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Oxidation of amides by laccase-generated aminoxyl radicals

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Abstract

The enzyme laccase from the fungus *Trametes villosa* catalyses the oxidation of two hydroxylamines (>NO–H), i.e., HPI (*N*-hydroxy-phthalimide) and HBT (1-hydroxy-benzotriazole), into their corresponding aminoxyl radicals (>NO•) PINO and BTNO. The ensuing oxidation of a few amides and lactames by PINO and BTNO has been investigated in buffered water solution (pH 5) at room temperature. The results from this chemo-enzymatic approach have been compared with a literature method that generates the aminoxyl radical PINO by the HPI/Co(II)/O_2 chemical system, and uses it for the oxidation of similar amides. The merits of the aminoxyl radicals PINO and BTNO have been comparatively assessed in the chemo-enzymatic method, and the mechanism investigated. A Hammett treatment of the relative reactivity of oxidation of X-substituted-*N*-acetylbenzylamides in competition experiments supports a rate-determining H-abstraction route. With a few of the investigated substrates, stereoelectronic effects have been uncovered, and a rationalisation of their contribution to the reactivity of the H-abstraction route is offered, and supported by semiempirical calculations.

Keywords: Laccase; Mediators; Oxidation; Aminoxyl radicals; Amides; Semiempirical calculations

1. Introduction

The aminoxyl radicals are versatile paramagnetic species well documented in the literature in view of numerous applications [1–4]. In some cases they are stable and isolable, TEMPO (i.e., 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Chart 1) being a prominent example [3,5]. Stable aminoxyl radicals have found use as promoters of polymerisation reactions and inhibitors of free radical processes [1], or as spin probes for EPR investigations [6,7]. In contrast, unstable (transient) aminoxyl radicals embody reactive intermediates valuable as catalysts in chemical transformations, particularly for oxidation reactions endowed with low environmental impact [4,8].

Among these, PINO (phthalimide-*N*-oxyl) is perhaps the most know [9,10]. In principle, an aminoxyl radical ($R_2NO^•$) can be generated from the parent hydroxylamine (R_2NO–H) by H-atom transfer, or by recurring to suitable oxidants for electron-abstraction followed by deprotonation (Scheme 1).

Use of these aminoxyl radical to abstract hydrogen atom from a substrate (a HAT route) opens the way to further interaction with O_2, in this belonging (Scheme 2) to the growing field of 'green chemistry' reactions [4,8]. A viable experimental approach to the generation of PINO from precursor HPI (*N*-hydroxy-phthalimide) has been first described by Ishii et al. [10], and subsequently followed by Minisci et al. [9,11]. It requires catalytic amounts of a Co(II) salt, usually $Co(OAc)_2$, in the presence of *m*-Cl-benzoic acid (MCBA) and of O_2 as the terminal oxidant. PINO is generated in a catalytic cycle, involving also a Co(II) → Co(III) oxidation [11,12]. The reaction, which takes place under mild conditions, has been successfully applied to the oxidation of alcohols or even hydrocarbons to carbonyl derivatives [9–11], and mechanistic investigation supporting the radical HAT route reported [12,13]. Another application of the synthetic strategy concerns the aerobic oxidation of *N*-alkyl- or *N*-benzylamides or even lactams, affording carbonyl products

Abbreviations: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; HPI, *N*-hydroxyphthalimide; PINO, phthalimide-*N*-oxyl; HBT, 1-hydroxybenzotriazole; BTNO, benzotriazole-*N*-oxyl; VLA, violuric acid; NHA, *N*-hydroxyacetanilide; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) salt; MCBA, *m*-chloro-benzoic acid; BDE, bond dissociation energy; TvL, *Trametes villosa* laccase; NHE, normal hydrogen electrode; HAT, hydrogen atom transfer.

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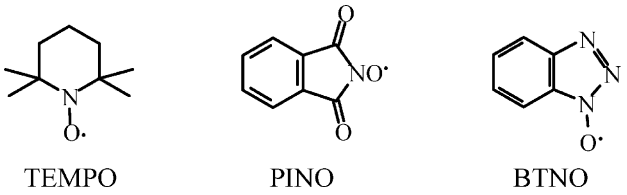
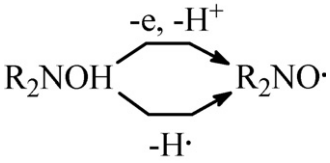
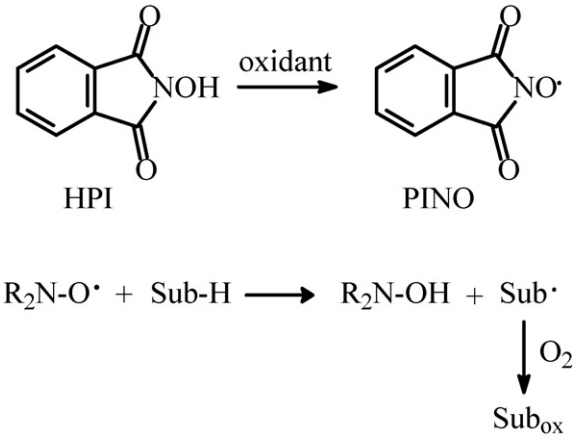


Chart 1. Examples of aminoxyl radicals.



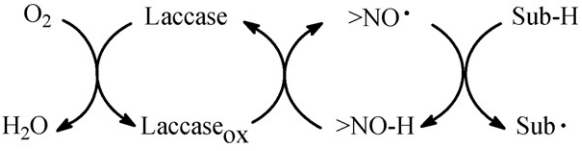
Scheme 1. Electron-transfer or radical pathways of oxidation for the generation of an aminoxyl radical.



Scheme 2. The HAT route of oxidation *via* an aminoxyl radical.

in good yields as mixtures of imides, aldehydes or carboxylic acids [14].

We and others have shown that the 'blue-copper' enzyme laccase (EC 1.10.3.2, *para*-benzenediol:oxygen oxidoreductase) is a valuable alternative to chemical oxidants for the preliminary generation of aminoxyl radicals, including PINO, from the parent hydroxylamines (Scheme 3) [15–19]. Fungal laccases, due to a redox potential in the 0.7–0.8 V/NHE range [20], can in fact catalyze the monoelectronic oxidation of hydroxylamines (Scheme 1) such as HPI, HBT (1-hydroxybenzotriazole), NHA (*N*-hydroxyacetanilide) or VLA (violuric acid), endowed with redox potentials in the 0.9–1.0 V/NHE range [21], to the corresponding >NO• species (plus H^+), by relying on O_2 as the terminal electron acceptor. The hydroxylamines are known as



Scheme 3. The chemo-enzymatic oxidation cycle of redox-recalcitrant substrates (Sub-H) by means of laccase and a >NO—H mediator.

redox mediator in this contest [22], because are able to turn the enzyme, which typically behaves as a redox catalyst, into the inducer of a radical HAT process towards redox-recalcitrant substrates [15,16,21].

