A study of the oxidation of ethers with the enzyme laccase under mediation by two N–OH–type compounds

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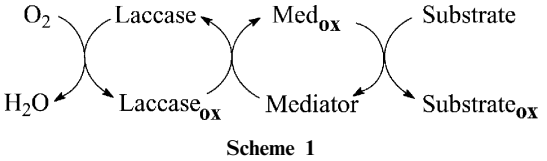
Received (in London, UK) 7th November 2002, Accepted 25th November 2002
First published as an Advance Article on the web 6th December 2002

The oxidation of ethers of various structure by O_2 with two laccase/>N–OH systems (*i.e.*, HBT, 1-hydroxybenzotriazole, and HPI, N-hydroxyphthalimide) is described. The process affords carbonylic products in reasonable-to-good yields. The oxidation is carried out by the intermediate >N–O$^{\bullet}$ species of the mediators, through a radical H-atom abstraction (HAT) route that represents an elaboration of the HAT route followed with benzyl alcohols. Alternative mechanisms have been considered and dismissed. A comparison with a similar oxidation of ethers by the laccase/TEMPO system, reported previously, is made. A clear-cut specialisation of the mediator *vs.* the substrate emerges, *i.e.* the laccase/HBT system is more competent for the oxidation of ethers, whereas the laccase/TEMPO system was most proficient in the oxidation of benzyl alcohols.

Introduction

White-rot fungi in nature are able to degrade wood by secreting ligninolytic enzymes.[1] Laccase, one of these enzymes, is more precisely a family of 'blue-copper' oxidase proteins, containing four copper ions in the active site; these enzymes have a molecular mass of about 70 000 Da, and perform the oxidation of a broad range of substrates, including lignin, with the concomitant reduction of O_2 to water.[1,2] Laccase cooperates with other enzymes, such as lignin peroxidase (LiP)[3] and manganese peroxidase (MnP),[4] in this task. LiP and MnP are heme-based peroxidase enzymes. Laccase has a lower redox potential (*ca.* 0.7–0.8 V/NHE)[2,5] than LiP and MnP, which are more powerful oxidants (more than 1.3 V/NHE), and therefore is able to catalyse mono-electronic oxidation steps only with the easy-to-oxidise phenolic components of lignin (phenol-oxidase activity).[1,2] Lignin is a three-dimensional, insoluble aromatic polymer that constitutes 15–30% of biomass. Its structure encompasses a number of different types of links between its constituents, among which ether and C–C diaryl linkages represent more than 70% of all the functional groups present.[6] It was an important discovery, therefore, to find that the action of laccase, a readily available and easier to manipulate enzyme than LiP and MnP, can be expanded towards more difficult to oxidise non-phenolic substrates through the use of appropriate mediators.[7–9] The role of a mediator in laccase oxidations is outlined in Scheme 1.

Once oxidised by laccase, the mediator (*i.e.*, Med_{ox}) performs a non-enzymatic oxidation of the substrate, by using mechanisms that may be unavailable to laccase, thereby explaining the possibility to oxidise non-phenolic substrates, and widening the usefulness of a purely enzymatic method.[9]



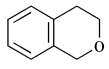
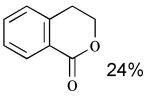
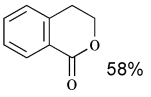
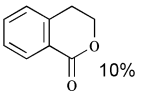
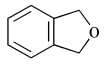
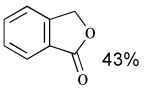
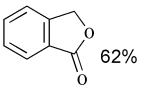
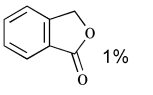
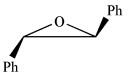
Scheme 1

