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Aryl substituted *N*-hydroxyphthalimides as mediators in the laccase-catalysed oxidation of lignin model compounds and delignification of wood pulp

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Abstract

Aryl substituted *N*-hydroxyphthalimides (NHPIs) have been tested as mediators in the laccase-promoted oxidation of non-phenolic monomeric and dimeric lignin model compounds and in the delignification of kraft pulp samples. In the oxidation of the model compounds a significant increase in the product yields was observed upon increasing the electron donating properties of the NHPI ring substituent. Product yields also increased, but to a smaller extent, by increasing the electron donating properties of the aromatic substituent in the lignin models. These results suggest the contribution to the overall reactivity of both the oxidation of the aryl substituted NHPI to the *N*-oxyl radical (X-PINO) by laccase and the hydrogen atom transfer step from the substrate to the X-PINO, with a major contribution of the former process. When applied to the delignification of kraft pulps again the mediation efficiency increased by increasing the electron donating properties of the NHPI aryl substituent, the best mediators being 4-Me-NHPI and 4-MeO-NHPI. Thus, the use of non-phenolic lignin model compounds in the oxidation promoted by the laccase/X-NHPI system is well suited to mimic the behaviour of the lignin polymer.

Keywords: *N*-Hydroxyphthalimide; Laccase; Mediator; Oxidation; Lignin

1. Introduction

The use of molecular oxygen in catalytic oxidation systems is a major challenge in organic chemistry; as a matter of fact O_2 is the only economically and environmentally feasible oxidant for large-scale processing [1]. The development of molecular oxygen-based catalytic systems has a particular relevance in the context of the oxidative degradation of lignin. This process is of fundamental importance because it can convert lignin into low molecular weight aromatic compounds, thus making this polymer a renewable source for the industrial preparation of a number of chemicals [2]. Moreover, the selective degradation of lignin and its removal from the carbohydrate component of wood is a key process in the pulp and paper industry [3,4]. The replacement of conventional polluting chlorine-based procedures for the industrial preparation of paper is necessary for the protection of the environment [5]. Recently, particular attention has been given to the possibility of degrading lignin by fungi, which would represent a process with low environmental impact and energy consumption [6,7]. Most of the basidiomycetes that cause white rot of wood produce laccases as ligninolytic enzymes. Laccases (EC 1.10.3.2) are multi-copper oxidases that catalyze the direct oxidation of electron-rich aromatic substrates, like phenols and anilines, with the concomitant reduction of O_2 to water [8,9]. In the presence of appropriate low molecular weight compounds (mediators) laccases are also able to cat-

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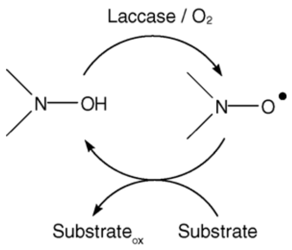
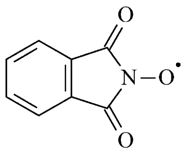


Scheme 1. Catalytic cycle in oxidation promoted by the laccase/N—OH mediators/O_2 systems.

alyze the oxidation of non-phenolic substrates, characterized by a relatively high redox potential [10–12].

Several good mediators have been found that share the structural feature of being N–OH derivatives: 1-hydroxybenzotriazole (HBT), violuric acid, *N*-hydroxyacetanilide, and *N*-hydroxyphthalimide (NHPI) [13–15]. Previous studies have indicated that these compounds are oxidised by the type 1 (T1) Cu center of laccase to the corresponding *N*-oxyl radicals (M_{ox}), which are the active species in the oxidation of the substrate (Scheme 1) [16–19].

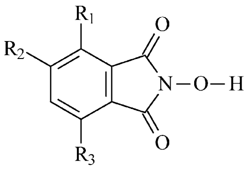
Among the N–OH mediators NHPI is of particular interest in consideration of the fact that this compound, in combination with molecular oxygen and metal salt co-catalysts like $Co(OAc)_2$ or $Co(acac)_2$, is able to efficiently catalyze the oxidation of a large variety of organic compounds under mild conditions at moderate oxygen pressure and temperature [20]. Also in this case the *N*-oxyl radical, phthalimide-*N*-oxyl (PINO), is considered to be the active oxidant.



