A Mechanistic Survey of the Oxidation of Alcohols and Ethers with the Enzyme Laccase and Its Mediation by TEMPO

Francesca d'Acunzo,[a] Paola Baiocco,[a] Maura Fabbrini,[a] Carlo Galli,*[a] and Patrizia Gentili*[a]

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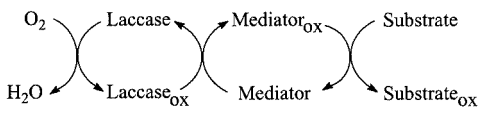
The oxidation of alcohols and ethers by O_2 with the enzyme laccase, mediated by the stable *N*-oxyl radical TEMPO, affords carbonylic products. An ionic mechanism is proposed, where a nucleophilic attack of the oxygen lone-pair of the alcohol (or ether) onto the oxoammonium form of TEMPO (generated by laccase on oxidation) takes place leading to a transient adduct. Subsequent deprotonation of this adduct α to the C–O bond leads to the carbonylic product. Additional mechanistic considerations for the laccase-mediated oxidation of ethers and thioethers are offered. The proposed mechanism is supported by: (i) investigating the inter- and intramolecular selectivity of oxidation with appropriate substrates, (ii) thermochemical considerations, and (iii) attempting a Hammett correlation for the oxidation of a series of 4-X-substituted benzyl alcohols, wherein a shift of the rate-determining step as a function of the 4-X-substituent results. Based on the above points, the lack of mediation efficiency of another stable *N*-oxyl radical (viz., IND-O·) can be explained.

Introduction

The use of enzymes in organic chemistry presents several features of interest because, for example, they may confer remarkable regio- or stereoselectivity to a reaction, or render polluting processes environmentally friendly.[1] For these reasons enzymes are seen as a new frontier in organic synthesis, and are gradually becoming useful tools in the hands of chemists. The case of the enzyme laccase presents distinct peculiarities. Although the natural substrates of this multi-Cu oxidase are the phenolic residues of lignin in wood,[2] the inclusion of appropriate mediators, even in catalytic amounts, makes the oxidation of non-phenolic substrates accessible to laccase.[3−5] We have recently reported the synthetic value of a new procedure for oxidation of alcohols by oxygen, catalysed by laccase with mediation by the stable *N*-oxyl radical TEMPO (2,2′,6,6′-tetramethylpiperidine-*N*-oxyl).[6] The likely role of this mediator is outlined in Scheme 1.



Scheme 1. The role of a mediator of laccase activity

[a] Dipartimento di Chimica and Centro CNR Meccanismi di Reazione, Università "La Sapienza",
P. le A. Moro 5, 00185 Roma, Italy
Fax: (internat.) + 39-06/490421
E-mail: carlo.galli@uniroma1.it

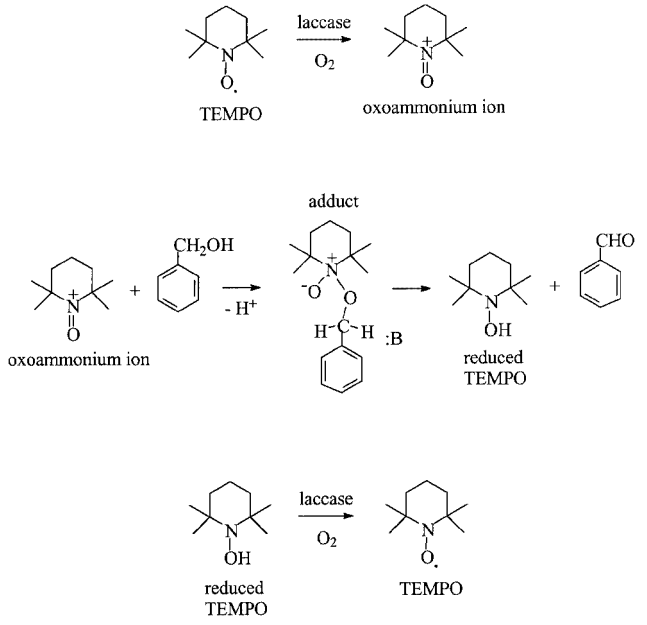
In general, the mediator is capable of utilizing oxidation mechanisms not available to laccase, thereby expanding the enzyme's synthetic usefulness. The mediator can also solve problems of solubility and/or access of encumbered substrates to the active site of the enzyme.[2] We have undertaken an evaluation of the efficiency of a number of mediators of laccase in the oxidation of benzylic alcohols, taken as non-phenolic lignin models: TEMPO proved most efficient.[7] TEMPO is independently known to catalyse the oxidation of alcohols to carbonyl products in the presence of a number of chemical oxidants,[8−9] including copper salts.[10−11] In this paper, new advances in our understanding of the mechanistic features of the laccase/TEMPO oxidising system are presented where alcohols, ethers, and thioethers have been considered as substrates.

