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Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase–mediator systems

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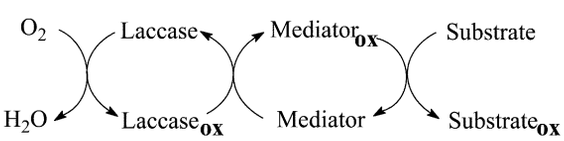
The oxidation of benzyl alcohols with the enzyme laccase, under mediation by appropriate mediator compounds, yields carbonylic products, whereas laccase can not oxidise these non-phenolic substrates directly. The oxidation step is performed by the oxidised form of the mediator (Med_{ox}), generated on its interaction with laccase. The Med_{ox} can follow either an electron transfer (ET) or a radical hydrogen atom transfer (HAT) route of oxidation of the substrates. Experimental evidence is reported that enables unambiguous assessment of the occurrence of either one the oxidation routes with each of the investigated mediators, namely, ABTS, HBT, HPI and VLA. Support to the conclusions is provided by (i) investigating the intermolecular selectivity of oxidation with appropriate substrates, (ii) attempting Hammett correlations for the oxidation of a series of 4-X-substituted benzyl alcohols, (iii) measuring the kinetic isotope effect, (iv) investigating the product pattern with suitable probe precursors. Based on these points, a HAT mechanism results to be followed by the laccase–HBT, laccase–HPI and laccase–VLA systems, whereas an ET route appears feasible in the case of the laccase–ABTS system.

Introduction

Laccase is a family of 'blue-copper' oxidase proteins containing four copper ions, and excreted by white-rot fungi under ligninolytic conditions. Laccase cooperates with other enzymes in the degradation of lignin in wood. With respect to lignin peroxidase (LiP)[1] and manganese peroxidase (MnP),[2] that are more powerful oxidants, laccase has a lower redox potential (*ca.* 0.7–0.8 V/NHE)[3,4] and therefore is able to catalyse single-electron oxidation steps only with the easy-to-oxidise phenolic constituents of lignin, with the concomitant reduction of O_2 to water. However, the activity of laccase can be fostered and expanded towards more difficult to oxidise non-phenolic substrates by the use of appropriate mediators.[5–7] The role of mediators in laccase oxidations is outlined in Scheme 1.



Scheme 1 The role of a mediator on laccase activity.

In general, a mediator could be a sort of 'electron shuttle' that, after being oxidised by the enzyme, diffuses away from the active site to oxidise any substrate that, for its size, could not enter the enzymatic pocket directly.[8,9] In addition, the oxidised form of the mediator (Med_{ox}), being structurally 'diverse' from the enzyme, might rely on a different mechanism of oxidation, thereby extending the range of substrates susceptible to the enzymatic action.[7,10] For example, 3-hydroxyanthranilic acid (HAA) has been reported to promote the laccase activity of the fungus *Pycnoporus cinnabarinus* towards non-phenolic lignin structures.[11] Suitable enzyme–mediator systems could also enable the environmentally benign (*i.e.*, chlorine free) bleaching process of kraft pulps for the paper industry.[5b,12] Understanding the role and mechanism of action of these mediators is a practical issue, that however requires a knowledge of fundamental reactivity features; the latter can be addressed by the typical experimental approach of physical organic chemistry.

We have undertaken a systematic investigation of the mediation phenomenon with laccase, and have already reported on a comparative evaluation of the efficiency of a number of mediators in the oxidation of non-phenolic lignin model compounds, such as the benzylic alcohols.[7] The mediators ABTS, HBT, HPI, VLA (structures and abbreviated names in Fig. 1) and TEMPO, for various reasons, presented the more

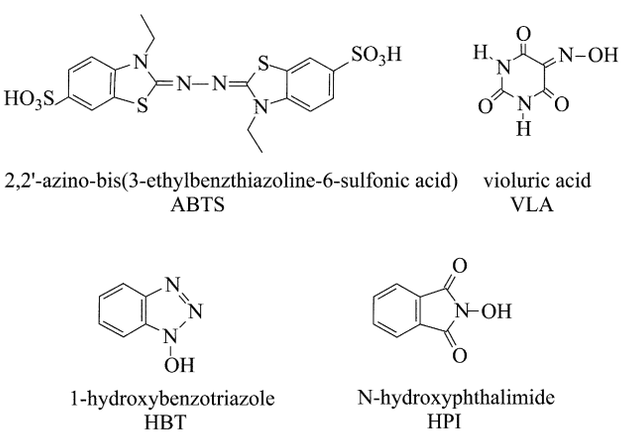


Fig. 1 Structure and abbreviated name of the mediators employed.

promising features as catalysts in this oxidation. A simple and synthetically-attractive oxidation of alcohols to carbonyl compounds by oxygen, induced by the laccase–TEMPO system, has been developed from the previous investigation.[13]