PINO

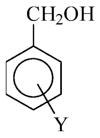
Even though the NHPI/O_2/Co(II) is an efficient catalytic system, it requires the use of an organic solvent [20]. The laccase/NHPI/O_2 system represents instead a convenient way of carrying out oxidation processes, like those involved in the degradation of lignin or environmental pollutants, in water [15,18].

The laccase/NHPI system has been applied with some success in the delignification of kraft pulp samples, however, the mediation efficiency was significantly lower than that observed with another classical mediator, 1-hydroxybenzotriazole [17]. In this context, we have considered worthwhile to investigate the possibility of increasing the mediation efficiency by the introduction of aryl substituents in the NHPI. To this purpose we have synthesised several aryl substituted NHPI (**1a**–**1h**) containing either electron withdrawing (4-MeOCO, 3-F) or electron donating groups (3-Me, 4-Me, 4-MeO, 3-MeO, 3,6-(MeO)$_2$).

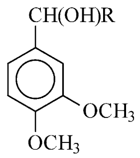


1a	4-MeOCO-NHPI	$R_1 = H$	$R_2 = CH_3OCO$	$R_3 = H$
1b	3-F-NHPI	$R_1 = F$	$R_2 = H$	$R_3 = H$
1c	NHPI	$R_1 = H$	$R_2 = H$	$R_3 = H$
1d	3-Me-NHPI	$R_1 = CH_3$	$R_2 = H$	$R_3 = H$
1e	4-Me-NHPI	$R_1 = H$	$R_2 = CH_3$	$R_3 = H$
1f	4-MeO-NHPI	$R_1 = H$	$R_2 = CH_3O$	$R_3 = H$
1g	3-MeO-NHPI	$R_1 = CH_3O$	$R_2 = H$	$R_3 = H$
1h	3,6-(MeO)$_2$-NHPI	$R_1 = CH_3O$	$R_2 = H$	$R_3 = CH_3O$

The effect of the NHPIs aryl substituents on the mediation efficiency has been evaluated both in the oxidation of non-phenolic lignin model compounds (primary and secondary benzylic alcohols **4**–**7**) and in the delignification of kraft softwood pulps. Benzyl alcohol (**2**) and 4-trifluoromethylbenzyl alcohol (**3**), that are not proper lignin model compounds, have been included in the list of substrates in order to investigate the electronic effect of the aromatic substituent on the mediation efficiency. Secondary benzylic alcohols **5**–**7** differ in the steric requirements of the α-substituent group. It has to be noted that compound **7**, 1-(3,4-dimethoxyphenyl)-2-phenoxyethanol, is a dimeric non-phenolic lignin model containing a β-*O*-4 aryl linkage, which is the most abundant in lignin [3].



2 Y = H
3 Y = 4-CF_3
4 Y = 3,4-$(CH_3O)_2$



5 R = CH_3
6 R = $C(CH_3)_3$
7 R = $CH_2OC_6H_5$

2. Results and discussion

The oxidations of lignin models were carried out for 15 h under oxygen (filled balloon) by adding 20 units of purified laccase from *Trametes villosa* (viz. *Poliporus pinsitus*), 10 μmol of the mediator and 30 μmol of the substrate at room temperature to 6 mL of a stirred buffered water solution (0.1 M sodium citrate, pH 5.0 containing 25% of dioxane as cosolvent) purged with O_2 for 30 min before the addition of the reagents. The oxidation of primary benzylic alcohols **2**–**4** with all the mediators tested leads to the formation of the corresponding aromatic aldehydes as the only reaction products. The yields of the aldehydes, referred to the initial amount of the substrate, were determined by GC analysis and are reported in Table 1.

