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New mediators for the enzyme laccase: mechanistic features and selectivity in the oxidation of non-phenolic substrates

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New mediators of laccase have been comparatively evaluated and ranked towards the benchmark aerobic oxidation of *p*-MeO-benzyl alcohol. The mechanism of oxidation of this non-phenolic substrate by each mediator, which is initially oxidised by laccase to the Med_{ox} form, has been assessed among three alternatives. The latter make the phenoloxidise laccase competent for the indirect oxidation of non-phenolic (and thus 'unnatural') substrates. Experimental characterisation of the mediators, by means of spectrophotometric, electrochemical and thermochemical survey, is reported. Clear-cut evidence for the formation of a benzyl radical intermediate in the oxidation of a particular benzyl alcohol with laccase and a >N–OH mediator is attained by means of a trapping experiment. The selectivity of the laccase-catalysed oxidation of two competing lignin and polysaccharide model compounds has been assessed by using the highly proficient 4-MeO-HPI mediator, and found very high in favour of the former model. This evidence is in keeping with the operation of a radical hydrogen-abstraction process that efficiently cleaves the benzylic rather than the aliphatic C–H bond of the two models. Significant is the finding that catechol, *i.e.*, a model of recurring phenolic structures in lignin, once oxidised to aryloxyl radical by laccase is capable to mediate a radical oxidation of non-phenolic compounds. This supports a fully-fledged role of laccase as a delignifying enzyme in nature by way of no other mediators than the very phenolic groups of lignin. Finally, an evaluation of the dissociation energy of the NO–H bond of HBT, which is not accessible experimentally, is provided by the use of a thermochemical cycle and theoretical calculations.

Introduction

The world of biochemistry offers many examples of the role of a messenger. For example, the RNA-messenger delivers the genetic information to the proteins factory embodied by the ribosome, whereas cyclic-AMP promotes the metabolism of lipids and sugars. A messenger solves communication needs, but it may also act as a catalyst.[1] Peculiarities of the role may require turning the name messenger into that of mediator: this becomes apparent in the field of enzymatic oxidations and, in particular, in the degradation of wood. Basidiomycete 'white-rot' fungi perform the delignification of dead wood through a complex but efficient strategy, requiring specialised extracellular enzymes.[2–4] The enzymes oxidise lignin in woody tissue, in order to make cellulose available to the metabolism of fungi. Lignin, however, the largest alkylaromatic polymer in nature, is so structurally complex that a direct interaction with the active site of an enzyme is sterically unfeasible. The problem is solved by appropriate messengers.[4,5] These low-molecular-weight metabolites, once oxidised by the enzymes, sneak away into the woody fibres and deliver oxidative equivalents to appropriate functional groups of the lignin polymer. For example lignin peroxidase,[6] a heme enzyme endowed with a redox potential of 1.3–1.4 V/NHE and with a small active site, oxidises the metabolite veratryl alcohol (*i.e.*, 3,4-dimethoxybenzyl alcohol; E°_{ox} 1.36 V) to its radical cation by electron abstraction. The latter, in turn, removes electrons from lignin, causing its degradative oxidation and consequently solving the communication problems between enzyme and substrate. Another enzyme involved in the biodelignification of rotten wood is laccase, a family of multicopper oxidases endowed with redox potential in the 0.5–0.8 V/NHE range.[4,7,8] Due to the lower redox potential,[9] laccase can oxidise monoelectronically only the phenolic groups of lignin (phenoloxidase activity),[4,10] but these represent less than 20% of all the functional groups of the polymer.[11] The benzyl alcohol and ether groups, summing up to about 70% of the residues but being more resistant to monoelectronic oxidation (redox potentials > 1.5 V/NHE), cannot be oxidised by laccase directly. Use of appropriate redox metabolites, or of purposedly added compounds, enables laccase to oxidise indirectly even non-phenolic groups in a catalytic cycle.[1,4,5,12] Following monoelectronic interaction with the enzyme, the oxidised messenger (Med_{ox}) oxidises 'unnatural' non-phenolic substrates according to mechanisms not available to laccase, as sketched in Scheme 1 in very general terms. In this way the messenger expands the oxidation ability of the enzyme, and more specifically fulfils the role of a 'mediator'.[1,13]

Several laccase/mediator systems have been investigated and found valuable in the aerobic oxidation of non-phenolic lignin models (such as benzyl alcohols),[14] and of lignin itself from industrial wood pulp.[15–17] Our contribution to this field has been to provide a consistent way to evaluate and compare the efficiency of a number of laccase mediators: the oxidation of a specific non-phenolic substrate, *i.e.* 4-methoxybenzyl alcohol (*viz.* *p*-anisyl alcohol), under strictly reproducible conditions represents the benchmark reaction over which the merits of several mediators can be assessed unambiguously.[14] This

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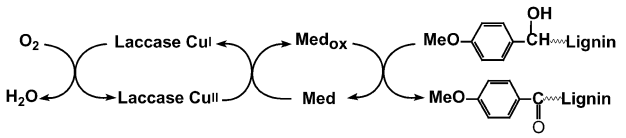


Scheme 1 The oxidation cycle of a laccase/mediator system towards non-phenolic substrates.

approach has further enabled us to characterise three different oxidation routes that the mediators of laccase can undertake depending on their structure.[1,13]

In the present paper we (i) report on new mediators, including a few that the recent literature has brought to our attention, (ii) evaluate their efficiency and merits with respect to those already investigated, and (iii) characterise their operating mechanism. Relevant redox, spectrophotometric and thermochemical data of the mediators have been acquired, aiming at finding a relationship between reactivity features and structure. The selectivity of one of the most proficient mediators of laccase has been assessed in the competitive oxidation of a lignin model with a polysaccharide model compound. Finally, the benzyl radical intermediate, resulting from the oxidation of a suitable benzyl alcohol with a laccase/mediator system, has been intercepted for the first time.

Results and discussion

Determination of the efficiency of new mediators

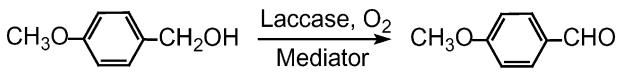
The aerobic oxidation of *p*-anisyl alcohol, catalysed by laccase from *Trametes villosa*, is taken up in order to rank the efficiency of the new mediators.[14] The reaction is run at room temperature for a fixed time span (24 h), in buffered water solution (3 mL; pH 5) previously purged with O_2 (Scheme 2).

The experimental conditions adopted, namely, [*p*-anisyl alcohol] 20 mM, [mediator] 6 mM, with 3 U mL^{-1} of laccase, are such as to comply with the 'ideal' scheme of a mediated oxidation (Scheme 1) where the mediator, *in deficiency* with respect to the substrate, turns over between its natural and oxidised (Med_{ox}) states, due to the intervention of laccase and O_2, while carrying out the oxidation of the non-phenolic substrate in a catalytic cycle.[14,18]

In Table 1 the conversion into product, investigated with 15 new potential mediators, is given and compared with that of other mediators already examined under these conditions.[14] No other oxidation products besides *p*-anisaldehyde appear, the rest of mass balance being the recovered *p*-anisyl alcohol. Significant thermochemical data (bond dissociation energy, BDE) of the mediators are listed in the Table, either experimental or calculated ones.[19,20] The redox potential of some mediators has been determined by cyclic voltammetry here, while already available values for other mediators are added.[14,21,22] On the basis of the data in Table 1, we can recognise three groups of mediators according to their behaviour and structure, and a more satisfactory mechanistic assessment of the mediation phenomenon now becomes possible.

