Isotope-Effect Profiles in the Oxidative *N*-Demethylation of *N,N*-Dimethylanilines Catalysed by Lignin Peroxidase and a Chemical Model

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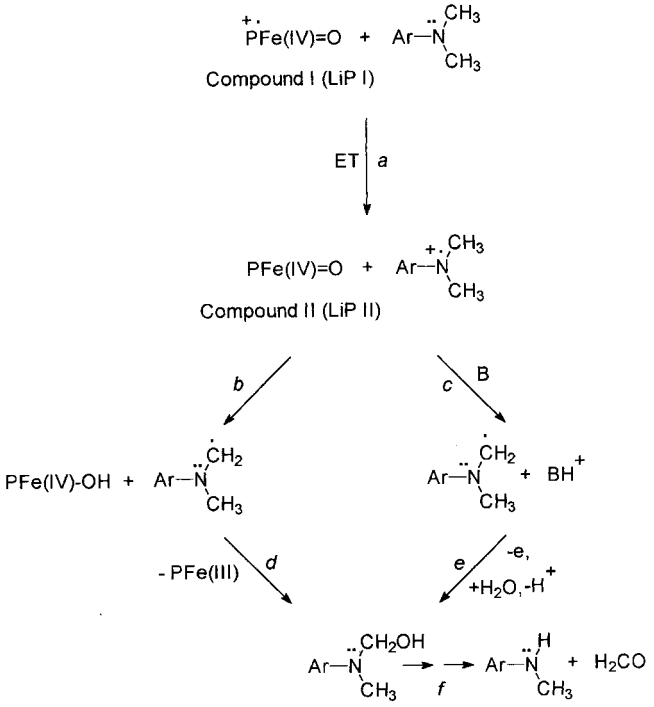
Lignin peroxidase catalyses the oxidative *N*-demethylation of ring-substituted *N,N*-dimethylanilines by an electron-transfer mechanism whereby an anilinium radical cation is formed which is then deprotonated by the enzyme. Information on the nature of the basic centre which deprotonates the radical cation has been obtained by determining the KDIE profile (plot of k_H/k_D vs. the pK_a of the aniline radical cations) for a number of ring-substituted *N,N*-bis(dideuteriomethyl)-anilines. From the bell-shaped curve it has been estimated that the pK_a of the proton-abstracting base is about 7. Interestingly, almost the same value has been obtained when the same type of study has been carried out using a water-soluble model compound: 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine-*p*,*p*′,*p*″,*p*‴-tetrasulfonic acid iron(III) chloride. This is a strong indication that the radical cation is deprotonated by the same species in the enzymatic and in the chemical reactions. It is suggested that this species is the reduced iron-oxo complex.

Introduction

Lignin peroxidase (LiP), a heme-containing glycoprotein isolated from the ligninolytic cultures of the white-rot fungus *Phanerochaete chrysosporium*, is one of the most important enzymes involved in the biodegradation of lignin.[1] LiP is also able to catalyse the oxidation of phenolic and non-phenolic electron-rich aromatic lignin model compounds[1c,1d,2] as well as the oxidation of different classes of easily oxidisable organic compounds.[3]

Recently, we have reported on the ability of LiP to catalyse the oxidative *N*-demethylation of *N,N*-dimethylanilines,[4] a process of great biological importance. For this reaction it has been suggested an initial electron transfer (ET) between the *N,N*-dimethylaniline and the active species of the enzyme (compound I or LiP I),[5] formed by oxidation of the native enzyme with H_2O_2 and described as an iron(IV)-oxo porphyrin radical cation (Scheme 1, path *a*). Such an ET leads to an anilinium radical cation and to the reduced form of LiP I (compound II or LiP II).[5] The anilinium radical cation should then undergo deprotonation to give an α-amino carbon radical,[6] this reaction being promoted by LiP II or whichever base is present in the medium. In the former case the carbon radical may be converted into a carbinolamine by oxygen rebound (path *d*). In the second case the carbinolamine may be formed by oxidation of the carbocation followed by reaction with H_2O (path *e*). The carbinolamine is finally converted into the *N*-demethylated product and CH_2O (path *f*).[9]