The oxidation of secondary benzylic alcohols **5**–**7** with all the mediators tested leads to the formation of the corresponding aromatic ketones as the only reaction products. The yields of the ketones, referred to the initial amount of

Table 1
Yields of aromatic aldehydes in the oxidation of ring substituted benzyl alcohols **2**–**4** by the laccase/X-NHPI/O_2 systems[a]

Mediator	Substrates		
	2	**3**	**4**
1a	1.8 ± 0.1	0.5 ± 0.1	6.2 ± 0.2
1b	2.5 ± 0.1	1.2 ± 0.1	9.4 ± 0.3
1c	11.6 ± 0.5	7.4 ± 0.3	31 ± 1
1d	13.5 ± 0.5	14.6 ± 0.5	43 ± 2
1e	22 ± 1	22 ± 1	50 ± 2
1f	30 ± 1	27 ± 1	68 ± 2
1g	14.2 ± 0.6	12.8 ± 0.5	28 ± 1
1h	7.2 ± 0.3	3.0 ± 0.2	18.2 ± 0.8

[a] At room temperature in 0.1 M sodium citrate buffered solution, pH 5.0, with 25% dioxane as cosolvent. Reaction time 15 h. The yields (%) are referred to the initial amount of substrate. Average of three determinations.

Table 2
Yields of aromatic ketones in the oxidation of secondary benzylic alcohols **5**–**7** by the laccase/X-NHPI/O_2 systems[a]

Mediator	Substrates		
	5	**6**	**7**
1a	2.0 ± 0.1	0.2 ± 0.1	1.0 ± 0.1
1b	2.8 ± 0.1	1.5 ± 0.1	3.1 ± 0.1
1c	13.1 ± 0.5	8.3 ± 0.3	15.6 ± 0.5
1d	18.6 ± 0.6	12.7 ± 0.5	22 ± 1
1e	20 ± 1	18.6 ± 0.5	43 ± 2
1f	37 ± 1	29 ± 1	44 ± 2
1g	12.0 ± 0.5	12.8 ± 0.5	36 ± 1
1h	4.6 ± 0.2	9.7 ± 0.3	23 ± 1

[a] At room temperature in 0.1 M sodium citrate buffered solution, pH 5.0, with 25% dioxane as cosolvent. Reaction time 15 h. The yields (%) are referred to the initial amount of substrate. Average of three determinations.

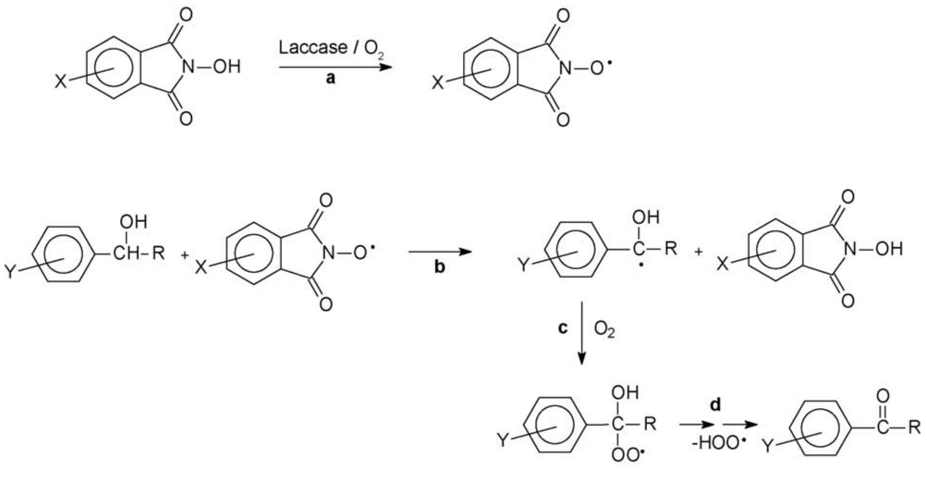
the substrate, were determined by GC and ^{1}H NMR analysis and are reported in Table 2. A good material balance (>95%) was observed in all the experiments. In the absence of the mediator or the enzyme no products have been observed in significant amounts (<0.1%).

From the data reported in Tables 1 and 2 we can observe that the NHPI aryl substituent exerts a very significant effect on the catalytic efficiency in the oxidation of all the lignin models, the reactivity increasing as the electron donating properties of the aryl substituent increase. Accordingly, the reaction yields are negligible or very small with NHPIs substituted with the electron withdrawing groups 4-CH_3OCO and 3-F, while 4-MeO-NHPI appears to be a very efficient mediator, the aldehydes and ketones yields being up to four times higher than those obtained with unsubstituted NHPI. It is interesting to note that in several cases yields in excess of 100%, calculated versus the molar amount of the mediator and implying an oxidation process with turnover, were obtained.The mechanism proposed in the literature for the NHPI mediated oxidation of benzylic alcohols by laccase [15,18] is reported in Scheme 2. The oxidation of X-NHPI by laccase leads to the *N*-oxyl radical (X-PINO) (path a) that can abstract a benzylic hydrogen atom from the substrate (path b) regenerating the X-NHPI and leading to an α-hydroxyl benzyl radical. Reaction of the latter species with O_2 leads to the formation of the α-hydroxyperoxyl radical (path c) from which the carbonyl product is formed by release of hydroperoxyl radical $HOO^\bullet$ (path d) [21].