Oxygen re-oxidises laccase, thereby closing the catalytic cycle. Laccase/mediator systems may be considered for various applications, such as textile dye bleaching,[10] or environmentally-friendly Kraft pulp delignification in the paper industry,[7b,11] or also in selective organic transformations.[12–15] It is clear that a better understanding of the mechanistic role of the mediator, and of the spectrum of substrates accessible to laccase/mediator systems, has both practical and fundamental relevance. We have undertaken an evaluation of the efficiency of a number of mediators of laccase in the oxidation of benzylic alcohols, taken as non-phenolic models of lignin:[9,16] TEMPO (*i.e.*, 2,2,6,6-tetramethylpiperidin-1-yloxy free radical) proved the most efficient mediator.[9,13] However, when we considered benzylic ethers, which are even more representative of the lignin structure, TEMPO gave poorer performances.[17] We explained this partial failure towards oxidation of ethers on the basis of the *ionic* oxidation mechanism that the Med_{ox} form of TEMPO, *i.e.*, an oxoammonium ion,[18] follows.[9,17] Other mediators of laccase, notably those presenting a N–OH structure, such as 1-hydroxybenzotriazole (HBT) and N-hydroxyphthalimide (HPI), have been found to follow a different, *radical* mechanism of oxidation of benzyl alcohols.[5b,7c,8,9,16,19–22] We therefore deemed it worthwhile to explore the efficiency of these laccase/N–OH-type mediator systems towards benzyl ethers. The present study reports our results obtained in the mediated oxidation of some ether-type model compounds, both lignin and non lignin-related. The foreseeable application to enzymatic methods of Kraft pulp delignification for paper making will be discussed.

Results and discussion

Several ether-type substrates were investigated, which were considered representative of structural motifs present in the lignin polymer, or also significant from a mechanistic viewpoint. They include: diaryl ethers, benzyl ethers, alkyl ethers and an epoxide. These precursors were oxidised with the laccase/HBT and laccase/HPI systems, at room temperature for a 24 h reaction time. The solvent was a 0.1 M citrate buffer

Table 1 Results of the oxidation reactions of ethers with two laccase/N–OH-type mediators, compared with previous results obtained with the laccase-TEMPO system

Run	Substrate	Reaction products (yield, %) with Laccase-HPI	Laccase-HBT	Laccase-TEMPO [a]
1	$PhCH_2OPh$	PhCHO (6%) PhCOOPh (4%)	PhCHO (8%) PhCOOPh (6%)	no reaction
2	$PhCH_2OCH_2Ph$ [b]	PhCHO (16%) $PhCOOCH_2Ph$ (2%)	PhCHO (21%) $PhCOOCH_2Ph$ (8%)	PhCHO (16%) $PhCOOCH_2Ph$ (3%)
3	PhOPh	no reaction	no reaction	no reaction
4	*p*-MeO–C_6H_4–C_6H_4–OMe-*p*	—	no reaction	no reaction
5	 isochroman	 24%	 58%	 10%
6	 phthalan	 43%	 62%	 1%
7	4-MeO–$C_6H_4CH_2OMe$	—	ArCHO (32%) ArCOOMe (43%)	—
8	3,4-dimethoxy-$C_6H_3CH_2OMe$	ArCHO (38%) ArCOOMe (15%)	ArCHO (67%) ArCOOMe (25%)	ArCHO (18%)
9	cyclo-Hex-CH_2OMe	—	no reaction	no reaction
10	 *trans*-stilbene oxide	—	no reaction	no reaction
11	$PhCH_2OCH_2Ph$ [c]	—	PhCHO (50%) $PhCOOCH_2Ph$ (16%)	—

[a] From ref. 17. [b] In this compound, part of PhCHO also originates by further oxidation of the alcohol fragment, *i.e.*, $PhCH_2OH$ (see text). [c] Employing a substrate:mediator 1:1 ratio.

