The radical rate-determining step in the oxidation of benzyl alcohols by two N–OH-type mediators of laccase: the polar *N*-oxyl radical intermediate

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Determination of the effect of substituents in the aerobic oxidation of X-substituted benzyl alcohols by laccase, with mediation by HPI or HBT, confirms the H-atom abstraction from the benzylic C–H bond as the rate-determining step (HAT route), and supports a polar nature for the *N*-oxyl radical intermediate originating from the two N–OH mediators.

Introduction

Ecologically benign oxidation procedures have received much attention in recent times. An efficient aerobic oxidation of alcohols into aldehydes and ketones has been published,[1] that makes use of catalytic amounts of a Cu^{I}–phenanthroline complex. Another aerobic procedure requires $Co(OAc)_2$ as the catalyst, in combination with *N*-hydroxyphthalimide (HPI).[2] By this approach, not only alcohols[3,4] but also *N*-alkylamides[5] and even alkanes[6] were oxidised to carbonyl compounds under mild conditions. In these HPI-dependent oxidations, the phthalimide-*N*-oxyl (PINO) radical (*i.e.*, $>N–O^\bullet$), generated *in situ* in the initiation stage from the N–OH precursor (eqn. 1), was reported to play the key role of abstracting an H-atom from the substrate,[2–6] eventually leading to oxidation products with the intervention of O_2 (eqn. 2).

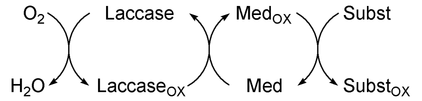
$$Co^{II} + O_2 \rightarrow Co^{III}OO^\bullet \xrightarrow{HPI} Co^{III}OOH + PINO \quad (1)$$

$$PINO + ArCH_2OH \rightarrow HPI + ArC^\bullet HOH \xrightarrow{O_2} ArCHO \quad (2)$$

We have independently postulated the intermediacy of PINO as the key reactive intermediate in an aerobic oxidation of benzyl alcohols catalysed by the enzyme laccase with mediation by HPI.[7] Although laccase is a phenol-oxidase,[8] the inclusion of oxidizable low molecular weight compounds (*viz.*, mediators) enables the activity of this enzyme to be expanded towards non-phenolic substrates.[9] The H-abstracting PINO would be generated *in situ* by laccase through a preliminary redox step, followed by deprotonation (eqn. 3).[7]

$$HPI + laccase \rightarrow HPI^{\bullet +} \rightarrow PINO + H^+ \quad (3)$$

The intermediacy of PINO appears as the common feature between our enzymatic process[7] and the non-enzymatic ones of the literature.[2–6] Another N–OH derivative, *i.e.* 1-hydroxybenzotriazole (HBT), has been analogously found to efficiently mediate laccase in the oxidation of non-phenolic lignin model compounds,[7] such as benzyl alcohols, in enzymatic attempts at bleaching kraft pulp.[9–12] In analogy to PINO, it has been suggested that the benzotriazole-1-oxyl (BTO) radical (*i.e.*, Med_{ox} in Scheme 1), generated from HBT by laccase (compare with eqn. 3),[7] removes H-atoms from the substrate (as in eqn. 2)[7,10–14] and gives the oxidation products.



Scheme 1 The role of a mediator of laccase activity.

It is important that the mediator can be easily oxidised by laccase to the Med_{ox} status: the structure of the latter is crucial for the mechanism of the ensuing non-enzymatic oxidation of the substrate. Then, two different hypotheses do confront in the literature for the mechanism of the latter step. Either the Med_{ox} intermediate should perform a one-electron oxidation (ET) of the substrate to a radical cation,[9,10,12,15] or it should abstract H-atom (HAT), converting the substrate into a radical.[7,14,16] End-products of oxidation would be formed from either one of these two short-lived intermediates of the substrate.

Because the oxidising power of these N–OH-derived Med_{ox} intermediates (*i.e.*, $>N–O^\bullet$) is not strong enough[7] to remove electrons efficiently from benzyl alcohols, we rather favour the HAT route,[7] in keeping with the radical mechanism proposed for the non-enzymatic HPI-dependent oxidations.[2–6] This HAT route has received recent support from kinetic isotope effect determinations.[17] Here we provide further evidence for the radical HAT pathway in laccase oxidations with N–OH mediators, by resorting to the determination of the effect of substituents on the reactivity of the process.