The occurrence of an electron transfer (ET) mechanism from the substrate to the X-PINO can be excluded on the basis of the absence of the C_α–C_β bond cleavage product, 3,4-dimethoxybenzaldehyde, in the oxidation of both 1-(3,4-dimethoxyphenyl)-2,2-dimethyl-1-propanol (**6**) [15,22–24] and the dimeric lignin model compound **7** [25].

On the basis of the mechanism reported in Scheme 2, the overall reactivity should depend on the ease of the oxidation of X-NHPI by laccase (path a) as well as on the reactivity of the X-PINO (path b). The former process should be favoured by electron donor X-substituents that lower the mediator redox potential and increase the rate of path a [26].

The influence of the NHPI aryl substituents on the redox potential has been determined, in this study, in aqueous buffered solution (pH 5.0) by cyclic voltammetry. In Fig. 1, as an example, is reported the cyclic voltammogram of NHPI. The $E_{1/2}$ value, determined as $(E_{pa} + E_{pc})/2$ for the one-electron reversible wave, is 1.08 V versus NHE.



Scheme 2. Mechanism of oxidation of benzyl alcohols promoted by the laccase/X-NHPI/O_2 systems.

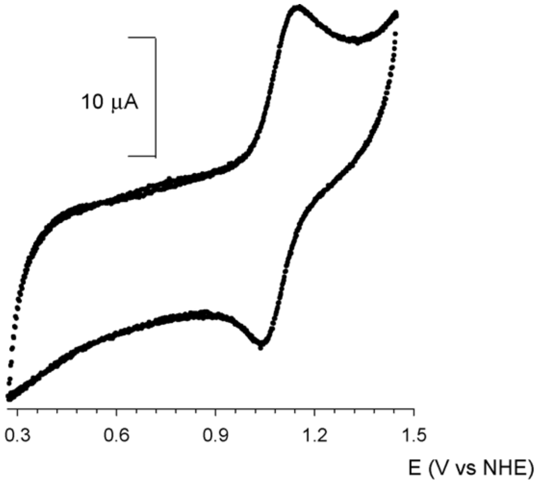


Fig. 1. Cyclic voltammogram of NHPI (0.50 mM) in 50 mM sodium citrate buffer solution, pH 5.0 containing $LiClO_4$ (0.12 M) as the supporting electrolyte. Voltage sweep rate 200 mV/s^{-1}.

In Table 3 are reported the $E_{1/2}$ values of aryl substituted NHPIs. A similar effect of the NHPI aryl substituents on the $E_{1/2}$ was observed in CH_3CN in the presence of collidine. $E_{1/2}$ values were found to correlate linearly with the Hammett σ constants of the substituent, a more positive σ-value (a more electron withdrawing substituent) corresponding to a higher $E_{1/2}$ [27].

An opposite electronic effect should be exerted by the NHPI aryl substituent in the hydrogen atom abstraction from the benzylic alcohol by the X-PINO (Scheme 2, path b). In fact, this process should be favoured by electron withdrawing substituents that increase the O–H bond dissociation enthalpies of X-NHPIs [28].

The significant increase in the NHPIs mediation efficiency observed with the electron donating substituent would seem to indicate that the effect of the NHPI aryl substituent on step a is stronger than that on step b, even though a minor role of the latter can be envisaged (vide infra). The opposite electronic effect of the NHPI aryl substituents has been observed in the oxidation of benzylic alcohols with molecular oxygen and $Co(OAc)_2$ in CH_3CN. In that case, the HAT from the substrate to the PINO played the major kinetic role [28,29].