Results and Discussion

Substrate Reactivity

The laccase/TEMPO system converts benzyl alcohols into carbonylic products without side-products, at room temperature and in buffered water solution (pH = 5).[6] The oxidation occurs efficiently regardless of the number of electron-donor substituents on the benzyl alcohols. In the series $C_6H_5CH_2OH$, 4-$MeOC_6H_4CH_2OH$, and 3,4-$(MeO)_2C_6H_3$-CH_2OH the electronic activation of the substrate increases in the order given, as shown by the E^p redox potentials of 2.4, 1.8, and 1.4 V, respectively.[7] This has a mechanistic significance − if the substrates were oxidised through a

single-electron-transfer process, one would expect the product yield to be affected by the number of methoxy substituents, but this is not observed. Therefore, an ionic oxidation mechanism that is not dependent on the redox features of the substrate is suggested with the laccase/TEMPO system (Scheme 2).[6,7] In this route, the actual oxidant is the oxoammonium ion (i.e., $Mediator_{ox}$ in Scheme 1), easily generated from TEMPO on oxidation by laccase.[6]

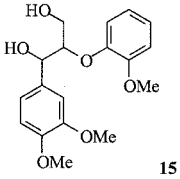
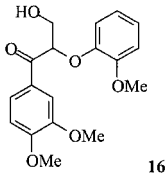
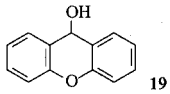
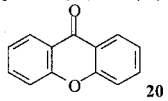


Scheme 2. The oxidation of alcohols with laccase/TEMPO

Following this preliminary oxidation, a nucleophilic attack of the lone-pair of the alcohol onto the TEMPO-oxoammonium ion takes place to form an adduct. Deprotonation of the adduct at the α-C−H benzylic bond, either intra- (from N−O⁻) or intermolecularly (from the base form of the buffer, i.e. B),[8,12] gives rise to the carbonylic product and to the reduced form of TEMPO (i.e., N−OH). Laccase oxidises the latter to regenerate TEMPO, and further oxidation leads to the oxoammonium ion (Scheme 2).[7,13] The enzyme itself is finally oxidised by dioxygen, thereby completing the catalytic cycle (Scheme 1). The mechanism reported in Scheme 2 matches the well-established one reported for the oxidation of alcohols by catalytic amounts of TEMPO with stoichiometric amounts of co-oxidants [e.g., manganese/cobalt salts, bis(acetoxy)iodobenzene, KBr/NaClO].[8−11,14] In all these cases the oxoammonium form of TEMPO is involved. In our particular case, the catalytic oxidant of TEMPO would be the copper-enzyme laccase.[6]

Further support for the ionic mechanism of Scheme 2 has been sought, as opposed to the electron transfer (ET) route suggested for the laccase/ABTS system,[4,15−17] or for the radical H-abstraction (HAT) route suggested for the laccase/HBT and laccase/HPI systems [ABTS: 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); HBT: 1-hydroxybenzotriazole; HPI: *N*-hydroxyphthalimide].[5,7,18,19] Oxidation of 4-MeO-benzyl alcohol (**1**) and 1-(4-MeO-phenyl)ethanol (**3**) in a competition experiment with the laccase/TEMPO system (Table 1)[7] afforded the carbonylic products **2** and **4**, the relative amounts of which allowed for calculation of the relative reactivity of oxidation of a primary (**1**) vs. a secondary (**3**) benzylic alcohol. The k_1/k_3 ratio obtained (i.e., 0.7) is consistent with the slightly higher nucleophilicity of a secondary vs. primary alcohol,[20] thereby supporting the theory of a nucleophilic attack of the alcohol onto the oxoammonium ion (Scheme 2). In contrast, a k_1/k_3 ratio greater than 1 is expected in case of the operation of an ET mechanism of oxidation, which proceeds through the radical cation of the substrate, because stereoelectronic effects, as previously reported,[21] are known to retard C−H deprotonation of the radical cation of an encumbered (secondary) alkylaromatic substrate compared to a primary one.