Scheme 1. Electron transfer mechanism for the oxidative *N*-demethylation of *N,N*-dimethylanilines catalysed by LiP

Concerning the basic centre responsible for the anilinium radical cation deprotonation, strong evidence in favour of an enzyme-promoted process (Scheme 1, path *b* or *c*) was recently provided by the complete masking [k_H/k_D = 1.04 (± 0.06)] of the intramolecular kinetic deuterium isotope effect (KDIE) observed for the LiP-catalysed *N*-demethylation of 2,4,6-trichloro-*N*-methyl-*N*-trideuteriomethylaniline.[4] However, the actual nature of the proton-abstracting base remained uncertain.

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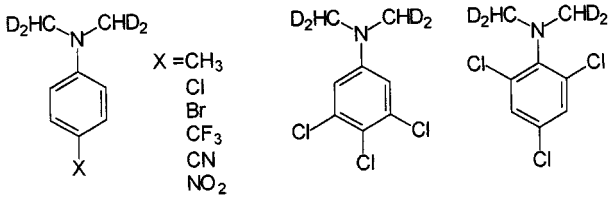
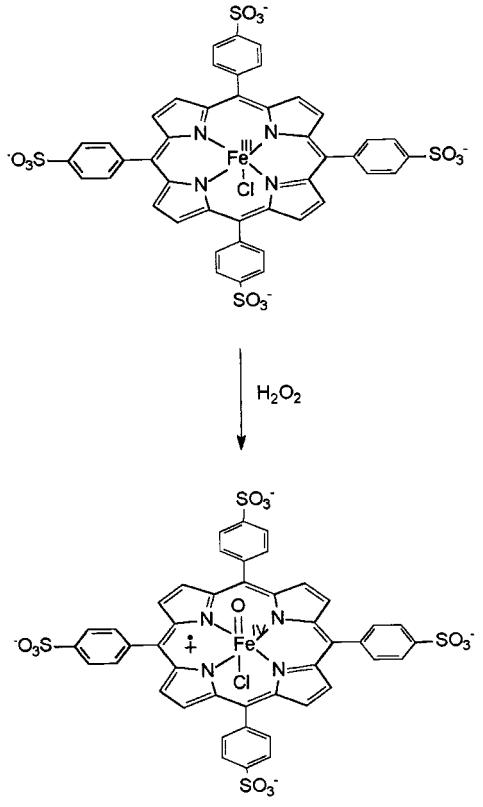
In order to acquire more information in this respect an intramolecular KDIE study of the oxidative *N*-demethylation of ring substituted *N,N*-dimethylanilines catalysed by LiP has been undertaken. The idea was that the KDIE dependence on the radical cation pK_a might give useful information on the strength and the nature of the deprotonating base. As substrates, the *N,N*-bis(dideuteriomethyl)anilines reported in Scheme 2 were used. With these substrates, masking effects should be minimised since H and D are bonded to the same carbon atom.



Scheme 2. Ring substituted *N,N*-bis(dideuteriomethyl)anilines used in this work

The results of the LiP-catalysed *N*-demethylation reactions have also been compared with those obtained using a chemical model compound: FeTPPSCl [5,10,15,20-tetraphenyl-21*H*,23*H*-porphine-*p*,*p*′,*p*″,*p*‴-tetrasulfonic acid iron(III) chloride]. Water-soluble metalloporphyrins like FeTPPSCl are known to mimic LiP reactivity,[10] since the active species, formed by reaction with a suitable oxygen donor (e.g. H_2O_2), can be described as an iron(IV)-oxo porphyrin radical cation and closely resemble the active species of LiP (LiP I) (Scheme 3).



