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Oxidation of non-phenolic substrates with the laccase/*N*-hydroxyacetanilide system: Structure of the key intermediate from the mediator and mechanistic insight

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We have investigated the reactivity and mechanistic features in the oxidation of non-phenolic substrates by the enzyme laccase under mediation by *N*-hydroxyacetanilide, NHA. A radical route of hydrogen-abstraction by the >N–O• reactive intermediate of the mediator, formed in a preliminary oxidative interaction with the enzyme, seems the more viable oxidation mechanism, in keeping with analogous conclusions reached for other laccase/N–OH-type mediators. The evaluated value of the energy of the O–H bond of NHA corroborates the occurrence of hydrogen-abstraction from the benzylic substrates by the >N–O• intermediate of NHA. The occurrence of an alternative ionic route through the oxoammonium ion (>N=O$^+$) of mediator NHA is ruled out by experimental evidence acquired through an Hammett structure/reactivity correlation in the oxidation of substituted benzyl alcohols, as well as by kinetic isotope effect determinations. The >N=O$^+$ species of NHA, being a one-electron oxidant of moderate strength, could in principle even be responsible for an alternative electron-transfer route of oxidation of the substrates. This hypothesis could also be dismissed through the use of probe substrates and intermolecular selectivity determinations.

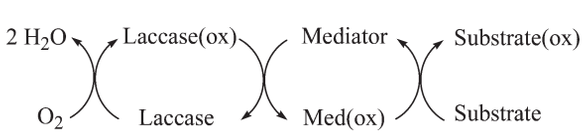
Introduction

Laccase, a family of 'blue-copper' oxidases containing four copper ions, is excreted by white-rot fungi under ligninolytic conditions.[1,2] It cooperates with other enzymes, in particular with lignin peroxidase (LiP)[3] and manganese peroxidase (MnP),[4] in the degradation of the biopolymer lignin in woody tissues. With respect to LiP and MnP, that are more powerful oxidants,[1] laccase has a lower redox potential (0.7–0.8 V/NHE)[1,5] 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. Advantages of laccase are that it is more readily available and easier to manipulate than LiP and MnP, and that its substrate specificity is low, as long as a good match of oxidation potentials is provided.[1,2,5*a*,6] Additionally, the use of appropriate mediators makes non-phenolic compounds, that are more difficult to oxidise, to become substrates of laccase.[5*d*,7] The role of mediators in laccase oxidations is outlined in Scheme 1.

The mediator needs to be easily oxidised by laccase to the Med_{ox} status: the structure of the latter is then crucial for the mechanism of the ensuing non-enzymatic oxidation of the substrate. In fact the Med_{ox} species may rely on a mechanism of oxidation, radical for example, which differs from that of the direct laccase oxidation.[8] In this way the mediators turn laccase into a much more versatile enzyme, as already exemplified by various biotechnological applications, *e.g.* in the textile dye bleaching,[9] in selective organic transformations,[10] in bioremediation of soils and water,[5*c*,11] or for an environmental-benign bleaching process of kraft pulps for paper making,[7*c*,7*d*,12] which hopefully will enable to circumvent the use of polluting chlorine-based treatments.

We have undertaken a systematic mechanistic investigation on the mediation phenomenon with laccase,[8] and have already reported on a comparative evaluation of a number of mediators in the oxidation of benzylic alcohols, taken as non-phenolic lignin models.[7*e*] The mediators HBT, HPI, VLA (structures and shortened names in Fig. 1), all endowed with the >N–OH functionality, or TEMPO (2,2′,6,6′-tetramethylpiperidine-*N*-oxyl), a stable >N–O• radical, presented the more promising features as catalysts in this oxidation.[8,13] For example, a simple and synthetically-attractive oxidation of alcohols to carbonyl compounds by oxygen, induced by the laccase/TEMPO system, has been developed.[10*f*]

In the present paper we investigate on *N*-hydroxyacetanilide (NHA), another N–OH-type mediator of laccase. At variance with the other mediators, NHA is not commercially available



Scheme 1 Catalytic cycle of a laccase/mediator oxidation system.

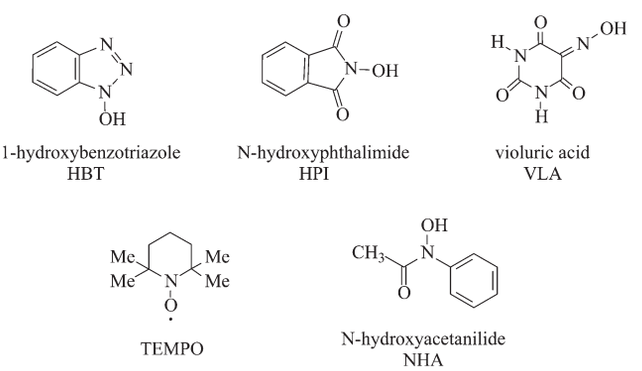


Fig. 1 Structures and abbreviated names of the mediators.

