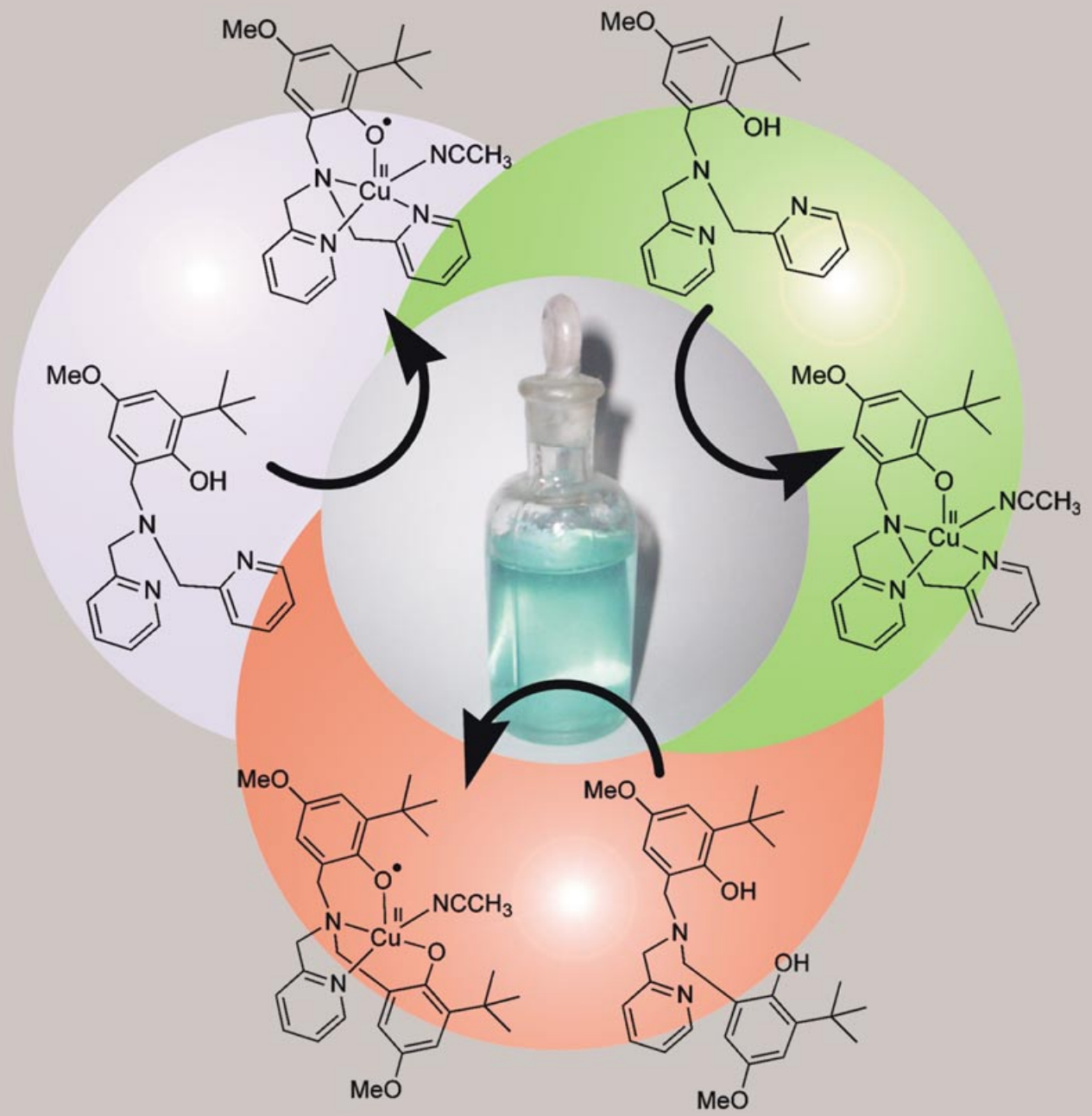
# Galactose Oxidase Models




For more information see the following pages.

 **DOI: 10.1002/chem.200400099**  

# Galactose Oxidase Models: Solution Chemistry, and Phenoxyl Radical Generation Mediated by the Copper Status

**Fabien Michel,[a] Fabrice Thomas,*[a] Sylvain Hamman,[a] Eric Saint-Aman,[b] Christophe Bucher,[b] and Jean-Louis Pierre[a]**

**Abstract:** Galactose oxidase (GO) is an enzyme that catalyzes two-electron oxidations. Its active site contains a copper atom coordinated to a tyrosyl radical, the biogenesis of which requires copper and dioxygen. We have recently studied the properties of electrochemically generated mononuclear $Cu^{II}$-phenoxyl radical systems as model compounds of GO. We present here the solution chemistry of these ligands under various copper and dioxygen statuses: $N_3O$ ligands first chelate $Cu^{II}$, leading, in the presence of base, to $[Cu^{II}(ligand)(CH_3CN)]^+$ complexes (*ortho-tert*-butylated ligands) or $[(Cu^{II})_2(ligand)_2]^{2+}$ complexes (*ortho*-methoxylated ligands). Excess copper(II) then oxidizes the complex to the corresponding mononuclear $Cu^{II}$-phenoxyl radical species. $N_2O_2$ tripodal ligands, in the presence of copper(II), afford directly a copper(II)-phenoxyl radical species. Addition of more than two molar equivalents of copper(II) affords a $Cu^{II}$–bis(phenoxyl) diradical species. The donor set of the ligand directs the reaction towards comproportionation for ligands possessing an $N_3O$ donor set, while disproportionation is observed for ligands possessing an $N_2O_2$ donor set. These results are discussed in the light of recent results concerning the self-processing of GO. A path involving copper(II) disproportionation is proposed for oxidation of the cross-linked tyrosinate of GO, supporting the fact that both copper(I) and copper(II) activate the enzyme.

**Keywords:** copper · enzyme models · galactose oxidase · phenoxyl radicals · tripodal ligands

## Introduction

Galactose oxidase (GO) is an extracellular copper-containing enzyme that catalyzes the oxidation of primary alcohols to their corresponding aldehydes with concomitant reduction of molecular dioxygen to hydrogen peroxide. The apparent paradox, a two-electron oxidation catalyzed by a single copper atom, is explained by the presence of an additional redox center, namely a tyrosyl radical from the protein chain, coordinated to the metal.[1] An unusual feature is the cross-linking between this residue and a neighboring cysteine, presumably to modulate its redox properties.[2] Prior to the work of Rogers et al.,[3a,b] Firbank et al.,[3c] and Whittaker et al.,[4] very little was known about this post-translational modification and the oxidation of the tyrosine residue into the tyrosyl radical. It has recently been shown that in vitro processing of GO results from its reaction with copper and molecular oxygen.[3] Three mechanisms have been proposed. The first involves an initial tyrosine-to-copper(II) electron transfer (i.e., the copper is coordinated and initiates the self-processing) and $O_2$ is required to achieve the cross-linking. An alternative second mechanism involves an initial oxidation of Cys-228 to a sulfenate group in the presence of $O_2$ and free copper; the S atom, which is more electrophilic, is subject to a nucleophilic attack by the tyrosinate and the copper(II) atom is then incorporated. Both of these mechanisms afford a cross-linked tyrosinate-$Cu^{II}$ intermediate that is proposed to be oxidized by $O_2$ to the active tyrosyl-$Cu^{II}$ form.[3] In the third pathway, copper(I) is chelated by the unprocessed tyrosinate and then $O_2$ induces a two-electron oxidation, affording the $Cu^{I}$-cross-linked tyrosinate; an additional two-electron oxidation leads to the mature $Cu^{II}$-cross-linked tyrosinyl form.[4]