Scheme 3. Formation of the active oxidant by reaction of FeTPPS with H_2O_2

Results

N,N-dimethylanilines (10 μmol) were reacted in the presence of LiP (0.96 units, 1.16 nmol) or FeTPPSCl (0.3 μmol) with an equimolar amount of hydrogen peroxide added, over a period of 1 h, by an infusion pump, to an argon-degassed 50 mM sodium-tartrate-buffered solution, pH = 4.0, containing 2% of CH_3CN as the cosolvent, at 25 °C. A clean *N*-demethylation reaction occurred with formation of the corresponding *N*-methylaniline and formaldehyde, which was detected by GC-MS after conversion into the dimedone adduct. Reaction products were characterised by GC-MS and ^{1}H NMR spectroscopy. Yields were determined by GC and ^{1}H NMR spectroscopy and were referred to the starting material. The results are reported in Table 1 where the reduction potentials of the $ArN^{+\cdot}(CH_3)_2$/$ArN(CH_3)_2$ couple are also displayed. Some experiments with FeTPPSCl were also carried out in the presence of imidazole, but the results were practically the same as in its absence.

Table 1. Yields of *N*-demethylated products ($ArNHCH_3$) in the LiP or FeTPPSCl catalysed oxidation of ring substituted *N,N*-dimethylanilines [$ArN(CH_3)_2$] by H_2O_2 and reduction potentials of the $ArN^{+\cdot}(CH_3)_2/ArN(CH_3)_2$ couple

Ar	$E°$ vs. NHE (V)[a]	Yields (%)[b] LiP[c]	FeTPPSCl
2,4,6-$Cl_3C_6H_2$	1.58[c]	25	7
4-$NO_2C_6H_4$	1.46	20	11
3,4,5-$Cl_3C_6H_2$	1.40[c]	27	5
4-CNC_6H_4	1.37	62	30
4-$CF_3C_6H_4$	1.33[d]	28[e]	23
4-BrC_6H_4	1.15[d]	16	3
4-ClC_6H_4	1.11	n.d.	12
4-$CH_3C_6H_4$	0.94	–	10

[a] Values estimated in water (ref.[11]) from the corresponding values in CH_3CN (ref.[12]). – [b] Referred to the starting material, equimolar to H_2O_2. Average of at least two determinations, the error is in all cases less than ± 1. – [c] From ref.[4] – [d] Value estimated from a Hammett correlation. – [e] A small amount of 4-trifluoromethylaniline (5%) was also detected.

KDIE values were determined by GC-MS analysis of the formaldehyde-dimedone adduct by the ratio of the intensity of the molecular peaks m/z = 294 and 293, corrected for a statistical factor and the ^{13}C contribution. The KDIE values are reported in Table 2, together with the pK_a of the *N,N*-dimethylaniline radical cations, which were estimated by the usual thermochemical cycle.[13]

Discussion

With both the enzymatic and the biomimetic systems the yields of the *N*-demethylated product do not follow any regular trend upon increasing the electron-donating properties of the ring substituent, probably due to the variable

Table 2. KDIE values for LiP or FeTPPSCl catalysed oxidative *N*-demethylation of ring substituted *N,N*-bis(dideuteriomethyl) anilines [$ArN(CHD_2)_2$] by H_2O_2

Ar	KDIE[a]		pK_a radical cation[b]
	LiP	FeTPPSCl	
2,4,6-$Cl_3C_6H_2$	3.4 (1)	3.4 (1)	2.2
4-$NO_2C_6H_4$	5.3 (2)	5.3 (2)	4.1
3,4,5-$Cl_3C_6H_2$	5.6 (1)	5.5 (1)	5.2
4-CNC_6H_4	5.9 (1)	5.7 (2)	5.7
4-$CF_3C_6H_4$	5.8 (1)[c]	5.6 (2)	6.4
4-BrC_6H_4	5.3 (1)	5.7 (2)	9.4
4-ClC_6H_4	5.1 (2)	5.1 (1)	10.1
4-$CH_3C_6H_4$	n.d.	4.0 (2)	13.0

[a] Average of at least four determinations. The error (standard deviation) in the last significant digit is given in parentheses. – [b] In water. – [c] A lower amount of LiP (0.64 units, 0.77 nmol) was used to avoid the formation of 4-trifluoromethylaniline.

