potential (0.8 V), can oxidise the N—OH mediators to the same extent as appropriate chemical oxidants; the slightly uphill redox process would be driven to the right by deprotonation of the initially formed radical cation. The intermediate aminoxyl radical subsequently removes hydrogen atoms from the non-phenolic subunits of lignin, as the benzylic lignin model in Scheme 3 shows, thereby overcoming oxidation restrictions that benzyl alcohol residues, or similarly difficult to oxidise substrates, would cause to laccase in electron transfer (ET) routes.

We have demonstrated this radical hydrogen-atom transfer (HAT) route of oxidation with laccase and N—OH mediators through several mechanistic tests.[8,12]

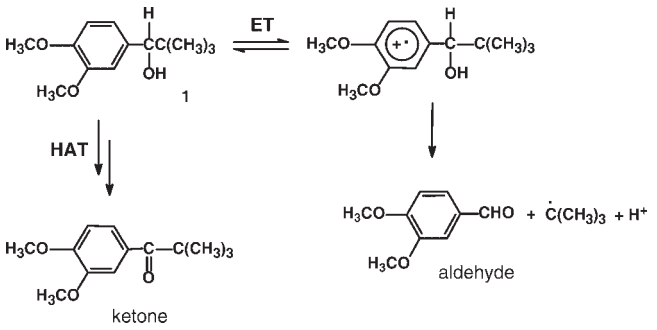
Evidence is, for example, provided by the oxidation of a particular benzylic alcohol (**1**, Scheme 4), which laccase cannot oxidise on its own.[12]



Scheme 1



Scheme 3



Scheme 4

In a genuine ET route with chemical oxidants or by anodic oxidation, **1** gives the radical cation that undergoes exclusive cleavage of the Cα—-Cβ bond, with the formation of an aldehyde product only. In contrast, in a genuine radical process, **1** gives the exclusive cleavage of the Cα—H bond, with the formation of a ketone having an intact side-chain. Probe substrate **1** was then oxidised with laccase and N—OH mediators, and the ketone was obtained exclusively, in keeping with a radical route through the intermediate aminoxyl radical (Scheme 3); the remainder of the mass balance in Table 1 consists of recovered **1**. In order to stress the contrast, the *bona fide* ET oxidation of **1** with a CoIII complex gave the exclusive formation of the aldehyde product. Hence, obtaining two different products is clear-cut evidence for the operation of two different oxidation mechanisms.

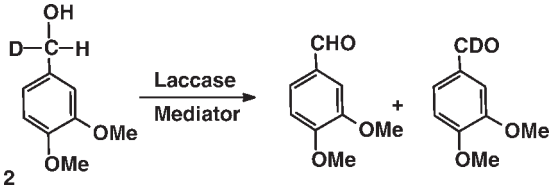
Another piece of evidence in favour of the radical HAT mechanism of oxidation with laccase and N—OH mediators comes from the determination of the intramolecular kinetic isotope effect in the oxidation of the α-deuteriated benzyl alcohol **2** (Scheme 5 and Table 2).[12]

Mass analysis provided the relative amounts of the two aldehydes produced, and Table 2 shows that the k_H/k_D ratio is large and close to the maximum value with the

Table 1. Laccase-mediated oxidations of probe substrate **1**

Mediator[a]	Oxidation product (%)
HBT	Ketone, 50
HPI	Ketone, 70
VLA	Ketone, 20
CoIII	Aldehyde, 15

[a] [**1**] 20 mM [Med] 6 mM, [laccase] 3 U ml^{-1}, under O_2, 24 h, pH = 5, T = 25 °C.