In the present paper we report on new advances in the interpretation of the reactivity features of laccase–mediator systems, and will particularly focus on the mechanism that the oxidised form of the mediator can employ in the oxidation of non-phenolic substrates. The possibility exists that the Med_{ox} species abstracts either one-electron (ET route) or a H-atom (HAT route) from the substrate, depending on the reactivity propensity of the latter.[10] In order to clarify this important point, the following experimental evidence has been acquired: (i) reactivity trends or intermolecular selectivity with significant substrates (key features: redox potential, stereoelectronic effects); (ii) product patterns with suitable probe precursors, which unambiguously indicate either an electron-transfer or a radical mechanism of oxidation; (iii) effect of substituents in

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Table 1 Oxidations with laccase–mediator systems: products and yields (see eqns. (1) and (2))

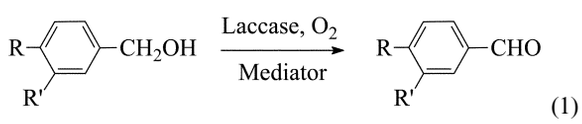
Substrate ($E°$, in V/NHE, in H_2O)[a]	Product yield (% *vs.* substrate)			
	With ABTS	With HBT	With HPI	With VLA
Benzyl alcohol, **1** (2.4)	**2**, 2	**2**, 30	**2**, 54	**2**, 42
4-MeO-benzyl alcohol, **3** (1.8)	**4**, 22	**4**, 76	**4**, 65	**4**, 65
Veratryl alcohol, **5** (1.4)	**6**, 37	**6**, 92	**6**, 74	**6**, 61
1-(4-MeO-phenyl)ethanol, **7** (1.4)	**8**, 15	**8**, 49	—	**8**, 82
Methyl veratryl ether, **9** (*ca.* 1.7)	0	**6**, 57; **10**, 25	**6**, 38; **10**, 15	**6**, 24; **10**, 1
4-MeO-ethylbenzene, **11** (*ca.* 1.7)	0	**7**, 8; **8**, 20	**7**, 4; **8**, 11	**7**, 1; **8**, 1
k_3/k_1 relative rate[b]	*30*	*4.1*	*10*	*2.2*
k_3/k_7 relative rate[b]	*1.5*	*0.7*	*0.8*	*0.4*

[a] The source of the $E°$ data,[22] and a few experimental details, were already given.[7] [b] In competition experiments.

the oxidation of benzyl alcohols (Hammett correlation); (iv) determination of the kinetic isotope effect. The results obtained allow us to come to conclusions about the oxidation mechanism adopted by the various laccase–mediator systems. The case of mediator TEMPO, in view of its peculiar and *ionic* oxidation route, is the object of a separate investigation.[14] Our previous experience in the case of competing electron-transfer *vs.* radical routes of oxidation was certainly helpful in the approach to the present problem.[15]

Results and discussion

Purified laccase from *Trametes villosa* (*viz. Poliporus pinsitus*)[7] was employed in the experiments. These were performed at room temperature in a stirred water solution, 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.[7] A first set of substrates was investigated, that could provide information about the oxidation mechanism, as well as hints about any possible substrate-specialisation of the mediator. The yield of oxidation (Table 1) was determined by GC analysis, and calculated with respect to the molar amount of the *substrate*. It must be stressed that, in contrast to similar reports in the literature, a *deficiency* of mediator is used in our experiments, in combination with a very small amount of laccase, so that the overall oxidation of the substrate by oxygen (eqn. (1) and Scheme 1) is truly *catalysed* by the laccase–mediator system adopted.[7,16] In fact, yields in excess of 100% are obtained if calculated *vs.* the molar amount of the *mediator*, thereby implying an oxidation process with turnover.



(1)