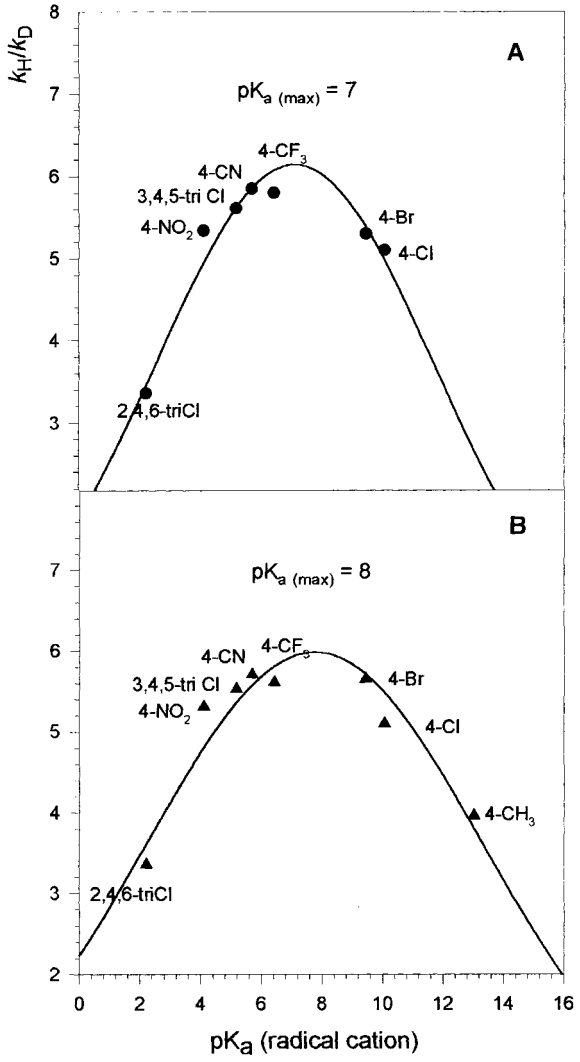


Figure 1. Bell-shaped curve for the LiP (A) and FeTPPSCl (B) catalysed oxidative *N*-demethylation of ring substituted *N,N*-bis(dideuteriomethyl)anilines

extent of substrate protonation. In this respect, it should be noted that 4-methyl-*N,N*-dimethylaniline reacted only with FeTPPSCl. LiP I is probably a weaker oxidant than the active species of FeTPPSCl[17] and is not able to promote the oxidation of this substrate, which is almost completely protonated at the low pH required for the enzymatic catalysis.[18]

Examining the data reported in Table 2, a very remarkable observation is that the KDIE values are very similar for both the enzymatic and the biomimetic systems. Moreover, increasing the electron-donating properties of the ring substituents (and the pK_a of the *N,N*-dimethylaniline radical cations) the KDIE values increase up to a maximum value and then decrease for both systems. Thus, a plot of the KDIE values as a function of the pK_a of the substituted *N,N*-dimethylaniline radical cations presents the bell-shaped profile depicted in Figure 1 for the enzymatic (Figure 1, A) and biomimetic (Figure 1, B) systems. The solid curves reported are the theoretical ones obtained from Equation (1), [where $\Delta pK_a = pK_a - pK_{a\ (max)}$] based on the Marcus theory as applied to acid-base reactions.[19]

$$\lg\left(\frac{k_H}{k_D}\right) = \left[1 - \frac{(2.3RT\Delta pK_a)^2}{\lambda_H\lambda_D}\right]\lg\left(\frac{k_H}{k_D}\right)_{max} \quad (1)$$