Experimental

Enzyme preparation

Laccase from a strain of *Trametes villosa* (*viz. Poliporus pinsitus*) (Novo Nordisk Biotech) was employed. It was purified by ion-exchange chromatography on Sephadex by elution with phosphate buffer,[18] and an activity of 9000 U mL^{-1} determined spectrophotometrically by the standard reaction with ABTS.[19]

Chemicals

The substituted benzyl alcohols, as well as the corresponding aldehydes, were reagent grade commercial samples (Aldrich).

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Enzymatic reactions

The reactions were performed at room temperature in stirred water solution (6 mL, containing 10% of dioxane), 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. Competition experiments were run between a substituted benzyl alcohol and $PhCH_2OH$, where the concentrations of the reagents were: $[ArCH_2OH]$, 20 mM; $[PhCH_2OH]$, 20 mM; [mediator], 6 mM, with 10 units of laccase. Reaction times of 4–5 h were adopted. The yield of the oxidation products (ArCHO and PhCHO) was determined by GC analysis with respect to an internal standard (acetophenone or *p*-methoxyacetophenone), suitable response factors being determined from authentic products. A VARIAN 3400 Star instrument, fitted with a 20 m × 0.25 mm methyl silicone gum capillary column, was employed in the GC analyses. The standard integrated formula for competitive reactions was adopted (eqn. 4),[20] in order to calculate the k_X/k_H ratios from the yields of the oxidation products:

$$k_X/k_H = \frac{\log\dfrac{[ArCH_2OH]_o - [ArCHO]}{[ArCH_2OH]_o}}{\log\dfrac{[PhCH_2OH]_o - [PhCHO]}{[PhCH_2OH]_o}} \qquad (4)$$

Results and discussion

Hammett structure/reactivity correlation

In order to gain a better understanding of the nature of the rate-determining step in the laccase-mediated oxidations, we attempted the determination of the effect of substituents by resorting to a Hammett correlation. The influence of the inductive and electronic effects of the substituents, in making the rate-determining step faster or slower, was to emerge from this treatment.[20] A series of X-substituted benzyl alcohols (*i.e.*, $ArCH_2OH$), featuring NO_2, Cl, Me, and MeO as X-substituents, was therefore oxidised with the laccase/HPI and laccase/HBT systems. Each of these precursors was pitted with the unsubstituted benzyl alcohol in separate competition experiments, and the k_X/k_H relative reactivity ratios determined (eqn. 4) from the amounts of the two resultant aldehydes (ArCHO and PhCHO, respectively, eqns. 5 and 6) by GC analysis (Table 1), for reaction times that would ensure only a modest conversion into the carbonylic products.[20] No other products form, besides ArCHO and PhCHO.

$$ArCH_2OH + Med_{OX} \xrightarrow{k_X} ArCHO + Med \qquad (5)$$

$$PhCH_2OH + Med_{OX} \xrightarrow{k_H} PhCHO + Med \qquad (6)$$

The log k_X/k_H ratios were then plotted *vs.* the pertinent σ constant of the substituent X,[20] according to the Hammett equation:

$$\log k_X/k_H = \rho\sigma \qquad (7)$$

Table 1 Determination of the ρ parameter for the laccase-mediated competitive oxidations of $X{-}C_6H_4CH_2OH$ *vs.* $PhCH_2OH$, with HPI or HBT as mediators

	HPI	HBT
$k_{p\text{-}NO_2}/k_H$	0.47	0.68
$k_{m\text{-}NO_2}/k_H$	0.44	0.59
$k_{p\text{-}Cl}/k_H$	1.48	1.70
$k_{p\text{-}Me}/k_H$	3.80	2.57
$k_{p\text{-}MeO}/k_H$	10.96	6.61
$\boldsymbol{\rho}(r^2)$	**−0.89** (0.99)	**−0.64** (0.98)

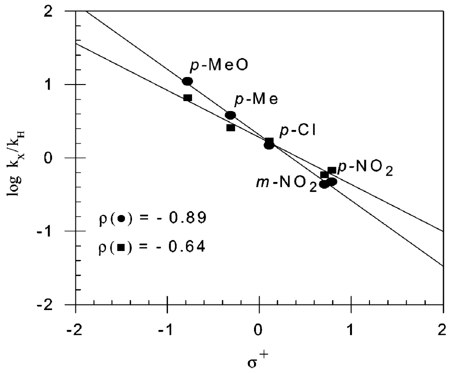

Fig. 1 Hammett correlation for the laccase/HPI (●) and laccase/HBT (■) induced oxidation of X-substituted benzyl alcohols.