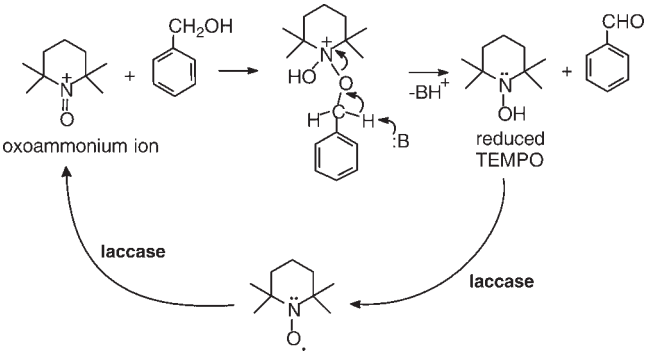


Scheme 5

Table 2. Intramolecular kinetic isotope effect determinations for the oxidation of substrate **2**

Oxidant	k_H/k_D[c]
CoIII[a]	3.8
Laccase + VLA	6.4
Laccase + HBT	6.4
Laccase + HPI[b]	6.2

[a] [CoIII]:[**2**] 2:1, pH 5, reaction time 24 h at 25 °C.
[b] [**2**] 20 mM, [Med] 6 mM, [laccase] 3 U ml^{-1}, pH 5, reaction time 24 h at 25 °C, under O_2.
[c] Determined by GC-MS.



Scheme 6

three N—OH mediators we suggested for the radical oxidation route. This large value is as expected, since removal of either H$^{\cdot}$ or D$^{\cdot}$ is the only and rate-determining step of the radical route. In contrast, in the *bona fide* ET route, the k_H/k_D ratio has a smaller value because mono-electronic oxidation of the substrate occurs first, followed by loss of either H$^+$ or D$^+$ (Scheme 4).[12]

TEMPO, another laccase mediator,[8] will now be discussed. This aminoxyl radical takes a different oxidation route with non-phenolic substrates. The route is ionic (Scheme 6), and has precedents in the efficient oxidation procedures of alcohols by TEMPO with chemical oxidants.[14] The chemical oxidants, as well as laccase, oxidise TEMPO to the oxammonium ion, which is attacked by the substrate as a nucleophile, to give an adduct. Removal of an α-proton leads to the oxidation product and to reduced-TEMPO (N—OH), which is oxidised back to TEMPO (N—O$^{\cdot}$) and then to the oxoammonium ion.

What evidence is there in favour of this ionic route with laccase/TEMPO? By running competitive oxidations of a substituted [Eqn (1)] vs unsubstituted benzyl alcohol pairwise, we found that the relative rates (k_X/k_H) determined on product formation (i.e. aldehydes ArCHO and PhCHO) correlate with the inductive σ-parameter (σ_I) in a bell-shaped plot (Fig. 2).[15]

$$ArCH_2OH + Med_{ox} \xrightarrow{k_X} ArCHO + Med \qquad (1)$$

In the region of the electron-withdrawing (E.W.) substituents, the nucleophilicity of the alcohol towards

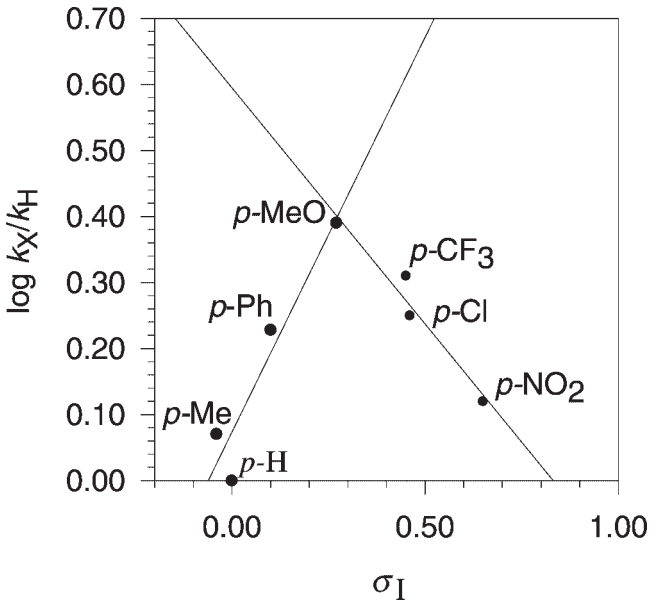


Figure 2. Hammett plot for the oxidation of 4-X-substituted benzyl alcohols with the laccase/TEMPO system, in competitive experiments with benzyl alcohol