The $>$N–O$^{\bullet}$ species

The negligible oxidation of the substrate documented in entries 1–4 (Table 1) points out that the aminoxyl radicals are not efficient mediators of laccase, with the exception of TEMPO and its cognates (entries 5,6,8), and a twofold explanation is necessary here. Direct hydrogen-atom transfer (*viz*., the HAT mechanism)[13,14] from the benzylic C–H bond of *p*-anisyl alcohol is precluded to all of these $>$N–O$^{\bullet}$ compounds on enthalpic grounds (Scheme 3).[19,20] In fact, the $>$NO–H bond that ought to be formed is weaker than the benzylic C–H bond to break (*ca*. 83 kcal mol^{-1}),[19a] as the BDE_{NO-H} values ($\leq$72 kcal mol^{-1})[19b] of the parent hydroxylamines indicate (Table 1).



Scheme 2 The benchmark aerobic oxidation of *p*-anisyl alcohol by laccase/mediator/O_2.

The reason why the aminoxyl radical TEMPO (entry 5)[13,14] performs instead so well as mediator is that its monoelectronic oxidation to the oxoammonium form ($>$N$=$O^{+}) occurs at a redox potential (*ca*. 0.7 V/NHE)[14] that matches the one of *Trametes villosa* laccase.[9] Thus the ionic mechanism of oxidation (Scheme 4), where laccase recycles reduced-TEMPO to the active species by electron-transfer oxidation,[13,14,23] takes over.

This route *via* the $>$N$=$O^{+} ion can be undertaken with equal success by 4-substituted TEMPO derivatives (*cf*. entry 6)[14] or by the 5-membered analogue PROXYL (entry 8), in view of the comparable $>$N$=$O^{+}/$>$N–O$^{\bullet}$ redox values. Accordingly, in the oxidation of α-monodeuteriated *p*-MeO-benzyl alcohol with laccase/PROXYL we give here a value of the intramolecular kinetic isotope effect (k_H/k_D = 2.4) equal to that (k_H/k_D = 2.2)[1] reported for the oxidation by laccase/TEMPO. This is in keeping with the operation of the ionic oxidation mechanism, where deprotonation of the TEMPO–alcohol (or PROXYL–alcohol) adduct *en route* to the oxidation product is rate determining with electron-donor substituted benzyl alcohols.[1,23] The ionic route has precedents whenever TEMPO is oxidised to the $>$N$=$O^{+} form by suitable oxidants (*e.g*., $PhI(OAc)_2$; cupric salts; Mn–Co salts)[24] other than the enzyme laccase. In contrast, a radical hydrido-metal mechanism is documented when TEMPO is used in combination with $RuCl_2(PPh_3)_3$, and in fact a different and larger kinetic isotope effect value (k_H/k_D = 5.1) is reported for α-monodeuteriated benzyl alcohol.[25]

The ionic oxidation route available to TEMPO is instead precluded to the aminoxyl radicals in entries 1, 3 and 4, because conversion to their corresponding $>$N$=$O^{+} ion takes place at a redox potential higher ($\gg$1.1 V;[22a,f] for entry 3 we obtain here 1.2 V/NHE) and not attainable to laccase. The lack of efficiency with (t-Bu)$_2$N–O$^{\bullet}$ (entry 2), which is an open-chain structural counterpart of TEMPO, and has a redox potential (E^{p} 0.6 V)[22b,g] that could enable conversion into the $>$N$=$O^{+} ion by laccase, would seem puzzling. However, the instability of the oxidised form of (t-Bu)$_2$N–O$^{\bullet}$ has been already discussed,[22g,h] and explains the irreversible electrochemical peak determined, as opposed to the case of TEMPO. Finally, the commercially available polymer-bound TEMPO comes out inactive as a laccase mediator (entry 7): it is indeed covalently linked to the polymeric support through its N–O bond, and therefore conversion into the oxoammonium ion, and operation of the ionic oxidation route, are precluded.

The $>$N–OH mediators

Leading examples in this class are violuric acid (VLA), *N*-hydroxyphthalimide (HPI) and 1-hydroxybenzotriazole (HBT) (entries 14–16), which perform with laccase well in the oxidation of non-phenolics *via* the HAT mechanism (*cf*. Scheme 3).[1,13,14] In general terms, monoelectronic oxidation of $>$N–OH by laccase generates the $>$N–OH$^{\bullet+}$ species that deprotonates to the aminoxyl radical ($>$N–O$^{\bullet}$) as the reactive intermediate. However, at the pH of the benchmark oxidation reaction (*i.e*., pH 5) many $>$N–OH derivatives are already present as $>$N–O^{-}, and directly converted to $>$N–O$^{\bullet}$ by laccase. Independent monoelectronic generation of the aminoxyl radicals from both HBT and HPI by the use of suitable oxidants, such as Ce^{IV} or $Pb(OAc)_4$,[26] is reported and confirms this point. The experimental BDE_{NO-H} value (88.1 kcal mol^{-1})[19b] of HPI supports an exothermic HAT process by its corresponding $>$N–O$^{\bullet}$ species. For entries 12 and 13, avail-

Table 1 Benchmark aerobic oxidation of *p*-anisyl alcohol with laccase/mediator systems (Scheme 2) in 3 mL buffered water solution (0.1 M sodium citrate, pH 5) saturated with O_2.[a] Yields of the oxidation product are determined by GC after a 24 h reaction time at room temperature. Redox and thermochemical data of the mediators are added, either determined here or already available