Table 3
Redox potentials of aryl substituted *N*-hydroxyphthalimides determined in aqueous buffered solution by cyclic voltammetry

Mediator	$E_{1/2}$[a]
1a	1.13
1b	1.12
1c	1.08
1d	1.06
1e	1.06
1f	1.04
1g	1.05
1h	1.04

[a] $E_{1/2}$ (V vs. NHE) were determined by cyclic voltammetry in 50 mM sodium citrate buffer solution, pH 5.0, containing $LiClO_4$ (0.12 M) as the supporting electrolyte. [X-NHPI] = 0.50 mM. Voltage sweep rate 200 mV/s^{-1}.

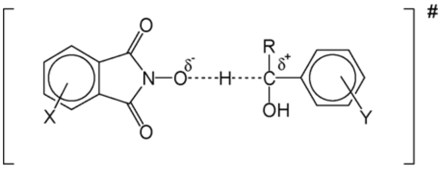


Fig. 2. Polar transition state for the HAT from the benzylic alcohol to the X-PINO.

It is interesting to note that a decrease of only 90 mV of the redox potential on going from 4-CH_3OCO-NHPI ($E^\circ = 1.13$ V versus NHE) to 4-MeO-NHPI ($E^\circ = 1.04$ V versus NHE) resulted in a remarkable increase of the carbonyl product yields. The maximum effect is observed with the least reactive substrate **6** where the yields of 1-(3,4-dimethoxyphenyl)-2,2-dimethyl-1-propanone increase from 0.2% with **1a** to 29% with **1f**. The lowest effect was instead observed with the most reactive substrate **4** where the yields of veratraldehyde increase from 6.2 to 68%. Such a result, however, is not too surprising if we consider that in the laccase promoted oxidation of aryl substituted *N*-hydroxyacetanilides and other mediators containing the –N(OH)– moiety a difference of less than 100 mV in the mediator redox potentials resulted in a variation of almost two orders of magnitude in k_{cat} [26].

The data in Tables 1 and 2 also show that NHPIs with similar redox potentials can give rise to significantly different product yields. Thus, **1f**, **1g** and **1h** have very similar redox potential ($E^\circ = 1.04$–1.05 V versus NHE), however **1f** is by far the most efficient mediator. The same consideration can be done for **1d** and **1e** which are characterized by the same redox potential ($E^\circ = 1.06$ V versus NHE), but different reaction yields (**1d** is always less efficient than **1e**). Looking at the structure of these mediators, it can be noted that the less efficient systems are characterized by the presence of a substituent in the 3-position. Tentatively, it might be suggested that this structural feature causes a lower enzyme affinity for the 3-substituted NHPIs as compared to the 4-substituted ones.

Passing to consider the effect of the benzyl alcohols structure on the reaction efficiency, it can be noted that with all the mediators the carbonyl product yields decrease in the order **4** > **2** > **3** that is as we move from electron donating (**4**) to electron withdrawing substituents (**3**). This trend is in accordance with a partial contribution of the hydrogen atom transfer step, from the substrate to X-PINO, to the overall reaction rate. The higher yields of carbonyl products observed by increasing the electron donating properties of the ring substituent in the benzylic alcohol can be rationalized by the stabilization of the transition state of the hydrogen atom transfer which has a polar character (Fig. 2) [28–30].

The reaction is also sensitive to the steric bulkiness of the alcohol α-substituent that evidently makes more difficult the approach of the H atom abstracting species X-PINO to the benzylic C–H bond. Accordingly, the reactivity of 3,4-dimethoxybenzyl alcohol (**4**) is much higher than that of the dimethoxylated secondary alcohols (**5**–**7**). Moreover, the yields of 1-(3,4-dimethoxyphenyl)ethanone in the oxidation of **5** and of 1-(3,4-dimethoxyphenyl)-2-phenoxy-1-ethanone in the oxidation of **7** are higher than those of 1-(3,4-dimethoxyphenyl)-2,2-dimethyl-1-propanone in the oxidation of **6**.