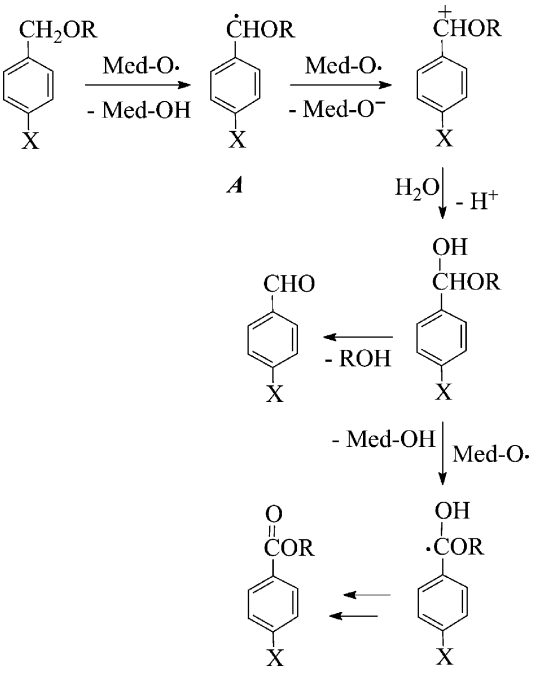
(pH 5) solution containing 4% MeCN, in order to ensure sufficient solubility of the substrates.[23] The reaction conditions employed, and in particular the molar defect of mediator with respect to the substrate, are consistent with Scheme 1, where the mediator shuttles back and forth between its natural and oxidised states (Med_{ox}), due to the intervention of laccase and oxygen, while carrying out the non-enzymatic oxidation of the substrate in a catalytic cycle.

Many of the investigated substrates were converted into carbonyl products, and the oxidation yields are reported in Table 1; a comparison is provided with the results previously obtained with the laccase/TEMPO system.[17] It must be observed that the yields are calculated with respect to the substrate, the molar amount of the mediator being only one-third of that. Accordingly, some yields of product are greater than 100%, if evaluated with respect to the mediator, thereby supporting an oxidation process with turnover. No other oxidation products were found, besides those indicated; the amount of substrate recovered in each experiment is not indicated, but always complements the amount of product detected. The structure of the products was confirmed by GC-MS and by comparison with authentic samples. No conversion to products was obtained in the absence of laccase, nor of the mediator, with any of these non-phenolic substrates.

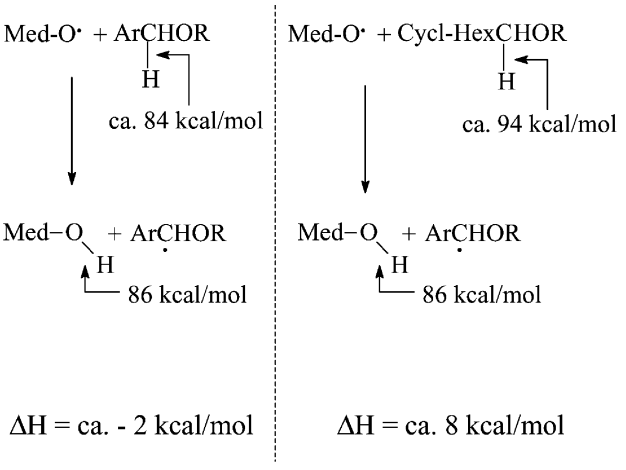
Reactivity of substrate

A first glimpse at Table 1 shows that mediators HBT and HPI behave similarly, as far as reactivity with substrate and extent of conversion is concerned, and clearly differ with respect to the behaviour of mediator TEMPO.[17] In general, conversion into oxidised products is higher with HBT and HPI. In particular, both HBT and HPI enable the laccase-mediated oxidation of $PhCH_2OPh$, which did not react with laccase-TEMPO.[17] These results are reasonably accounted for if we consider that the oxidised forms of the mediators (*i.e.* the oxammonium ion form of TEMPO on the one hand, and the >N–O• radical form of HBT and HPI on the other) oxidise the substrates through different mechanisms. Whereas an ionic mechanism of oxidation is followed by the TEMPO-oxoammonium,[9,13,17,18] where the nucleophilic character of the substrate plays an important role,[17] the >N–O• species follows a hydrogen atom transfer (HAT) route.[8,9,16,19–22] The HAT mechanism, in the case of benzylic alcohols as substrates, has been supported by the determination of the effect of substituents (Hammett correlation),[16,20] by kinetic isotope effect measurements,[16,19] and by the product distribution with a suitable probe molecule.[16,19] In order to account for the oxidation of ethers, particularly with respect to the formation of ester-like products, the HAT mechanism needs to be somewhat implemented, as reported in Scheme 2.