Synthetic application of this approach to a HAT route of oxidation of benzyl alcohols and ethers, which are difficult to oxidise by electron-abstraction, and based on aminoxyl radicals enzymatically generated, has been reported [23,24]. We wanted to extend the scope of the environmental friendly approach of Scheme 3, and describe here the aerobic oxidation of substrates even more reluctant to undergo electron-loss, such as the amides (redox potentials above 1.8 V [25]), by recurring to HPI or HBT as precursors of the aminoxyl radicals, in combination with laccase from *Trametes villosa*. Points to address were: (i) a comparison of efficiency of the chemo-enzymatic approach with respect to the chemical method that resorts to HPI/$Co(OAc)_2$/MCBA/O_2 for the oxidation of equal amides [14]; (ii) a comparison of efficiency between the aminoxyl radicals PINO (from HPI) and BTNO (from HBT) (Chart 1) in the chemo-enzymatic method; (iii) an assessment of the mechanism of oxidation of amides induced by aminoxyl radicals. In fact, no specific clue had been brought so far about the mechanism of oxidation of amides according to the Ishii/Minisci approach [14]. Our experimental attempts to resolve these three points are here described.

2. Experimental

2.1. Materials

Solvents, salts, mediators HPI and HBT, besides a few substrates and products were commercially available (Aldrich). Required starting amides were synthesized from the parent amines by conventional acetylation with Ac_2O in pyridine [26], and characterised by NMR and GC-MS.

$PhCH_2NHCOMe$, mp 62–64 °C (lit. 64–65 °C) [27], MS (*m*/*z*) 149 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.3–7.4 (m, 5 H, ArH), 5.9 (bs, 1 H, NH), 4.4 (d, 2 H, $PhCH_2N$), 2.0 (s, 3 H, $COCH_3$).

4-MeO-$C_6H_4CH_2NHCOMe$, mp 96 °C. (lit. 95–96 °C) [28], MS (*m*/*z*) 179 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 6.8–7.3 (AA'XX', 4 H, ArH), 5.9 (bs, 1 H, NH), 4.3 (d, 2 H, $PhCH_2N$), 3.8 (s, 3 H, OCH_3), 2.0 (s, 3 H; $COCH_3$).

4-Me-$C_6H_4CH_2NHCOMe$, mp 112–114 °C (lit. 112–113 °C) [29], MS (*m*/*z*) 163 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.1–7.3 (m, 4 H, ArH); 5.8 (bs, 1 H, NH), 4.4 (d, 2 H, $PhCH_2N$), 2.3 (s, 3 H, $ArCH_3$), 2.0 (s, 3 H, $COCH_3$).

4-Cl-$C_6H_4CH_2NHCOMe$, mp 106–108 °C, MS (*m*/*z*) 183 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.2–7.3 (m, 4 H, ArH), 5.9 (bs, 1 H, NH), 4.4 (d, 2 H, $PhCH_2N$), 2.0 (s, 3 H, $COCH_3$) [30].

4-CF_3-$C_6H_4CH_2NHCOMe$, mp 101–103 °C, MS (*m*/*z*) 217 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.3–7.6 (m, 4 H, ArH), 5.9 (bs, 1 H, NH), 4.5 (d, 2 H, $PhCH_2N$), 2.0 (s, 3 H, $COCH_3$). ^{13}C NMR δ ($CDCl_3$) 170.5 (CO), 142.7 ($ArC_{ipso}CH_2$), 130.4 ($ArC_{ipso}CF_3$), 128.2, 127.0 (CF_3), 125.9, 43.5 (CH_2NH), 23.6 (CH_3).

4-NO_2-$C_6H_4CH_2NHCOMe$, mp 131–133 °C, MS (*m/z*) 194 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.4–8.2 (m, 4 H, ArH), 6.0 (bs, 1 H, NH), 4.5 (d, 2 H, $PhCH_2N$), 2.1 (s, 3 H, $COCH_3$). ^{13}C NMR δ ($CDCl_3$) 170.6 (*C*O), 146.2 (Ar$C_{ipso}NO_2$), 139.0 (Ar$C_{ipso}CH_2$), 128.6, 124.2, 43.3 (*C*H_2NH), 23.5 (CO*C*H_3).

PhCH(Me)NHCOMe, mp 75–76 °C (lit. 75–77 °C) [31], MS (*m/z*) 163 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.2–7.3 (m, 5 H, ArH), 5.8 (bs, 1 H, NH), 5.2 (m, 1 H, ArC*H*Me), 1.9 (s, 3 H, $COCH_3$), 1.5 (d, 3 H, CHC*H*$_3$) [32].

N-Acetyl-tetrahydroquinoline, MS (*m/z*) 175 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.1–7.2 (m, 4 H, ArH), 3.8 (t, J = 6.6 Hz, 2 H, NCH_2), 2.7 (t, J = 6.6 Hz, 2 H, $ArCH_2$), 2.2 (s, 3 H, $COCH_3$), 1.9 (m, 2 H, CH_2) [33].

N-Acetyl-tetrahydroisoquinoline, MS (*m/z*) 175 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.2–7.5 (m, 4 H, ArH), 4.7 (bs, 2 H, $ArCH_2N$), 3.8 (t, 2 H, CH_2N); 3.0 (bs, 2 H, $ArCH_2$), 2.2 (s, 3 H, $COCH_3$) [34].

$PhCH_2CH_2NHCOMe$, mp 44–46 °C (lit. 48–50 °C) [35], MS (*m/z*) 163 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.2–7.3 (m, 5 H, ArH), 5.6 (bs, 1 H, NH), 3.5 (m, 2 H, CH_2N), 2.8 (t, 2 H, $ArCH_2$), 1.9 (s, 3 H, $COCH_3$).

2.2. Instrumentation

^{1}H and ^{13}C NMR spectra have been recorded on a Bruker AC 200 instrument (200 and 50 MHz, respectively). GC analyses have been run on a Varian CP 3800 instrument, equipped with either methyl silicone gum or 5% phenyl silicone 30 m × 0.25 mm × 25 μm capillary columns. GC–MS analyses have been run on a HP 5892 series II GC, equipped with a 30 m × 0.25 mm × 25 μm 5% phenyl silicone capillary column and coupled to a HP 5972 MSD instrument operating at 70 eV.

2.3. Enzyme purification

Crude laccase from *Trametes villosa* (TvL) was a kind gift of Novo Nordisk Biotech. The enzyme was purified by anion-exchange chromatography on Q-Sepharose Fast Flow as previously reported [21]. The purified sample had an absorption ratio A_{280}/A_{610} of 20–30 and its activity, determined by the spectrophotometric ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) salt) assay [36], was 6000 U/mL. One unit (U) is defined as the amount of enzyme producing 1 μmol of product per min under the assay conditions.