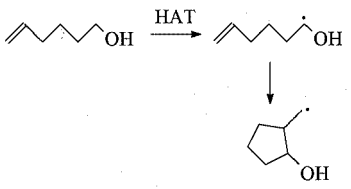
Table 1. Oxidations of alcohols with the laccase/TEMPO system in buffered (pH = 5) water at room temperature for 24 h: products and yields[a]

Substrate	Product	Yield (% vs. subst.)
4-MeO-benzyl alcohol (**1**)	4-MeO-benzaldehyde (**2**)	98
1-(4-MeO-phenyl)ethanol (**3**)	4-MeO-acetophenone (**4**)	92
1 + **3**[b]	**2** + **4**	**2**: 13; **4**: 8.3 $k_1/k_3 = 0.78$[b,c]
5-Hexen-1-ol (**5**)	5-Hexen-1-al (**6**)	19[d]
Benzyl alcohol (**7**) and (hydroxymethyl)cyclohexane (**9**)[b]	Benzaldehyde (**8**) and cyclohexancarbaldehyde (**10**)	**8**: 8.1; **10**: 0.92 $k_7/k_9 = 9$[b]
Benzyl alcohol (**7**) and decanol (**11**)[b]	Benzaldehyde (**8**) and decanal (**12**)	**8**: 52; **12**: 8 $k_7/k_{11} = 7$[b]
Benzyl alcohol (**7**) and cinnamyl alcohol (**13**)[b]	Benzaldehyde (**8**) and cinnamaldehyde (**14**)	**8**: 22; **14**: 58 $k_{13}/k_7 = 2.6$[b]
 **15**	 **16**	10[e]
p-$MeSC_6H_4CH_2OH$ (**17**)	*p*-$MeSC_6H_4CHO$ (**18**)	87
 **19**	 **20**	70
p-$HOC_6H_4CH_2OH$ (**21**)	Not identified[f]	

[a] In the absence of either TEMPO or laccase, no conversion of any substrate to product is observed (ref.[6]). [b] Competition experiment. [c] Corrected for the 2:1 statistical factor. [d] Unchanged substrate is recovered (45%). [e] Unchanged substrate is recovered (85%). [f] The substrate is completely oxidised.

Thermochemical Considerations

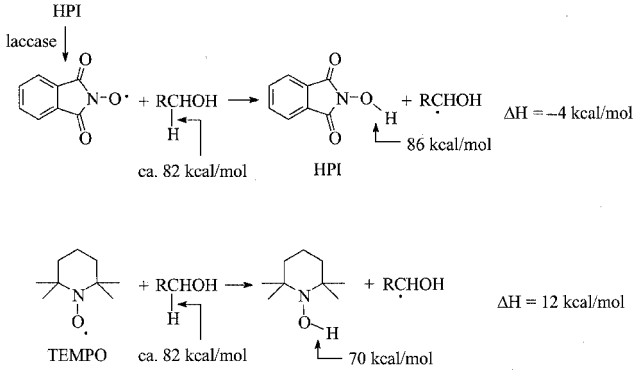
Treatment of another substrate with the laccase/TEMPO system provided additional support for the ionic route of



Scheme 3. Radical rearrangement

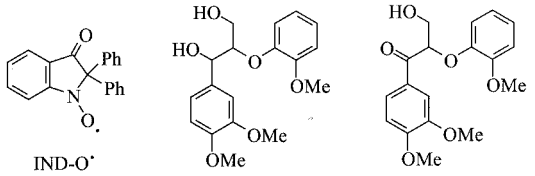
Scheme 2. Oxidation of 5-hexen-1-ol (**5**) afforded only 5-hexen-1-al (**6**) (Table 1). A different outcome would be expected in the case of the alternative HAT route. In fact, removal of an H atom α to the hydroxy group of **5** is known to lead to a cyclopentyl alcohol through an intramolecular radical rearrangement/addition (Scheme 3).[22]