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and this has limited so far studies aimed at investigating its efficiency and mechanism of oxidation.[5d] Previous work had given firm indication that a radical route is followed by mediators HBT, HPI and VLA with laccase,[5d,7b,7e,8,14] in their oxidation of benzyl alcohols (and ethers) to carbonyl products. We wished to confirm this behaviour with NHA, in view of the common N–OH functionality. To this aim, the following experimental evidence has been acquired in the oxidations with the laccase/NHA system: (i) reactivity trends with significant substrates; (ii) product patterns with suitable 'scout' precursors; (iii) effect of substituents in the oxidation of benzyl alcohols (Hammett correlation); (iv) determination of the kinetic isotope effect. An electrochemical investigation on the redox features of NHA has been preliminarily carried out, and also the energy of the O–H bond of its N–OH functionality evaluated, in order to acquire additional clues in support of the various possible oxidation mechanisms. Purified laccase from *Trametes villosa* (*viz. Poliporus pinsitus*) has been employed, and mediator NHA synthesized according to the literature.

Results and discussion

Electrochemical study

The electrochemical oxidation of NHA had been previously investigated by cyclic voltammetry (CV), and two oxidation steps detected.[15] The first step was attributed to a quasi-reversible one-electron transfer, occurring at 0.62 V, followed by a second irreversible electron transfer at 0.97 V, both values being obtained at a 20 mV s^{-1} scan rate *vs.* NHE, in buffered (pH 7.4) water/methanol (9/1 v/v) solution. This evidence was attributed to the following electrochemical/chemical events (Scheme 2).[15]

In the first electrochemical step the $NHA^{\bullet+}$ species is generated, but its deprotonation to the amino-oxyl radical competes with back-electron transfer, thereby making the E_1 value largely irreversible. We have repeated this CV experiment and scanned the potential up to sweep rates as fast as 5 V s^{-1}, certainly faster than the 20 mV s^{-1} rate reported in the literature,[15] without obtaining a well-structured reverse CV wave, and therefore no full reversibility. We tentatively provide a 0.8 V *vs.* NHE value for the *quasi* E°_1 of NHA in sodium acetate buffer (pH 4.7), and point out that the $NHA^{\bullet+}$ species seems more short-lived than reported.[16] In another electrochemical investigation on NHA and derivatives, an E_1 value of 0.72 V/NHE (pH 6, phosphate buffer) was in fact given, at scanning rates of about 20–500 mV s^{-1}, but described to be reversible.[16] A similar E_1 value of 0.75 V/NHE at pH 4.5 was obtained in a recent CV determination, at a slower scan rate (100 mV s^{-1}).[17]

A second oxidation wave is appreciable in the CV scan of NHA, and it has been attributed to the oxidation of the >N–O$^{\bullet}$ species formed in the first electrochemical/chemical stage (Scheme 2).[15–17] The transient formation of an oxoammonium ion has been postulated, which easily loses acetylium ion thereby explaining the irreversibility of the E_2 value. Once again, because the sweep scan employed in the study was rather slow (20 mV s^{-1}),[15] we wanted to repeat the measurement at a faster scan rate (up to 5 V s^{-1}), in order to verify how this could affect the irreversibility/reversibility of the second electrochemical stage. Unfortunately, serious adsorption problems of NHA on the surface of the glassy carbon electrode prevented us from any reliable determination. We could detect only the presence of an irreversible anodic peak at about 1.1 V/NHE, at 5 mV s^{-1}.

This electrochemical discussion has general relevance with respect to the mediation phenomenon by N–OH compounds with laccase. In fact, mediators HBT, HPI and VLA are all oxidised in CV experiments according to the first step of Scheme 2,[7e,18] with E_1° values of 1.09, 1.08, and 0.92 V/NHE, respectively, that are reversible at sweep scan faster than 1 V s^{-1}.[7e] Further oxidation of the corresponding >N–O$^{\bullet}$ species to the oxoammonium ion (second stage in Scheme 2) ought to occur (if any) at E_2 values more positive than 1.3 V, that cannot be experimentally attained due to the discharge of the electrolytes in solution. Clearly, these higher redox potentials are not accessible either to the oxidation capacity of laccase (0.7–0.8 V),[1,5] and therefore the oxidation mechanism followed by these laccase/>N–OH systems with non-phenolic substrates (*e.g.*, benzyl alcohols) is associated with the unique formation of the >N–O$^{\bullet}$ species, and proceeds through the radical (HAT; Scheme 3) route extensively documented.[5d,7b,7e,8,14]