Several model compounds for the active site of GO have been described in the last decade.[5] We have recently studied the electrochemically generated mononuclear $Cu^{II}$-phenoxyl radical complexes of *ortho-tert*-butylated $N_2O_2$ and $N_3O$ ligands (Figure 1a,b) that appeared to be valuable structural and functional model compounds of GO.[6,7] In

[a] F. Michel, Dr. F. Thomas, Dr. S. Hamman, Prof. Dr. J.-L. Pierre
Laboratoire de Chimie Biomimétique
LEDSS, UMR CNRS 5616, Université J. Fourier, BP 53
38041 Grenoble Cedex 9 (France)
Fax: (+33) 4 76 51 48 36
E-mail: Fabrice.Thomas@ujf-grenoble.fr

[b] Prof. Dr. E. Saint-Aman, Dr. C. Bucher
LEOPR, UMR CNRS 5630, Université J. Fourier, BP 53
38041 Grenoble Cedex 9 (France)

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.



DOI: 10.1002/chem.200400099

order to better understand the way in which copper and dioxygen are implied in GO activation, we present herein the solution chemistry of these ligands under various copper and dioxygen statuses. The study is extended to *ortho*-methoxylated $N_3O$ ligands (Figure 1c), in which the *ortho* group does not provide enough steric hindrance around the chelated copper atom to prevent the formation of phenolato bridges leading to dinuclear complexes.

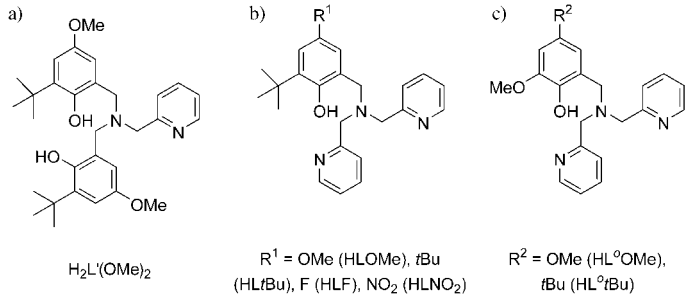



Figure 1. Ligands used in this work: a) $H_2L'(OMe)_2$, b) HLOMe ($R^1$ = OMe), HL*t*Bu ($R^1$ = *t*Bu), HLF ($R^1$ = F), $HLNO_2$ ($R^1$ = $NO_2$), c) HL$^o$OMe ($R^2$ = OMe), HL$^o$*t*Bu ($R^2$ = *t*Bu).

## Results and Discussion

**Preparation of the ligands**: The ligand denoted L′ possesses two *ortho-tert*-butyl-phenol moieties and one pyridine moiety; that denoted L possesses one *ortho-tert*-butyl phenol and two pyridine moieties, and that denoted L$^o$ possesses one *ortho*-methoxyphenol and two pyridine moieties. Synthesis of the ligands HLOMe, HL*t*Bu, HLF, $HLNO_2$, and $H_2L'(OMe)_2$ has been described previously.[7,8] HL$^o$OMe was obtained by way of a one-pot Mannich synthesis by mixing bis(2-pyridylmethyl)amine and 2,4-dimethoxyphenol in the presence of one molar equivalent of formaldehyde. Alkylation of bis(2-pyridylmethyl)amine by 2-hydroxy-3-methoxy-5-*tert*-butylbenzaldehyde was the preferred method for obtaining HL$^o$*t*Bu.

**Solution chemistry of the $N_3O$ ligands (up to one molar equivalent of copper(II) added)—Copper complexation**: When one molar equivalent of copper(II) triflate is added to a solution of HLOMe in acetonitrile, an absorption band at 600 nm, corresponding to a d-d transition, is observed in the visible spectrum (Figure 2a), and EPR reveals the presence of a mononuclear copper(II) complex. These spectroscopic features closely resemble those reported for the isolated $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ complex bearing an axially coordinated phenol and an equatorially coordinated exogenous acetonitrile ($\lambda_{max}$ = 590 nm, $\varepsilon$ = 230 $M^{-1}cm^{-1}$).[7] Likewise, the addition of one molar equivalent of copper(II) to HL*t*Bu, HLF, and $HLNO_2$ affords $[Cu^{II}(HLtBu)(CH_3CN)]^{2+}$, $[Cu^{II}(HLF)(CH_3CN)]^{2+}$, and $[Cu^{II}(HLNO_2)(CH_3CN)]^{2+}$, respectively (Table 1).

However, closer examination of the complexation process shows that the formation of these protonated copper(II)

Table 1. Electronic and EPR properties of the copper(II) complexes in acetonitrile solution.

| Complexes | Spin state | $\lambda_{max}$ [nm]/$\varepsilon$ [$M^{-1}cm^{-1}$] |
|---|---|---|
| | HLOMe | |
| +1 equiv copper(II) | ($S = 1/2$) | 600 (110)[a] |
| +0.5 equiv copper(II) | ($S = 1/2$) | 586 (460)[b] |
| +1 equiv copper(II) and $NEt_3$ | ($S = 1/2$) | 579 (1190)[a] |
| +2 equiv copper(II) and 1 equiv of $NEt_3$ | ($S = 1$) | 426 (4300), 552 (470)[a] |
| | HL*t*Bu | |
| +1 equiv copper(II) | ($S = 1/2$) | 590 (170)[a] |
| +0.5 equiv copper(II) | ($S = 1/2$) | 560 (540), 960 (120)[b] |
| +1 equiv copper(II) and $NEt_3$ | ($S = 1/2$) | 550 (1220), 900 (200)[a] |
| +2 equiv copper(II) and 1 equiv of $NEt_3$ | ($S = 1$) | 416 (1900), 650 (500)[a] |
| | HLF | |
| +1 equiv copper(II) | ($S = 1/2$) | 580 (120)[a] |
| +0.5 equiv copper(II) | ($S = 1/2$) | 535 (665), 940 (90)[b] |
| +1 equiv copper(II) and $NEt_3$ | ($S = 1/2$) | 523 (1080)[a] |
| | $HLNO_2$ | |
| +1 equiv copper(II) | ($S = 1/2$) | 608 (100)[a] |
| +0.5 equiv copper(II) | ($S = 1/2$) | 517 (740)[b] |
| +1 equiv copper(II) and $NEt_3$ | ($S = 1/2$) | 515 (600)[a] |
| | $H_2L'(OMe)_2$ | |
| +2 equiv copper(II) and 2 equiv $NEt_3$ | ($S = 0$) | 416 (3370), 530 (1510), 850 (740)[a] |
| +3 equiv copper(II) and 2 equiv $NEt_3$ | ($S = 1/2$) | 426 (3600), 650 (540)[a] |
| | HL$^o$OMe | |
| +1 equiv copper(II) | ($S = 1/2$) | 590 (85)[a] |
| +0.5 equiv copper(II) | ($S = 1$) | 470 (220), 870 (120)[b] |
| +1 equiv copper(II) and $NEt_3$ | ($S = 1/2$) | 470 (230)[a] |
| +2 equiv copper(II) and 1 equiv $NEt_3$ | n.d. | 418 (2300), 605 (400)[a] |
| | HL$^o$*t*Bu | |
| +1 equiv copper(II) | ($S = 1/2$) | 610 (170)[a] |
| +0.5 equiv copper(II) | ($S = 1$) | 466 (560), 870 (380)[b] |
| +1 equiv copper(II) and $NEt_3$ | ($S = 1$) | 462 (500), 870 (300)[a] |
| +2 equiv copper(II) and 1 equiv $NEt_3$ | n.d. | 420 (3200), 620 (350)[a] |