The absence of rearrangement products confirms that the laccase/TEMPO system does not follow a radical route. However, a discussion is in order as to why a *stable N*-oxyl radical, such as TEMPO, does not react through H-abstraction, whereas other *transient N*-oxyl radicals, generated in situ by laccase from the oxidation of N−OH mediators such as HBT and HPI,[7] do react by an HAT route. First of all, TEMPO is more easily oxidised to the oxoammonium form[13] than HBT and HPI are.[7] Moreover, another important point is the energy of the O−H bond (BDE_{OH}) that these *N*-oxyl radicals would form on H-abstraction, as indicated in Scheme 4.



Scheme 4. Thermochemical data

The HAT process in the case of HPI (and HBT) is exothermic by ca. 4 kcal/mol, whereas with TEMPO it is endothermic by ca. 12 kcal/mol, in view of a BDE_{OH} that is ca. 86 kcal/mol in the first case and ca. 70 kcal/mol in the second case.[23] Thus, there is a sound thermochemical reason why TEMPO does not react as a radical but rather reacts along the ionic route outlined in Scheme 2. Indeed, radical reactions of TEMPO with benzylic or allylic C−H bonds are reported to be very slow.[24] Whenever an oxidant is present, the alternative oxidation of TEMPO to oxoammonium ion can take precedence over the slow HAT route. In full agreement with this explanation, the stable 2,2′-diphenyl-3-oxoindol-*N*-oxyl radical (viz., IND-O·)[25] does not perform as a mediator of laccase in the oxidation of test substrate **1**.



In fact, neither the ionic (Scheme 2), nor the radical HAT mechanism (as in Scheme 4) are accessible to IND-O·. It has a higher redox potential (E° = 1.18)[25] than TEMPO,[13] so that its oxidation to the corresponding oxoammonium ion by laccase is disfavoured, and the ionic mechanism is hampered. The radical HAT route is also prevented because, by H-abstraction, IND-O· forms a weak O−H bond (BDE_{OH} similar to that of the O−H bond of TEMPO),[23,26] so that the HAT route is again endothermic. Thus, IND-O· does not mediate the oxidation of **1**.

Substrate Selectivity

The chemoselectivity of the ionic oxidation process with laccase/TEMPO was investigated either by competitive reactions (*intermolecular*), or by means of substrates that present two functional groups susceptible to oxidation (*intramolecular*; Table 1). In a competitive oxidation of benzyl alcohol (**7**) vs. (hydroxymethyl)cyclohexane (**9**, cyclohexylmethanol), the *intermolecular* selectivity between the two primary alcohols was calculated by determining the relative amount of the two corresponding aldehydes (**8** and **10**) by GC analysis. Benzyl alcohol was nine times more reactive than the alkyl analogue. Similarly, in a competitive oxidation of **7** vs. decanol (**11**), **7** was seven times more reactive. Finally, the competitive oxidation of **7** and cinnamyl alcohol (**13**) indicated that the allylic substrate was 2.6-times more reactive than the benzylic one.

The *intramolecular* chemoselectivity was investigated with four substrates. Adlerol (**15**) is a well-known model compound of lignin;[2,4,27,28] it bears a secondary benzylic moiety and a primary aliphatic alcohol moiety, as well as two ethereal functions. On laccase/TEMPO oxidation of **15** only Adlerone (**16**) was detected as a product, in agreement with the higher reactivity of a benzylic vs. aliphatic alcohol (see the k_7/k_9 and k_7/k_{11} ratios in Table 1), and with the good reactivity of the secondary alcohol (see k_1/k_3 ratio). In 4-(methylthio)benzyl alcohol (**17**), the laccase/TEMPO system could, in principle, oxidise either the alcohol or the thioether group of the substrate. Only the former was oxidised, as 4-(methylthio)benzaldehyde (**18**) was the unique product. Analogously, 9-hydroxyxanthene (**19**; viz. xanthydrol) contains both a benzhydrylic alcohol and an ether functional group; only the former was oxidised, affording xanthone (**20**). No direct oxidation of **19** to **20** by TEMPO takes place in the absence of laccase. Finally, 4-hydroxybenzyl alcohol (**21**) contains a phenolic group as a substituent. This substrate was completely oxidised within 1 h. Despite the high reactivity of the laccase/TEMPO system towards benzyl alcohols, 4-hydroxybenzaldehyde was not formed. Clearly, laccase "recognises" a phenolic substrate in this precursor and reacts with it selectively, most likely by inducing dimerisation and oligomerisation through the intermediacy of the phenoxy radical, as is normal for this enzyme.[2,5]