2.4. Enzymatic oxidations

The oxidation reactions were performed under magnetic stirring in 3 mL 0.1 M citrate buffer solution at pH 5; the sodium citrate buffer was purged with O_2 for 30 min prior to the addition of the reagents. The substrate (60 μmol, in 120 μL of MeCN), the mediator (60 μmol, or 20 μmol in the initial experiments) and 60 U of TvL laccase (or 15 U in the initial experiments) were added with syringes from mother solutions. Incubations were carried out at room temperature for 24 h under oxygen (filled latex balloon). After a conventional work-up with ethyl acetate (containing an internal standard), the molar amount of the oxidation products was measured by GC analysis with respect to an internal standard (acetophenone or 4-MeO-acetophenone), suitable response factors being determined with authentic compounds, and the yields reckoned with respect to the molar amount of the substrate. The identity of the products was confirmed by GC–MS analysis.

The oxidation of pairs of substrates in competition was carried out under similar conditions, by dissolving 60 μmol of each competing substrate in 6 mL 0.1 M citrate buffer solution at pH 5 with the aid of 240 μL of MeCN, in the presence of 60 μmol of mediator and 60 U of TvL. The molar amount of the detected products was employed for reckoning the relative reactivity ratios; these ratios were plotted in logarithmic form *versus* the Hammett σ parameters.

2.5. Synthesis of reaction products

The Minisci procedure was followed for the preparation of authentic specimens of the imide products [14]. As an example, 41 mg of HPI (0.25 mmol), 20 mg of *m*-Cl-benzoic acid (MCBA, 0.013 mmol), 3 mg of $Co(OAc)_2{}^{\bullet}4H_2O$ (0.013 mmoli) and 2.5 mmol of *N*-benzylacetamide ($PhCH_2NHCOCH_3$) were dissolved in 5 mL of MeCN and kept under stirring at room temperature for 24 h under oxygen (filled latex balloon). The reaction crude was filtered over a silica gel pad in order to remove the cobalt salts, and the solvent evaporated. *N*-Benzoylacetamide ($PhCONHCOCH_3$) was obtained as a solid and recrystallized from a 1:1 (v/v) diethyl ether:EtOH mixture, 20% yield; mp 116 °C (lit. 115–116 °C) [37]. ^{1}H NMR δ ($CDCl_3$) 8.8 (bs, 1 H, NH), 7.5–7.6 (m, 5 H, ArH), 2.6 (s, 3 H, $COCH_3$). MS (*m/z*) 163 (molecular ion). In a similar way the following imides were obtained and characterised:

4-NO_2-$C_6H_4CONHCOMe$, 33% yield, mp 220 °C (from MeCN and EtOH), MS (*m/z*) 208 (molecular ion). ^{1}H NMR δ (DMSO-d_6) 11.3 (bs, 1 H, NH), 8.2–8.4 (AA'XX' system, 4 H, ArH), 2.3 (s, 3 H, $COCH_3$). ^{13}C NMR δ (DMSO-d_6) 170.0 (*C*OMe), 165.6 (Ar*C*O), 149.7 (Ar$C_{ipso}NO_2$), 139.1 (ArC_{ipso}CO), 129.9, 123.5, 25.6 (CO*C*H_3).

4-CF_3-$C_6H_4CONHCOMe$, 45% yield, mp 142–144 °C (from 1:1 AcOEt:hexane), MS (m/z) 231 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 9.4 (bs, 1 H, NH), 7.7–7.9 (AA'XX' system, 4 H, ArH), 2.6 (s, 3 H, $COCH_3$). ^{13}C NMR δ ($CDCl_3$) 174.3 (*C*OMe), 165.0 (Ar*C*O), 136.0 (Ar$C_{ipso}CF_3$), 134.6 (ArC_{ipso}CO), 128.5, 126.2, 123.6 (*C*F_3), 25.9 (CO*C*H_3).

4-Cl-$C_6H_4CONHCOMe$, 40% yield, mp 140–142 °C (from diethyl ether; lit. 143 °C [38]). MS (*m/z*) 197 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 8.9 (bs, 1 H, NH), 7.7–8.0 (AA'XX' system, 4 H, ArH), 2.6 (s, 3 H, $COCH_3$).

1-Acetyl-tetrahydroquinolin-4-one, MS (*m/z*) 189 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.2–7.5 (m, 4 H, ArH), 4.2 (t, J = 6.2 Hz, 2 H, NCH_2), 2.8 (t, J = 6.2 Hz, 2 H, $COCH_2$), 2.7 (s, 3 H, $COCH_3$). ^{13}C NMR δ ($CDCl_3$) 194.2, 169.7, 144.0, 134.2, 127.9, 126, 126.2, 124.2, 44.1, 39.6, 23.2 [39].

2-Acetyl-tetrahydroisoquinolin-1-one, MS (*m/z*) 189 (molecular ion). ^{1}H NMR δ ($CDCl_3$) 7.9–6.9 (m, 4 H, ArH), 4.1 (t, J = 6.3 Hz, 2 H, NCH_2), 2.9 (t, J = 6.3 Hz, 2 H, $ArCH_2$), 2.6 (s, 3 H, $COCH_3$) [40].

1-Benzoyl-pyrrolidinone + N-benzyl-succinimide; these products could not be separated by column chromatography nor by crystallisation, and the mixture was characterised by ^{1}H NMR; δ ($CDCl_3$) 7.3–7.5 (m, 5 H, ArH), 4.0 (t, 2 H, CH_2N), 2.6 (t, 2 H, $COCH_2$), 2.1 (m, 2 H, $CH_2CH_2CH_2$), 4.7 (s, 2 H, $PhCH_2N$), 2.7 (s, 4 H, CH_2CH_2) [41,42]. MS (*m/z*) 189 (molecular ion).

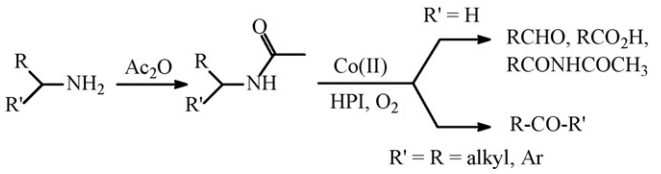
2.6. Semiempirical calculations

The structure of the amides investigated has been optimized by a semi-empirical method (PM3 level) by using the Hyperchem program [43]. Then, to calculate the energy of other conformers, the desired conformation has been imposed and the $\Delta_f H_{298}$ calculated (single point).