Correlation with the Okamoto–Brown σ^+ constants[20] for the two electron donor Me and MeO substituents gave better fits (Fig. 1), and the resulting ρ parameters are reported in Table 1 for the two laccase/mediator systems. The small ρ values obtained (*i.e.*, −0.89 and −0.64) offer experimental support for the radical nature of the slow step of the laccase-mediated oxidations, in keeping with the HAT route (eqn. 2). In fact, small ρ values are consistent with those obtained in unambiguous radical processes, such as the reactions of substituted toluenes with bromine or NBS[21] or with $Cl_3C^\bullet$,[22] where ρ values in the range of −0.3 to −1.5 were reported. As a fitting example of the mechanistic significance of a ρ value, with respect to a HAT *vs.* ET dichotomy, the oxidation of a series of substituted benzyltrimethylsilanes is recalled. The ρ value of −1.15 obtained in a procedure that makes use of PhIO and $TFPPMn^{III}Cl$ [where TFPP stands for tetrakis(pentafluorophenyl)porphyrin],[23] was considered to be convincing evidence for the occurrence of H-atom abstraction from the silane by the intermediate $TFPPMn^{V}{=}O$ species. In contrast, when the same series of substituted benzyltrimethylsilanes was oxidised[24] by the strong electron transfer agent $(NH_4)_2Ce^{IV}(NO_3)_6$ (*viz.*, CAN; E° 1.5 V),[25] a much larger value of ρ (*i.e.*, −5.4) was found; this indicated that the substituents play a more substantial role in making easier, or more difficult, the generation of the radical cation of the silanes in the ET rate-determining step of the process. A final consideration, with respect to the small ρ values we have obtained with HPI and HBT as mediators, concernes the possibility that the H-abstraction step (eqn. 2) by the PINO and BTO radicals is *only partially* rate determining. If the very generation of the PINO and BTO radicals by laccase concurs, in a pre-equilibrium, to make up the rate determining step, this could (also) justify the small ρ values obtained. While we thank one of the referees for taking up this point, we add that in an ongoing study we find evidence that the generation of the *N*-oxyl radical from a substituted HPI precursor by laccase may become rate determining only if HPI is substituted with an electron-withdrawing substituent.

Polarity of the $>N{-}O^\bullet$ intermediate

Interestingly, in all the above mentioned HAT processes,[21–23] the Hammett correlations were found to give better fits *vs.* the σ^+ substituent constants. This was taken as an indication of the relevance of stabilising contributions from charge-separated resonant structures to the radical transition state,[26,27] and also of the electrophilic character of the attacking radical species ($Br^\bullet$ or $Cl_3C^\bullet$).[21,22] It is worth mentioning that the importance of polar effects in radical processes has been recently stressed[4,5] for the non-enzymatic oxidation reactions occurring through the PINO radical,[2–6] an electrophilic radical. In this context, the electron-richness of the substrate ought

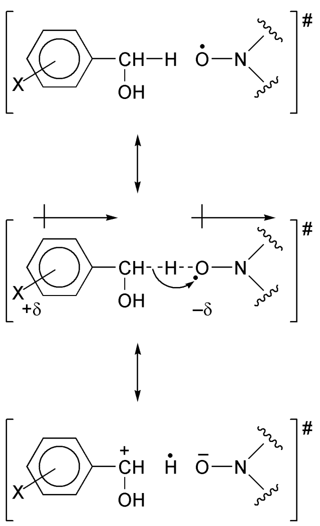

Fig. 2 Polar effects in the radical H-abstraction step.

to provide stabilisation for the radical transition state of H-abstraction,[4] particularly with the electron-donor groups. Consistently, the relevance of polar effects upon the radical route of our laccase-mediated oxidations would explain the better fit obtained *vs.* the σ^+ constants, and would be responsible for making 'rather significant' in value the ρ determined with HPI (and HBT). The relevance of polar effects in the stabilisation of the radical HAT transition state,[26,27] and the electrophilic character of the $>N–O^\bullet$ intermediate,[4,5] are described in Fig. 2.