This result is fully consistent with the proposed ET mechanism (Scheme 1), with the KDIE being determined in the step involving the dimethylaniline radical cation deprotonation (Scheme 1, path *b*, or *c*). A hydrogen-atom transfer (HAT) mechanism appears unlikely since the strength of the NCH_2-H bond in the neutral aniline is almost insensitive to the presence of ring substituents[14] and the same should hold for the KDIE values. Accordingly, only a small, steady decrease of the KDIE values was observed upon decreasing the electron-withdrawing properties of the ring substituents in the oxidative *N*-demethylation of *N,N*-dimethylanilines promoted by cytochrome P450, a reaction suggested to occur by an HAT mechanism.[20]

The pK_a values corresponding to the maximum of the profiles reported in Figure 1 are 7 and 8. According to the theory, the maximum of the bell-shaped curve should be reached when the pK_a of the acid equals that of the deprotonating base.[19,21] It follows that bases of very similar strength act in both the enzymatic and biomimetic reactions. Thus, unless we are dealing with an extraordinary coincidence, the most simple conclusion is that the same deprotonating base, with a pK_a value around 7, is operating in the two reactions. In this situation, the most reasonable suggestion is that the deprotonation of the intermediate radical cation is promoted by the reduced iron-oxo complex (LiP II). Very interestingly, a pK_a greater than that of pyridine (and therefore >5, in water) has already been proposed for the $P-Fe^{IV}=O$ species on the basis of a study of the *N*-demethylation of 9-*tert*-butyl-*N*-methylacridane catalysed by an iron porphyrin.[22,23]

This suggestion, if correct, would be very interesting since it would seem to contrast the current views about the

restricted accessibility of the heme in LiP,[25] thereby the substrate should have access only to the heme edge, as very crudely shown in Figure 2, where the electron-transfer process can still take place, but not the deprotonation of the radical cation by the ferryl oxygen. Accordingly, the possibility that compound II would act as a base in the *N*-demethylation reactions was suggested for CPO and cytochrome P450, but not for HRP, whose access to the heme is much more restricted than in the other two enzymes and similar to that in LiP.[9] However, the structure depicted in Figure 2 might not be rigid and it cannot be excluded that the substrate radical cation may move to put the α-hydrogen close enough to the oxygen of the reduced form of the iron-oxo complex, as suggested by the present results.[26] On the other hand, it should be remembered that LiP (and HRP) is able to effect sulfoxidation with the oxygen-transfer process occurring from the oxoferryl species of the enzyme.[27]

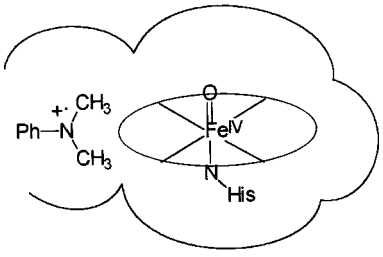


Figure 2. Rough visualisation of the heme-edge approach of *N,N*-dimethylaniline in the LiP active site

Finally, it should be noted that the hypothesis of a base with pK_a 7−8 operating at pH = 4 may be reasonable only if this base is a transient species, as is the case with P−Fe^{IV}=O, which should form in close association with the radical cation.

Conclusion

LiP and its model compound FeTPPSCl catalyse the oxidative *N*-demethylation of ring substituted *N,N*-dimethylanilines by an ET mechanism leading to the formation of *N*-methylanilines and formaldehyde. The intermediate anilinium radical cation thus formed is then deprotonated by the enzyme. Bell-shaped curves are obtained in the KDIE profiles (plot of k_H/k_D vs. the pK_a of the radical cations) both for the enzymatic and biomimetic oxidations. From these curves, pK_a values of 7 (enzymatic reaction) and 8 (biomimetic reaction) have been estimated for the proton-abstracting base. The close similarity of the two cases is a strong indication that the radical cation is deprotonated by the same species in the enzymatic and in the chemical reactions. It is therefore most likely that this species is the reduced iron-oxo complex.

Experimental Section

General: ^{1}H NMR spectra were recorded on a Bruker AC300P spectrometer in $CDCl_3$ using TMS as the internal standard. GC-MS analyses were performed on an HP5890 GC (OV1 capillary column, 12 m × 0.2 mm) coupled with an HP5970 MSD. GC analyses were performed on a Varian 3400 GC (OV1 capillary column, 25 m × 0.2 mm) and Varian Vista 6000 (OV1701 capillary column, 30 m × 0.35 mm).