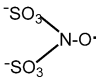
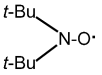
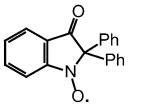
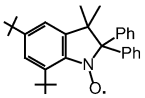
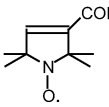
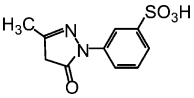
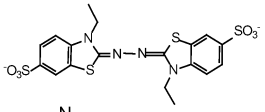
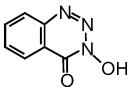
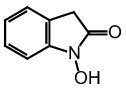
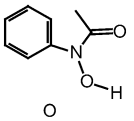
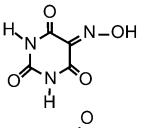
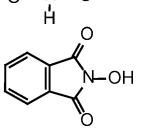
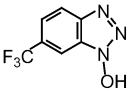
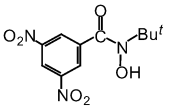
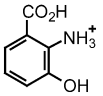
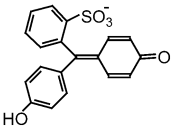
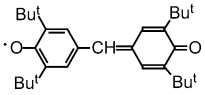
No.	Mediator	Yield of *p*-anisaldehyde (%)[b]	BDE_{NO-H} /kcal mol^{-1} (from ref. 19*a*)	E°/V *vs.* NHE [c] in H_2O[14,21,22]
1	 Fremy salt	<1	—	1.36 (ref. 22*f*)
2	 Di-*tert*-butylnitroxide	1	≤69	0.6 (E^p value) (refs. 22*b*,*b*)
3	 INDO	0[d]	71 (ref. 19*b*)	1.2 (in MeCN)
4		<1	*ca.* 71 (*cf.* entry 3).	*ca.* 1.2 (*cf.* entry 3)
5	 TEMPO	99[e]	69.6	0.73 (0.92 in MeCN)
6	4-OH-TEMPO	98[e]	72	0.85 (ref. 22*c*)
7	TEMPO-polymer bound	0	—	—
8	 PROXYL	83	70	1.1 (in MeCN)
9	 3-MPO	22	—	>1.1
10	 ABTS	21[e]	—	0.69 and 1.1 (ref. 14)
11	 HBO	19	≥ 80 (*cf.* ref. 19*b*)	1.1 (E^p value; E° 1.2 in MeCN)
12		7	78–79 (*cf.* ref. 19*b*)	0.8 (also in MeCN)
13	 NHA	54[f]	80	0.8 (ref. 21)
14	 VLA	85	*ca.* 80–85 (*cf.* ref. 19*c*,*d*)	0.92 (0.95 in MeCN)
15	 HPI	70[e]	88.1 (ref. 19*b*)	1.09 (0.92 in MeCN)
16	 HBT	76[e]	(*cf.* ref. 20)	1.08 (0.97 in MeCN)
17	 TFBT	33	(*cf.* ref. 20)	1.11 (in MeCN)
18	 HOAT	38	—	1.14 (in MeCN)
19	 NBI	11	80 (in ref. 19*b*)	>1.2 (this work and ref. 22*i*)

Table 1 (*continued*)

No.	Mediator	Yield of *p*-anisaldehyde (%)[b]	BDE_{NO-H} /kcal mol^{-1} (from ref. 19*a*)	E°/V *vs.* NHE [c] in H_2O[14,21,22]
20	 HAA	2	—	—
21	2-NH_2-purine	14	—	—
22	 Phenol red	20[g]	*ca.* 85–90 (as BDE(O–H)	0.8 (ref. 22*e*)
23	Cresol red	20[g]	See above	—
24	Dichlorophenol red	55[g]	See above	0.7 (ref. 22*e*)
25	Phenolphthalein	5[g]	See above	—
26	Catechol (1,2-dihydroxybenzene)	15	See above	—
27	 Galvinoxyl	<1	79	—

[a] Conditions: [*p*-anisyl alcohol] 20 mM, [mediator] 6 mM, laccase 3 U mL^{-1}. [b] Yields (±2%) are reckoned *vs.* the molar amount of alcohol, the rest of mass balance being recovered starting material; no other products besides *p*-anisaldehyde are detected. [c] In buffered water or in MeCN with Py (see Experimental). The E° data pertain to the $>N{=}O^{+}/>N{-}O^{\bullet}$, $R^{\bullet+}/R$ or $ArOH^{\bullet+}/ArOH$ couples. [d] From ref. 23*a*. [e] From ref. 14. [f] From ref. 21. [g] From ref. 22*e*.

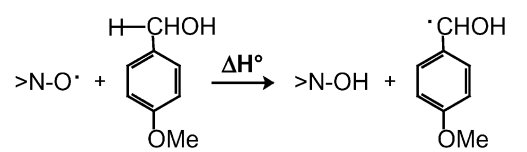
able BDE_{NO-H} values are analogously $\geq$ 79 kcal mol^{-1},[19,20] and compatible with mediation efficiencies ranging from moderate to good (Table 1). In this respect, the lower efficiency displayed (in entry 12) by the cyclic counterpart of the proficient mediator *N*-hydroxyacetanilide (NHA, entry 13),[21] could be due to the slightly lower BDE_{NO-H} value.

Commercially available aryl-substituted HBTs, bearing the electron-withdrawing CF_3– (entry 17) or aza-groups (entry 18), show a mediation efficiency lower than HBT itself (entry 16). Their redox potentials have been determined here by cyclic voltammetry, and found higher in value than that of HBT. As a consequence, oxidation by laccase to the corresponding $>N{-}O^{\bullet}$ form is relatively more difficult and consistent with a lower efficiency as mediators. Furthermore, the aryl-substituent is likely also to affect the BDE_{NO-H} value and, in the H-abstraction step from the substrate, a lower BDE_{NO-H} value would be detrimental to the efficiency of the HAT route.[20] Unfortunately, BDE_{NO-H} values could not be determined for the HBTs family, due to the short life-time of the involved $>N{-}O^{\bullet}$ species that prevents the use of the EPR equilibration method.[19b,c] However, this point could be investigated experimentally within the family of the aryl-substituted HPIs (see below); moreover, use of a thermochemical cycle (and theoretical calculations) allows us to extract a BDE_{NO-H} value for HBT and even for other $>N{-}OH$ mediators (*vide infra*).

Another mediator (entry 19; *N*-*t*-Bu-3,5-dinitrobenzohydroxamic acid, NBI) of this group shows a modest efficiency in spite of a BDE_{NO-H} value that could be adequate.[19b] However, the redox potential that we attempted to measure appears to be beyond the reach of laccase, in keeping with literature evidence for structurally similar compounds (E° values $\geq$ 1.3 V),[22i] and consequently hampers the generation of the Med_{ox} form.

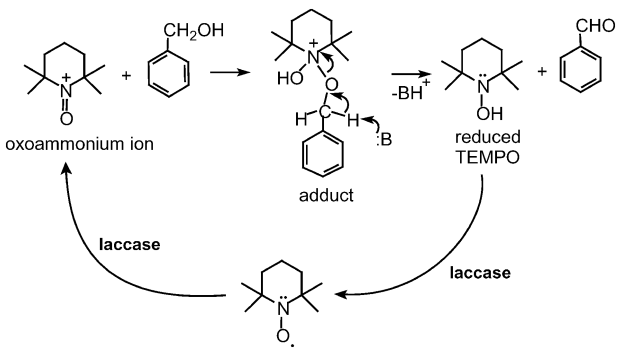
Large values of the kinetic isotope effect (k_H/k_D in the 5.2–6.4 range)[1,13,21] are obtained for this group of mediators on using α-deuteriated benzyl alcohols, as expected for a rate-determining H-abstraction mechanism of oxidation (Scheme 3). The operation of the ionic route documented for TEMPO (Scheme 4)[13,23] would be an unlikely alternative to the HAT route of the $>N{-}OH$ mediators, because further oxidation of their $>N{-}O^{\bullet}$ intermediate to the corresponding $>N{=}O^{+}$ species takes place at a E° value high (>1.3 V) and inaccessible to laccase.