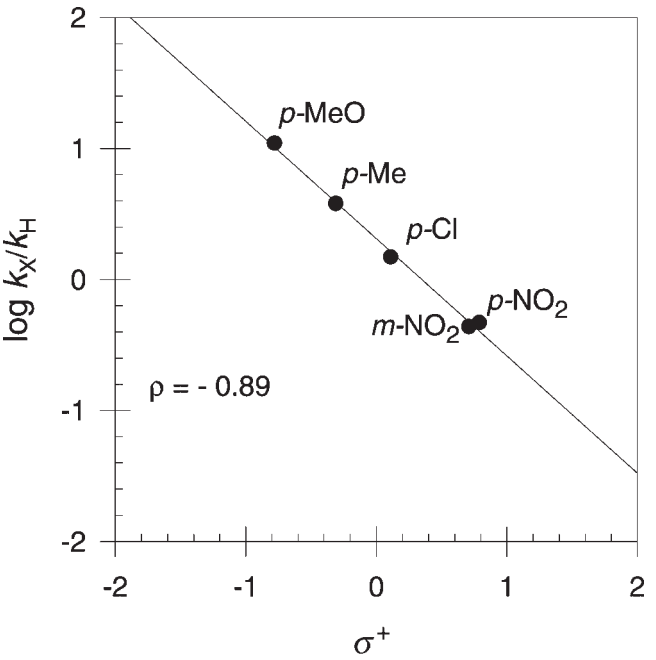


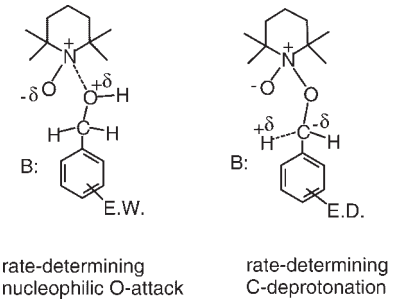
Figure 3. Hammett plot for the oxidation of 4-X-substituted benzyl alcohols with the laccase/HPI system, in competitive experiments with benzyl alcohol

TEMPO-oxoammonium is depressed more (Scheme 6) the stronger the substituent effect. On moving to the electron-donor (E.D.) substituents, the nucleophilicity of the alcohol increases and its addition becomes faster, so that the rate-determining step is the deprotonation of the adduct; this is made more difficult the more electron-donating the substituent is (Scheme 7). The kinetic isotope effect for the deprotonation of the monodeuteriated benzyl alcohol substituted in the *para*-position by the E.D. methyl group is consistently 2.2.[15]

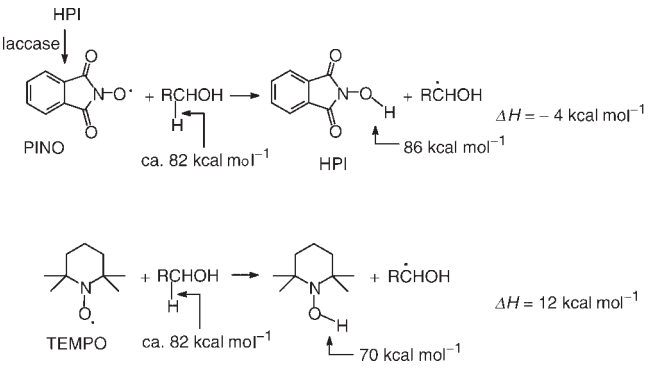
It is appropriate to comment here on the contrast with similar Hammett treatments obtained for the oxidation of substituted benzyl alcohols with laccase and the N—OH mediators:[12] these gave linear plots vs the σ^+ parameter (shown in Fig. 3 for HPI), in keeping with the slight electrophilic character of the N—O$^{\cdot}$ reactive intermediate.

A reasonable question then arises. Why does TEMPO, a stable aminoxyl radical, not follow the same HAT radical route (Scheme 3) pursued by the transient aminoxyl radicals deriving from the N—OH mediators? Thermochemical considerations help to understand this difference (Scheme 8). The enthalpic variation for removal of a hydrogen atom by, for example, the aminoxyl radical PINO is favourable, owing to the fairly strong O—H bond that is formed. In contrast, a radical route is not enthalpically favourable for TEMPO with the same substrate, because of the much weaker O—H bond that would be formed; and as the oxidation of TEMPO to oxoammonium ion by laccase takes place easily, the ionic mechanism takes over. Why does this not apply to the other aminoxyl radicals? Because their oxidation to the corresponding oxoammonium ion would occur at a higher redox potential (>1.3 V), not attainable by laccase. This explains the difference between the two types of mediated oxidations.