Hammett Correlation

The mechanism described in Scheme 2 is a two-step process. The first step requires *nucleophilic attack* by the sub-

strate onto TEMPO-oxoammonium, to form the adduct. The adduct is *deprotonated* at the α-C−H bond in the second step. In order to ascertain which one of the two steps is the rate-determining step in the oxidation mechanism, we resorted to the determination of a Hammett correlation, where the influence of the electronic effects of the substituents was studied. The oxidation of a series of 4-X-substituted benzyl alcohols ($ArCH_2OH$), where X is NO_2, Cl, CF_3, Me, Ph, or MeO, was investigated with the laccase/TEMPO system. Each one of these precursors was treated with the unsubstituted benzyl alcohol (**7**) in separate competition experiments, and the k_X/k_H relative reactivity ratio calculated by determining the relative amounts of the two resultant aldehydes (ArCHO and PhCHO, respectively) by GC analysis (Table 2). Reaction times of 2−4 h were chosen to ensure only a modest conversion[29] of the substrates into the carbonylic products.

$$ArCH_2OH + Med_{ox} \xrightarrow{k_X} ArCHO + Med$$

$$PhCH_2OH + Med_{ox} \xrightarrow{k_H} PhCHO + Med$$

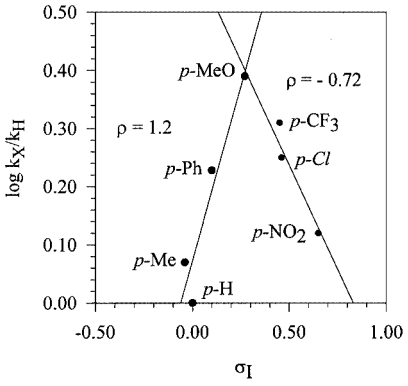
$$k_X/k_H = \frac{\log\frac{[ArCH_2OH]_o - [ArCHO]}{[ArCH_2OH]_o}}{\log\frac{[PhCH_2OH]_o - [PhCHO]}{[PhCH_2OH]_o}}$$

Table 2. Competitive oxidations of a 4-X-substituted benzyl alcohol vs. benzyl alcohol, with the laccase/TEMPO system: Hammett correlation

Competing substrates	Products (yield [%])[a]	k_X/k_H	σ_I [b]
p-$O_2NC_6H_4CH_2OH$, $C_6H_5CH_2OH$	*p*-$O_2NC_6H_4CHO$ (12) C_6H_5CHO (10)	1.3	0.65
p-$ClC_6H_4CH_2OH$, $C_6H_5CH_2OH$	*p*-ClC_6H_4CHO (13) C_6H_5CHO (7.0)	1.8	0.46
p-$F_3CC_6H_4CH_2OH$, $C_6H_5CH_2OH$	*p*-$F_3CC_6H_4CHO$ (19) C_6H_5CHO (10)	2.0	0.45
p-$PhC_6H_4CH_2OH$, $C_6H_5CH_2OH$	*p*-PhC_6H_4CHO (21) C_6H_5CHO (13)	1.7[c]	0.10
p-$MeC_6H_4CH_2OH$, $C_6H_5CH_2OH$	*p*-MeC_6H_4CHO (10) C_6H_5CHO (8.0)	1.2	−0.04
p-$MeOC_6H_4CH_2OH$, $C_6H_5CH_2OH$	*p*-$MeOC_6H_4CHO$ (41) C_6H_5CHO (19)	2.5	0.27

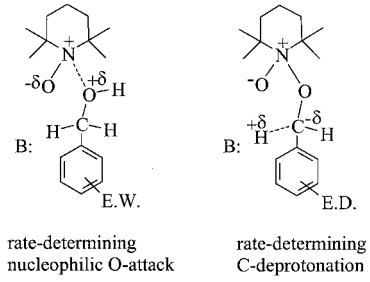
[a] The yields were determined by GC and calculated vs. the mmol of substrate (typical errors: ±4%). Runs in triplicate. [b] Substituent constant σ_I: inductive effect. See p. 139 in ref.[29] for σ_I values. [c] Run in a mixed solvent containing 20% dioxane, for solubility reasons.