The effect of the NHPIs aryl substituents on the mediation efficiency in the delignification of wood pulp has been determined by treating samples of post-oxygen delignified softwood kraft pulps (kappa number = 16, viscosity = 987 ml/g) with the laccase/mediator system in sodium citrate buffer (pH 5) at 50 °C for 20 h. The enzymatic treatment was followed by a further oxygen delignification stage in alkaline conditions (NaOH 1.2%) at 100 °C and 0.7 MPa oxygen pressure for 80 min. The results have been compared with those obtained in a blank experiment carried out with laccase, in the absence of the mediator, and with those obtained using the laccase/1-hydroxybenzotriazole system. The effect of mediators on the extent of delignification was inferred from the delignification degree (DD) [31], defined as:

$$DD = (K_0 - K_f)/K_0$$

where K_0 is the kappa number of the starting pulp, and K_f the kappa number after the treatment with the laccase/mediator system or with the laccase/mediator plus the O_2/alkali stage. The effect of mediators on the selectivity was evaluated by the viscosity parameter since the lower is this value after the laccase/mediator treatment, the more extensive degradation of the carbohydrates has occurred.

Table 4
Delignification degree (DD %) and viscosity after the laccase/mediator or laccase/mediator plus O_2/alkali treatment of post-oxygen delignified softwood kraft pulps (initial kappa number: 16, initial viscosity: 987 ml/g) employing no mediator, X-NHPIs and HBT

Mediator	DD (%)		Viscosity (ml/g)	
	Laccase	Laccase + O_2/OH^-	Laccase	Laccase + O_2/OH^-
No mediator	9.3 ± 0.1	37.5 ± 0.3	970 ± 10	940 ± 10
1b	9.4 ± 0.1	37.7 ± 0.3	970 ± 10	940 ± 10
1c	15.5 ± 0.2	42.5 ± 0.4	970 ± 10	940 ± 10
1e	21.2 ± 0.2	48.1 ± 0.5	950 ± 10	920 ± 10
1f	20.0 ± 0.2	47.5 ± 0.5	950 ± 10	920 ± 10
HBT	25.6 ± 0.2	50.2 ± 0.5	940 ± 10	910 ± 10

The results, together with that obtained from a pulp treated without mediator (blank), are reported in Table 4 and displayed in Fig. 3 (DD) and Fig. 4 (viscosity). Figs. 3 and 4 clearly show that, with the exclusion of 3-F-NHPI (same DD of the blank experiment), all the mediators induce a significant delignification of the pulps (DD ranging between 15.5 and 21.2% after the treatment with the laccase/X-NHPIs system and between 42.5 and 48.1% with the laccase/X-NHPIs plus the O_2/alkali stage) leaving almost unaltered the cellulose fibers (after the treatment with the laccase/X-NHPIs system more than 95 and 92% of the original pulp viscosity is retained before and after the O_2/alkali stage). It is interesting to note that the reduction of the kappa number in the final oxygen stage after the laccase/X-NHPIs treatment ($\Delta K \approx 4$) was higher than that observed when the same oxygen stage was applied to reference oxydelignified pulp samples possessing the same kappa number of the samples obtained after the laccase mediator treatments ($\Delta K \approx 2.5$). Thus, the laccase/mediator treatment induced a chemical modification of the pulps that has a beneficial effect in the following oxidative degradation step.

The presence of electron donating aryl substituents has a positive effect on the efficiency of pulp delignification. The DD increases from 15.5% for NHPI to 21.2 and 20.0% for 4-Me-NHPI and 4-MeO-NHPI, respec-

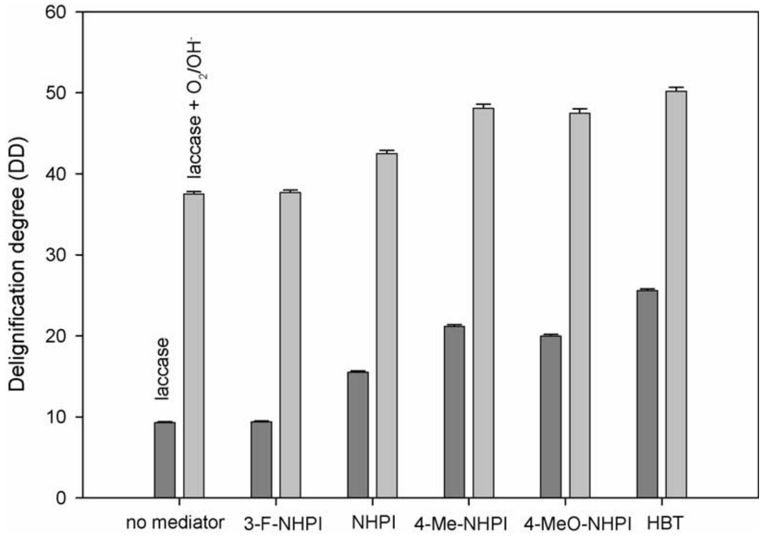


Fig. 3. Delignification degree after the laccase/mediator or laccase/mediator plus O_2/alkali treatment of post-oxygen delignified softwood kraft pulps (initial kappa number: 16) employing no mediator, X-NHPIs and HBT.