3. Results and discussion

3.1. Oxidation of amides by the chemo-enzymatic approach

Previous investigation by Minisci et al. [14] had shown that simple amines interact with and degrade HPI by nucleophilic attack, thereby hampering any generation of PINO from HPI according to Scheme 2. The problem was circumvented by protecting the amino group through acylation, because the resulting amide does not deactivate HPI, but rather behaves as a substrate for the PINO-induced oxidation. This strategy led to develop a novel synthetic transformation of amines to carbonyl derivatives through the amides, based on the PINO reactive intermediate generated *in situ* from HPI and Co(II) salts in the presence of O_2, in MeCN solution under mild conditions (80 °C, or even room temperature) (Scheme 4) [14]. A few results of this approach are given in Table 1 for significant structures.

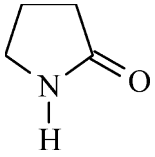
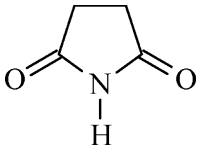
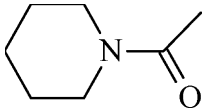
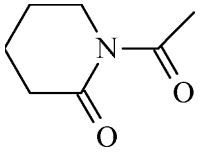
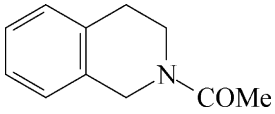
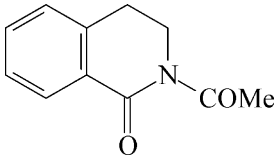


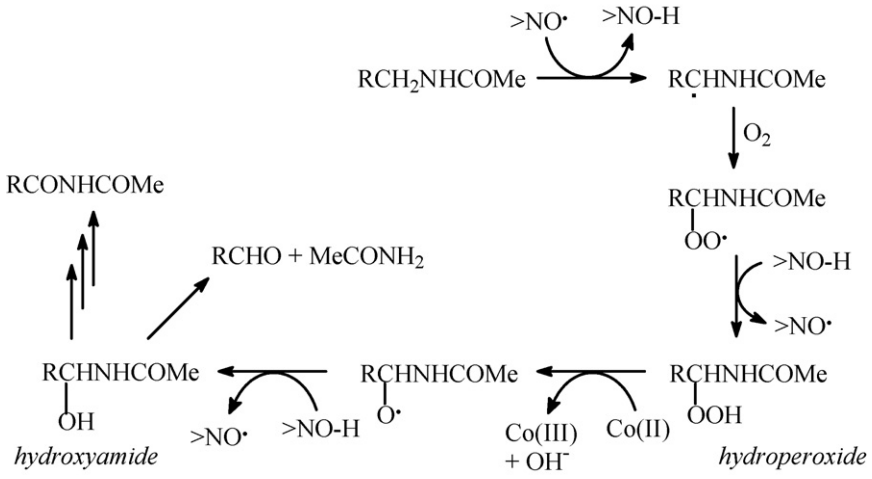
Scheme 4. Oxidation strategy of amines through amides. Redrawn with permission from Minisci et al. [14]. Copyright (2002) American Chemical Society.

This oxidation turns the CH_2 group in α to the nitrogen atom of lactames or amides into an intermediate hydroxyamide, which either gives hydrolysis to the aldehyde product or undergoes a further cycle of oxidation to afford the imide product (Scheme 5), as rationalised by Minisci et al. [14]. MCBA enhances the solubility of the cobalt salts in MeCN solution, thereby ensuring better efficiency to the redox decomposition of the hydroperoxide intermediate of the substrate. The conversion is particularly good with benzylic CH_2 groups; open-chain substrates, and particularly aliphatic ones, yield a more pronounced overoxidation of the aldehyde to carboxylic acid. Additional examples were provided in the original paper [14].

We have re-investigated this reaction by shifting to laccase in order to oxidise HPI into PINO. This enzymatic approach had already enabled us to accomplish the aerobic oxidation of benzylic alcohols or ethers into carbonyl derivatives in water solution at room temperature [23,24]. Here we have selected a few amides among those investigated by Minisci et al. [14], plus other ones, and subjected them to the aerobic oxidation

Table 1
Selected results of the oxidation of amides after Minisci et al. [14], by the HPI/$Co(AcO)_2$/MCBA/O_2 method in MeCN solution

Amide	*T* (°C)	*t* (h)	Product(s)	Yield (%)
	80	5		90
	80	4		42
	20	1		87
$PhCH_2NHCOCH_3$	20	4	PhCHO	19
			$PhCONHCOCH_3$	69
$C_6H_{13}NHCOCH_3$	80	4	$C_4H_9CO_2H$	14
			$C_5H_{11}CO_2H$	13
			$C_5H_{11}CONHCOCH_3$	67

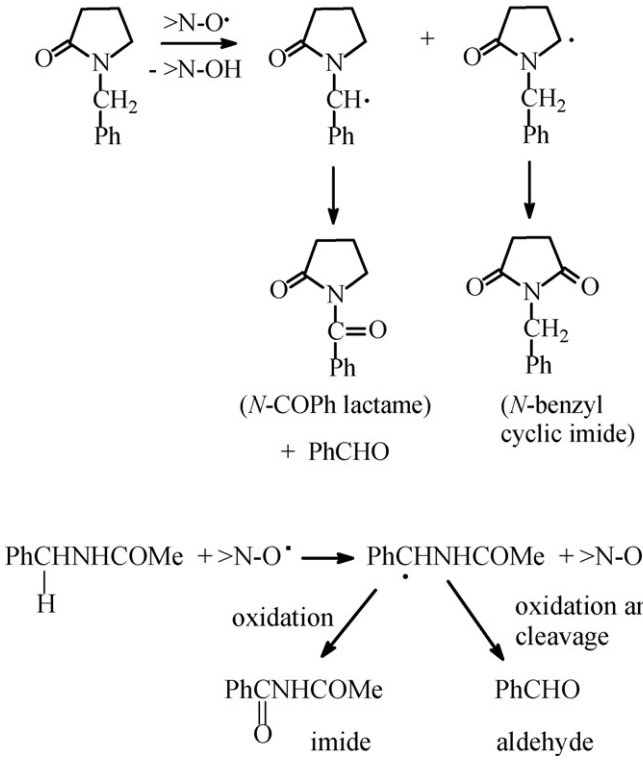


Scheme 5. The plausible mechanism of oxidation of amides by the HPI/Co(II)/MCBA/O_2 chemical system, from [14].

catalysed by *Trametes villosa* laccase (TvL) in the presence of HPI. Duplication of the experiments in the alternative presence of HBT, as precursor of the aminoxyl radical BTNO, has also been performed; in fact, BTNO had given even better results than PINO in previous studies of radical oxidation towards other substrates [15,24]. The operating conditions of the chemo-enzymatic oxidation, i.e., buffered water (pH 5, 0.1 M sodium citrate) solution and room temperature, for a reaction time of 24 h under O_2 atmosphere, are very mild. The enzyme is present in catalytic amounts, while equimolar amounts of substrate and redox mediator (either HPI or HBT) are used (60 μmol each); O_2 enables to re-oxidise laccase in a catalytic cycle (Scheme 3) for further redox oxidation of the mediator to the aminoxyl radical intermediate.