Bi-distilled water and CH_3CN (CARLO ERBA-HPLC grade) were used as solvents. High purity commercial samples of tartaric acid, 4-methoxyacetophenone and dimedone (Aldrich) were used as received. LiP was prepared and purified as described in the literature.[28] The concentration of the enzyme solution was determined spectrophotometrically (ε_{409nm} = 169 mM^{-1} cm^{-1}).[29] The concentration of H_2O_2 (Carlo Erba Reagents) was determined by titration with permanganate.[30] 4-Chloro-*N,N*-bis(dideuteriomethyl)aniline, 4-cyano-*N,N*-bis(dideuteriomethyl)aniline and 4-nitro-*N,N*-bis(dideuteriomethyl)aniline were prepared according to the literature procedure.[20] 5,10,15,20-Tetraphenyl-21*H*,23*H*-porphine-*p,p′,p″,p‴*-tetrasulfonic acid tetrasodium salt dodecahydrate (Aldrich) was metallated according to the literature procedure.[31]

General Procedures for Substrate Preparation: The ring-substituted *N,N*-bis(dideuteriomethyl)anilines were prepared by reaction of the corresponding anilines with [D_2]paraformaldehyde and sodium borohydride according to the literature procedure.[20] A Schlenk tube charged with [D_2]paraformaldehyde (Aldrich, 99%, 7.5 mmol) was heated at 200 °C under a stream of argon and the evolved gas collected in sulfuric acid (3 M, 12 mL). The formaldehyde/sulfuric acid solution was cooled in an ice-water bath and a slurry of aniline (2.5 mmol) and sodium borohydride (20 mmol) in tetrahydrofuran (30 mL) was added dropwise over a period of 10 min. The reaction mixture was allowed to warm to room temperature and stirred for 40 min. After recooling in an ice-water bath, the reaction mixture was made basic by dropwise addition of a sodium hydroxide solution. The organic layer was separated and the aqueous solution was extracted with diethyl ether. The combined organic layers were washed with water and then brine and dried over anhydrous Na_2SO_4. Each compound was purified by chromatography on silica gel. The purity of all these compounds (>99%) was checked by GC, GC-MS and ^{1}H NMR spectroscopy. The isotopic composition of the substituted *N,N*-bis(dideuteriomethyl)anilines was determined by GC-MS in the selected ion monitoring mode from the D_4/D_0 ratios. In all cases the percentage of the deuterated product was >98%.

3,4,5-Trichloro-*N,N*-bis(dideuteriomethyl)aniline: This material was prepared using the procedure described above. Purification on silica gel with petroleum ether/diethyl ether (9:1) as the eluent gave, after solvent removal, a pale yellow solid (70%). ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 6.67 (s, 2 H), 2.88−2.92 (m, 2 H).

2,4,6-Trichloro-*N,N*-bis(dideuteriomethyl)aniline: This material was prepared using the procedure described above. Purification on silica gel with petroleum ether as the eluent gave, after solvent removal, a colourless liquid (54%). ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 7.26 (s, 2 H), 2.81−2.85 (m, 2 H).

***N,N*-Bis(dideuteriomethyl)-4-methylaniline:** This material was prepared using the procedure described above. Purification on silica gel using *n*-pentane/chloroform (5:1) as the eluent gave, after solvent removal, a colourless liquid (70%). ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 7.07 (d, *J* = 8.2 Hz, 2 H), 6.74 (d, *J* = 8.3 Hz, 2 H), 2.87 (m, 2 H), 2.26 (s, 3 H).

***N,N*-Bis(dideuteriomethyl)-4-trifluoromethylaniline:** This material was prepared using the procedure described above. Purification on silica gel using petroleum ether as the eluent gave, after solvent

removal, a white solid (75%). ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 7.45 (d, *J* = 8.6 Hz, 2 H), 6.70 (d, *J* = 8.6 Hz, 2 H), 2.97 (m, 2 H).