To this group belongs 3-hydroxybenzotriazin-4-one (HBO, entry 11) that was suggested to be a valuable mediator of laccase.[20,22d] The objective comparison of efficiency through the benchmark oxidation (Table 1) sets instead HBO on a modest level among the $>N{-}OH$ mediators. Anyhow, its $>N{-}O^{\bullet}$ form (dubbed here BONO) has been independently generated by us with a stoichiometric amount of the mono-electronic oxidant cerium(IV)ammonium nitrate, CAN, and observed spectrophotometrically in MeCN solution (Fig. 1). It presents UV spectral features (λ_{max} = 524 nm, ε = 1100 M^{-1} cm^{-1}) similar to those of the currently investigated aminoxyl radicals from HBT (*i.e.*, BTNO) and HPI (*i.e.*, PINO),[26] and a



$\Delta H^{\circ} = \Delta H^{\circ}(products) - \Delta H^{\circ}(reactants) = BDE(C_{\alpha}\text{-}H) - BDE(NO\text{-}H)$

Scheme 3 The thermochemical scheme of the radical H-atom transfer (HAT) mechanism between a $>N{-}O^{\bullet}$ species and a C–H donor substrate.



Scheme 4 The ionic mechanism of oxidation of benzyl alcohols by an oxoammonium ion.

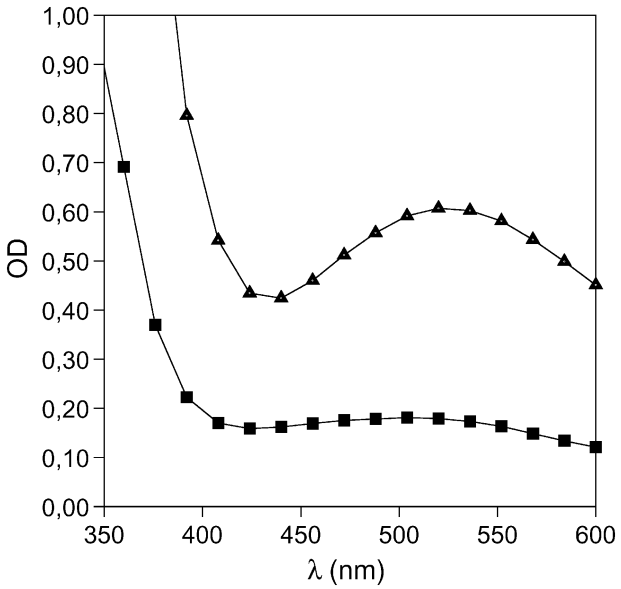


Fig. 1 UV-Vis spectrum of BONO in MeCN: (a) immediately after the generation with CAN (curve ▲), (b) after 2 h (curve ■).

kinetic investigation of its H-abstraction proficiency is planned. The formation of the BONO radical is followed by its spontaneous decay, which is faster (first half-life: 400 s) than that of PINO (first half-life: 7900 s)[26a,b] but slower than that of BTNO (110 s).[26c]

The redox potential of HBO has been determined by cyclic voltammetry (Table 1). An EPR spectrum of BONO has been acquired in MeCN, from reaction of HBO with CAN (Fig. 2).

Redox mediators

Among the artificial mediators of laccase, ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) was the first to be employed.[1,4,5,7,12,14,27] It undergoes one-electron oxidation to the relatively stable (half-life of 90 min) blue-coloured radical cation $ABTS^{\bullet+}$ (λ_{max} = 420 nm, $\varepsilon = 3.5 \times 10^4$ M^{-1} cm^{-1}),[28] at a redox potential (0.69 V)[14] that almost matches that of laccase,[9] so to provide the standard assay of the activity of this enzyme.[29] Further oxidation of $ABTS^{\bullet+}$ to dication $ABTS^{2+}$ occurs electrochemically at higher potential (1.1 V),[5,14,28] but this red-coloured species is less stable.[28] The laccase/ABTS system has been reported to oxidise non-phenolic compounds,[4,5,7,12,27] but a precise assessment of the structure of the intervening Med_{ox} form (whether $ABTS^{\bullet+}$ or $ABTS^{2+}$), as responsible for the monoelectronic oxidation of suitably electron-rich substrates, has not been reported yet.[30] Anyhow, Table 1 points out that the mediation efficiency of laccase/ABTS towards *p*-anisyl alcohol is moderate (entry 10).[14] A new mediator, *i.e.*, 1-(3′-sulfophenyl)-3-methylpyrazolone-5 (3-MPO), has recently been described to be comparable in both mediation efficiency and oxidation mechanism to ABTS.[31] This is confirmed by the result of our benchmark oxidation (entry 9). Furthermore, the addition of a stoichiometric amount of the Ce^{IV} oxidant to a water solution of 3-MPO in a spectrophotometric cuvette gives rise to a broad absorption band having λ_{max} = 314 nm and ε = 1100 M^{-1} cm^{-1}. It spontaneously decays with a half-life of only 8 s, which certainly undermines the value of 3-MPO as a potential diffusible mediator of laccase.[5,12] An attempt to determine the redox potential of 3-MPO has been made by cyclic voltammetry but without success: no distinct oxidation wave appears on scanning the potential up to 1.1–1.2 V.

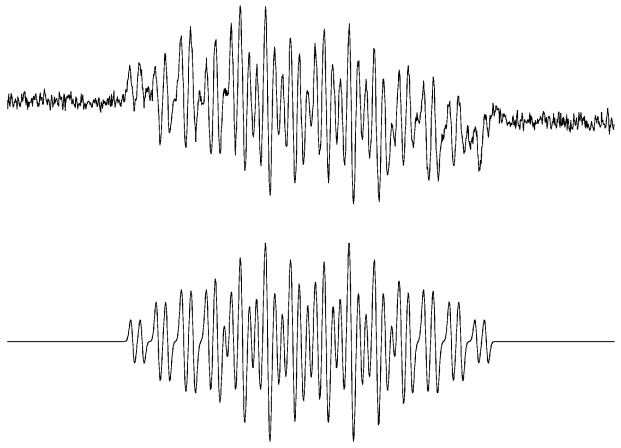


Fig. 2 Experimental EPR spectrum from HBO with CAN in MeCN (upper) and its computer simulation (lower); EPR data: a(N) = 1.38 G, a(N) 2.74 G, a(N) = 4.60 G; a(H) = 0.53 G, a(H) 1.38 G; g = 2.0062.

Natural mediators

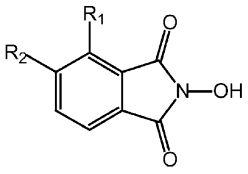
In keeping with the concept of redox mediators delineated in the Introduction,[1,4,5,12] natural metabolites of laccase have been actively searched, but not many have been found and moreover these give contrasting results.[18,32] Phenol or aniline derivatives were at times indicated as potential natural mediators of laccase, two exemplary cases being 3-hydroxyanthranylic acid (HAA) and 2-aminopurine;[33,34] however, our benchmark oxidation gives negligible or modest mediation efficiency with them (entries 20 and 21). The dimerisation reaction of HAA to cinnabarinic acid ensuing oxidation by laccase is indeed well established,[32] and proceeds through a typical phenoxyl–radical coupling; the dimer is not active in any oxidation mechanism of non-phenolics.[32] As a matter of fact, reports of successful mediation with those 'natural' compounds relied on peculiar mediator-to-substrate molar ratios as high as 40 or even 200.[34] At those high concentrations of 'mediator' it is conceivable that any degradation route of the Med_{ox} species, taking place in the reaction medium and leading to side-products of unknown structure, might concur to the onset of oxidation routes of unidentified nature.[18,30c]