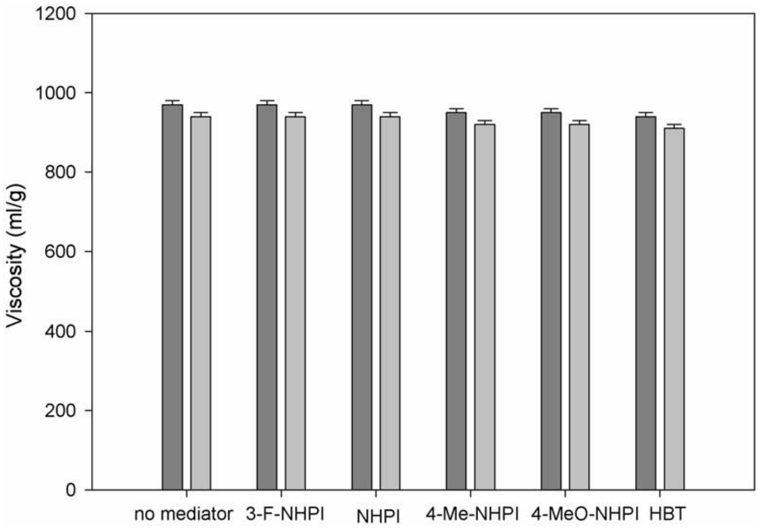


Fig. 4. Viscosity after the laccase/mediator or laccase/mediator plus O_2/alkali treatment of post-oxygen delignified softwood kraft pulps (initial viscosity: 987 ml/g) employing no mediator, X-NHPIs and HBT.

tively. The same substituents effect is observed after the O_2/alkali stage. These results differ from those obtained in the delignification of kraft pulp samples promoted by the laccase/1-hydroxybenzotriazoles system where a net decrease of the mediation efficiency was observed by the introduction of either electron donating or withdrawing aryl substituents [17].

Despite the positive effect of electron donating NHPI aryl substituents on the mediation efficiency, this effect is not sufficient to obtain a delignification degree higher than that observed with HBT (25.6 and 50.2%, respectively, before and after the O_2/alkali stage), even though the latter mediator induced a slightly higher loss of viscosity (Table 4).

On the other side, the similar effects of the NHPIs aryl substituents on the mediation efficiency observed by us in the oxidation of both non-phenolic lignin model compounds and in the delignification of softwood kraft pulps demonstrate that, in our series of structurally related mediators, the use of simple non-phenolic lignin model compounds is well suited to mimic the behaviour of the lignin polymer in enzymatic oxidative degradation studies.

3. Conclusions

The introduction of electron donating substituents in the aromatic ring of *N*-hydroxyphthalimide has a beneficial effect in the yields of the oxidation products of non-phenolic lignin model compounds promoted by laccase. This result can be rationalized considering that the major contribution to the overall reaction rate is given by the oxidation of the aryl substituted NHPI to the *N*-oxyl radical (X-PINO) by laccase. 4-MeO-NHPI appears to be the most efficient mediator with all the lignin models. When applied to the delignification of wood pulp samples again the higher mediation efficiency was displayed by the NHPIs containing electron donating aryl substituents. Possible applications of NHPI substituted with electron donating groups in combination with laccase, either for synthetic purposes or for the oxidative delignification of wood pulps, are thus worth to be considered.

4. Experimental

4.1. Instrumentation

^{1}H NMR spectra were recorded on a Bruker AC300P spectrometer in $CDCl_3$. GC–MS analyses were performed on a HP5890 GC (OV1 capillary column, 12 m × 0.2 mm) coupled with a HP5970 MSD. GC analyses were carried out on a Varian 3400 GC (OV1 capillary column, 25 m × 0.2 mm). UV–vis measurements were performed on a Perkin-Elmer Lambda 18 spectrophotometer. Cyclic voltammetry measurements were carried out using an Amel 5000 potentiostat, the cell was fitted with a glassy carbon working electrode (Ø3 mm) in combination with an aqueous SCE reference electrode.