[a] Based on ligand concentration. [b] Based on copper(II) concentration.

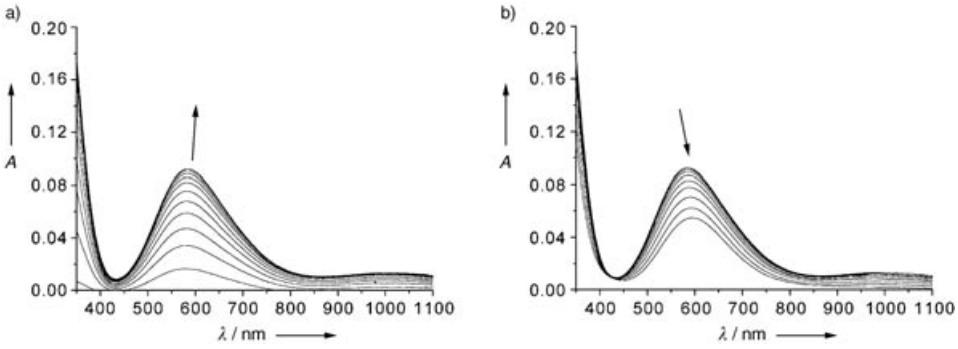
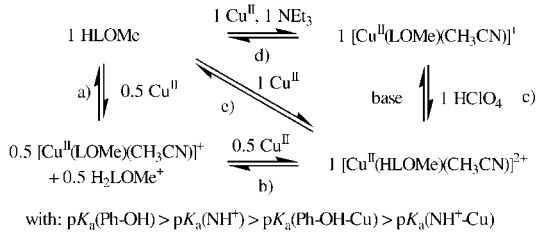



Figure 2. Titration of HLOMe (0.4 mM) with copper(II) triflate: a) 0 to 0.5 molar equivalents of copper(II) and b) 0.5 to 1 molar equivalents of copper(II). Arrows indicate spectral changes upon addition of copper; spectra recorded in $CH_3CN$ at 298 K.

complexes is more complicated than a simple reaction between the ligands and copper(II). From 0 to 0.5 molar equivalents added, the solution turns from colorless to purple and a linear increase in absorption is observed at 586 nm (for HLOMe, Figure 2a), 560 nm (for HL*t*Bu), 535 nm (for HLF), and 517 nm (for $HLNO_2$). The $\lambda_{max}$ is shifted according to the electronic properties of the *para* substituent (the lower the electron-donating character of the group, the lower the $\lambda_{max}$), indicating that this transition arises from an LMCT (Table 1). These results suggest that once the copper is chelated by part of the available amount of ligand, the remaining free ligand acts as a base and deprotonates the weakly coordinating phenol (Scheme 1, path a). Owing to




Scheme 1. Solution chemistry of HLOMe (the same behavior was observed with HL*t*Bu, HLF, and $HLNO_2$); the base may be either $NEt_3$ or the ligand. Path e has been described previously.[7]

the higher p$K_a$ values of tertiary amines relative to pyridines, the amine is proposed to play the acid-base role. The intensity of the 586 nm absorption of a solution of HLOMe in acetonitrile with 0.5 molar equivalents of copper(II) added is intermediate between that of a solution of isolated $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ and that of $[Cu^{II}(LOMe)(CH_3CN)]^+$ (Table 1). The former solution thus comprises a mixture of the phenol-copper $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ and the phenolate-copper $[Cu^{II}(LOMe)(CH_3CN)]^+$ complexes. The p$K_a$ of the coordinating methoxyphenol ($7.31 \pm 0.04$, see Supporting Information) is thus close to that of the free tertiary amine. In contrast, the intensity of the 535 nm absorption of a solution of HLF in acetonitrile with 0.5 molar equivalents of copper(II) added is close to that of a solution of $[Cu^{II}(LF)(CH_3CN)]^+$. This higher ratio of deprotonated to protonated complexes is consistent with the lower p$K_a$ of the coordinating fluorophenol ($6.66 \pm 0.02$, see Supporting Information).

From 0.5 to one molar equivalent of copper(II) added to HLOMe, the purple solution turns deep blue (the same behavior is observed for all the ligands, thus only the results obtained with HLOMe are described in detail here). The spectrum of the deprotonated complex (characterized by its LMCT transition) vanishes and is progressively replaced by that of the protonated complex (characterized by d–d transitions at 600 nm, Table 1). Complexation of copper(II) by the protonated ligand $H_2L^+$ induces deprotonation of its tertiary ammonium group. The proton is transferred to the coordinating phenolate of $[Cu^{II}(LOMe)(CH_3CN)]^+$. Excess ligand thus acts as a base in path a of Scheme 1 and as an acid in path b. To confirm these results, solutions of the ligands HLOMe, HL*t*Bu, HLF, and $HLNO_2$ in acetonitrile were titrated with copper(II) in the presence of one molar equivalent of triethylamine as an exogenous base. The deprotonated complex was formed directly, as reflected by a linear increase in the intensity of the LMCT band from 0 to one molar equivalent of copper(II) added (Scheme 1, path d, Figure 3a). Its formation is quantitative at one molar equivalent of copper(II) added (as judged by the $\varepsilon$ value of $1190\ M^{-1}cm^{-1}$ at $\lambda_{max} = 579$ nm). Similarly, addition of one molar equivalent of HLOMe or $NEt_3$ to $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ induces a hypsochromic shift of $\lambda_{max}$ and an increase in the intensity of the absorption, consistent with deprotonation of the weakly coordinating phenol (Scheme 1, path c). The solution chemistry of these $N_3O$ ligands is summarized in Scheme 1.