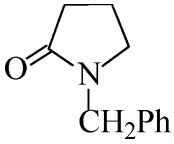
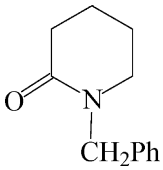
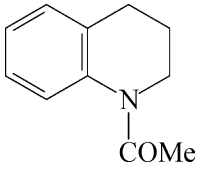
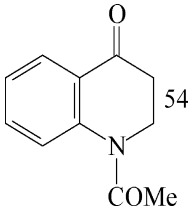
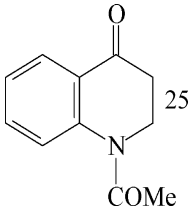
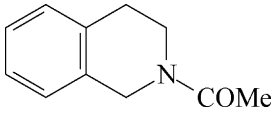
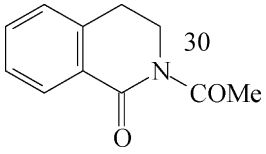
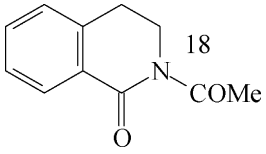
Preliminary experiments run with lower amounts of both mediator (20 μmol) and enzyme (15 U) gave lower conversions. The results of the chemo-enzymatic oxidations are reported in Table 2 as gas-chromatographic yields of product(s). Recovery of unreacted starting material (not given in Table 2) represents the rest of mass balance in each case within the experimental errors (3–8%); the structure of the products could be confirmed by comparison with authentic samples, either available in the laboratory or independently synthesized.

Table 2 reports lower conversion into products than in the Minisci study (Table 1) [14] for equal amides, as the *N*-acetyltetrahydroisoquinoline and *N*-benzylacetamide cases show, even though the nature and structure of the products obtained is the same (cf. Scheme 6 and Table 2). In fact, we analogously find that the oxidation of substrates having benzylic methylene groups is particularly successful, as indicated by the cases of the quinoline- and isoquinoline-derivatives. This is a useful mechanistic clue, because a radical HAT route is expected to be fostered whenever the frangible C–H bond undergoing oxidation is the weak benzylic one [21], and supports our belief that >NO• is the reactive intermediate both in the chemical and chemo-enzymatic oxidations. However, we find it difficult to explain why the chemo-enzymatic procedure is less efficient than the chemical one, if the reactive intermediate is the same. In both procedures the formation of >NO• takes place in a catalytic cycle. One could speculate that PINO (or BTNO), once generated by laccase from the >NO–H precursor, as outlined in Scheme 3, is present at very low concentration throughout the oxidation process: this low concentration would kinetically disfavour any bimolecular step of oxidative functionalisation. In contrast, the generation of PINO from HPI under the Ishii/Minisci conditions [9,10,14] could possibly be more efficient, thereby providing better chances to the oxidation of the amides. Because the Ishii/Minisci procedure takes advantage from use of catalytic amounts of MCBA for fostering the cleavage of hydroperoxide intermediates (Scheme 5), we have similarly added MCBA in one experiment (Table 2), but no significant improvements in our



Scheme 6. Conceivable pathways of formation of products.

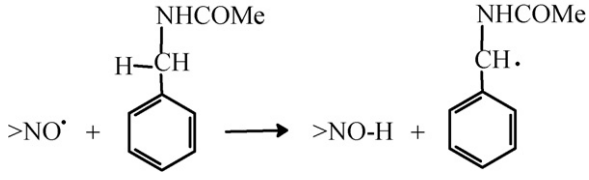
Table 2
Products and yields from the aerobic oxidation of amides catalysed by *Trametes villosa* laccase (TvL) with two different redox mediators (cf. Scheme 6)[a]

Substrate	Products with HPI		Products with HBT	
	ArCHO (%)	Imide[b] (%)	ArCHO (%)	Imide[b] (%)
4-MeO-C_6H_4-CH_2NHCOMe[c]	14	4.5	15	3.5
4-MeO-C_6H_4-CH_2NHCOMe	10	53	26	15
$PhCH_2$NHCOMe[c]	3	12	0	0
$PhCH_2$NHCOMe	6	18	3	17
$PhCH_2$NHCOMe[d]	2	15	–	–
	0.8	Cyclic imide-*N*-CH_2Ph 19, *N*-COPh lactame 4	0.9	Cyclic imide-*N*-CH_2Ph 0, *N*-COPh lactame 0
	0.6	Cyclic imide-*N*-CH_2Ph 8	0.9	Cyclic imide-*N*-CH_2Ph 0.6
	0	 54	0	 25
	0	 30	0	 18
PhCH(Me)NHCOMe	0	PhCOMe 3	0	PhCOMe 1.5
$PhCH_2CH_2$NHCOME	2.5	0	0.9	0
4-CF_3-C_6H_4-CH_2NHCOMe	1	24	2	0
4-Me-C_6H_4-CH_2NHCOMe	12	65	16	11
4-Cl-C_6H_4-CH_2NHCOMe	10	28	7	2.8
4-NO_2-C_6H_4-CH_2NHCOMe	1	10	3	0

[a] Reaction conditions: substrate, 60 μmol, mediator, 60 μmol, 60 U of TvL, in 3 mL of buffered water (pH 5; 0.1 M sodium citrate), at 25 °C for 24 h under O_2. The yields (%) were determined by GC analysis with respect to an internal standard.
[b] Structure of products in Scheme 6.
[c] Run with a lower molar amount (20 μmol) of mediator, and with 15 U of TvL only.
[d] With the additional presence of 30 μmol of MCBA (cf. Table 1).

results were recorded. Finally, the solvent system is also different between the chemical [9,10,14] and the chemo-enzymatic conditions, and this could affect the stability of the aminoxyl radicals [44] as reactive intermediates responsible for the conversion to products, lowering it the more in water solution, or enabling a more pronounced incursion from undesired side-reactions.