In contrast, we reported that Phenol Red and Dichlorophenol Red mediate laccase efficiently in the oxidation of *p*-anisyl alcohol (entries 22–24).[22e] This was attributed (i) to the low pK_a value of these phenolic compounds (*i.e.*, 7 or below), that makes easy both their conversion into the ArO^- form, as well as the subsequent monoelectronic oxidation to $ArO^{\bullet}$ by laccase, but also (ii) to their fair stability as aryloxyl radicals. The $ArO^{\bullet}$ radical in fact must be sufficiently 'stable' to enable the H-removal from non-phenolics before disappearing in undesired phenol-coupling routes.[22e] The BDE_{O-H} of phenol derivatives appears appropriate to a moderately exothermic H-abstraction route as in Scheme 3,[19a] in keeping with the case of the >N–O$^{\bullet}$ intermediates. Phenolphthalein (entry 25) is less acid (pK_a 8) than the cognate compounds of entries 22–24, and this could explain its lower efficiency at the pH of the benchmark reaction. Finally galvinoxyl (entry 27), although a persistent phenoxyl radical, is endowed with a lower BDE_{O-H} that makes the H-abstraction route endothermic.[19a] It also suffers from the steric hindrance of two *t*-Bu groups *ortho* to the phenoxyl radical center, possibly impairing the interaction with potential substrates. Catechol (entry 26) is instead a newly found active mediator, and this has an important implication because the *ortho*-diphenolic moiety is a recurring structural feature in lignin,[11] and benzenediols are typical substrates of laccase.[4,8] The concluding message is that phenolic derivatives, including suitable phenolic moieties of lignin, once oxidised by laccase to phenoxyl radicals, can behave as mediators for the radical oxidation of non-phenolic groups of lignin, provided that they are both easily oxidisable and sufficiently stable as phenoxyl radicals to enable the occurrence of the H-abstrac-

tion route. This conclusion, and the case of catechol in particular, supports a proficient delignifying role for the phenoloxidase laccase in nature even in the absence of added 'foreign' mediators.[1,10,22e,35]

Derivatives of HPI in reaction with laccase

Among the >N–OH mediators, HPI is of particular synthetic interest because, in combination with co-catalysts like $Co(OAc)_2$ or $Co(acac)_2$,[36,37] it efficiently catalyses the oxidation of a large variety of organic compounds under mild conditions, at moderate oxygen pressure and temperature. The above mentioned phthalimide-*N*-oxyl radical (PINO) is the active oxidant, which oxidises the substrate by the radical H-abstraction route, regenerating HPI.[19b,26b,36–38] In this context we considered that it would be worthwhile understanding how the introduction of substituents in HPI can affect the mediation efficiency with laccase. To this purpose, several aryl-substituted HPI's containing either electron-withdrawing (4-MeOCO, 3-F) or electron-donating groups (4-Me, 4-MeO) have been synthesised and investigated.[26b]



4-MeOCO-HPI	$R_1 = H$, $R_2 = CH_3OCO$
3-F-HPI	$R_1 = F$, $R_2 = H$
HPI	$R_1 = H$, $R_2 = H$
4-Me-HPI	$R_1 = H$, $R_2 = CH_3$
4-MeO-HPI	$R_1 = H$, $R_2 = CH_3O$

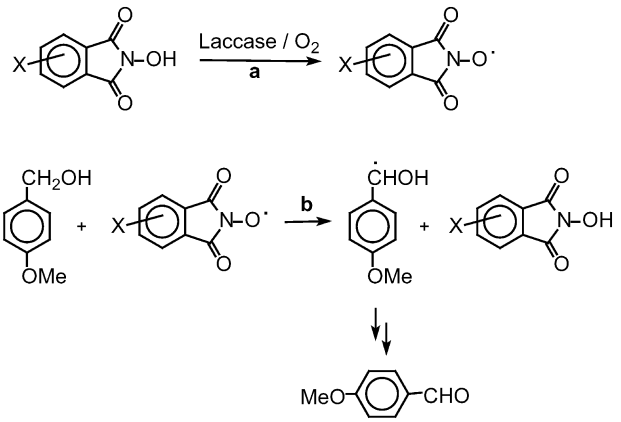
The effect of these substituents on the mediation efficiency by the HPIs towards laccase has been assessed here in the benchmark oxidation (Scheme 2). The reactions were run at room temperature for a shorter (15 h) time, in order to better discriminate these mediators in efficiency. The buffered water solution (0.1 M sodium citrate, pH 5.0), previously purged with O_2, contained 25% dioxane as a co-solvent for better solubility of the substituted HPIs. Once again, the only oxidation product observed in all cases was *p*-anisaldehyde, the rest of mass balance being the recovered substrate. The results are given in Table 2 and compared with those obtained (under the same conditions, *i.e.*, 15 h) with two of the best mediators of Table 1, namely HBT and VLA.[14] Available BDE_{NO-H} and redox data of the mediators are listed,[26b,39] to help in the interpretation.

A clear trend of increasing oxidation efficiency on increasing the electron-richness of the HPIs emerges in Table 2. In fact, negligible or very low yields of *p*-anisaldehyde are obtained with the electron-withdrawing substituted HPIs (4-CH_3OCO and 3-F), whereas 4-MeO-HPI gives a yield more than double that of the unsubstituted HPI, and also decidedly higher than those with HBT and VLA.

Table 2 Yields of *p*-anisaldehyde in the oxidation of *p*-anisyl alcohol by laccase/mediator/O_2 systems in buffered water solution (0.1 M sodium citrate, pH 5), with 25% dioxane as co-solvent, for 15 h at room temperature

Mediator	4-MeO-C_6H_4CHO yield (%)[a]	BDE_{NO-H}/ kcal mol^{-1}[b]	E°/V *vs.* NHE in H_2O[b]
4-CO_2Me-HPI	2.0	88.9	1.18
3-F-HPI	3.0	88.6	—
HPI	13	88.1	1.09
4-Me-HPI	24	88.2	—
4-MeO-HPI	33	87.3	0.99
HBT	16	(*cf.* ref. 20)	1.08
VLA	19	*ca.* 80–82[c]	0.92

[a] The yields % are referred to the initial amount of substrate, and are the average of three determinations (by GC); typical error: ±3%. [b] Refs. 26b and 39. [c] Ref. 19c.



Scheme 5 Mechanism of oxidation of *p*-anisyl alcohol promoted by laccase/X-HPI/O_2 systems.

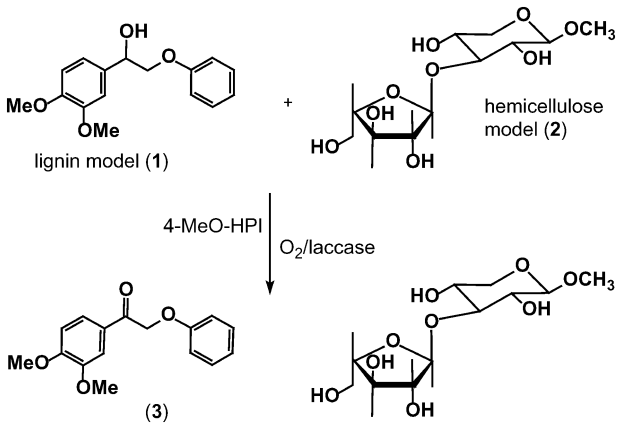
This result can be rationalised considering that the major contribution to the overall reaction rate is given by the oxidation of the aryl-substituted HPI to the *N*-oxyl radical (X-PINO) by laccase (Scheme 5, step a). This process should be favoured by electron-donor X-substituents that lower the mediator redox potential (*cf.* data in Tables 1 and 2).[26b] On the other hand, the H-abstraction process between *p*-anisyl alcohol and the X-PINO (Scheme 5, step b) should be favoured by electron-withdrawing X-substituents that give rise to stronger NO–H bonds (in Table 2).[26b]

Since 4-MeO-HPI emerges as the most efficient mediator, not only among the X-HPIs but even considering all those investigated here (excluding TEMPO and its derivates), it has been chosen for appraising the selectivity of the aerobic oxidation (see below).