The >N–OH mediator is converted into the Med-O• form by the enzyme (Scheme 1),[5b,7c,9] and subsequently it abstracts a H-atom from reactive C–H bonds, to give a radical intermediate (***A***). This explains why benzylic substrates ($ArCH_2OR$) can react (runs 1, 2, 5–8), whereas diaryl ethers (runs 3 and 4), lacking weak C–H bonds, cannot. In the case of an aliphatic



Scheme 2



Scheme 3

ether (run 9), the stronger C–H bond makes the abstraction step endothermic (Scheme 3)[24] to such extent that the HAT route slows down substantially.

The strength of vinyl-like C–H bonds (BDE, *ca.* 110 kcal mol^{-1})[25] in precursor epoxide (run 10), is such that the HAT route is no longer accessible, and no reaction takes place (*vide infra*).

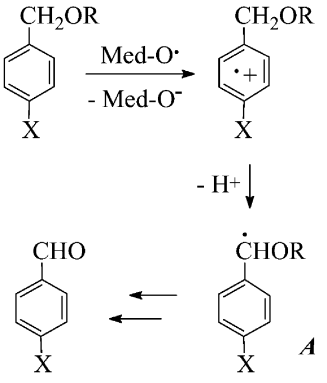
Radical intermediate ***A*** may be further oxidised to a carbocation,[26] either by the Med-O• or by laccase itself. Addition of water to the carbocation would give a hemiacetal, which gives rise to the observed aldehyde through loss of an alcohol molecule. The latter can also be oxidised: this is the case with $(PhCH_2)_2O$, where benzyl alcohol is initially produced along with benzaldehyde; benzyl alcohol is oxidised in turn to provide additional benzaldehyde. On the other hand, further oxidation of the hemiacetal by Med-O• yields the other observed product, that is the ester.[26,27] The second route occurs almost exclusively with the two cyclic precursors (runs 5 and 6), most likely due to the higher stability of the cyclic (sugar-analogue) hemiacetal intermediate, to afford the corresponding lactones in synthetically interesting yields. In contrast, oxidation to the ester-level was only a minor path in the laccase-TEMPO case.[13,17,18]

Finally, an increase of the molar amount of HBT, from a mediator:substrate ratio of 1:3 to 1:1, with an equal reaction time, causes a sizeable increase in the conversion of $(PhCH_2)_2O$ to products (run 11 *vs.* 2), as expected for a process where the oxidation efficiency depends on the nature and amount of the mediator (Scheme 1).

HAT *vs.* ET mechanism?

The possibility that the Med-O• species carries out the oxidation of the substrate to intermediate ***A*** by an electron transfer (ET) route,[28] and not by a HAT route, has been considered (Scheme 4).

Both HBT and HPI, in the >N–O• form, have redox potentials in the order of 1.0–1.1 V/NHE,[9,29–31] that would be perhaps sufficient to remove an electron, at least from those substrates[9] endowed with the lower redox potential, which are easier to oxidise. The feasibility of this alternative hypothesis has been tested independently, through the use of a *bona fide* ET agent. Oxidation of isochroman with $(NH_4)_2$-$Ce^{IV}(NO_3)_6$ (*viz*, CAN; E° 1.5 V),[32] a strong mono-electronic oxidant, has been performed in H_2O:MeCN 2:1 solution at room temperature: no oxidation product (*i.e.*, the lactone 3,4-dihydroxycoumarin) was obtained, and the ether was quantitatively recovered, even though CAN is certainly a stronger monoelectronic oxidant than the Med-O• species. Only at the reflux temperature of the mixed solvent, and employing twice the stoichiometric amount of CAN, a modest



Scheme 4

(5%) conversion into the lactone was observed. It was accompanied by a C_α–C_β bond cleavage product (*ca.* 10%, by GC-MS), which is a typical outcome of radical cations of ethers.[26,27] The difference with the efficient oxidation of isochroman to 3,4-dihydroxycoumarin under mild condition, with the laccase-HBT and laccase-HPI systems, is striking and confirms the operation of a different, radical in nature, mechanism of oxidation by the Med-O• species, at least with substrates endowed with a redox potential equal or higher than 1.4–1.5 V/NHE.

Further along this line, the anodic oxidation of *trans*-stilbene oxide to the radical cation intermediate has been found to give rise to benzophenone and diphenylacetic acid;[26] neither of these two products was found in our case, because *trans*-stilbene oxide was recovered unreacted from incubation with laccase-HBT. Once again, no ET route appears to be pursued by the Med-O• species.