The experiments reported in Table 2 enable instead to compare the relative efficiency of HPI and HBT as mediators in the chemo-enzymatic procedure, and HPI appears to give slightly better performances than HBT. The limited stability of the aminoxyl radicals PINO and BTNO is likely to matter here, and PINO is known to be more stable [45]. However, because the aminoxyl radical is continuously re-formed in the catalytic cycle of Scheme 3, in that mending for the limited stability, another point to take into consideration is the energy value of the O–H bond that the two aminoxyl radicals form upon H-abstraction from a given substrate (Scheme 7). The BDE(O–H) of HPI is larger than that of HBT, i.e., 88 kcal/mol *versus* 85 kcal/mol respectively [13,21], and therefore the H-abstraction oxidation with HPI is thermodynamically more favoured. This thermodynamic issue is indeed corroborated by kinetic data, showing that



Scheme 7. Relevance of NO–H bond making *vs*. C–H bond cleavage upon the HAT route.

$$ArCH_2NHCOMe + >NO\cdot \xrightarrow{k_X} ArCHO + ArC(O)NHCOMe + >NOH$$

$$PhCH_2NHCOMe + >NO\cdot \xrightarrow{k_H} PhCHO + PhC(O)NHCOMe + >NOH$$

Scheme 8. Competitive oxidation experiments by laccase/mediator systems.

H-abstraction by PINO [12] proceeds at least one order of magnitude faster than by BTNO [45] for equal H-donor substrates.

The outcome of the oxidation reactions of *N*-benzyl-2-pyrrolidinone and *N*-benzyl-2-piperidone sets up a puzzling question instead (cf. Scheme 6 and Table 2). Why is the exocyclic benzylic CH_2 moiety turned into a carbonyl in the 5-membered lactame, but not as much in the 6-membered analogue? We suspected a stereoelectronic effect, and will come back to this point later. A few other pieces of evidence deserve an explanation. Oxidation of the benzylic C–H bond in PhCH(Me)NHCOMe is more difficult than in $PhCH_2NHCOMe$, indicating an unfavourable effect from the α-Me substituent. Strangely enough, this effect offsets the otherwise favourable contribution that cleaving the weaker tertiary C–H bond in the former compound, as opposed to the secondary C–H bond in the latter, ought to have on the HAT route of oxidation [46]. Furthermore, oxidation of the benzylic CH_2 in substrate $PhCH_2CH_2NHCOMe$, being it β to the N-atom, gives a worse result with respect to $PhCH_2NHCOMe$, where the benzylic CH_2 and NH groups are adjacent. Finally, in the homologous series of 4-X-substituted *N*-benzylacetamides it is found that the nature of the 4-X-substituent appreciably affects the extent of oxidation into the products imide and aldehyde (Scheme 6).

3.2. Hammett correlation

This effect of the substituents upon the reactivity of oxidation has been investigated more closely in competitive experiments, where 4-X-substituted *N*-benzylacetamides were in turn pitted *versus* the unsubstituted *N*-benzylacetamide in pairs, for the aerobic oxidation by TvL with either HPI or HBT (Scheme 8).

Analysis of the reaction products by GC enabled to sort out the products of oxidation from the two competing precursors and to assess their extent (Table 3). Different sampling times had to be adopted for the various reacting pairs, in the attempt to enhance the conversion to products for analytical purposes. For each substrate the molar amount of product imide was added to that of product aldehyde, because both originate from the common benzyl-radical intermediate initially formed (cf. Schemes 5 and 6), and the relative reactivity k_X/k_H was calculated according to Eq. (1). It can be qualitatively observed that electron-donor substituents enhance the conversion to products, whereas electron-withdrawing ones depress it. The relative proportion of aldehyde and imide from each precursor is not easy to rationalise. It is going to be the complex outcome of both the effect of the substituents and the sampling time, owing to the different routes (and timing) of formation of the two products from the common hydroxyamide intermediate (in Scheme 5): chain-cleavage for the aldehyde *versus* further oxidation for the imide. This is undoubtedly an interesting point but, because the actual formation of the oxidation products takes place after the H-abstraction step, which is the focus of our kinetic analysis, no efforts to further clarify it were made.

$$\frac{k_X}{k_H} = \frac{\log([ArCH_2NHAc]_o - ([ArCHO]_t + [ArCONHAc]_t))/[ArCH_2NHAc]_o}{\log([PhCH_2NHAc]_o - ([PhCHO]_t + [PhCONHAc]_t))/[PhCH_2NHAc]_o} \quad (1)$$

The log k_X/k_H ratios were plotted *versus* the σ parameter of the X-substituent according to the Hammett equation ($\log k_X/k_H = \rho\sigma$), slightly better linear fits being obtained with the σ^+ parameters for both HPI and HBT, and the ρ values (i.e., −0.88 and −1.23) are accordingly given in the plots (Fig. 1).

If one concedes that the k_X/k_H relative reactivities are reckoned by combining the molar amounts of two products for each reacting substrate in the pairs, the quality of the plots can be judged more than reasonable. Because the conversion into products obtained with mediator HBT is lower, the inherent error on its ρ value is presumably larger. Nevertheless, we can compare the present ρ data with those acquired in the oxidation of X-substituted benzyl alcohols, analogously run with TvL under mediation by HPI or HBT, where ρ values of −0.8 and −0.6, respectively, had been obtained in competition experiments [15,16]. Small ρ values are indeed expected for a radical route of oxidation that proceeds by rate-determining H-abstraction from the benzylic position; this is indeed experimentally found with both the benzyl alcohols and the present *N*-benzylacetamides as substrates. Uniform support to the HAT mechanism of oxidation is thereby gained. The negative sign of ρ is consistent with the electrophilic nature of the aminoxyl radicals PINO and BTNO, as already pointed out [47]. No Hammett treatment had been reported by Minisci et al. in their study of the oxidation of amides [14], and therefore our investigation allows for a deeper mechanistic assessment.

Table 3
Yield (%) of products (aldehyde and imide) in the competitive chemo-enzymatic oxidation of 4-X-substituted *N*-benzylacetamides *vs*. the unsubstituted one, with two TvL/mediator systems at 25 °C in buffered (pH 5) water solution

4-X	TvL with HPI				TvL with HBT			
	ArCHO	ArCONHCOMe	PhCHO	PhCONHCOMe	ArCHO	ArCONHCOMe	PhCHO	PhCONHCOMe
OMe	29	23	3.2	6.2	19	4.1	0.95	3.1
Me	12	23	3.3	8.6	8.5	10	2.0	4.3
Cl	8.4	14	3.0	11	4.6	2.4	1.3	8.8
CF_3	2.1	9.1	5.6	15	2.0	0.58	3.7	9.5
NO_2	1.7	5.9	2.8	15	1.5	0	1.4	12

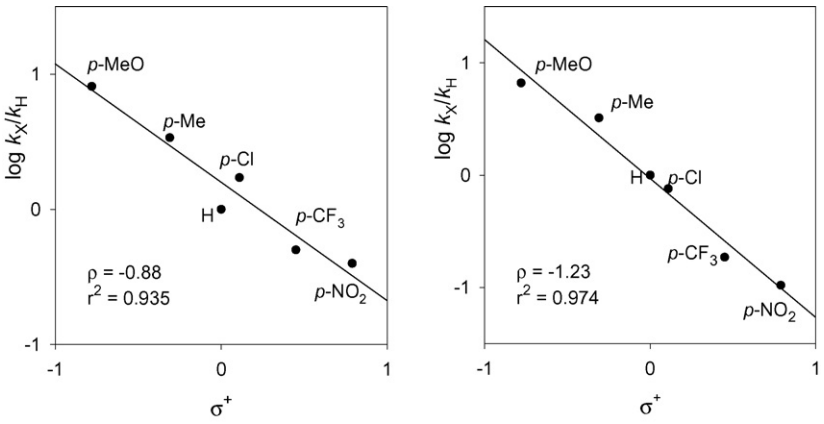


Fig. 1. Hammett plots for the aerobic oxidation of 4-X-substituted *N*-benzylacetamides catalyzed by TvL with mediation by HPI (left) or HBT (right).