Selectivity between lignin and polysaccharides

In a search for new oxidation strategies that take place in an environmental friendly way in water solution,[40] and might enable a selective degradation of lignin in wood pulp for paper manufacture,[2,4,7,41] 4-MeO-HPI has been evaluated as the mediator of laccase in the oxidation of two model compounds of woody tissue. These were a dimeric model of lignin (**1**),[15a,42] having a secondary benzylic alcohol residue, and a dimeric model of hemicellulose (**2**),[43] which is believed to 'tie' lignin to cellulose.[11] Oxidation of a 1 : 1 mixture of these two models with laccase and 4-MeO-HPI in the presence of O_2 was performed at room temperature for a 24 h reaction time (Scheme 6).

Our system oxidised selectively model **1** to the carbonyl derivative **3** (58% yield, referred to the initial amount of **1**), whereas it did not oxidise the aliphatic alcohol groups present in model **2**, which was recovered unchanged (see Experimental Section). The lower BDE of the benzylic C–H bonds (*ca.* 83–87 kcal mol^{-1}),[19] when compared to that of the aliphatic C–H bonds (*ca.* 94–98 kcal mol^{-1}),[19] explains why this >N–O$^\bullet$ radical (BDE_{O-H} of 4-MeO-HPI = 87.3 kcal mol^{-1})[26b] abstracts the *weaker* benzylic C–H bonds geminal to the OH bond in **1**, but not the *stronger* aliphatic C–H bond geminal to the OH bond in **2**. We have therefore a highly selective HAT process, endowed with interesting potential applications in the pulp and paper industry. In contrast, the most efficient mediator of laccase in the oxidation of alcohols, *i.e.*, TEMPO,[44] is not suitable for use in the industrial biodelignification because it lacks selectivity. It oxidises not only benzylic but even aliphatic alcohols,[24] in view of its oxidation mechanism that



Scheme 6 Competitive oxidation of a lignin model (**1**) and of a polysaccharide model (**2**).

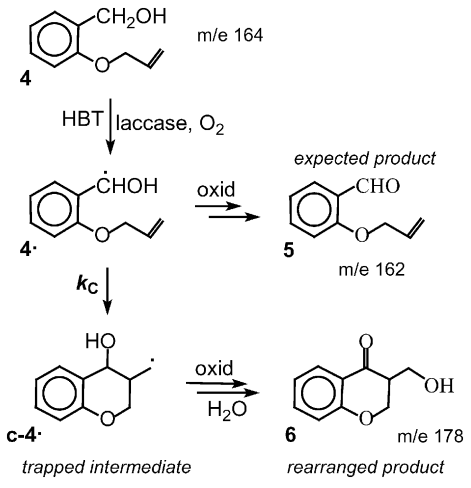
is ionic and not radical, and consequently oxidises even the carbohydrate component of wood, as already demonstrated.[45]

Intercepting the radical intermediate

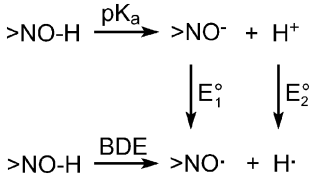
The radical HAT route of oxidation available to >N–OH mediators (Scheme 3) requires H-abstraction from the benzylic position of a non-phenolic substrate, with the consequent formation of a benzyl radical intermediate.[13,46] Further reaction with dioxygen leads to the observed carbonyl end-products.[26b] Capture of an intermediate in a multistep reaction is a classic clue for elucidating a mechanism, but no attempts to intercept the benzyl radical intermediate of the HAT oxidation with laccase/>N–OH mediators have ever been made. We wanted to acquire this particular evidence, in addition to the other evidence already consistent with the HAT route.[13]

A suitable probe substrate was foreseen in 2-allyloxybenzyl alcohol (**4**), in keeping with the radical-clock strategy.[47] The expected oxidation product of **4** is the corresponding 2-allyloxybenzaldehyde (**5**), but the intermediate benzyl radical (**4**$^{\bullet}$) could be diverted and trapped in a fast 6-membered ring formation according to an intramolecular 6-*exo-trig* process (k_c),[48] with ensuing functionalisation (Scheme 7).

Precursor **4** was synthesised in an uneventful route (see Experimental), and subjected to oxidation with laccase/HBT under the usual conditions. As projected, the formation of aldehyde **5** was accompanied by a product that MS and NMR analyses showed to be the 4-chromanone derivative **6**. This is the reasonable outcome of further oxidation of the cyclic radical (**c-4**$^{\bullet}$), deriving from the 6-*exo-trig* ring-closure of the



Scheme 7 Intercepting the radical intermediate of the HAT oxidation mechanism.



Scheme 8 Thermochemical cycle for determining BDE_{NO-H} values.

original benzyl radical intermediate (**4**$^{\bullet}$). Conclusive evidence for the occurrence of the radical HAT route is thereby provided. It is apt to remind that oxidation of a similar cyclisable probe with laccase/TEMPO failed to produce a rearranged product;[23] in fact, no radical intermediate is expected in the ionic route of Scheme 4.[24]

Bond energies from a thermochemical cycle and theoretical calculation

On the basis of the approach delineated in a seminal *Accounts* paper,[49] we have exploited a thermochemical cycle in order to have access to the BDE_{NO-H} value of >NO–H mediators that are not experimentally accessible by means of the EPR equilibration method.[19b,c] The cycle is given in Scheme 8, and requires the knowledge of pK_a[50] and E°[14] data.

Both pK_a and E° values have the dimensions of free energies, whereas an entropy correction must be applied to BDE (an enthalpic value), and also the solvation of the $H^+/H^{\bullet}$ couple must be taken into account:[49] these details are dealt with in the above paper.[49] We have calibrated the cycle in the case of HPI, for which pK_a, E_1° and BDE data are all known (Table 3), in order to confine and extract both the entropy correction and the solvation term in the E_2° parameter, which we then use in the calculation of the BDE_{NO-H} for the other structurally similar >NO–H mediators.

From our calculations it clearly emerges that all of these >N–OH mediators have a BDE_{NO-H} value above 80 kcal mol^{-1},[20] thereby sufficient for making the H-abstraction step in Scheme 3 possible. These numbers support the relative proficiency of mediation given in Table 1 and, in particular, HBT comes out efficient even from a thermochemical viewpoint.