The UV/Vis profiles for titration of each ligand with zinc(II) triflate instead of copper(II) triflate show an isobestic

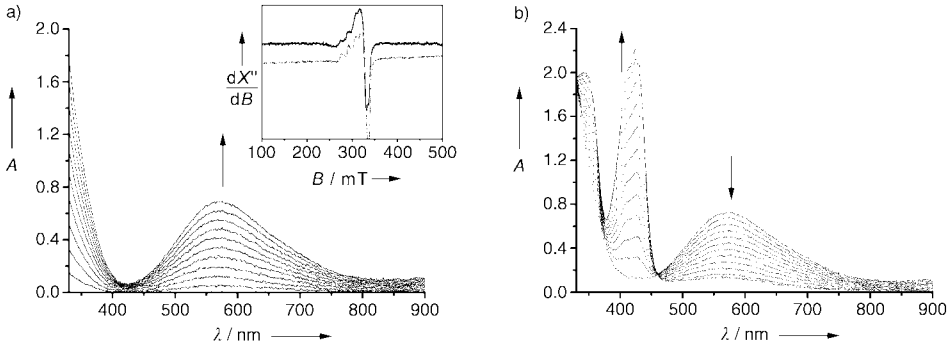



Figure 3. Titration of HLOMe (0.55 mM) with copper(II) triflate in the presence of 0.55 mM triethylamine: a) 0 to 1 molar equivalent of copper(II) and b) 1 to 2 molar equivalents of copper(II) added. Arrows indicate spectral changes upon addition of copper; spectra recorded in $CH_3CN$ at 233 K in order to avoid degradation of the radical species (see text); inset: X-band EPR spectra recorded at 100 K: bold line: 1 mM solution of HLOMe with one molar equivalent of copper(II) added in the presence of $NEt_3$; gray line: 1 mM solution of the isolated $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ complex in $CH_3CN$ in the presence of one molar equivalent of $NEt_3$.

point between 0 and 0.5 molar equivalents of zinc(II) added, followed by a decrease in the absorbance at 299 nm (HLOMe, see Supporting Information), 283 nm (HL*t*Bu), or 288 nm (HLF) from 0.5 to 1 molar equivalent. As for copper(II) complexation, a first equilibrium involves the formation of a deprotonated zinc complex followed by its conversion into a protonated complex.

In contrast to the *ortho tert*-butyl group of the $N_3O$ ligands, the *ortho* methoxy group of HL$^o$*t*Bu or HL$^o$OMe does not provide sufficient steric bulk to prevent the formation of a dimeric structure in which two phenoxo oxygen atoms bridge the two copper ions (see Supporting Information). The titration profiles of HL$^o$*t*Bu and HL$^o$OMe with copper(II) are similar.

When one molar equivalent of copper(II) triflate is added to a solution of each ligand in acetonitrile (in the absence of triethylamine), an absorption band at around 600 nm, corresponding to d–d transitions, is observed in the visible spectra (Table 1). EPR measurements reveal the presence of a mononuclear copper(II) complex. This suggests the formation of the mononuclear $[Cu^{II}(HL^{o}OMe)(CH_3CN)]^{2+}$ and $[Cu^{II}(HL^{o}tBu)(CH_3CN)]^{2+}$ complexes, in which the phenol remains protonated and weakly coordinated to the copper(II). The titration of a solution of the ligand in acetonitrile with 0 to one molar equivalent of copper(II) triflate shows that the formation of these complexes, as monitored by the increase in the intensity of the d–d transition band, is nonlinear. As for the *ortho-tert*-butylated ligands, an LMCT transition band appears in the UV/Vis spectra up to 0.5 molar equivalents of copper(II) added (at 470 nm and 466 nm for $HL^{o}OMe$ and $HL^{o}tBu$, respectively). This band, which is the signature of an unprotonated complex, is progressively replaced by the d-d transition of the $[Cu^{II}(HL^{o}OMe)(CH_3CN)]^{2+}$ or $[Cu^{II}(HL^{o}tBu)(CH_3CN)]^{2+}$ species upon addition of 0.5 to one molar equivalent of copper(II) (Table 1). In contrast to the *ortho-tert*-butylated ligands, the EPR spectra, recorded at 0.5 molar equivalents of copper(II) added, reveal an $S = 1$ state for the unprotonated complexes. This can be attributed to a ferromagnetic coupling between two copper(II) atoms, mediated by two μ-phenoxo bridges:[5j] the unprotonated complexes thus have the dimeric structure $[Cu^{II}_2(L^{o}OMe)_2]$ or $[Cu^{II}_2(L^{o}tBu)_2]$.

In the presence of base, with up to one molar equivalent of copper(II) added, the electronic spectrum exhibits a linear increase in the intensity of the LMCT band (Figure 4a), while EPR spectroscopy reveals an $S = 1$ state for the formed species. The same spectra were obtained by dissolving single crystals of $[Cu^{II}_2(L^{o}OMe)_2]\cdot 2\,ClO_4$ or $[Cu^{II}_2(L^{o}tBu)_2]\cdot 2\,CF_3SO_3$ in $CH_3CN$ or $CH_2Cl_2$, strongly supporting the direct formation of $[Cu^{II}_2(L^{o}OMe)_2]^{2+}$ or $[Cu^{II}_2(L^{o}tBu)_2]^{2+}$ during the titration.

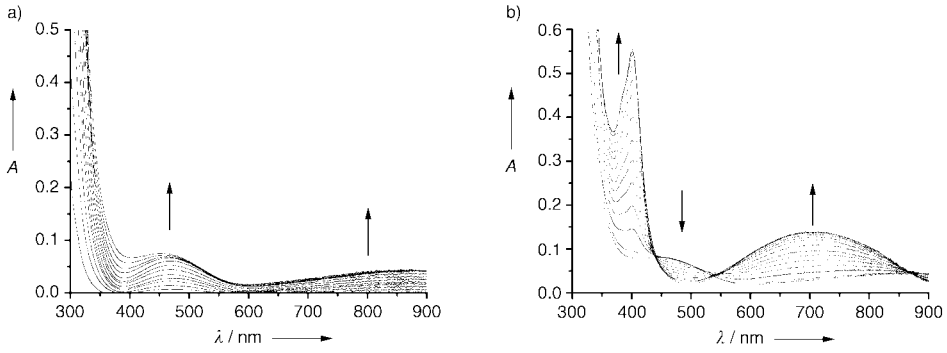



Figure 4. Titration of $HL^{o}tBu$ (0.3 mM) with copper(II) triflate in the presence of 0.3 mM triethylamine: a) 0 to one molar equivalent of copper(II) and b) one to two molar equivalents of copper(II) added. Arrows indicate spectral changes upon addition of copper; spectra recorded in $CH_3CN$ at 233 K in order to avoid degradation of the radical species (see text).