The $\log k_X/k_H$ ratios were then plotted vs. the pertinent σ constant of the substituent X[29] according to the Hammett equation[29] $\log k_X/k_H = \rho\sigma$. No linear or meaningful plots were obtained (not shown) with either σ_p or σ^+ values for the two-electron donor (E.D.) Me and MeO substituents.[29] We reasoned that if the nucleophilic attack of the alcohol onto TEMPO-oxoammonium (Scheme 2) plays an important role in the success of the laccase/TEMPO oxidation, use of the σ_I values, that provide the inductive contribution of a substituent, would be more appropriate.[29] With these parameters, a V-shaped plot was obtained (Scheme 5).



Scheme 5. Hammett plot for the oxidation of 4-X-substituted benzyl alcohols with the laccase/TEMPO system from competition experiments with benzyl alcohol

We observe that in the right branch of the plot all the aligned substituents are electron-withdrawing (E.W.) according to the σ_I parameter (including MeO), whereas the electron-donating (E.D.) substituents (Me and Ph) determine the left branch of the plot. One could infer that, as the E.W. effect of X becomes gradually weaker on progressing from NO_2 to MeO, the alcoholic oxygen atom becomes accordingly more nucleophilic. This speeds up the *addition* step, as indicated by the increasing k_X/k_H ratios, as long as this is the rate-determining step. The turning point is obtained when MeO is the substituent. In fact, on progressing towards the E.D. Me group, the addition step becomes so fast that the slow step is now the *deprotonation* of the adduct. An E.D. group ought to oppose such a deprotonation, in which a partial negative charge develops on the benzylic carbon atom, and is indicated by the decrease in reactivity in the left branch of the Hammett plot. The peculiar and unexpected outcome of a V-shaped plot can be reconciled within the ionic mechanism of Scheme 2, on the basis of this hypothesis of a change in the rate-determining step of the oxidation as a function of the substituent (Scheme 6).

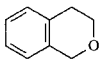
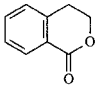
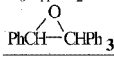


Scheme 6. The change of the rate-determining step as a function of the substituent

Oxidation of Ethers and Thioethers

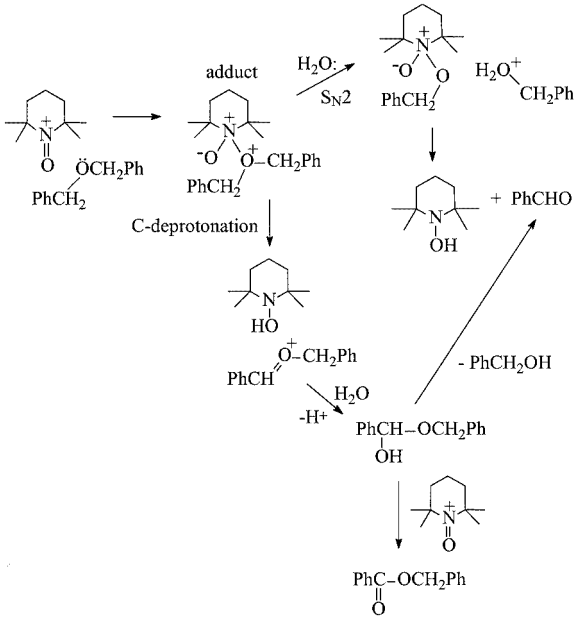
The lack of reactivity of the ether moiety in xanthydrol **19**, as well as the lack of reactivity of the methoxy group of substrates **1** and **3**, appeared in sharp contrast with the preliminary report of a moderate conversion of ethers into aldehydes by the laccase/TEMPO system.[6,7] This prompted us to further investigate other ethers as substrates, in an attempt to recognize any relationship between reactivity and structure, as well as to verify if the ionic mechanism of Scheme 2 was compatible with the oxidation of ethers. Table 3 collates our data. Diphenyl ether (**22**) was unreactive, in keeping with the lack of reactivity of the same structural moiety in **19**. In addition, 4,4′-dimethoxybiphenyl (**23**) and 2-methoxydibenzofuran (**24**) did not react. In contrast with the moderate conversion of methyl veratryl ether (**25**) into aldehyde **26**, as well as $(PhCH_2)_2O$ (**27**) to **8**,[6] benzyl phenyl ether (**28**) did not react. It can be concluded that *monoaryl* and *diaryl* ethers are not oxidised by this procedure. It must be also observed that, in the oxidation of **27**, production of benzaldehyde was accompanied by 3% of the ester $PhCOOCH_2Ph$ (**29**). Moreover, an ester (better, lactone **31**) was the *unique* oxidation product of isochroman (**30**). Moving to thioethers, even the methylthio substituent of the *aromatic* precursor **17** was not oxidised, whereas the *aliphatic* thioether (**32**) gave a minute conversion into the corresponding sulfoxide (**33**), in addition to some **8**. No oxidation was obtained with the aliphatic ether (**34**), nor with the epoxide of stilbene (**35**).