Determination of the BDE_{NO-H} of HBT has been also accomplished by quantum chemical calculations (Gaussian 98 package)[51] by the Density Functional approach (DFT). The geometries of the starting neutral HBT and of the corresponding N–O$^{\bullet}$ radical were optimized at the MPW1P86/6-311G**level. This functional was chosen because it is reported to yield the best agreement with experimental BDEs.[52]

Table 3 Relevant data for the calculation of BDE_{NO-H} values (see Scheme 8). Shortened names of the >NO–H mediators are from Table 1. In brackets, the values of pK_a and E°_1 are converted in kcal mol^{-1} by multiplying by 1.4 and 23.06, respectively

>NO–H	pK_a in H_2O[a]	E°_1[b]	E°_2[c]	BDE_{NO-H}/ kcal mol^{-1}
HPI	6.3 (8.8)	1.09 (25.1)	(54)	88.1[d]
HBT	4.6 (6.4)	1.08 (24.9)	(54)	85[e]
VLA	4.3 (6.0)	0.92 (21.2)	(54)	82[e]
HBO	4.0 (5.6)	1.2 (27.7)	(54)	85–87[ef]
TFBT	3.8 (5.3)	1.11 (25.6)	(54)	83–85[ef]
HOAT	3.5 (4.9)	1.14 (26.3)	(54)	83–85[ef]

[a] From ref. 50 (value in kcal mol^{-1} in brackets). [b] From Table 1 (value in kcal mol^{-1} in brackets). [c] In kcal mol^{-1}; obtained from Scheme 8 for HPI (ref. 49 gives 53 kcal mol^{-1}). [d] Experimental value (ref. 26*b*). [e] Obtained from Scheme 8 as: $BDE = (pK_a + E^{\circ}_1 + E^{\circ}_2)$, all data being in kcal mol^{-1}. [f] Uncertainty due to the solvent (MeCN instead of H_2O) used in the E°_1 determination.

Harmonic vibrational frequencies were calculated at the same level of theory to confirm that the optimized structures correspond to local minima, and to determine zero-point energy (ZPE) corrections. The BDE_{NO-H} of HBT results in this way to be 85.9 kcal mol^{-1}, in excellent agreement with the value (85 kcal mol^{-1}) obtained from the thermochemical cycle. This approach has been validated by back-calculating the experimental BDE_{NO-H} value of HPI (*i.e.*, 88.1 kcal mol^{-1}), and a very satisfactory result of 89.7 kcal mol^{-1} obtained.

Conclusions

A number of new potential mediators of laccase has been comparatively evaluated and ranked towards the benchmark aerobic oxidation of *p*-MeO-benzyl alcohol. The mechanism of oxidation of this non-phenolic substrate by each mediator has been assessed among three alternatives. An ionic mechanism results viable to persistent $>N–O^{\bullet}$ species (such as TEMPO) that can be converted into the $>N{=}O^{+}$ form (*i.e.*, Med_{ox}) on monoelectronic oxidation at a redox potential accessible to laccase, and therefore not exceeding 1 V. A radical H-abstraction mechanism (the HAT route) is instead followed by >N–OH mediators that laccase oxidatively converts into short-lived $>N–O^{\bullet}$ intermediates, provided that the energy of the NO–H bond they give rise to on abstracting H-atom from the substrate is at least 80 kcal mol^{-1} or higher. A redox mechanism may be finally accessible to species, such as ABTS, that are easily oxidised by laccase.[30c] Experimental characterisation of some of the mediators, by means of spectrophotometric, electrochemical and thermochemical survey, supports these assessments. Very significant is the finding that catechol, a model of phenolic moieties of lignin, once oxidised by laccase performs as a radical mediator in the oxidation of non-phenolic groups. This supports a stand alone role as delignifying enzyme for laccase in nature, without the need for added mediators. Clear-cut evidence for the formation of a benzyl radical intermediate in the HAT oxidation of a benzyl alcohol by laccase and >N–OH mediators is gathered through a trapping experiment with a cyclisable probe. The selectivity of the laccase-catalysed oxidation of competing lignin and polysaccharide model compounds has been determined by using the highly proficient 4-MeO-HPI mediator, and found very high in favour of the former model. This is in keeping with the operation of a radical HAT process that efficiently cleaves the benzylic C–H bond with respect to the aliphatic C–H bond of the two models. The selective HAT oxidation by laccase/>N–OH mediators shows therefore interesting promises for application in the pulp and paper industry. Finally, an evaluation of the BDE_{NO-H} value of HBT, which is not accessible experimentally, is provided by the use of a thermochemical cycle, and the approach is extended to other >N–OH mediators. DFT calculations do confirm this result, as 85 kcal mol^{-1}.

Experimental

General remarks

NMR spectra were taken on a AC 300 Bruker instrument. EPR spectra were recorded on a Bruker ESP 300 spectrometer. A VARIAN CP 3800 GC, fitted with a 30 m × 0.25 mm methyl silicone gum capillary column (CPSil5CB), was employed in the analyses. The identity of the products was confirmed by GC-MS analyses, run on a HP 5892 GC, equipped with a 30 m × 0.2 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV.

Materials

Most of the mediators or substrates were commercially available (Aldrich) or already present in the laboratory,[13,14,21] as for example α-monodeuteriated *p*-MeO-benzyl alcohol,[13] 1-(3,4-dimethoxyphenyl)-2-phenoxy-1-ethanol (**1**) and 1-(3,4-dimethoxyphenyl)-2-phenoxy-1-ethanone (**3**),[15] while 2-allyloxybenzaldehyde (**5**) is commercial (Aldrich). The seven-step synthesis of methyl-3-*O*-(α-L-arabinofuranosyl)-β-D-xylopiranoside (**2**), starting from methyl-β-D-xylopiranoside and methyl-α-D-arabinofuranoside, is from the literature.[43] We have repeated it obtaining **2** in an overall 3% yield, and NMR characterisation of **2** is consistent with the one published.[43] The synthesis of a few mediators (entries 3,4,12,19 in Table 1) had been described elsewhere.[19b,53] The substituted *N*-hydroxyphthalimides used in Table 2 were synthesised as reported before.[26b] Acetonitrile was dried over molecular sieves (4 Å) for at least 24 h under Ar prior to the electrochemical experiments. Commercial Bu_4NBF_4 (Fluka) was crystallised from ethyl acetate–petroleum ether, and dried under vacuum (10^{-2} Torr) at 60 °C for 6 h; commercial anhydrous $LiClO_4$ (Aldrich) was used as received. Buffers were prepared using ultrapure water obtained from a MilliQ apparatus.

Synthesis of 2-allyloxybenzyl alcohol (4)

2-Hydroxybenzyl alcohol (Aldrich; 3.2 g, 25 mmol), dissolved in 5 mL of acetone, was allylated with 2.2 mL of allyl bromide (25 mmol) in the presence of 3.6 mg of K_2CO_3 (26 mmol) under stirring for 14 h at room temperature. Acetone was removed, the residue taken-up in diethyl ether, extracted with Na_2CO_3 solution and dried (an. Na_2SO_4). Removal of the solvent, and filtration over a short-path silica gel column, gave **4** in 50% yield; *m*/*z* 164. ^{1}H NMR (200 MHz, $CDCl_3$) δ_H 2 (bs, 1H, O*H*), 4.55–4.50 (d, 2H, OCH_2), 4.65 (s, 2H, CH_2OH), 5.45–5.25 (ddq, 2H, CH_2=), 6.1–5.9 (m, 1H, C*H*=), 7.3–6.8 (m, 4H, Ar*H*); ^{13}C NMR (50 MHz, $CDCl_3$) δ_C 62 (*C*H_2OH), 69 (ArO*C*H_2), 117 (*C*H_2=), 111–127 (C^{Ar}-H), 129 (C^{Ar}–CH_2OH), 133 (*C*H=CH_2), 156 (C^{Ar}–OCH_2).