**Solution chemistry of the $N_3O$ ligands (more than one molar equivalent of copper(II) added)—Formation of a phenoxyl radical species**: A solution of HLOMe in acetonitrile was titrated with copper(II) up to a $[Cu^{II}]$:[HLOMe] ratio of 2, in the presence of one molar equivalent of triethylamine. When one to two molar equivalents of copper(II) were added, the LMCT transition at 579 nm progressively decreased and vanished, while a new intense transition appeared at 426 nm. An isobestic point was observed at 460 nm, indicating a simple equilibrium between the purple $[Cu^{II}(LOMe)(CH_3CN)]^{+}$ complex and this new deep-green species (Figure 3b). No changes were observed beyond two molar equivalents of copper(II) added. The UV/Vis spectrum of the final species exhibits an absorption at 426 nm ($\varepsilon = 4300\ M^{-1}\,cm^{-1}$ based on the ligand concentration), corresponding to a π–π* transition of the phenoxyl radical (no LMCT band is observed).[9] Moreover, its X-band EPR spectrum exhibits a $\Delta M_S = \pm 2$ transition at 150 mT, characteristic of a phenoxyl radical ferromagnetically coupled to a copper(II) atom (Figure 5).[7] These features are similar to those of the electrogenerated $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ species previously described.[7] In order to delineate the respective roles of dioxygen and copper(II) in radical generation, a solution of HLOMe in acetonitrile was titrated with copper(II) under an argon atmosphere (in a glove box). The same UV/Vis feature was observed, indicating that $O_2$ is not responsible for radical formation. Moreover, when the copper(II) triflate salt was replaced by the redox-inactive zinc(II) triflate salt, no formation of a phenoxyl radical was observed, indicating that it is the copper(II) that acts as the oxidizing agent.[10] $[Cu^{II}(LOMe)(CH_3CN)]^{+}$ is thus quantitatively oxidized by copper(II) to $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$.[11]

The titration process was further examined by cyclic voltammetry (CV) and rotating disc electrode (RDE) voltammetry using an acetonitrile-based electrolyte (0.1 M TBAP). The initial CV curve of HLOMe exhibits an irreversible peak at $E_p^{a} = +0.17$ V versus $Fc/Fc^{+}$, corresponding to the oxidation of the phenol subunit. When one molar equivalent of copper(II) is gradually added, a new reversible redox system at $E_{1/2} = +0.02$ V appears at the expense of the original irreversible one. The new redox system corresponds to the phenolate/phenoxyl redox couple. The shift in potential from +0.17 to +0.02 V is consistent with a lower oxidation potential of the phenolate-$Cu^{II}$ redox center compared to that of the phenol.[7] Accordingly, the RDE voltammetric wave is shifted (Figure 6) towards more negative potentials upon addition of copper(II). The quantitative generation of $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ upon further addition of copper(II) (one to two molar equivalents) was evidenced by RDE amperometric titration: the anodic wave at +0.02 V is progressively replaced by a cathodic wave at the same potential, demonstrating that the complex is in its oxidized state $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$.

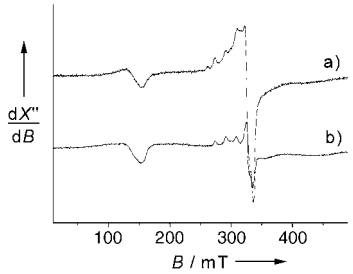



Figure 5. X-band EPR spectra of a 1 mM solution of HLOMe with two molar equivalents of copper(II) in the presence of $NEt_3$ (a) and a 1 mM solution of the electrochemically generated $[Cu^{II}(LOMe^{\bullet})(Pyr)]^+$ complex in $CH_3CN$ (+0.1 M TBAP) (b; from ref. [7]); microwave freq. 9.45 GHz, power: 0.25 mW; mod. freq. 100 KHz, amp. 0.5 mT; $T = 4$ K.

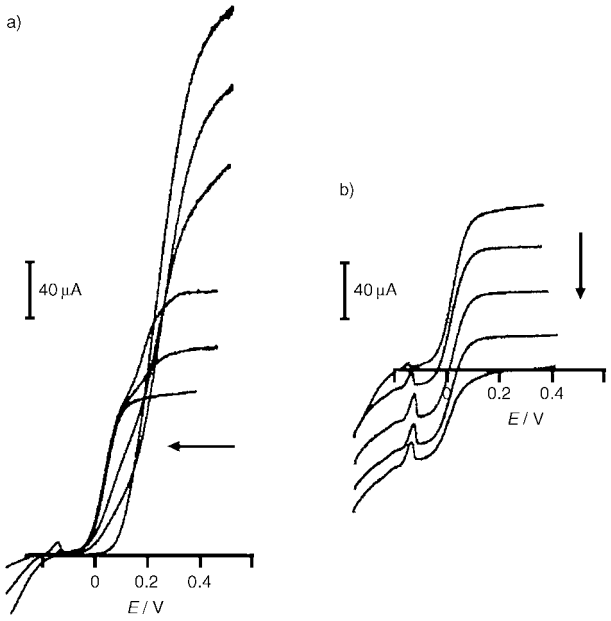
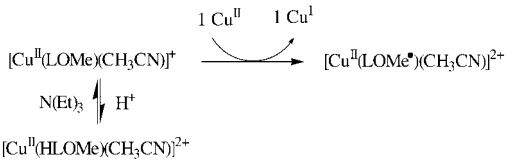



Figure 6. Voltammetric curves at a glassy carbon RDE (600 rpm, 2 mm in diameter) recorded for 1 mM HLOMe in acetonitrile (+1 mM $NEt_3$ + 0.1 M TBAP): a) from zero to one molar equivalent of copper(II) added and b) from one to two molar equivalents of copper(II) added. Arrows indicate changes when copper(II) is added. The potential values are referred to the $Fc/Fc^+$ couple.

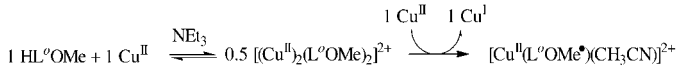
In the absence of base, when one to two molar equivalents of copper(II) are added to a solution of HLOMe in acetonitrile, the d-d transition of $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ at 600 nm decreases slightly while a weak absorption appears at 426 nm. Based on the molar extinction coefficient previously reported for the phenoxyl radical complex $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ (5170 $M^{-1}cm^{-1}$ at 427 nm),[7] we estimate that less than 8% of the $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ is converted into the radical species.[12] This result is consistent with an oxidation potential of the phenol in $[Cu^{II}(HLOMe)(CH_3CN)]^{2+}$ that is too high to allow its oxidation by copper(II) into the corresponding phenoxyl radical.[7] Only a small amount of the $[Cu^{II}(LOMe)(CH_3CN)]^+$ present in solution is oxidized (Scheme 2). The same behavior was observed with HL*t*Bu (with $\lambda_{max} = 417$ nm).




Scheme 2. Generation of the $Cu^{II}$-phenoxyl radical complex of HLOMe by copper(II) oxidation; the same behavior was observed for HL*t*Bu.