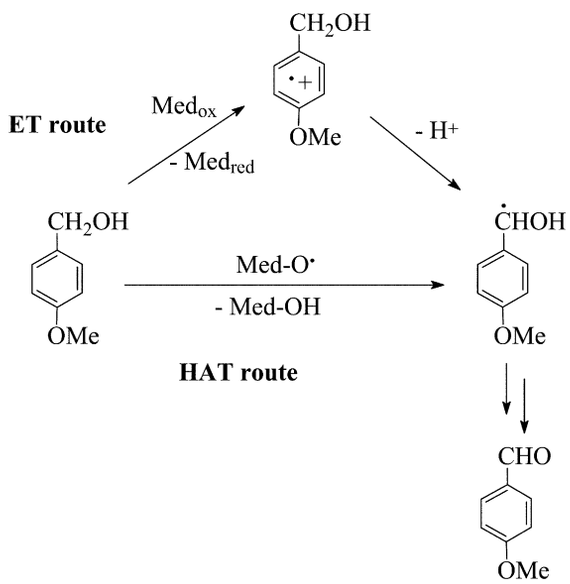
1, R=R'=H
3, R=OMe, R'=H
5, R=R'=OMe

2, R=R'=H
4, R=OMe, R'=H
6, R=R'=OMe

Reactivity of substrate

The concentration of the reagents was: [substrate], 20 mM; [mediator], 6 mM, with 10 units of laccase. The reaction time was 24 h, even though a reaction time of 7–8 h was previously found sufficient in most cases.[7] Three benzylic alcohols (**1**, **3** and **5**) were considered as significant structural models of lignin (eqn. (1)). It must be stressed that, in the absence of mediators, laccase alone does not oxidise these non-phenolic precursors, as expected, nor can the mediators alone do it, in the absence of the enzyme.[3,7,17] The three benzyl alcohols are mechanistically significant in that they differ in redox potential ($E°$ in V/NHE in H_2O given in Table 1),[7] as brought about by the different number of electron–donor methoxy substituents in the aromatic ring.

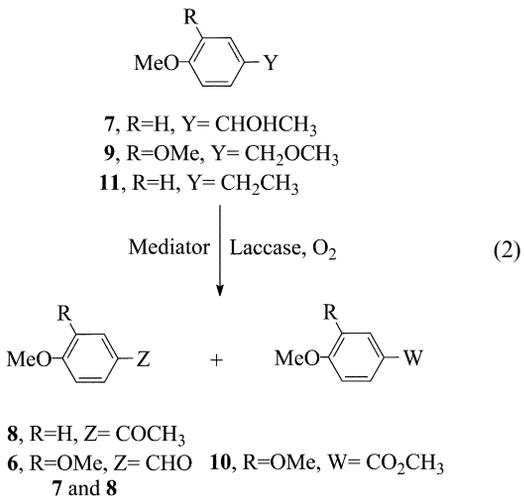
ABTS–laccase. ABTS is the most common mediator of laccase activity (Scheme 1),[5c,18] but not the most efficient one.[7] In our experiments (Table 1) the yields in oxidised products (*i.e.*, aldehydes **2**, **4** and **6**) strongly depend on the redox feature of the substrate, the extent of conversion with the easier to oxidise **5** being higher than that with **1** and **3**, when the reaction times are the same. A more quantitative determination of this intermolecular selectivity was attempted in a competitive oxidation of **1** and **3** by laccase–ABTS, where a k_3/k_1 relative rate of 30 was obtained. This consistency between redox potential of the substrate and conversion to products supports the operation of an ET mechanism (Scheme 2), where the initial monoelectronic



Scheme 2 Mechanisms of oxidation.

oxidation of the benzylic alcohol by the oxidised mediator (being the faster with the precursor of lower redox potential) is followed by a fast C–H deprotonation of the intermediate radical cation of the alcohol,[19] that drives the conversion to aldehyde.

Competition of a primary (**3**) *vs.* secondary (**7**) benzylic alcohol (eqn. 2) was additionally carried out, and a k_3/k_7 relative rate of 3 obtained. This result is also consistent with the ET route of Scheme 2, because stereoelectronic effects are known to retard deprotonation from the radical cation of an encumbered (secondary) alkylaromatic substrate,[15] such as **7**. As for the nature of the Med_{ox} species deriving from ABTS on interaction with laccase,[20,21] either $ABTS^{\bullet+}$ or $ABTS^{++}$ ($E°$ = 0.69 and 1.1 V/NHE, respectively)[7] could be generated by the enzyme, whose redox potential is around 0.7–0.8 V.[4] Clearly, if the Med_{ox} were the $ABTS^{++}$ species, the subsequent monoelectronic oxidation of appropriate substrates, such as **3**, **5** and **7** would occur in a moderately endoergonic ET step, whereas it had to be much more endoergonic if the Med_{ox} were the weaker $ABTS^{\bullet+}$.[16] It appears therefore *likely* that the Med_{ox} form of ABTS is the dication.[10,20] It is finally worth emphasizing the



lack of reactivity of the laccase–ABTS system with a benzylic ether (**9**) or with an alkylbenzene (**11**) (eqn. (2)), despite the fact that the redox potential of **9** and **10** is close to that of **3**[22] which reacted satisfactorily. A specificity of this mediator towards benzylic alcohols would therefore emerge.[16]