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.[54] The collected fractions were concentrated by dialysis in cellulose membrane tubing (Sigma) against poly (ethylene glycol) to a final activity of 8000 U mL^{-1}, as determined spectrophotometrically by the standard assay with ABTS.[29]

Enzymatic reactions

The aerobic oxidations reported in Table 1 were performed at room temperature in stirred water solution (3 mL), buffered at pH 5 (0.1 M in sodium citrate) and purged with O_2 for 30 min prior to the addition of the reagents.[13,14] In general, the initial concentration of the reagents was: [*p*-anisyl alcohol] 20 mM, [mediator] 6 mM, with 3 U mL^{-1} of laccase, and the reaction time was 24 h at room temperature, an atmosphere of oxygen being kept in the reaction vessel by means of a hemi-inflated latex balloon. The oxidation reactions reported in Table 2 were similarly performed at room temperature, but a 4 : 1 buffered water : dioxane mixed solvent (5 mL) was employed to ensure full solubility of the HPIs mediators. The initial concentrations were: [*p*-anisyl alcohol] 5 mM, [mediator] 1.7 mM, with 1 U mL^{-1} of laccase, and the reaction time was shorter (15 h) than in Table 1. The reaction mixture was extracted with ethyl acetate or with CH_2Cl_2, dried over Na_2SO_4 and characterised by GC-MS. The yields of oxidation (Table 1 and 2) were reckoned by GC analysis from multiple injections with respect to an internal standard (acetophenone or 4-methoxyacetophe-

none or 4-methylbenzophenone), using suitable response factors determined from authentic products. A good material balance (>95%) was observed in all the experiments. In the absence of the mediator or the enzyme, no products were observed in significant amounts (<0.1%).

Competitive aerobic oxidation of model compounds 1 and 2

Mediator 4-MeO-HPI (18 μmol), laccase (20 units) and 40 μmol of each of the models **1** and **2** were added to 6 mL of a buffered solution (0.1 M sodium citrate, pH 5.0) with 5% MeCN as co-solvent, which had been purged with O_2 for 30 min before the addition of the reagents. The mixture was magnetically stirred at room temperature for 15 h under oxygen (filled balloon), and then worked-up with ethyl acetate. The solvent was removed under reduced pressure, the reaction mixture acetylated with Ac_2O in pyridine and finally analysed by ^{1}H NMR. Model **1** underwent selective oxidation to 1-(3,4-dimethoxyphenyl)-2-phenoxy-1-ethanone (**3**) (58% yield, referred to the initial amount of **1**). 39% of **1** was recovered as the acetyl derivative (referred to the starting material, and calculated from the integrated signal of the benzylic CHOAc at 6.09 ppm). 96% of model **2** was recovered as the per-acetylated derivative (the recovery is referred to the starting material, and calculated from the integrated signal of the H-1_{ara} at 5.09 ppm). ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.91–2.20 (s, 15H, $COCH_3$), 3.30 (dd, J = 5.0, 10.5, 1H, H-5_{xyl}), 3.78 (m, 1H, H-5_{xyl}), 4.05 (m, 1H, H-3_{xyl}), 4.20 (m, 1H, H-4_{ara}), 4.25 (m, 2H, H-5_{ara}), 4.28 (d, J = 7.5, 1H, H-1_{xyl}), 4.85 (m, 1H, H-2_{xyl}), 4.85 (m, 1H, H-4_{xyl}), 4.81–4.90 (m, 2H, H-2_{ara} and H-3_{ara}), 5.09 (s, 1H, H-1_{ara}).

Laccase/HBT aerobic oxidation of 4

To a solution of 100 mg of **4** (0.6 mmol) in 10 mL of 0.1 M citrate buffer (pH 5), containing 30% dioxane and purged with O_2, HBT was added (36 mg, 0.2 mmol) followed by 100 U of laccase, and the solution was stirred at room temperature for 24 h under oxygen (filled balloon). After work-up with ethyl acetate, GC-MS analysis gave evidence of three peaks. One was residual **4** (m/z 164); the second peak, having m/z 162 and an intense M-1 fragment, was identified as **5** by comparison of the retention time and fragmentation pattern with those of commercial **5**. The third peak had m/z 178. Short-path chromatography of the crude residue of this reaction, run over silica gel with a hexane : ethyl acetate 9 : 1 eluent, gave 11 mg of this product, and NMR spectroscopy confirmed the structure of **6**. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 2.95–3.05 (m, 1H, C*H*CO), 4.0–3.8 (ddd, 2H, CH_2OH), 4.6–4.3 (m, 2H, OCH_2), 5.6 (bs, 1H, O*H*), 8.1–6.9 (m, 4H, Ar*H*); ^{13}C NMR ($CDCl_3$) δ_C 47 (*C*HCO), 69 (*C*H_2OH), 71 (ArO*C*H_2), 115 (C^{Ar}–CO), 135–120 (C^{Ar}–H), 162 (C^{Ar}–OCH_2), 195 (*C*=O); DEPT confirmed signals 71 and 69 as CH_2, signals 135–120 and 47 as CH, signals 162 and 115 as quaternary carbons.

Kinetic isotope effect determination

As described previously,[13] α-monodeuteriated *p*-anisyl alcohol (Ar–CH(D)OH) was oxidised aerobically with laccase/PROXYL under the experimental conditions given in the 'Enzymatic reactions' section. After a 5 h reaction time, determination of the relative amount of the Ar–CHO and Ar–CDO oxidation products was done by GC-MS analyses in the SIM mode, and this enabled to reckon the intramolecular k_H/k_D ratio.

Electrochemical determinations

Cyclic voltammetry at the steady disc electrode (glassy-carbon disc, 1.5 mm in diameter)[14] was carried out at 25 °C in MeCN containing 0.1 M Bu_4NBF_4, or alternatively in 0.01 M sodium acetate buffer containing 0.1 M $LiClO_4$. A three-electrode circuit was used with either an electrochemical computerized system (Amel System 5000, version 2.1), or a potentiostat with positive feedback ohmic-drop compensation,[55] and a MPLI Hardware (Vernier Software & Technology) controlled by a program written in C++ language for Windows 95/98.[56] The auxiliary electrode was a platinum wire (surface 1 cm^2), and either a saturated calomel electrode (SCE) or a Ag/AgCl/KCl 3 M electrode was used as reference. Redox potential values are referred to NHE. The scanning rate ranged from 0.005 V s^{-1} to 50 V s^{-1}. The investigated mediator was 1–2 mM, and a twofold excess of pyridine in MeCN was used to deprotonate the >N–OH mediators into the more easily oxidisable >N–O^- form (see pK_a values in Table 3).

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