With HLF and $HLNO_2$, the progressive appearance of the absorption of a phenoxyl radical is not observed upon addition of one to two molar equivalents of copper(II) (with or without exogenous base). Only a decrease in the intensity of the LMCT transition is seen, suggesting the formation of a transient radical species that decomposes on the time scale of the measurement.[13] This phenoxyl radical (which immediately decays) is formed more slowly with $HLNO_2$ than with HLF, in keeping with the higher oxidation potential of the nitrophenolate moiety and thus the lower driving force for the reaction ($E_{1/2} = 0.70$ V versus $Fc/Fc^+$ for $[Cu^{II}(LNO_2)(CH_3CN)]$ compared to 0.02, 0.15, and 0.22 V for $[Cu^{II}(LOMe)(CH_3CN)]$, $[Cu^{II}(LtBu)(CH_3CN)]$, and $[Cu^{II}(LF)(CH_3CN)]$, respectively).[7]

Addition of one molar equivalent of copper(II), in the presence of $NEt_3$, to either HL$^o$OMe or HL$^o$*t*Bu affords the dimeric structures $[Cu^{II}_2(L^oOMe)_2]$ and $[Cu^{II}_2(L^otBu)_2]$, respectively. Further addition of copper(II) up to two molar equivalents leads to a progressive disappearance of the LMCT transition. The characteristic $\pi$–$\pi^*$ transition of the phenoxyl radical appears at 420 (HL$^o$*t*Bu) or 418 nm (HL$^o$OMe). The absorption at this wavelength is maximal when two molar equivalents of copper(II) are added, indicating quantitative oxidation of the $\mu$-phenolate moieties. In addition, oxidation by copper(II) affords the same final species as the electrochemical oxidation of $[Cu^{II}_2(L^oOMe)_2]^{2+}$ or $[Cu^{II}_2(L^otBu)_2]^{2+}$, leading to a mononuclear $Cu^{II}$-phenoxyl complex (see Supporting Information). The excess copper is thus able to oxidize the phenolates in $[Cu^{II}(LOMe)(CH_3CN)]^+$ (or $[Cu^{II}(LtBu)(CH_3CN)]^+$) as well as the bridging phenolates in $[Cu^{II}_2(L^oOMe)_2]^{2+}$ (or $[Cu^{II}_2(L^otBu)_2]^{2+}$) to phenoxyl radicals (Scheme 3).




Scheme 3. Solution chemistry of HL$^o$OMe; the same behavior was observed for HL$^o$*t*Bu.

**Titration of copper(II) with HLOMe—Evidence for a comproportionation reaction**: The ligand reactivity was probed through the titration of copper(II) with HLOMe in the presence of one molar equivalent of $NEt_3$ (based on the ligand concentration). Upon addition of 0 to 0.5 molar equivalents of HLOMe, an absorption corresponding to a phenoxyl radical increases linearly at 426 nm (Supporting Information). Addition of 0.5 molar equivalents of HLOMe affords 0.5 molar equivalents of $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ and 0.5 molar equivalents of solvated $Cu^I$. Further addition of

ligand to the resulting solution up to one molar equivalent induces a color change from deep green to purple, with quantitative formation of $[Cu^{II}(LOMe)(CH_3CN)]^+$, as judged from the molar extinction coefficient of the LMCT band observed in the final solution (see Supporting Information). Therefore, once the 0.5 molar equivalent of free copper(I) is chelated by HLOMe, it is readily oxidized by $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ to $[Cu^{II}(LOMe)(CH_3CN)]^+$.[14] This unambiguously demonstrates the occurrence of a comproportionation reaction (Scheme 4), which can be more easily visualized using the $Cu^{III}$-phenolate canonical form instead of the $Cu^{II}$-phenoxyl form.[15]

$$[Cu^{I}(LOMe)(CH_3CN)] + [Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+} \longrightarrow 2\,[Cu^{II}(LOMe)(CH_3CN)]^+$$

Scheme 4. Comproportionation of the copper complexes of HLOMe.

**Solution chemistry of the $N_2O_2$ ligands--Direct formation of the copper(II)-phenoxyl complex**: A solution of $H_2L'(OMe)_2$ in acetonitrile was titrated at −40 °C with copper(II) triflate in the presence of two molar equivalents (one per phenol) of $NEt_3$. As soon as the copper(II) had been added an absorption at 416 nm attributed to the π-π* transition of a phenoxyl radical ($\varepsilon$ = 3370 $M^{-1}cm^{-1}$ based on the copper(II) concentration), an absorption at 530 nm attributed to the phenolate-to-copper charge-transfer transition ($\varepsilon$ = 1510 $M^{-1}cm^{-1}$ based on the copper(II) concentration), and an absorption at 850 nm attributed to an LLCT transition ($\varepsilon$ = 740 $M^{-1}cm^{-1}$ based on the copper(II) concentration) appeared in the visible spectrum (Figure 7a, Table 1). These absorptions increase linearly up to two molar equivalents of copper(II) added. These results are consistent with the formation of a phenolate-copper(II)-phenoxyl complex and a redox role for the copper(II) atom.[16] It is noticeable that, in the absence of base, the obtained phenoxyl radical species are much less stable and decompose during titration.

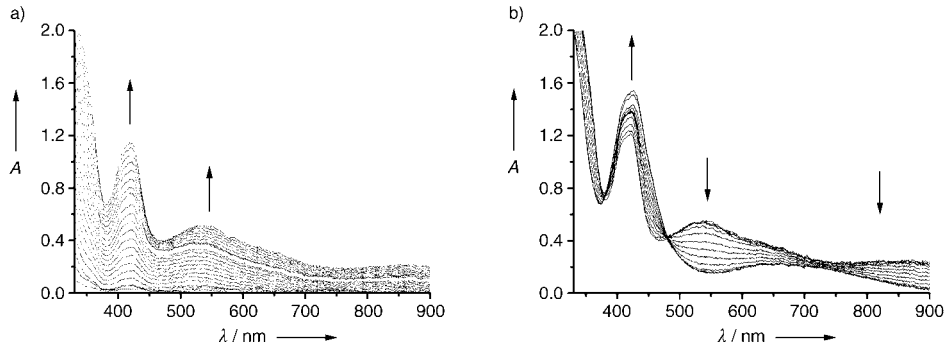



Figure 7. Titration of $H_2L'(OMe)_2$ (0.4 mM) with copper triflate in the presence of 0.8 mM triethylamine: a) 0 to two molar equivalents of copper(II) and b) two to three molar equivalents of copper(II) added. Arrows indicate spectral changes upon addition of copper(II); spectra were recorded in $CH_3CN$ at 233 K.