Laccase–N–OH mediators. The mediators HBT, HPI and VLA share the structural feature of being N–OH derivatives (Fig. 1). Our previous investigation had documented their good mediation efficiency with laccase, even though not as good as that of TEMPO.[7] These mediators, *at variance* with ABTS, are able to react even with the difficult to oxidise **1**, the conversion gradually increasing with **3** and **5**. A radical H-abstraction route of oxidation can be inferred (HAT, in Scheme 2),[7] where the redox features of the substrate have marginal importance.[10] The initial step of the HAT process would be the conversion of the mediator into a radical cation by monoelectronic enzymatic oxidation. The $E°$ redox potential of HBT, HPI and VLA (1.08, 1.09 and 0.92 V/NHE, respectively)[7] supports the likelihood of such oxidation by laccase ($E°$ 0.7–0.8 V).[4] Deprotonation of the radical cation of the mediator then follows, to give the corresponding *N*-oxyl radical (Med-O$^{\bullet}$).[7] The latter abstracts the benzylic hydrogen from the substrate, thereby giving rise to the aldehyde.[23–25] It is worth noting that the bond dissociation energy (BDE) of the α C–H bond in a benzyl alcohol (*ca*. 85 kcal mol^{-1})[26] is much lower than the BDE of the O–H bond (*ca*. 104 kcal mol^{-1}),[27] and therefore occurs exclusively.[25] In fact, the corresponding ether (**9**), that obviously lacks the O–H bond of **5**, can be oxidised by the laccase–N–OH mediator systems (*vide infra*) equally well.

In the HAT radical route (Scheme 2), the redox potential of the substrate ought to have a negligible relevance on reactivity. However, effects arising from the polarity of the *N*-oxyl radical, an electrophilic radical,[24–26] and from the electron-richness of the substrate, could provide a dipolar stabilisation to the radical transition state of H-abstraction, due to the important contribution from a charge-separated resonance structure,[28,29] thereby explaining the slightly higher oxidation yields obtained with substrates **3** and **5**, that bear electron-donor groups. Anyhow, such polar effects are small, as the k_3/k_1 relative rates of 10, 4.1 and 2.2 with HPI, HBT and VLA, respectively, do confirm (compare with the value of 30 for ABTS). Support to the HAT route also comes from the value of the primary *vs*. secondary (k_3/k_7) selectivity, which is *lower than 1* with HBT, HPI and VLA (0.7, 0.8 and 0.4, respectively), rather than 1.5 as obtained with ABTS. An easier cleavage of a secondary *vs*. primary benzylic C–H bond is indeed expected in a radical route, based on the pertinent $BDE_{(C-H)}$ values of 88.0 and 85.4 kcal mol^{-1}, respectively.[15] Finally, the conversion into oxidised products from both the ether **9** and alkylbenzene **11** is appreciable, and opens up synthetically interesting possibilities.[16] Peculiar is the additional formation of the methyl ester **10** from **9**, besides the aldehyde **6**. Once again, a hint to a possible mediator-to-substrate specialisation emerges, as HBT proves more competent towards the ether moiety, whereas VLA proves more competent towards a secondary alcohol.

A final comment on these sections is that the observed variety of results not only provides support to the operation of two different mechanisms of oxidation (ET *vs*. HAT, in Scheme 2), but also confirms the dominant role of the Med_{ox} species (rather than that of laccase; see Scheme 1) in the interaction with the substrate. Clearly, the 'natural' phenol-oxidase activity of laccase is profoundly affected from its interaction with the mediators.[16]

Hammett correlation

A more extensive and quantitative treatment of the effect of substituents upon reactivity in the oxidations induced by laccase–mediator systems was sought through a Hammett-type correlation. Five X-substituted benzyl alcohols, *i.e.*, 4-NO_2- (***p*-12**), 3-NO_2- (***m*-12**), 4-Cl- (**14**), 4-Me- (**16**), 4-MeO–$C_6H_4CH_2OH$ (**3**), were oxidised pair-wise in competition experiments *vs*. the unsubstituted precursor (**1**) as the relay compound (eqn. (3)),[25] and the k_X/k_H ratios determined by determining the relative amounts of the corresponding aldehydes (***p*-13**, ***m*-13**, **15**, **17**, **4**, and **2**, respectively) by GC analysis (Table 2). The log k_X/k_H ratios gave better fits when plotted *vs*. the $\sigma+$ rather than the σ of the substituents,[30] and the resulting ρ correlation parameters of the four laccase–mediator systems are given in Table 2; the example of the laccase–ABTS correlation is presented in Fig. 2.