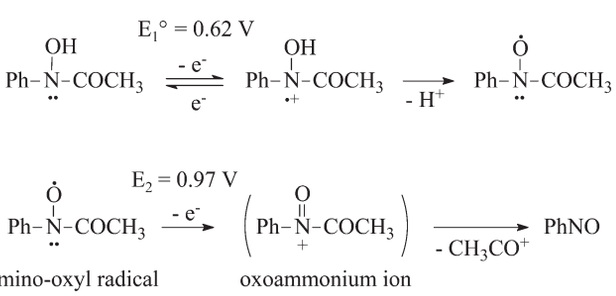
On the other extreme, TEMPO is a *stable* >N–O$^{\bullet}$ species,[19] whose oxidation to a >N=O^{+} ion is easier and occurs at 0.7 V.[13,20] Such oxidation is possible for laccase, in view of its redox potential,[1,5] and consistently the oxidation of benzyl alcohols with the laccase/TEMPO system can take place through the oxoammonium ion, and it is ionic (Scheme 4).[7e,8,13]

The case of NHA could be somewhat intermediate. Its E_1 value (0.7–0.8 V) is the lowest among the >N–OH mediators and certainly at easy reach of laccase, but even the second oxidation step to the >N=O^{+} ion (E_2 of *ca.* 1 V) could be accessible to the enzyme. Even though the oxoammonium ion of NHA is short-lived (as suggested by the second anodic peak, which is irreversible at 5 mV s^{-1}), the possibility could exist that in a laccase/NHA oxidation of a non-phenolic substrate the transient >N=O^{+} intermediate is partially stabilised by proximity with the negatively charged enzymatic pocket.[21] This could extend its life-time and make it responsible for either the occurrence of an ionic oxidation route (according to Scheme 4), or even an electron transfer (ET) route (*cf.* Scheme 5) with suitably oxidisable non-phenolic substrates. This could occur in combination/superposition with the radical HAT route (Scheme 3) *via* the >N–O$^{\bullet}$ species.

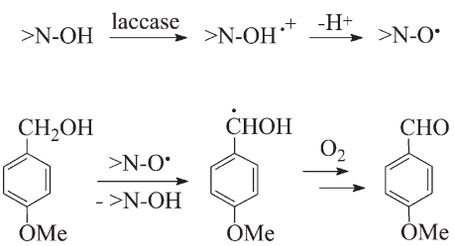
We planned to investigate these possible mechanistic alternatives, which depend on different reactive intermediates originating from the NHA mediator.

Thermochemical considerations

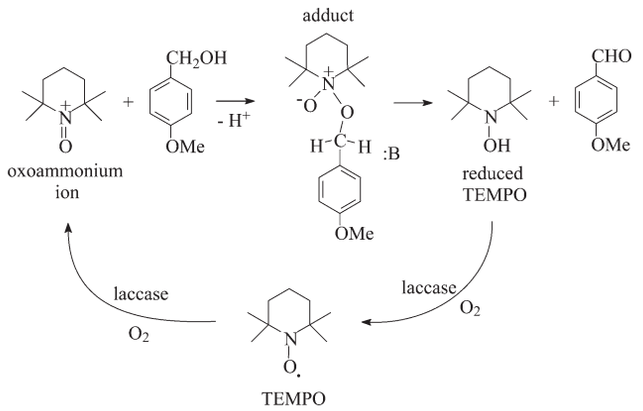
Thermochemical data concerning the enthalpy of the O–H bond (BDE_{OH}) of N–OH mediators are accessible for a few >N–OH compounds structurally similar to NHA (Fig. 2).[22]



Scheme 2 Electrochemical/chemical steps in the oxidation of NHA.



Scheme 3 The radical HAT route.



Scheme 4 The ionic route.

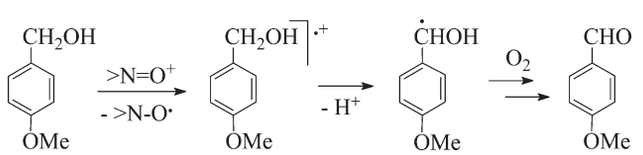
Because the nitrogen atom of NHA is analogously bonded to a single carbonyl group, and not to two carbonyl groups that confer a BDE_{OH} as high as 88.1 kcal mol^{-1} to HPI (*cf.* Fig. 1),[23] it is legitimate to infer a BDE_{OH} value of roughly 79 kcal mol^{-1} for NHA as well. Accordingly, abstraction of a benzylic H-atom from a benzyl alcohol (HAT route of Scheme 3) by the amino-oxyl radical of NHA would be approximately thermoneutral (Scheme 6), and possibly proceed at an acceptable rate.