Table 3. Oxidations of ethers and thioethers with the laccase/TEMPO system in buffered (pH = 5) water at room temperature for 24 h: products and yields

Substrate	Product	Yield (% vs. subst.)
PhOPh (**22**)	No reaction	_[a]
p-$MeOC_6H_4C_6H_4OMe$-*p* (**23**)	No reaction	_[a]
2-MeO-dibenzofuran (**24**)	No reaction	_[a]
3,4-Dimethoxy-$C_6H_4CH_2OMe$ (**25**)	3,4-Dimethoxy-C_6H_4CHO (**26**)	18[b,c]
$PhCH_2OCH_2Ph$ (**27**)	**8** and $PhCOOCH_2Ph$ (**29**)	16[b,c] and 3
$PhCH_2OPh$, **28**	No reaction	_[a]
 **30**	 **31**	10[c]
$PhCH_2SMe$ (**32**)	$PhCH_2S(O)Me$ (**33**) and **8**	1 and 5[c]
$C_6H_{11}CH_2OMe$ (**34**)	No reaction	_[a]
 **35**	No reaction	_[a]

[a] The substrate is quantitatively recovered. [b] Data from ref.[6] [c] The substrate is largely recovered unchanged.

It must be remembered that the yields are calculated with respect to the molar amount of substrate, the molar amount of the mediator being only one-third that of the substrate. Nevertheless, these results are certainly not very rewarding from a synthetic viewpoint, but they do comply with the ionic mechanism of Scheme 2. In the rate-determining formation of the adduct between the TEMPO-oxoammonium and the substrate, the nucleophilic requisite is hardly met by the *aryl* ethers (such as **22**−**24** and **28**), as a result of the resonance of the oxygen lone-pair on the aromatic group. Conversely, *benzylic* ethers are almost as nucleophilic as the benzyl alcohols, and the oxidation does take place. Clearly, no *O*-deprotonation of the adduct can ensue with an ether, in contrast to an alcohol, and the mechanism of oxidation of Scheme 2 requires an implementation, as outlined in Scheme 7 for dibenzyl ether **27**.



Scheme 7. Oxidation mechanism of an ether with laccase/TEMPO

S_N2 attack by water (or other nucleophiles) onto the oxoammonium/substrate adduct, which is an oxonium ion, may remove one alkyl group as an alcohol; this provides an additional reason why diaryl ether **22** cannot react through this route. Then, the remaining part of the adduct may undergo the usual α-*C* deprotonation, to yield the aldehyde. Alternatively, α-*C* deprotonation occurs first, and leads to the detachment of an oxonium ion. Then addition of water to this oxygen-stabilised carbocation would lead to a hemiacetal that, being generally unstable, splits into the aldehyde and alcohol constituents. Alternatively, with relatively stable cyclic hemiacetals, further oxidation of the alcoholic moiety by the TEMPO-oxoammonium would afford the ester product,[8] which is indeed the unique product of the oxidation of **30**. Esters are often obtained as side-products in the oxidation of ethers through the intermediate hemiacetal.[30] This ionic mechanism for the TEMPO-mediated oxidation of ethers seems more likely to us than the ET route that has been previously suggested.[31] Since the redox potential of an ether is ca. 1.4−1.6 V/SCE,[32] one-electron oxidation by the TEMPO-oxoammonium (a very modest oxidant[13]) would hardly be significant; it would be also in contrast with our experimental evidence that disfavours an ET route for the structurally similar benzyl alcohols as substrates.[7]