4.2. Substrates and reagents

Benzylic alcohols (**2**–**5**) and *N*-hydroxyphthalimide (**1c**) are commercially available and were used as received without further purification. 1-(3,4-Dimethoxyphenyl)-2,2-dimethyl-1-propanol (**6**) and 1-(3,4-dimethoxyphenyl)-2-phenoxyethanol (**7**) were prepared according to a previously described procedure [22,23,25]. 4-Methoxycarbonyl-*N*-hydroxyphthalimide (**1a**), 3-fluoro-*N*-hydroxyphthalimide (**1b**), 3-methyl-*N*-hydroxyphthalimide (**1d**), 4-methyl-*N*-hydroxyphthalimide (**1e**), 4-methoxy-*N*-hydroxyphthalimide (**1f**) and 3-methoxy-*N*-hydroxyphthalimide (**1g**) were synthesised as described in the literature [27,30]. 3,4-Dimethoxy-*N*-hydroxyphthalimide (**1h**) was synthesised by reacting 3,6-dimethoxyphthalic anhydride with NH_2OH, in anhydrous pyridine [32]. For the synthesis of 3,6-dimethoxyphthalic anhydride 1,4-dimethoxy-1,3-cyclohexadiene was reacted with dimethyl acetylenedicarboxylate to give dimethyl 3,6-dimethoxyphthalate [33] which was heated for 2 h under reflux in methanol/water with KOH to give 3,6-dimethoxyphthalic acid [34]. 3,6-Dimethoxyphthalic anhydride was then obtained by reaction of 3,6-dimethoxyphthalic acid with acetic anhydride [32]. Physical properties are identical to those reported in the literature [35]. Laccase from a strain of *T. villosa* (viz. *P. pinsitus*) (Novo Nordisk Biotech) was employed. It was purified by ion-exchange chromatography on Q-Sepharose by elution with phosphate buffer [36] and its activity (9000 U/ml) was determined spectrophotometrically by the standard reaction with ABTS [37].

4.3. Measurement of the redox potential

The redox potentials of aryl substituted NHPIs have been determined in aqueous solution by cyclic voltammetry. The measurements were carried out at 25 °C, in argon degassed 50 mM sodium citrate buffer solution, pH 5.0, with 3% dioxane as cosolvent, containing $LiClO_4$ (0.12 M) as the supporting electrolyte and the substrate (0.50 mM). Using a sweep scan up to 200 mV/s^{-1} reversible oxidation waves were obtained.

4.4. Enzymatic oxidations of benzyl alcohols

The mediator (10 μmol), laccase (20 units) and the substrate (30 μmol) were added to a buffered solution (0.1 M sodium citrate, pH 5.0) with 25% dioxane as cosolvent, 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). Reaction products were extracted with CH_2Cl_2, dried over Na_2SO_4 and characterised by GC–MS and ^1H NMR. A good material balance (>95%) was observed in all the experiments. In the absence of the mediator or the enzyme no products have been observed in significant amounts (<0.1%).

4.5. Delignification of wood pulps

Industrial softwood kraft pulp samples were subjected to a first step of oxygen delignification after a treatment with diethylenetriaminepentaacetic acid (DTPA), which lowered the concentration of transition metals in the pulps. The initial kappa number was 16 and the initial viscosity 987 ml/g. The moist pulp (80 g = 22 g dry pulp) was incubated with the mediator (2.5 mmol) and laccase (2000 units), in 0.1 M sodium citrate buffer (pH = 5) at 50 °C for 20 h. The pulp was then filtered, washed with distilled water and analysed for kappa number and viscosity according to standards approved by the Scandinavian pulp, paper and board testing committee. The repeatability (coefficient of variation) for both the kappa number (SCAN-C 1:00) and the viscosity (SCAN-CM 15:99) is 1.0%. The enzymatic treatment was followed by a further oxygen delignification stage in alkaline conditions (NaOH = 1.2%) at 100 °C and 0.7 MPa oxygen pressure for 80 min, kappa number and viscosity were determined with the same methods applied after the laccase/mediator treatment.

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