The EPR spectrum of a solution consisting of $H_2L'(OMe)_2$ in the presence of two molar equivalents of copper(II) shows a diamagnetic ground state for the resulting species (Figure 8a). Since a ferromagnetic exchange between

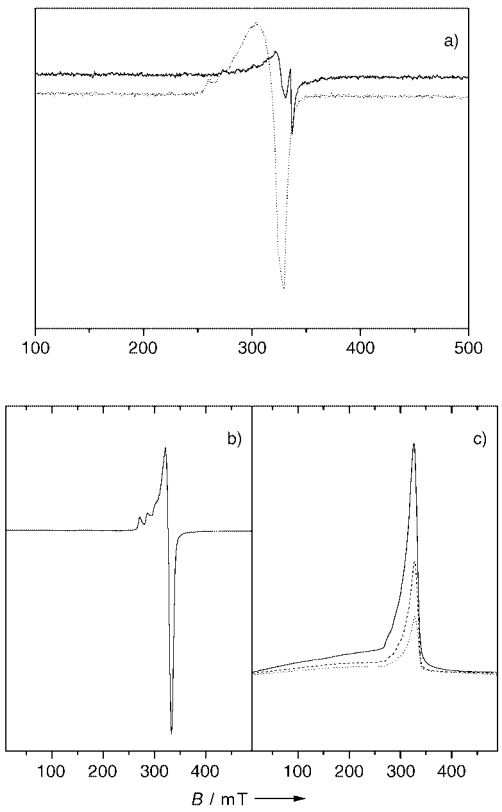
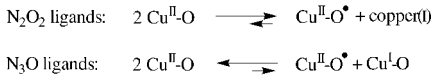



Figure 8. X-band EPR spectra of a) a 1 mM solution of $H_2L'(OMe)_2$ in $CH_3CN$ with 1.8 molar equivalents of copper(II) triflate in the presence of 2 mM triethylamine (solid line); dotted line: blank containing a 1.8 mM solution of copper(II) triflate in $CH_3CN$; b) 1.1 mM solution of $H_2L'(OMe)_2$ in $CH_3CN$ with 2.8 molar equivalents of copper(II) triflate in the presence of 2.2 mM triethylamine immediately after mixing at 233 K; c) integrated spectra: immediately after mixing (solid line), after 30 s at 293 K (dashed line), after 60 s at 293 K (dotted line). Microwave freq. 9.45 GHz, power: 10 mW; mod freq. 100 KHz, amp. 0.4 mT; $T$ = 4 K.

the two spins of the radical and the metal is expected for a mononuclear species,[7] a dimeric structure in which two μ-phenolato oxygens bridge two copper(II) atoms[16] is proposed for the diamagnetic copper-phenoxyl radical complex.[17,18] The reaction of $H_2L'OMe$ with copper(II) under anaerobic conditions (in the presence of two molar equivalents of $NEt_3$) at −40 °C was freeze-quenched at one second. The EPR spectrum of this sample exhibited a low intensity axial copper(II) signal overlapped by a radical signal (no hyperfine coupling constant could be resolved) at $g$ = 2.005, which may be attributed to a dicopper(II)-phenoxyl radical species, in contrast to the bis(copper(II)-phenoxyl) species, which is EPR-silent. In a second sample, freeze-quenched at 30 s, this radical signal had disappeared, suggesting that it was due to an intermediate in the reaction. Similarly, Shimazaki et al. have recently

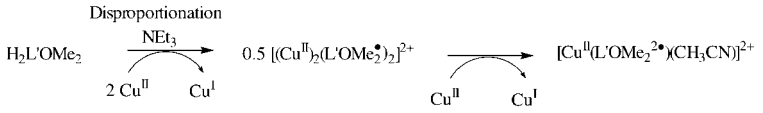
reported that mixing $N_2O_2$ tripodal ligands with stoichiometric amounts of copper perchlorate affords a $Cu^{II}$-phenoxyl radical species and $Cu^{I}$ in a disproportionation reaction,[17,19] while Stack et al. have prepared $Cu^{III}$ complexes by disproportionation of copper(II).[20] Disproportionation is thus proposed to occur in the copper(II) + $H_2L'(OMe)_2$ system. Therefore, a change in the copper coordination sphere from $N_3O$ to $N_2O_2$ could direct the reaction from comproportionation towards disproportionation (Scheme 5).




Scheme 5. Comproportionation/disproportionation reactions tuned by the coordination spheres of the ligands.

It has been suggested that a dimeric intermediate plays a key role in the copper(II) disproportionation.[19] The results obtained with our $N_2O_2$ ligands support this hypothesis, but those obtained with the $N_3O$ ligands HL$^o$OMe and HL$^o t$Bu do not. In addition, the titration of $H_2L'(OMe)_2$ in the presence of 50 molar equivalents of pyridine instead of $NEt_3$ (under these conditions, the dimeric non-radical complex is quantitatively converted to a monomer, pyridine acting as exogenous ligand)[6,7] again affords the phenoxyl-radical complex, at a somewhat lower yield. This suggests a crucial need for two phenol moieties in the same ligand to initiate the reaction; its ability to form stable dimeric structures does not seem to be of prime importance.[21]

Another interesting result was obtained by titrating $H_2L'(OMe)_2$ with up to three molar equivalents of copper(II). When two to three molar equivalents of copper(II) are added to $H_2L'(OMe)_2$, the 416 nm transition is shifted to 426 nm and its intensity increases ($\varepsilon = 3600\ M^{-1}cm^{-1}$ based on the ligand concentration) while the LMCT and LLCT transitions vanish. An isobestic point is seen at 480 nm, while an absorption is observed at 680 nm ($\varepsilon = 540\ M^{-1}cm^{-1}$). These spectral changes support the formation of a copper bis(phenoxyl) core. EPR shows a broad ($S = {}^1/_2$) signal for this species (Figure 8b), reflecting an intramolecular antiferromagnetic coupling between the two phenoxyl radicals (the ligand is consequently diamagnetic) in the copper(II) diradical complex.[5f] We rule out the possibility that this signal arises from free copper(II) since its spin Hamiltonian parameters ($g_{zz} = 2.296$, $A_{zz} = 15.3$ mT) differ significantly from those of free copper. Moreover, its intensity is dramatically decreased if the sample is allowed to warm to room temperature for 30 s prior to the measurement, suggesting that it arises from a highly reactive species that decomposes quickly at room temperature into a diamagnetic complex (Figure 8c). Based on the fact that oxidation of the bridging phenolate in $[Cu^{II}_2(L^o OMe)_2]^{2+}$ and $[Cu^{II}_2(L^o tBu)_2]^{2+}$ destabilizes the dimers and induces their breakage (see Supporting Information), the expected structure for the copper bis(phenoxyl) radical complex of $H_2L'(OMe)_2$ is monomeric. The solution chemistry of the $N_2O_2$ ligands is summarized in Scheme 6.




Scheme 6. Solution chemistry of the $N_2O_2$ ligand.