3.3. Stereoelectronic effects

Griller et al. [48] have convincingly demonstrated that hyperconjugation from the lone-pair of a heteroatom weakens an adjacent C–H bond, and stabilizes the intervening C-radical generated by H-abstraction. In the case of the nitrogen lone-pair of an amine, the contribution from this effect to the ease of cleavage of an adjacent C–H is the greater the more co-linear are the C–H bond and the lone-pair (Fig. 2).

This interaction reportedly accounts for a 7–9 kcal/mol weakening of the C–H bond. For diedral angles (θ) wider than 30°, the effect drastically fades away [48]. In keeping with this point, we have found a comparable stereoelectronic contribution from the lone-pair of the oxygen atom, which makes the adjacent benzylic C–H bond of $PhCH_2OH$ weaker (i.e., 79 kcal/mol) than that of $PhCH_3$ (88.1 kcal/mol) by 9 kcal/mol [45], as also predicted in the literature [49]. We accordingly expected a C–H bond in α to the N-atom of an amide to be similarly weakened, even though the concomitant conjugation of nitrogen with the carbonyl group might reduce the importance of this effect for amides with respect to amines (or alcohols). Unfortunately, pertinent thermochemical data are lacking or scanty for comparison.

In the present study the efficiency of oxidation of $PhCH_2NHCOMe$ by laccase/HPI (Table 2), to the corresponding imide PhCONHCOMe plus PhCHO, is found higher than for the homologous $PhCH_2CH_2NHCOMe$, which gives only traces of PhCHO by C_α–C_β bond cleavage. In fact, H-removal from $PhCH_2NHCOMe$ yields a benzyl radical stabilised by both hyperconjugation from the lone-pair of nitrogen and conjugation with the aromatic p-orbitals. In contrast, the spacer CH_2 in $PhCH_2CH_2NHCOMe$ makes this sort of ditopic stabilisation impossible, and lowers the reactivity of the latter compound in the HAT route.

In order to investigate the ditopic stabilisation more quantitatively, we resorted to a competitive aerobic oxidation of *N*-acetyl-tetrahydroisoquinoline and $PhCH_2NHCOMe$ (Scheme 9). HBT was preferred as mediator in this case owing to the simpler pattern of products obtained.

Although this cyclic substrate could in principle undergo H-abstraction and oxidation both at the 1-and 3-positions, the NMR spectrum of the obtained imide unequivocally indicated oxidation at position 1 only. One concludes that the BDE(C–H) of benzylic C-1, although unknown for this cyclic compound (being it 88 kcal/mol in toluene) [50], is lower than that of the C–H bond in position 3 (94 kcal/mol is reported for the α-CH_2 in β-phenylethylamide [50]).

In the competition outlined in Scheme 9, the relative efficiency of H-abstraction from the benzylic CH_2 adjacent to the N-atom in the cyclic *versus* the open-chain counterpart is assessed as $k_C/k_O = 8.3$. The higher reactivity of the former can be attributed to the operation of a stereoelectronic effect already illustrated for benzyl alcohols [51]. In the cyclic substrate, in fact, the scissile benzylic C–H bond is almost co-linear with the p-orbitals of the aromatic system, due to conformational restrictions caused by the ring. This weakens the C–H bond and stabilizes the intermediate benzyl radical (Fig. 3) more than in the open-chain analogue $PhCH_2NHCOMe$, where free rotation between the aromatic system and the benzylic CH_2 opposes to an equally significant stabilisation from π-conjugation.

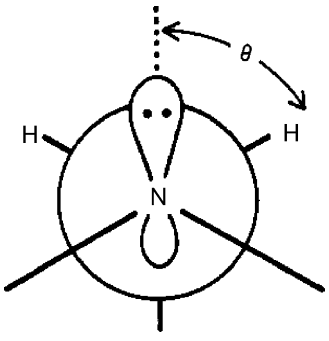
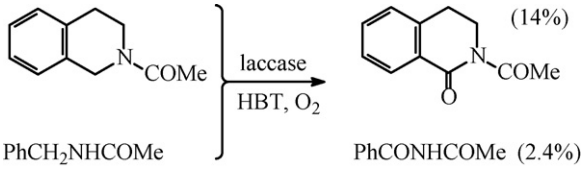


Fig. 2. Stereoelectronic interaction from a lone-pair. Reprinted with permission from Griller et al. [48]. Copyright (1981) American Chemical Society.



Scheme 9. Relative reactivity of a cyclic *vs*. an open-chain substrate: combination of two stabilizing effects (see text).

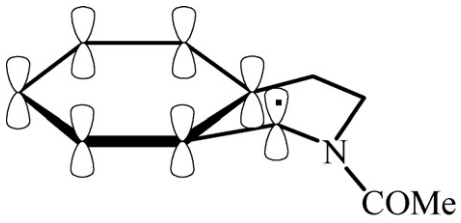


Fig. 3. Stabilizing interaction with both the π-system and the nitrogen lone-pair for the C-radical intermediate from oxidation of the cyclic substrate shown in Scheme 9.

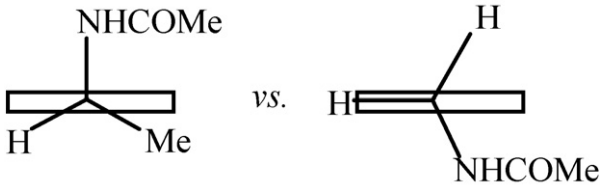


Fig. 4. Limiting conformations of PhCH(Me)NHCOMe and $PhCH_2NHCOMe$. The plane of the aromatic ring is represented as a rectangle.