The corresponding HAT process is instead *exothermic* by *ca.* 6 kcal mol^{-1} for the >N–O$^{\bullet}$ species deriving from HPI (BDE_{OH} of 88.1 kcal mol^{-1}),[23] and indeed it occurs easily,[22] whereas it would be *endothermic* by *ca.* 12 kcal mol^{-1} with TEMPO (BDE_{OH} of 69.6 kcal mol^{-1}),[24] and therefore does not occur and the ionic route (Scheme 4) takes over.[13] Thermochemistry (Scheme 6) confirms an intermediate position of NHA among its congeners, and indicates the HAT route (through >N–O$^{\bullet}$) as only a possible mechanistic alternative to the ET or ionic routes (through >N=O^{+}) for this mediator. Reactivity data for laccase/NHA reactions were indeed strongly needed in order to solve this mechanistic conundrum, and be able to rule out the last two mechanistic hypotheses.

Reactivity of substrate

In keeping with our previous studies, it was deemed mechanistically significant to investigate the efficiency of oxidation by the laccase/NHA system with three specific benzyl alcohols, *i.e.*, $PhCH_2OH$ (**1**), *p*-anisyl alcohol (**3**) and veratryl alcohol (**5**). Because these compounds widely differ in redox potential, as reported in Table 1 (E° in V/NHE, in H_2O),[7e] this should cause a sizeable difference in reactivity among them, if the oxidation of the substrate by the laccase/NHA system were to proceed by the ET route (Scheme 5). In contrast, the redox features of the substrate should have marginal importance on reactivity both in the radical (Scheme 3) and ionic (Scheme 4) routes of oxidation.[8,13,14c,14d]

These mediated oxidations were performed at room temperature in a stirred water solution buffered at pH 5, and purged with O_2 prior to the addition of the reagents.[7e,8] A small amount of MeCN (*i.e.*, 4% MeCN) was also added, in order to ensure solubility of both substrate and mediator.



Scheme 5 The ET route.

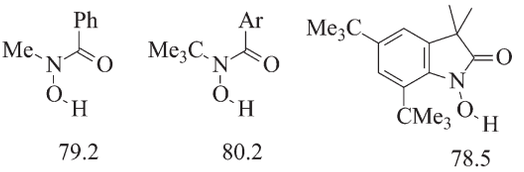


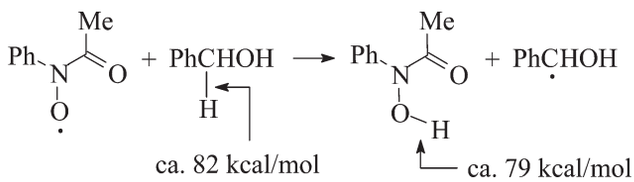
Fig. 2 BDE_{OH} data, in kcal mol^{-1}.

However, and at variance with the results obtained with other >N–OH mediators,[8,25] the extent of oxidation by laccase/NHA in this 4% MeCN mixed solvent resulted very meagre. A 1:1 buffered-water:dioxane mixed solvent (*i.e.*, 50% dioxane) was then adopted,[26] where the extent of oxidation became higher and comparable with that of the other laccase/N–OH-type systems towards the same substrates. Therefore, a specific solubility problem of mediator NHA does emerge here.

Apart from the marked difference in conversion between the two mixed solvents, the 50% dioxane solvent clearly shows that the laccase/NHA system is able to oxidise even the unsubstituted precursor **1** (to **2**) to a significant extent (32%), the conversion slightly increasing with the electron-donor substituted substrates **3** and **5**, to the corresponding aldehydes **4** and **6**. Even the oxidation of an electron-poorer substrate, such as 4-chlorobenzyl alcohol (**7**), occurs appreciably under these conditions (30%). Comparison between the yield of product and the amount of substrate recovered (bracketed values, in Table 1) ensures that the mass balance is good in all cases, and that no other products are formed besides those indicated. It must be stressed that, in the absence of NHA, laccase does not oxidise the non-phenolic substrates on its own, nor does the mediator in the absence of the enzyme: it is therefore the whole laccase/NHA system that is responsible for the oxidation of the substrates, in consistency with the operation of Scheme 1.[7]

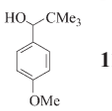
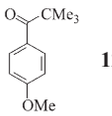
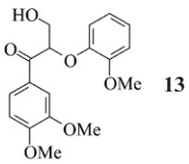
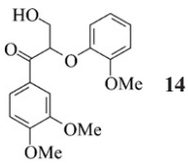
The negligible relevance of substrate electron-richness upon reactivity in these laccase/NHA oxidations is confirmed by a competition experiment of **3** and **1**, giving a k_3/k_1 relative rate as small as 1.9. This complies with the operation of the radical mechanism of oxidation (Scheme 3) common to the other N–OH mediators.[8] Further support to the operation of the HAT route comes from use of another mechanistic test.[27] Oxidation of secondary benzyl alcohol **9** by laccase/NHA gives the ketone **10** in good yield (67%), and **9** results easier to oxidise than **3** in a competition experiment ($k_3/k_9 = 0.72$). Abstraction of H-atom from secondary substrate **9** is indeed expected to be easier than from primary **3** in a radical process, due to the C–H bond energy value that is lower (85.4 kcal mol^{-1}) for **9** than for **3** (88.0 kcal mol^{-1}).[8,27] Consistently, k_3/k_9 values *lower than* 1 were obtained with all the other >N–OH mediators HBT (0.7), HPI (0.8) and VLA (0.4) in the same mixed solvent, whereas the laccase/ABTS system, which follows an ET route of oxidation, gave a k_3/k_9 value *higher than* 1 (*i.e.*, 1.5).[8] However, because even the laccase/TEMPO system gave a k_3/k_9 value *lower than* 1 (*i.e.*, 0.78),[13] the incursion of the ionic route (Scheme 4) in competition with the HAT route cannot be ruled out on this basis only.