These ρ parameters are admittedly rather small and similar. Nevertheless, it cannot be denied that the ρ values of HBT, HPI and VLA are the smaller ones, as it ought to be for a HAT mechanism.[25] In fact, small ρ values are obtained in unam-

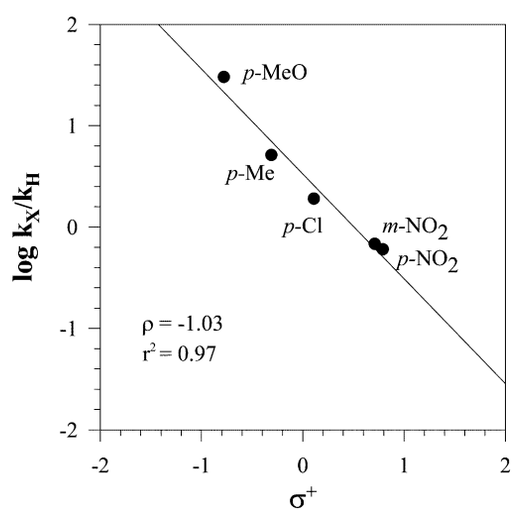
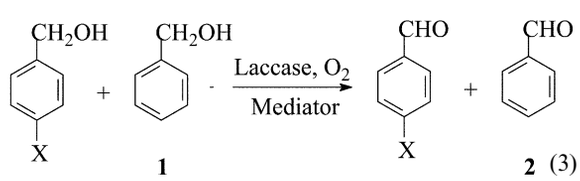


Fig. 2 Hammett correlation for the laccase–ABTS oxidation of substituted benzylic alcohols: competition experiments.



3, X=OCH_3 **4**, X=OCH_3
12, X=NO_2 **13**, X=NO_2
14, X=Cl **15**, X=Cl
16, X=CH_3 **17**, X=CH_3

Table 2 Determination of ρ values for the laccase-mediated oxidations of X–$C_6H_4CH_2OH$[a]

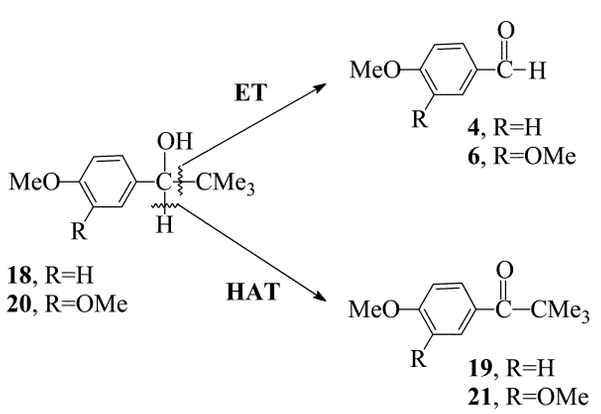
Mediator	$k_{p\text{-}NO_2}/k_H$	$k_{m\text{-}NO_2}/k_H$	k_{Cl}/k_H	k_{Me}/k_H	k_{MeO}/k_H	ρ
ABTS	0.60	0.67	1.9	5.1	30	−1.03
HBT	0.68	0.59	1.7	2.6	6.6	−0.64
HPI	0.47	0.44	1.5	3.8	11	−0.89
VLA	0.57	—	0.84	1.4	2.5	−0.41

[a] Typical error of the GC determinations: ±3%. Runs in triplicate.

biguous radical processes,[29] such as the reactions of substituted toluenes with bromine or NBS[28] or with $Cl_3C^{\bullet}$,[31] with ρ values in the range of −0.3 to −1.5. Indeed, the relevance[24–26] of polar effects on the Med–O$^{\bullet}$ electrophilic radicals, resulting from these N–OH mediators, would be responsible for making the substituent effects of these laccase-mediated oxidations more or less sensible,[25] and making the absolute values of the ρ parameters of VLA < HBT < HPI 'significant'. The smaller ρ value determined for VLA is consistent with the smaller k_3/k_1 and k_3/k_7 values (Table 1) obtained with this particular mediator, and would suggest that the corresponding Med–O$^{\bullet}$ intermediate is the one with the lower polar character. In the contest of polar radicals it is indeed common to find better correlations of the log k_X/k_H data with the σ^+, rather than with the σ of the substituent.[28,29,31] On the other hand, in the ET route of mediator ABTS (Scheme 2), the effect of the substituents on the reactivity of oxidation plays a divergent role in the two steps of the reaction.[19] In fact, removal of electrons from the substrate by Med_{ox} to form a radical cation is favoured by electron-donor substituents, whereas the ensuing deprotonation of the substrate radical cation is disfavoured by electron-donor substituents. The two effects tend to compensate each other, and this might explain the small ρ value obtained here with ABTS; anyway, small ρ values with ET processes are not always the case.[32,33] Support to the present mechanistic conclusions comes also from the following section.