The lower reactivity reported in Table 2 for PhCH(Me)NHCOMe *versus* $PhCH_2NHCOMe$ may find a rationalisation now. The former substrate is expected to be conformationally more stable (Fig. 4) whenever the larger Me- or NHCOMe-groups in α are co-linear with the aromatic p-orbitals, so that the aromatic C–H bonds in *ortho* cause less hindrance. As a consequence, the benzylic H-atom would be oriented in the ring plane, where no π-stabilisation of an incipient α-amido carbon radical resulting from H-abstraction would be possible. This is expected to slow down the oxidation with respect to the unsubstituted $PhCH_2NHCOMe$, where alignment of one α–C–H bond with the π-system is always feasible. Semiempirical calculations do confirm this expectation, because the lowest-energy conformation of PhCH(Me)NHCOMe is indeed found to be the one (Fig. 5, left structure) that precludes π-assistance to cleavage of the α-C–H bond, whereas such assistance is possible for $PhCH_2NHCOMe$ (right structure). The conformation of PhCH(Me)NHCOMe that would enable H-removal and π-stabilization of the benzylic carbon-radical (center structure) is instead 12 kcal/mol *less* stable than the one on the left.

A final comment on the peculiar and lower reactivity of oxidation of the exocyclic CH_2 group of *N*-benzyl-2-piperidone with respect to *N*-benzyl-2-pyrrolidinone (Table 2) is added. Semiempirical calculations for *N*-benzyl-2-piperidone show that the conformation where a benzylic C–H bond is co-linear with *both* the π-system and the nitrogen lone-pair is high in energy (Fig. 6, center structure), because the aromatic ring and the piperidone ring are forced to be *quasi*-coplanar, and hindrance arises. In contrast, the lowest energy conformation (left structure) has the exocyclic CH_2 group inappropriately oriented for any stabilizing contribution. This ought to retard H-abstraction from the benzylic C–H bond in the 6-ring with respect to the 5-ring, as it is indeed found. The corresponding calculations for *N*-benzyl-2-pyrrolidinone, in fact, show that a profitable conformation (right structure) for H-abstraction from the benzylic CH_2 is low in energy. Even the conformation where both rings are coplanar

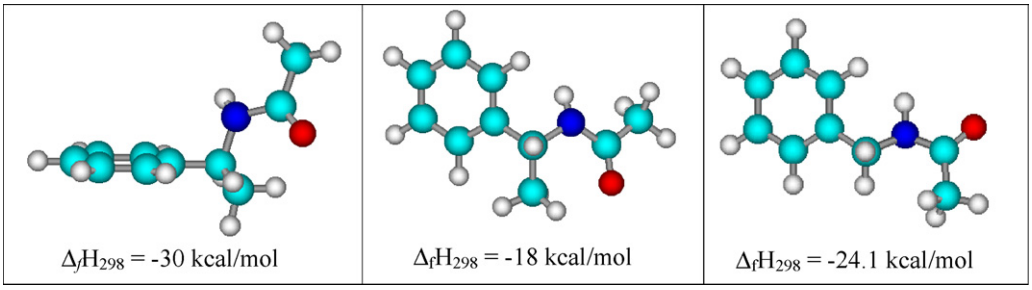


Fig. 5. Calculated heats of formation. Left: In the most stable conformation of PhCH(Me)NHCOMe, H-removal is hampered by lack of π-stabilisation to the 'equatorial' C–H bond. Center: In contrast, the conformation enabling H-removal and π-stabilisation of the benzylic C-radical is 12 kcal/mol *less* stable. Right: In $PhCH_2NHCOMe$, all the conformations have approximately the same energy, including the one enabling stabilisation to the C–H bond from *both* the π-system and the lone-pair of nitrogen.

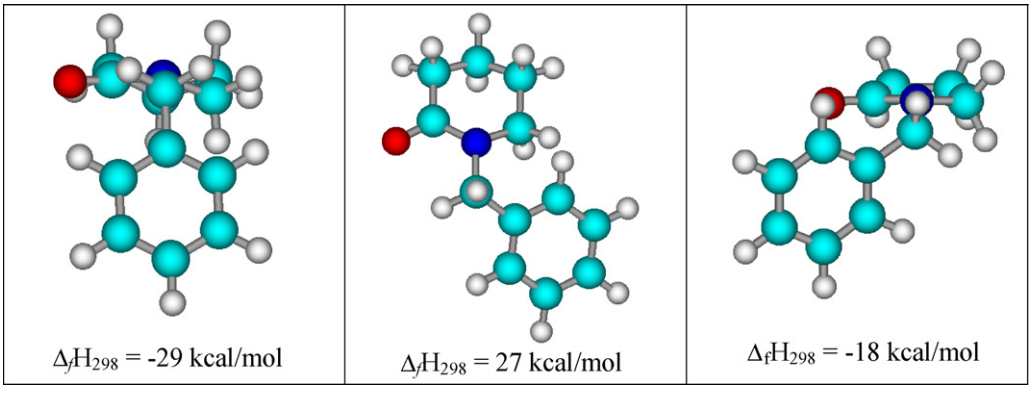


Fig. 6. Heats of formation of significant conformations of *N*-benzyl-2-piperidone (left and center structures) and *N*-benzyl-2-pyrrolidinone (right structure), from semiempirical calculations.

(not shown) is 25 kcal/mol more stable than the corresponding one (center) for the 6-membered piperidone compound. The calculations additionally confirm that, within the lactame ring, stabilisation from the nitrogen lone-pair to the adjacent C–H bonds is always feasible, even though better co-linearity and smaller θ angle (cf. Fig. 2) are reached for the C–H bonds of the 5-membered lactame. Accordingly, Table 2 shows appreciable yields of functionalisation for both lactams (i.e., cyclic imide-*N*-CH_2Ph) with mediator HPI, the conversion being higher with the 5- than for the 6-ring.

4. Conclusions

This investigation provides mechanistic insight on the aerobic oxidation of amides by two aminoxyl radical intermediates (PINO and BTNO), generated by laccase from oxidation of the corresponding hydroxylamines HPI and HBT. Unambiguous evidence in favour of a HAT route of oxidation of the amides by the aminoxyl radicals is provided by means of a Hammett treatment. The results of this chemo-enzymatic approach are compared with those from a literature study [14], where the aminoxyl radical is generated by chemical oxidation of HPI from cobalt salts. The chemical method results more efficient than the chemo-enzymatic one. Among the two hydroxylamines investigated by us, HPI performs better than HBT, and possible explanations have been advanced. The role of stereoelectronic effects has been investigated in the chemo-enzymatic oxidation, and their contribution to the reactivity of the H-abstraction route rationalised, even though only a qualitative explanation can be provided at present, due to lack of pertinent kinetic and thermochemical data. Future kinetic work upon the direct HAT oxidation of amides by *bona fide* aminoxyl radicals will hopefully put the investigation of these stereoelectronic effects on a more quantitative basis.

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