Oxidation of 'scout' substrates **11** and **13** with laccase/NHA gives the corresponding carbonyl derivatives **12** and **14**



Scheme 6 Hydrogen atom abstraction: thermochemical evaluation.

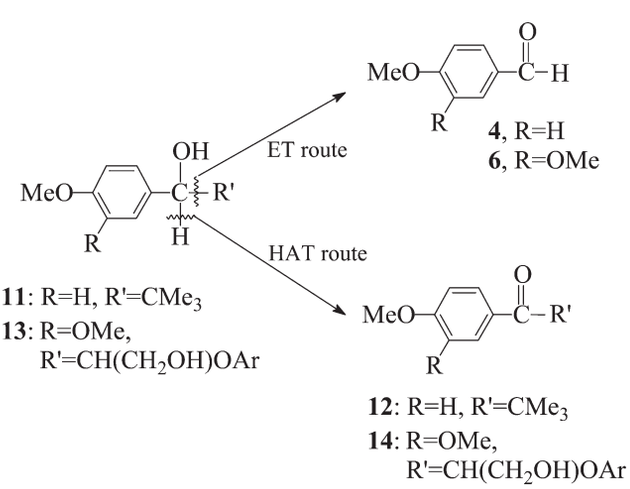
Table 1 Oxidation of selected substrates by the laccase/NHA system, at room temperature for 24 h

Substrate (E° in V/NHE, in H_2O)[a]	Oxidation product	Product yield, in % *vs.* substrate	
		Solvent: 4% MeCN	Solvent: 50% dioxane
benzyl alcohol, **1** (2.4)	benzaldehyde, **2**	3 (98)[b]	32 (67)[b]
4-MeO-benzyl alcohol, **3** (1.8)	4-MeO-benzaldehyde, **4**	4 (97)[b]	54 (45)[b]
3,4-dimethoxybenzyl alcohol, **5** (1.4)	3,4-dimethoxy-benzaldehyde, **6**	16 (84)[b]	59 (42)[b]
4-Cl-benzyl alcohol, **7**	4-Cl-benzaldehyde, **8**	–	30 (65)[b]
1-(4-MeO-phenyl)-ethanol, **9**	4-MeO-acetophenone, **10**	–	67 (31)[b]
 **11**	 **12**	4 (96)[b]	9 (91)[b]
 **13**	 **14**	–	25 (76)[b]
k_3/k_1 relative rate[c]		1.9	–
k_3/k_9 relative rate[c]		0.72	–

[a] Conditions: [substrate] 20 mM, [mediator] 6 mM, with 15 units of laccase. The yield of oxidation was determined by GC or HPLC analyses. [b] Recovered substrate (%). [c] In competition experiments.

as the *unique* products (Table 1). These two substrates are viewed as mechanistic probes because they give different products depending on the oxidation route,[8,14c,28] and give precisely the carbonyl products in a *bona fide* HAT process (Scheme 7), but give (mostly) the C_α–C_β cleavage products in a *bona fide* ET process. For example, with the laccase/ABTS system, which follows the ET route,[8] or with chemical mono-electronic oxidants,[28] or also under anodic oxidation,[29] **11** and **13** gave the cleavage products **4** and **6**, respectively, which are *not* observed with the laccase/NHA system nor with any other laccase/N–OH-type mediator.[8,14c] Comparable mechanistic dichotomies are known and have been reported with a few other substrates.[29,30]

While this would seem to unambiguously indicate the occurrence of the HAT route with laccase/NHA, we must observe that even the oxidation with laccase/TEMPO did give ketone **14** from precursor **13** as the unique product, albeit in low yield.[13] Because this took place through the ionic route (Scheme 4), the mechanistic ambiguity between the latter and the HAT route still persists!



Scheme 7 Performance of the mechanistic probes.