Probe substrates

Attention was turned towards the benzylic alcohol **18** (and **20**) (Scheme 3). Besides being very comparable in structure with



Scheme 3 Oxidation of probe substrates **18** and **20** by laccase–mediator systems: C_α–C_β *vs.* C_α–H cleavage.

other widely-employed lignin model compounds,[5c,34] **18** presents the distinct feature of giving rise to two diverse end-products depending on the oxidation mechanism.[10] In fact, under genuine ET conditions with chemical oxidants,[33] **18** gives the transient **18**$^{\bullet+}$ intermediate that cleaves at the C_α–C_β bond, to produce *p*-anysaldehyde **4** and *tert*-butyl radical.[22a] This cleavage is driven by steric and stereoelectronic factors,[35] that make the loss of the tertiary radical fragment easy.[22a,36] Conversely, under *bona fide* radical HAT conditions, **18** undergoes fragmentation of the C_α–H benzylic bond,[22a] and produces ketone **19** exclusively.[33,37] This clear-cut behaviour makes **18** a useful probe, capable of providing evidence for the oxidation mechanism from product analysis.[10,33] Use of probe **20** was additionally made whenever the conversion to products was low, as in the case of mediator ABTS (or, with some other mediator, for consistency); in fact **20** is structurally analogous to **18** but more viable to monoelectronic oxidation (to veratryl aldehyde **6**), in view of its additional methoxy-substituent.[38]

Table 3 Oxidations with laccase–mediator systems: use of probe substrates **18** and **20** (see Scheme 3)[a]

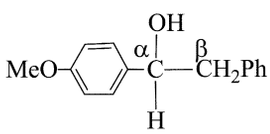
Mediator	Substrate	Product (% yield *vs.* subst.)[b]	
ABTS	**18 20**	**4**, 0[c]	**6**, 2[d]
HBT	**18 20**	**19**, 20	**21**, 50
HPI	**18 20**	**19**, 30	**21**, 70
VLA	**18 20**	**19**, 5[d]	**21**, 20
Co(III)W[e]	**18**	**4**, 15	
$ABTS^{++}$[f]	**20**	**6**, 4	

[a] Reaction conditions: [Subs.] = 20 mM, [Med] = 6 mM, [Lc] = 3 U ml^{-1}, pH 5, reaction time 24 h, at 20 °C, O_2 was purged for 30 min initially. [b] Determined by GC. [c] The substrate was quantitatively recovered. [d] Reaction time: 3 days. [e] [Co(III)W]: [Sub] = 2 : 1, pH 5, reaction time 24 h, at 20 °C; without laccase. [f] Generated *in situ* by using the Ce^{IV} salt, without laccase.

Test results (Table 3) show that reaction of **18** with a *bona fide* one-electron oxidant, such as $K_5Co^{III}W_{12}O_{40}$ (*viz*. Co(III)W; $E°$ 1.4 V),[10,39] causes C_α–C_β bond cleavage and yields aldehyde **4** *exclusively*. Analogous results are obtained on independent oxidation of **20** with $ABTS^{++}$ ($E°$ 1.1 V),[7] unambiguously generated from ABTS (in the absence of laccase) by use of the strong oxidant $(NH_4)_2Ce^{IV}(NO_3)_6$ (*viz*., CAN; $E°$ 1.5 V).[10,33] Consistent with this evidence for the ET route, the laccase–ABTS system gave only the aldehyde **6** from precursor **20** (no appreciable conversion from **18**), whereas the three laccase–NOH mediator systems gave only the ketone **19** or **21** from their corresponding precursors, in keeping with the HAT route. With respect to Table 3, it must be stressed that no other side-products are detected, and that the precursor probe is always recovered in amounts consistent with the yield of product, thereby providing a quantitative mass balance.

Two conclusions have been made concerning the results of Table 3. With reference to Scheme 3, the product obtained from the C_α–C_β cleavage (*i.e.*, **6**) with the laccase–ABTS system is in keeping with the oxidation pattern of the *bona fide* ET reagent Co(III)W, as well with that of the $ABTS^{++}$ species independently generated. Conversely, mediators HBT, HPI and VLA, that give the C–H cleavage product (**19** or **21**), follow the HAT pattern. Conversion is strangely low from **18** with mediators ABTS and VLA, in spite of the fact that they did catalyse the oxidation of the analogous mono-methoxylated substrate **3** (see Table 1) satisfactorily. The bulky *tert*-butyl group in **18** (and **20**) could be responsible for lowering the yield of oxidation with ABTS and VLA, perhaps for steric reasons. This does not seem to affect the reaction of the other mediators to a similar extent.

The virtue of probe **18** (and **20**) is its specificity, because it gives rise *uniquely* to either one (**4**) or the other (**19**) of the end-products, depending on the oxidation mechanism. Less specific probe substrates are also available,[33] however, and their products pattern is consistent with these mechanistic conclusions, although being less unambiguous. In fact, probe **22**, that features a benzylic residue rather than the *tert*-butylic residue of **18**, yields *both* the C_α–C_β cleavage product (*i.e.*, *p*-MeO-benzaldehyde, **4**) *and* the C_α–H fragmentation product (ketone **23**, as for **19**) under *bona fide* ET conditions (46% and 9%, respectively),[37] while giving rise to modest amounts (1%) of the aldehyde **4** in reaction with laccase–ABTS system. Conversely, ketone **23** *predominates* (30 : 1) over **4** in the oxidation of **22** with the laccase–HBT system.