C–H *vs.* O–H homolysis

We have evidence that the Med_{ox} intermediate of the two mediators, *i.e.*, the PINO and BTO radicals, abstracts H-atom from the benzylic C–H bond of the benzyl alcohols in the rate-determining step. A possible source of ambiguity could be provided by a concurrent H-abstraction from the O–H bond of these substrates. A benzyloxy radical would result,[28] that would then cooperate with either PINO or BTO in further HAT from the C–H bonds of the competing benzylic alcohols, so hampering the mechanistic significance of the ρ values obtained.

This alternative has no thermodynamic support. In fact, the dissociation energy of a benzylic C–H bond ($BDE_{C–H}$) is much lower (83–85 kcal mol^{-1})[4,5,29] than that of the O–H bond of an aliphatic alcohol (102–104 kcal mol^{-1}) (Fig. 3).[29,30]

While the homolysis of the weaker O–H bond of a *phenol* (82–85 kcal mol^{-1})[30,31] could well occur, that of a benzylic alcohol is energetically disfavoured over the benzylic C–H homolysis. In fact, homolysis of alcoholic O–H bonds, in competition with that of benzylic C–H bonds, has never been considered in HPI-based procedures,[2–6] nor in a recent review on radical reactions of aryl alkanols.[32]

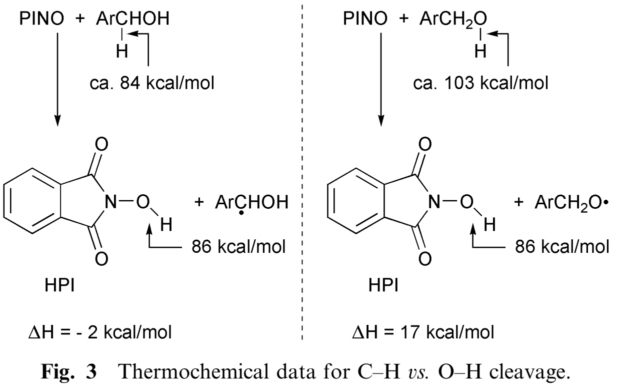

Fig. 3 Thermochemical data for C–H *vs.* O–H cleavage.

$$\mathrm{HBT + ArCH_2OMe} \xrightarrow[O_2]{\text{laccase}} \mathrm{ArCHO\ (75\%)}$$

$$\mathrm{HBT + ArCH_2OH} \xrightarrow[O_2]{\text{laccase}} \mathrm{ArCHO\ (76\%)}$$

Scheme 2

Neither does that alternative possibility have chemical support. In fact, the methyl ether of *p*-methoxybenzyl alcohol is converted into *p*-methoxybenzaldehyde by the laccase/HBT or laccase/HPI systems with the same efficiency found in the corresponding reactions of *p*-methoxybenzyl alcohol (Scheme 2), indicating that the O–H bond plays no role in the success of the oxidation process.[33]

Finally, we point out that the use of dioxane as the co-solvent (10%) causes no significant drawbacks in our reactivity/selectivity study. In the absence of this co-solvent the reaction solutions are somewhat cloudy, but comparable ρ values can be obtained. Therefore, although dioxane can be reactive with radicals, there appear to be no interference from its use during the generation of our *N*-oxyl radicals, nor with laccase/mediator systems.[34]

Conclusions

The present investigation offers additional experimental evidence that, combined with the results described in previous papers,[7,17] allows the assessment with sufficient confidence that a radical HAT mechanism operates in the laccase/HPI and laccase/HBT aerobic oxidations of benzyl alcohols. This conclusion parallels current mechanistic views available for the non-enzymatic HPI-dependent oxidation of benzyl alcohols.[2–6]

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