Hammett correlation

In view of these subtleties, and for a more exhaustive characterisation of the mechanism of oxidation with the laccase/NHA system, a quantitative treatment of the effect of substituents upon reactivity has been performed through a Hammett-type correlation. Four X-substituted benzyl alcohols, *i.e.*, 4-CF_3-, 4-Cl- (**7**), 4-Me-, 4-MeO–$C_6H_4CH_2OH$ (**3**), have been oxidised pair-wise in competition experiments *vs.* the unsubstituted precursor (**1**) as the relay compound,[8] and the k_X/k_H ratios reckoned by determining the relative amounts of the corresponding aldehydes by GC analysis (Table 2). For a closer comparison with the analogous studies performed with the other >N–OH mediators, the 4% MeCN mixed solvent has been employed.[8] The consequent lower conversion (*cf.* Table 1) is not a drawback in this contest, because a low extent of oxidation is required for a kinetically significant application of the competition treatment. The obtained log k_X/k_H ratios give a better fit when plotted *vs.* the σ^+ substituent parameters (Fig. 3) rather than *vs.* the σ ones,[31] and the resulting ρ correlation parameter is given in Table 2. For the sake of comparison, an analogous Hammett treatment has been conducted by using the ET oxidant $ABTS^{2+}$ (E° 1.1 V),[7e] independently generated by a chemical oxidant (see Experimental) without laccase,[14c] in view of the similarity of its redox potential with the E_2 value of NHA (1.0–1.1 V).

The ρ parameter for the laccase/NHA system (−0.42) is small in value and very comparable with those of the other laccase/>N–OH-type systems (ρ values in the −0.4 to −0.9 range),[8,14d] as expected for a HAT route through the >N–O$^\bullet$

Table 2 Determination of the ρ value for the laccase/NHA system, and with 'pre-formed' $ABTS^{2+}$, by oxidations of *p*-X-$C_6H_4CH_2OH$ *vs.* $PhCH_2OH$, in competition experiments at room temperature[a]

Oxidant	$k_{p\text{-}NO_2}/k_H$	$k_{p\text{-}CF_3}/k_H$	$k_{p\text{-}Cl}/k_H$	k_{Me}/k_H	k_{MeO}/k_H	ρ^b
laccase/NHA	—	0.59	0.96	1.3	2.2	−0.42
$ABTS^{++}$	0.35	—	0.85	8.7	26	−1.3

[a] Determined by duplicated GC analyses; typical error ±4%. See Experimental for conditions. [b] Obtained *vs.* the σ^+ parameters.

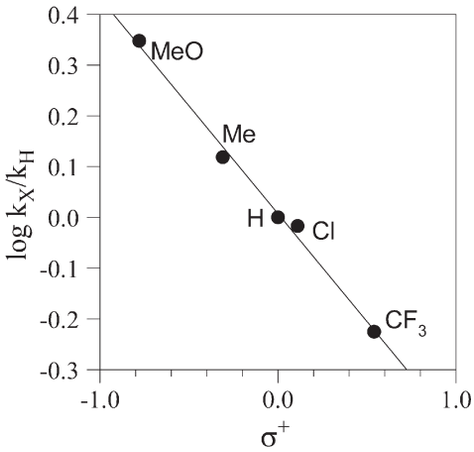
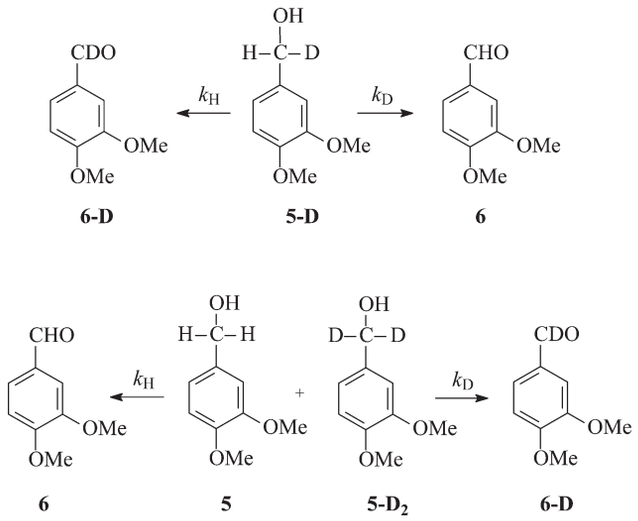


Fig. 3 Hammett structure–reactivity correlation with the laccase/NHA system: oxidation of 4-X-substituted-benzyl alcohols in competition experiments.