The described mechanistic investigation has enabled a possible application of laccase/mediator systems to paper



Scheme 7



Scheme 8

manufacture to be sought. There is in fact an interesting parallel with Nature. The ligninolytic enzymes degrade lignin in wood, and spare cellulose for the metabolism of the fungus, giving us a remarkable example of a 'green' unpolluting oxidation. Analogously, paper manufacture requires that lignin is removed from the wood pulp without endangering the fibre of the cellulose, but the industry uses polluting chemicals to attain this goal. It would be highly desirable if the paper industry could mimic Nature and use a laccase/mediator system that can selectively oxidise lignin from wood pulp in a clean way. Is this possible?

We have attempted the oxidation of gel beads containing pure cellulose and lignin, using laccase and mediators. Analysis of the products by size-exclusion chromatography revealed that the oxidation by laccase/TEMPO was not selective, because both cellulose and lignin were attacked. TEMPO–oxoammonium is indeed known to oxidise not only benzyl alcohols, which are simple chemical models of lignin, but also aliphatic alcohol,[14] which mimics the sugars, according to the ionic mechanism of Scheme 6. In contrast, the oxidation of the gel beads with laccase and N—OH mediators was more selective, because only lignin was attacked: VLA proved to be the most efficient mediator. This is consistent with the radical HAT route followed by the N—OH mediators (Schemes 3 and 8). In fact, the intermediate aminoxyl radical oxidises the benzylic alcohol (viz. lignin) while leaving the alkanol (viz. cellulose) unchanged, owing to the selective cleavage of the weaker [ca $82\,\text{kcal mol}^{-1}$ ($1\,\text{kcal} = 4.184\,\text{kJ}$)] benzylic C—H bond with respect to the stronger (ca $94\,\text{kcal mol}^{-1}$) aliphatic C—H bond of the sugar, as enthalpic evidence confirms.[15] Thus, this is a radical procedure that shows promise for a selective delignification of wood pulp, because it preserves the cellulose.

This has recently been confirmed by the direct oxidation of samples of wood chips from a mill, carried out with laccase and VLA.[16] Delignification was shown to be both extensive and selective, as assessed on the reacted sample by suitable industrial tests, such as the determination of the kappa number (which decreases in value) and of viscosity (which remains constant), respectively (Fig. 4). In contrast, oxidation of wood chips by laccase and TEMPO is again unselective, because the kappa number decreases with respect to the initial value, thereby confirming delignification, but the value of the viscosity also decreases, indicating a concomitant cleavage of the cellulose.

In conclusion, appropriate industrial pre-treatments of wood chips with laccase and a mediator that follows a radical oxidation route, such as VLA, can lead to significant and selective delignification. Subsequent modern stages of oxygen-delignification complete the paper making process, in an efficient and environmentally friendly alternative to conventional and polluting procedures. The accomplishment of this clean strategy, if it will ever find practical use, has been made possible by a fundamental physical-organic investigation of the mechanism operating.

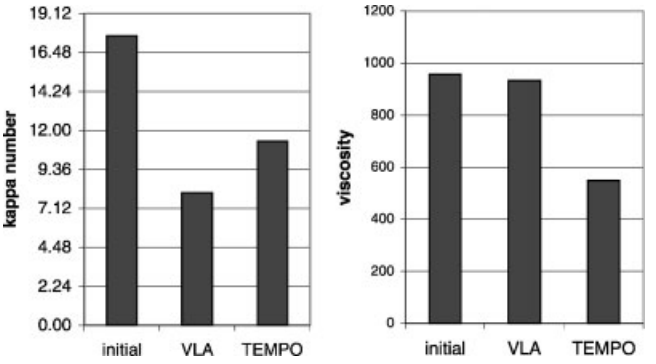


Figure 4. Effect of laccase/mediator treatments on wood chips: kappa number (lignin content) and viscosity (cellulose content) assays

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