Conclusion

The oxidation of ethers of various structure by two laccase/>N–OH systems is described. The oxidation is carried out by the intermediate >N–O• species according to a radical HAT route, which represents an elaboration of the HAT route followed with the benzyl alcohols;[8,9,16,19–22] alternative mechanisms have been considered and dismissed. Synthetically interesting results have been obtained in the oxidation of two cyclic ethers to the corresponding lactones. The present oxidation procedure appears more efficient than one recently described, which relies on ruminal microbes.[33]

A clear-cut specialisation of the mediator *vs.* the substrate emerges from the present study, because the laccase-HBT system is more competent for the oxidation of ethers than the laccase-TEMPO system. The latter, conversely, was the most proficient in the oxidation of benzyl alcohols, as we showed in a previous study.[9] Because the ether functional group is a widely recurring structural feature of lignin,[6] the development of mediator systems capable of cleaving the ether group efficiently and under mild conditions is promising for mediated enzymatic methods of Kraft pulp delignification for paper making.[34]

Experimental

General techniques

Characterisation of the structure of the reaction products was done by ^{1}H-NMR at 200 MHz on a Bruker AC200 NMR instrument; chemical shifts are reported in the δ scale (in ppm) relative to residual nondeuterated solvent signals ($CDCl_3$). 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 identity of the products was confirmed by GC-MS analyses, run on a HP 5892 GC, equipped with a 12 m × 0.2 mm methyl silicone gum capillary column, and coupled to a HP 5972 MSD instrument, operating at 70 eV. The substrates were either commercial (Aldrich) or available from a previous investigation.[9,13,16,17] Phthalan (*i.e.*, *o*-xylylene oxide; Aldrich) was oxidised to phthalide with periodic acid in 71% yield;[35] mp 72–74 °C (lit.[35] 72–74 °C). ^{1}H-NMR [δ (ppm) in $CDCl_3$: 5.3 (s, 2H, $ArCH_2O$), 7.4–7.5 (dd, 2H), 7.6–7.7 (d, 1H), 7.9 (d, 1H)].

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 Q-Sepharose by elution with phosphate buffer,[9] and an activity of 9000 U mL^{-1} was determined spectrophotometrically by the standard method with ABTS.[36]

Enzymatic reactions

The oxidation reactions were performed at room temperature in stirred water solution (3 mL), buffered at pH 5 (0.1 M in sodium citrate), containing 4% MeCN to improve the solubility of some of the substrates, and saturated by bubbling O_2 for 30 min prior to the addition of the reagents.[9] The concentration of the reagents was: [substrate], 20 mM; [mediator], 6 mM, with 15 units of laccase. The reaction time was 24 h, an atmosphere of O_2 being maintained over the reaction vessel. Following a conventional workup with ethyl acetate, the molar amount of the oxidation products was determined by GC analysis with respect to an internal standard (acetophenone or *p*-methoxyacetophenone), suitable response factors being determined from authentic compounds; the yields were calculated with respect to the molar amount of substrate.

Chemical oxidations

Reaction of 0.5 mmol of isochroman with 0.5 mmol of $(NH_4)_2Ce^{IV}(NO_3)_6$ (*viz*, CAN) in 7.5 mL of H_2O:MeCN 2:1 (v/v) at room temperature for 7 h did not afford any product,[27] and the ether was quantitatively recovered (GC). The reaction was repeated in the same mixed solvent, by doubling the molar amount of CAN (*i.e.*, 1 mmol) and refluxing the reagents for 7 h.[27] The lactone (*i.e.*, 3,4-dihydroxycoumarin) was obtained in a 5% GC-yield, and a C_α–C_β bond cleavage product also appeared at the GC-MS, in approximately 10% yield; the structure of this product was not further investigated. Unreacted isochroman was also recovered (75%).

Acknowledgements

Thanks are due to Novo Nordisk Biotech (Denmark) for a generous gift of laccase. We also thank the EU for financial support (grant QLK5-CT-1999-01277) to the OXYDELIGN project.

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