Scheme 8 Intramolecular (above) and intermolecular (below) kinetic isotope effect studies.

intermediate (Schemes 3 and 6); in contrast, the slightly larger ρ value of $ABTS^{2+}$ is more in line with ρ values obtained for *bona-fide* chemical ET oxidants.[14d] More importantly, however, the analogous Hammett treatment for the oxidation of the same substrates by the laccase/TEMPO system gave a V-shaped plot *vs.* the σ_I parameter, consistent with the operation of the ionic oxidation route (Scheme 4).[13] In this case the alcohol substrate behaves as a nucleophile in its rate-determining addition to the oxammonium ion, as long as the substituent is electron-withdrawing; moving to electron-donor substituents, the addition step becomes increasingly faster and the rate-determining step shifts to the deprotonation of the adduct, which is indeed retarded by electron-donor groups.[13] This outcome makes a noteworthy difference with the present linear plot of the NHA case (Fig. 3), and definitely rules out the operation of the ionic route.

Kinetic isotope effect determinations

In the HAT mechanism of Scheme 3, cleavage of a C_α–H bond is the rate-determining step of the oxidation process, whereas in the concurrent ET route C_α–H deprotonation of the radical cation of the substrate comes after the rate-determining monoelectronic oxidation of the substrate (*cf.* Scheme 5).[14c,27,32] This difference in rate-determining steps had already enabled us to corroborate the HAT route of oxidation with the other laccase/≥N–OH-type mediators.[8] The determination has been extended here to the laccase/NHA system on a suitable monodeuteriated benzyl alcohol, such as **5-D**. In the HAT route, either H• or D• ought to be intramolecularly abstracted from precursor **5-D**, with the concomitant formation of either Ar–CDO or Ar–CHO, respectively (Scheme 8).

Mass spectrometric determination of the relative amount of the two aldehydes (**6** and **6-D**) allowed to determine the *intramolecular* k_H/k_D selectivity (Table 3).[14c] The obtained value, *i.e.* 5.2, although being significantly *larger than* 1, and therefore consistent with a rate-determining C–H or C–D bond cleavage, is slightly smaller (close to the limits of the experimental errors) than the intramolecular k_H/k_D values obtained with the other HAT oxidants, ranging from 6.2 to 6.5.[8,14c] The determination was therefore repeated in an *intermolecular* mode, by competition of un-deuterated (**5**) and bis-deuterated (*i.e.*, **5-D_2**) precursors, and provided the slightly larger k_H/k_D value of 6.4, fully consistent with those of the other HAT mediators.[8,14c] For comparison, the intramolecular k_H/k_D value determined for the laccase/TEMPO system with substrate **5-D**, *i.e.* 2.6, is sizably different from that of laccase/NHA, well beyond the experimental errors. This smaller k_H/k_D value is indeed due to the deprotonation of the adduct of the ionic route (Scheme 4),[19] formed by the addition of the alcohol to the TEMPO-oxammonium ion, which is rate-determining (*cf.* previous section) with electron-rich alcohols,[13] such as **5**.

Conclusions

The present work investigates reactivity and mechanistic features of the laccase/NHA system in the oxidation of non-phenolic substrates (*i.e.*, benzyl alcohols) (*cf.* Scheme 1), and attempts to discriminate between the involvement of either one the oxidation forms (Med_{ox}) of mediator NHA, *i.e.*, the ≥N–O• or the ≥N=O^+ species (*cf.* Scheme 2). Experimental evidence acquired through the Hammett correlation, and the kinetic isotope effect (inter- and intra-molecular), contrasts with the possible occurrence of an ionic route (Scheme 4) of oxidation through the ≥N=O^+ species of NHA. The reactive behaviour of two mechanistic-probe substrates (**11** and **13**), as well as the value of the k_3/k_9 intermolecular selectivity, contrast with the occurrence of an ET route of oxidation (Scheme 5). The radical HAT route (Scheme 3) through the ≥N–O• species is therefore the more likely possibility for the laccase/NHA system, in keeping with the analogous conclusion reached in the oxidations with other laccase/N–OH-type mediators.[8] The evaluated enthalpy of the O–H bond of NHA is consistent with the occurrence of an almost thermoneutral radical H-abstraction route of oxidation *via* the ≥N–O• intermediate. It can be finally observed that laccase oxidations mediated by N–OH-type compounds, which proceed through the ≥N–O• species, appear promising for a selective delignification of wood pulp for paper manufacture, because they are respectful of the integrity of cellulose.[33] In contrast, the

Table 3 Kinetic isotope effect determinations[a]

Oxidant	k_H/k_D[b]
Laccase + HBT	6.4[c]
Laccase + HPI	6.2[c]
Laccase + NHA	5.2[d]
Laccase + TEMPO	2.6

[a] Approximate errors in the triplicated determinations: ±8%. See Experimental for conditions. [b] See Scheme 8. Intramolecular values, obtained with substrate **5-D**. [c] Data from ref. 8. [d] The intermolecular value, obtained from a mixture of **5** and **5-D_2**, is 6.4

laccase/TEMPO system oxidises not only the non-phenolic subunits of lignin but also the primary C6 alcohol functionality of the polysaccharide cellulose,[34] through the $\rangle N{=}O^+$ species,[35] thereby reducing the strength of the paper and resulting less valuable as a biotechnological alternative.