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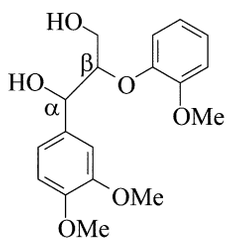

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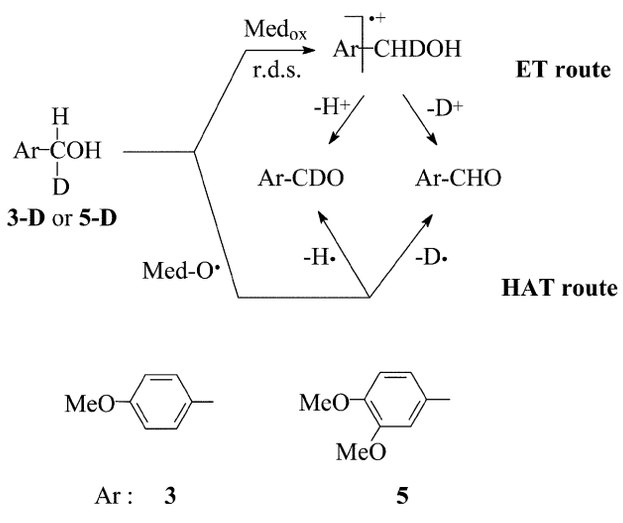
Analogously, another lignin model compound, *i.e.*, Adlerol (**24**),[40a] yields *predominantly* the C_α–C_β cleavage product **6** (*i.e.*, veratryl aldehyde) *vs*. the C_α–H fragmentation product (*i.e.*, ketone Adlerone, **25**) under *bona-fide* ET conditions, as for example with metalloporphyrins and $KHSO_5$,[40] or in an anodic oxidation,[41] whereas ketone **25** is almost exclusively obtained from laccase–mediator (*i.e.*, HBT, VLA) oxidations that follow the HAT route.[42] It can be concluded that the approach of the probe substrates, and the resulting mechanistic information, have a wide structure-generality.

Intramolecular isotope effect determination

In the ET mechanism of Scheme 2, a deprotonation at the C_α–H bond of the radical cation of the substrate is implied, but this is likely to be a *fast* step, following the rate-determining monoelectronic oxidation of the substrate by the Med_{ox} species.[15,19] Fragmentation of a C_α–H bond is instead the only and *rate-determining* step in the concurrent HAT mechanism of oxidation (Scheme 2).[15] On mono-deuteriation of a substrate (such as **5**) at the benzylic position, either $^1H^+$ or $^2H^+$ ought to be loss to the solvent, if the ET route operates, with the concomitant formation of either Ar-CDO or Ar-CHO, respectively (Scheme 4). Instead, in the HAT route, either $^1H^\bullet$ or $^2H^\bullet$ ought to be abstracted from precursor **5**, and the two corresponding aldehydes Ar-CDO or Ar-CHO formed. Mass spectrometric determination of the relative amount of the two aldehydes enabled determination of the intramolecular k_H/k_D selectivity (Table 4) for these oxidations, on a suitably synthesised **5-D** (or **3-D**) precursor.[10]



Scheme 4

Table 4 Intramolecular kinetic isotope effect determinations

Oxidant	k_H/k_D [d]	
	With 3-D	With 5-D
Co(III)W [a]	—	3.8
Laccase + ABTS [b]	3.1	3.6
Laccase + VLA [b]	—	6.4
Laccase + HBT [b]	6.2	6.4
Laccase + HPI [b]	6.4	6.2
$ABTS^{++}$ [c]	3.3	3.7

[a] [Co(III)W]: [Sub] = 2 : 1, pH 5, reaction time 24 h, 25 °C; without laccase. [b] [Sub] = 20 mM, [Med] = 6 mM, [Lc] = 3 U ml^{-1}, pH 5, reaction time 24 h, 25 °C, O_2 for 30 min. [c] Generated *in situ* by the Ce^{IV} salt, without laccase. Unreacted substrate is recovered (65%). [d] Determined by GC-MS.