Finally, the lack of reactivity of epoxide **35** is understandable, because the oxygen atom is a much weaker nucleophile in this case, due to the strain in the three-membered ring, giving sp^2-like character to the exocyclic C−H bonds as

well as to the oxygen lone-pairs of the molecule.[33] In contrast, the *aliphatic* ether **34** is a better O-nucleophile, and addition to TEMPO-oxoammonium can certainly occur. However, the ensuing deprotonation at the α-C−H is hampered by the reduced acidity of an *alkyl* vs. *benzyl* C−H bond (such as for **25** and **27**; cf. k_7/k_9 and k_7/k_{11} ratios in Table 1),[34] and the oxidation cannot proceed to completion.

The ether functional group is a widely recurring structural feature of natural polymer lignin,[27] and the development of laccase/mediator systems capable of cleaving this group efficiently would be beneficial for the success of enzymatic methods of kraft pulps delignification for paper making.[35] It will be interesting to ascertain if the laccase/HBT or laccase/HPI systems are more efficient than the laccase/TEMPO system in this specific task,[36] in view of their different and radical mechanism of oxidation.[7]

Experimental Section

General Remarks: NMR characterisation of the structure of the reaction products was performed with a 200 MHz Bruker instrument; chemical shifts are reported on 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 with an HP 5892 GC, equipped with a 12 m × 0.2 mm methyl silicone gum capillary column, and coupled to an HP 5972 MSD instrument, operating at 70 eV.

Materials: Many of the substrates are commercially available (Aldrich) and were used without further purification. Xanthydrol (**19**, viz. 9-hydroxyxanthene; Aldrich) was purified from xanthone (**20**, 9-xanthenone) by column chromatography (hexane/ethyl acetate, 6:1). Lactone **31** (viz. 3,4-dihydroxycoumarin) was synthesised by reaction of isochroman (**30**) with periodic acid.[37] Its ^{1}H NMR spectrum [in $CDCl_3$: δ = 3.0 (t, 2 H, $ArCH_2CH_2O$-), 4.5 (t, 2 H, $ArCH_2CH_2O$-), 7.15−7.60 (m, 3 H), 8.1 (d, 1 H) ppm] was consistent with the structure of **31**, but not with that of the conceivable isomer, 3-isochromanone. Benzyl phenyl ether (**28**) was synthesised according to the literature.[38] The methyl ether **34** of cyclohexylmethanol was prepared by methylation with MeI of the anion of $HOCH_2C_6H_{11}$,[39] generated by using NaH. ^{1}H NMR ($CDCl_3$): δ = 0.7−1.7 (m, 11 H, C_6H_{11}), 3.1 (d, 2 H, CH_2O-), 3.3 (s, 3 H, OCH_3) ppm. Benzyl methyl sulfoxide (**33**) was prepared from benzyl methyl sulfide (**32**) by treatment with $NaIO_4$.[40] Other precursors and products were available in the laboratory from previous work.[41,42] A sample of 2,2′-diphenyl-3-oxoindol-*N*-oxyl radical (viz., IND-O·) was kindly provided by Prof. L. Greci (University of Ancona, Italy).[25]

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,[7] and an activity of 9000 U/mL was determined spectrophotometrically by the standard method with ABTS.[43]

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, and purged with O_2 for 30 min prior to the addition of the reagents.[7] The concentration of the reagents was [substrate] = 20 mM, [mediator] = 6 mM, with 10 units of laccase. The reaction time was 24 h, in general. 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. In the absence of either laccase or TEMPO, conversion of substrates to their respective products was not achieved. The competition experiments were similarly run on a 60-μmol amount of each of the substrates, in 6 mL of citrate buffer containing 4% MeCN, and the yields of products determined after a suitable reaction time. Relative reactivity values were calculated by means of the standard integrated formula for competitive reactions.[29]

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