Experimental section

General remarks

NMR spectra were taken on a AC 200 Bruker instrument. A VARIAN CP 3800 instrument, fitted with a 30 m × 0.25 mm methyl silicone gum capillary column (CPSil5CB), was employed in the GC 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. A Hewlett-Packard 1050 HPLC system (pump, detector, and solvent delivery system), equipped with a Supelcosil LC-18-DB 25 cm × 4.6 mm column and a HP 3395B integrator, was employed in the HPLC analyses. These were carried out at 0.5–1 mL min^{-1} flow rate with a 3:2 water:MeOH mixed solvent as the eluent.

Materials

Many of the substrates and products were commercially available (Aldrich) and used without further purification. Other precursors and products were available from previous investigations,[7e,8,13,25,33a] including the mono- and bis-deuteriated alcohols (**5-D** and **5-D_2**).[36] A multi-step literature procedure was followed for the synthesis of NHA,[37] which requires the preliminary reduction of nitrobenzene to phenylhydroxylamine over Zn powder, followed by acetylation of the latter. The final compound was obtained in a 55% overall yield as a yellow solid, mp 63–65 °C (lit.[37] 65–66 °C), with the following ^{1}H-NMR spectrum [δ (ppm) in $CDCl_3$: 2.1 (s, 3H, PhN(OH)-COC*H*$_3$), 7.3–7.4 (m, 5H, *Ph*N(OH)COMe), 8.9 (broad, 1H, ArN(O*H*)COMe).

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.[6] The collected fractions were concentrated by dialysis in cellulose membrane tubing (Sigma) against poly(ethylene glycol) to a final activity of 9000 U/mL, as determined spectrophotometrically by the standard reaction with ABTS.[38]

Enzymatic reactions

The oxidation reactions were performed at room temperature.[7e,8] In order to ensure solubility of both substrate (60 μmol) and NHA (20 μmol), they were weighted in the same vial, dissolved in 120 μL of MeCN and added to 3 mL of the buffered water solution (pH 5; 0.1 M in sodium citrate), so that a final 4% MeCN mixed solvent resulted. The proper amount of a diluted solution of purified laccase was then added. The buffer solution had been purged with O_2 for 30 min prior to the addition of the reagents. Analogously, the 50% dioxane mixed solvent was prepared by employing 1.5 mL of dioxane to dissolve substrate and NHA, and then adding it to 1.5 mL of the buffer solution. The concentration of the reagents was: [substrate] 20 mM, [NHA] 6 mM, with 15 units of laccase. The reaction time was 24 h, in general, an atmosphere of oxygen being kept in the reaction vessel by means of a hemi-inflated latex balloon. 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, and calculated with respect to the substrate molar amount. With less volatile compounds, a HPLC analysis was employed, using benzophenone as the internal standard.[33a] The competition experiments for the Hammett correlation were similarly run on a 60 μmol amount of each of the substrates, dissolved in 0.25 mL MeCN, and added to 6 mL of citrate buffer (overall, 4% MeCN) along with 20 μmol of NHA and 10 units of laccase, and the yields of products determined after 3–6 h. Relative reactivity values were calculated by the use of the standard integrated equation for competitive reactions.[8] Unexpectedly, 4-NO_2-benzyl alcohol gave analytical problems with the laccase/NHA system, so that it was necessary to resort to another electron-withdrawing substituted benzyl alcohol, such as 4-CF_3-$C_6H_4CH_2OH$, for the Hammett treatment. In order to reckon the k_H/k_D ratios for the kinetic isotope effect determinations, both intra- and intermolecular ones, the relative amounts of the Ar-CHO and Ar-CDO oxidation products (Scheme 8) were determined by GC-MS analyses in the SIM mode, after a 5 h reaction time either from **5-D** or from an equimolar mixture of **5** and **5-D_2**.

Chemical oxidations

In the Hammett treatment with 'preformed' $ABTS^{2+}$, 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_4were added, and the red colour of $ABTS^{2+}$ developed immediately.[14c] A 60 μmol amount of each of the two competing substrates was 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.

Electrochemical measurements

Cyclic voltammetry (CV) experiments were carried out at 25 °C in a three-electrode circuit, using an electrochemical computerized system (Amel System 5000, version 2.1) and a glassy-carbon working electrode (planar disk, diameter 3 mm). A saturated calomel electrode (SCE) was used as reference and a platinum cylinder electrode (surface 1 cm^2) was employed as an auxiliary electrode. The E values are referred to NHE. The scanning rate range was from 0.005 V s^{-1} to 5 V s^{-1}. In the CV experiments, NHA was 1 mM in a 0.01 M sodium acetate buffer solution containing 0.1 M $LiClO_4$.

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