The results obtained support the stronger kinetic relevance of the H/D loss with mediators HBT, VLA and HPI, once again in keeping with the rate-determining H-abstraction step in the radical route (Scheme 2). Large k_H/k_D values are indeed documented for the oxidation of benzhydrols by the *N*-oxyl radical of HPI.[43] On the other hand, with laccase–ABTS we obtain a smaller k_H/k_D value, that is practically coincident with those from the *bona fide* ET oxidations with Co(III)W, and with $ABTS^{++}$ independently generated.[10] Small k_H/k_D values are consistently reported from enzymatic and biomimetic studies involving the radical cations of the alkylanilines.[44]

Conclusion

The present work reports on fundamental reactivity features of the mediation phenomenon with the enzyme laccase towards non-phenolic substrates, and offers a comprehensive mechanistic rationalisation of it. Experimental evidence is provided for the oxidation mechanism undertaken by the oxidised form (Med_{ox}) of four mediators, namely, ABTS, HBT, HPI and VLA. This evidence derives from determining the Hammett correlations and the kinetic isotope effect for each mediator. Additionally, experiments with some probe substrates, whose end-products of oxidation unambiguously indicate the occurrence of either the ET or the HAT route of oxidation, are described. Conclusive clues allowing to solve the dichotomy between ET *vs*. HAT oxidation routes are in this way obtained. In general, HBT and HPI prove to be the more efficient mediators in the HAT route. These laccase–mediator systems show promise for environmental friendly biotechnological applications to the delignification of kraft pulps for the paper industry. Tests of their efficiency towards samples of pulps are underway.

Experimental

General

Most of the precursors and products, and the solvents were commercially available (Aldrich). Others (**18**, **20**, **22**, and their oxidation products) were available from previous investigations.[7,22a,33,36,37] Monodeuteriated alcohols (**3D** and **5D**) were also available from previous work.[45] Laccase from a strain of *Trametes villosa* (*viz*. *Poliporus pinsitus*) (Novo Nordisk Biotech) was employed.[7] It was purified by ion-exchange chromatography on Q-Sepharose by elution with phosphate buffer,[46] and an activity of 9000 U mL^{-1} determined spectrophotometrically by the standard reaction with ABTS.[47]

Instrumentation

A VARIAN 3400 Star gas chromatograph, fitted with a 20 m × 0.25 mm methyl silicone gum capillary column, was employed in the GC analyses. GC-MS analyses were performed 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 acquisition of the MS signal was in the SIM mode for the Ar-CHO *vs*. Ar-CDO relative intensity determinations.

Enzymatic reactions

The oxidation reactions were performed at room temperature in 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.[7] In case of sparingly soluble substrates, 10% acetonitrile was added. The concentration of the reagents was: [substrate], 20 mM; [mediator], 6 mM, with 10 units of laccase. The incubation was carried out for 24 h under constant stirring, keeping a latex balloon half-filled with oxygen on top the reaction vessel. Following a conventional work-up, the yields of oxidation were determined by GC analysis with respect to an internal standard (acetophenone or *p*-methoxyacetophenone), suitable response factors being determined from authentic products. The identity of the products was also confirmed by GC-MS analyses. No other products, besides those indicated in the Tables, were detected.

Competition experiments

Competition experiments of two substrates, either for the Hammett's correlations (k_X/k_H), but also for the k_3/k_1 and k_3/k_7 relative rate determinations, were similarly run on a 40 mmol amount of each of the substrates, giving the following initial concentrations: [$ArCH_2OH$], 20 mM; [$PhCH_2OH$], 20 mM, [mediator], 6 mM, with 10 units of laccase. The yields of products were determined by GC, after a reaction time (4–5 h) that would ensure only a modest/moderate conversion into products (10–25%). In order to determine the k_H/k_D ratios, determination of the relative amount of the Ar-CHO and Ar-CDO oxidation products (Scheme 4) was done by GC-MS analyses after a 5 h reaction time.

Chemical oxidations

Oxidations with $K_5Co^{III}W_{12}O_{40}$ (*viz*. Co(III)W) (60 mmol) of a substrate (30 mmol) were conducted in 2 mL citrate buffer, at room temperature for 1 or 3 days; conventional work-up with $CHCl_3$ or ethyl acetate followed. In the oxidations with 'preformed $ABTS^{++}$', 20 μmol of ABTS were dissolved in 1.5 mL of 2 M H_2SO_4; 40 μmol of $(NH_4)_2Ce^{IV}(NO_3)_6$ (*viz*. CAN) dissolved in 1.5 mL of 2 M H_2SO_4 were added, and the red colour of the dication developed immediately; 20 μmol of substrate were added very quickly at this point, and the resulting solution stirred at room temperature for 3 min, or until the red colour had turned blue (*i.e.*, $ABTS^{\bullet+}$). Conventional work-up followed.

Abbreviations

HBT	1-Hydroxybenzotriazole
HPI	*N*-Hydroxyphthalimide
VLA	Violuric acid
ABTS	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

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