4-Bromo-*N,N*-bis(dideuteriomethyl)aniline: This material was prepared using the procedure described above. Purification on silica gel using petroleum ether/diethyl ether (4:1) as the eluent gave, after solvent removal, a white solid (72%). ^{1}H NMR ($CDCl_3$, 25 °C, TMS): δ = 7.30 (d, *J* = 9.0 Hz, 2 H), 6.58 (d, *J* = 9.0 Hz, 2 H), 2.88 (m, 2 H).

Enzymatic or Biomimetic Oxidation: H_2O_2 (10 μmol) was added, over a period of 1 h by an infusion pump, to a magnetically stirred argon-degassed solution of the substrate (10 μmol), LiP (0.96 units, 1.16 nmol) or FeTPPSCl (0.3 μmol) in 3 mL of 50 mM sodium-tartrate-buffered solution with 2% acetonitrile as cosolvent, pH = 4, at 25 °C. At the end of the reaction the mixture was made basic, the products of the reaction were extracted with CH_2Cl_2 and dried over Na_2SO_4. In the LiP-catalysed oxidation of 4-trifluoromethyl-*N,N*-bis(dideuteriomethyl)aniline a lower amount of LiP (0.64 units, 0.77 nmol) was used to avoid the formation of 4-trifluoromethylaniline.

Product Analysis: Yields were determined by GC and ^{1}H NMR spectroscopy (with 4-methoxyacetophenone as the internal standard) and referred to the starting material. A good material balance (>90%) was observed in all the experiments.

KDIEs Measurement: At the end of the enzymatic or biomimetic oxidation the reaction mixture was treated with 400 μL of a basic solution of dimedone (0.2 M) in order to allow the formation of the dimedone-formaldehyde adduct. After 30 minutes the mixture was extracted with CH_2Cl_2. All the KDIE values, averaged over at least four independent determinations, were determined by GC-MS analysis of the formaldehyde-dimedone adduct by the ratio of the intensity of the molecular peaks *m/z* = 294 and 293, corrected for the statistical factor and for the ^{13}C contribution, in the oxidation of *N,N*-bis(dideuteriomethyl)anilines.

Acknowledgments

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[6] The possibility that the radical cation reacts with P−Fe^{IV}=O by a HAT mechanism has also been considered at least for the biomimetic process.[7] However, the results reported by Lindsay-Smith seem clearly in favour of a deprotonation step.[8] Moreover, the HAT mechanism is unlikely also on energetic grounds, the homolytic C−H BDE free energy (180 kJ mol^{-1}, in MeCN) being much higher than the heterolytic one (70 kJ mol^{-1}).

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[13] The pK_a of the *N,N*-dimethylaniline radical cations were estimated by the following equation: pK_a = (2.3 RT)$^{-1}$ × [$\Delta G°_{hom}$ − 96.48 $E°_{ArN+\cdot(CH3)2/ArN(CH3)2}$ + 96.48 $E°_{H+/H\cdot}$]where $\Delta G°_{hom}$ is the standard free energy associated with the homolytic cleavage of the NCH_2−H bond, taken as 346 kJ mol^{-1} (at 25 °C), resulting from the difference between $\Delta H°_{hom}$ (380 kJ mol^{-1}), an average value for the NCH_2−H homolytic bond dissociation enthalpy for a number of *N,N*-dimethylanilines,[14] and $T\Delta S°_{hom}$, where $\Delta S°$ = 115 J K^{-1} is the standard entropy of the hydrogen atom.[15] $E°_{ArN+\cdot(CH3)2/ArN(CH3)2}$ is the standard redox potential (V vs. NHE in H_2O) of the couple $ArN^{+\cdot}(CH_3)_2/ArN(CH_3)_2$ (Table 1). $E°_{H+/H\cdot}$, the standard redox potential of the couple $H^+/H^\cdot$, is taken as −2.29 V vs. NHE in H_2O.[16]

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