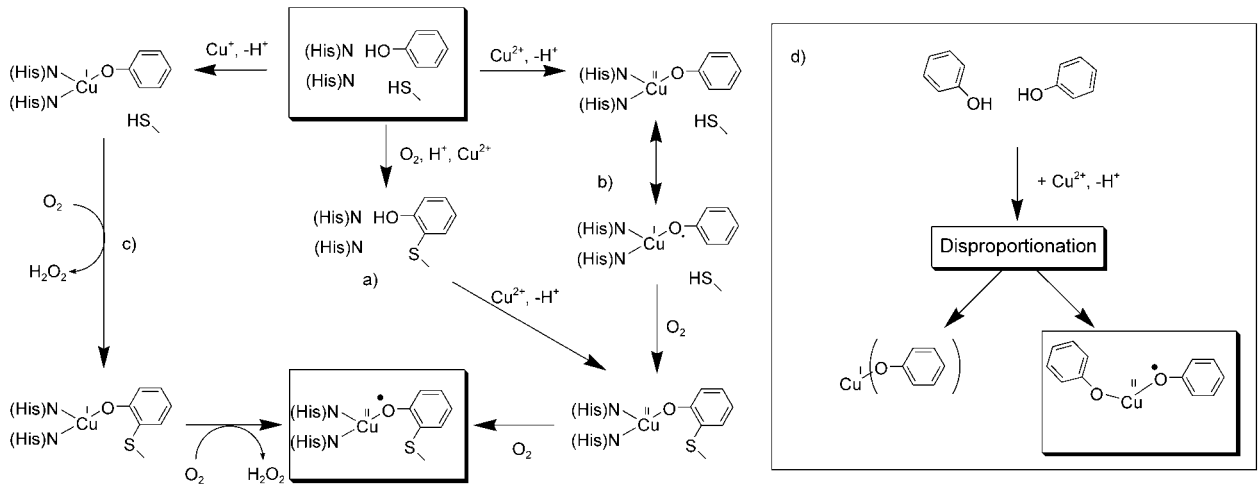
**Reaction with $Cu^I$**: Reaction of HLOMe (which affords the more persistent $Cu^{II}$-phenoxyl radical species) has been tested with $Cu^I$ (as $[Cu(CH_3CN)_4(ClO_4)]$). Upon exposure to air (at 233 K), the colorless solution of the $Cu^I$ complex (formation of the $Cu^I$ complexes was monitored by $^1H$ NMR)[21] turns violet within a minute in the absence of base or immediately in the presence of one molar equivalent of $NEt_3$. The UV/Vis feature of the final species ($\lambda_{max} = 565$ nm, $\varepsilon = 1160\ M^{-1}cm^{-1}$) is close to that reported for $[Cu^{II}(LOMe)(CH_3CN)]^+$,[7] indicating that $O_2$ does not oxidize the phenolate to a phenoxyl radical. Involvement of a proton in the reaction of $O_2$ with the copper(I) complex is evidenced through the phenol-to-phenolate conversion in the absence of base. Similar behavior (formation of a $Cu^{II}$-phenolate complex) is observed when HLOMe is replaced by HL$^o$OMe.

After bubbling dioxygen at 233 K, the solution of the copper(I) complex of $H_2L'(OMe)_2$ slowly changes from colorless to deep purple. No intermediate species could be detected during the transformation of the solution from colorless to purple. The final product exhibits a UV/vis feature ($\lambda_{max} = 500$ nm, $\varepsilon = 550\ M^{-1}cm^{-1}$) quite different from that of the isolated copper complexes of $H_2L'(OMe)_2$,[7] indicating that a highly reactive species is formed, which then decomposes. Even if the exact nature of this species remains unclear, these results highlight the dramatically different behavior of an $N_2O_2$ ligand relative to its $N_3O$ analogue. In the copper(I) complex, the $N_3O$ ligand is inert towards dioxygen while the $N_2O_2$ ligand is strongly reactive.

**Stability of the $Cu^{II}$-phenoxyl radical complexes**: The rate constant for the anaerobic self-decomposition of the copper(II) oxidized radical complex $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ at 298 K is 0.0076 $min^{-1}$ (similar to that reported for the electrochemically oxidized $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$),[7] while it is 0.0030 $min^{-1}$ in the presence of air (at 298 K). This result demonstrates that a shuttle between the free $Cu^{II}$ and $Cu^I$ redox states is achieved in the presence of $O_2$. The excess free $Cu^I$ is slowly oxidized by $O_2$ into $Cu^{II}$, which immediately reoxidizes the phenolate-based degradation products.

## Conclusion and Biological Relevance

$N_3O$ ligands chelate one molar equivalent of copper(II) leading, in the presence of base, to $[Cu^{II}(Ligand)(CH_3CN)]^+$ complexes (*ortho-tert*-butylated ligands) or $[Cu^{II}_2(\mu$-




Scheme 7. Simplified proposed pathways for the processing of GO: Paths a and b are adapted from ref. [3]) (Rogers et al. and Firbank et al.), path c from ref. [4]) (Whittaker et al.); path d: this work.

Ligand)$_2$]$^{2+}$ complexes (*ortho*-methoxylated ligands). An additional molar equivalent of copper(II) then oxidizes the complex to the corresponding mononuclear $Cu^{II}$-phenoxyl radical species. $N_2O_2$ tripodal ligands in the presence of copper afford directly a copper(II)-phenoxyl radical species. Addition of more than two molar equivalents of copper(II) affords a $Cu^{II}$-bis(phenoxyl) diradical species. The donor set of the ligand is crucial for tuning the reaction (Scheme 5).

It has recently been established that in vitro processing of GO results from its reaction with copper and molecular dioxygen, although controversial mechanisms have been proposed. In that proposed by Firbank et al.,[3] for example, a cross-linked tyrosinate-$Cu^{II}$ intermediate is oxidized by $O_2$ to the active tyrosyl-$Cu^{II}$ form (Scheme 7, paths a and b). In contrast, Whittaker et al.[4] proposed a main $Cu^{I}$-dependent path leading to a tyrosinate-$Cu^{I}$ intermediate, which then reacts with $O_2$ (Scheme 7, path c).

Our results[23] show that, in the path proposed by Firbank et al.,[3] an alternative mechanism involving disproportionation of copper(II)-tyrosinate could be considered for the oxidation of the cross-linked tyrosinate (Scheme 7, path d).[17] Since GO is extracellular the ratio [copper]:[apo-GO] might be poorly controlled, and so, if a path close to that observed for $N_3O$ ligands is considered, a comproportionation reaction consuming the radical species could occur, and the $N_2O_2$ coordination sphere of our ligands is likely to be close to that of GO. The controversy about the copper(II) or copper(I) activation could therefore be explained in terms of copper(II) disproportionation giving a tyrosyl-copper(II) species and copper(I), and reaction with dioxygen (Scheme 7, path d). Both copper(II) and copper(I) could thus activate GO. In this sense, our results reconcile the apparently contradictory pathways proposed by Rogers et al.,[3a,b] Firbank et al.,[3c] and Whittaker et al.[4] to explain the self-processing of GO (Scheme 6). This work may also pose new questions concerning the role of the metal status during processing of several metalloproteins.

## Experimental Section

**General**: All chemicals were of reagent grade and were used without purification. NMR spectra were recorded on a Bruker AM 300 ($^1$H at 300 MHz, $^{13}$C at 75 MHz) or a Bruker AC 200 ($^1$H at 200 MHz) spectrometer. Chemical shifts are quoted relative to tetramethylsilane (TMS). Mass spectra were recorded on a Thermofunnigen (EI/DCI) or a Nermag R 101 C (FAB+) apparatus. Microanalyses were performed by the Service Central d'Analyse du CNRS (Lyon, France).

**UV/Vis spectroscopy**: UV/Vis spectra were recorded on a Perkin–Elmer Lambda 2 spectrophotometer equipped with a temperature controller unit set at 298 K. The quartz cell pathlength was 1.000 cm. Low-temperature visible spectra were recorded on a Cary Varian 50 spectrophotometer equipped with a low-temperature Hellma immersion probe (1.000 cm pathlength).

**EPR spectroscopy**: X-band EPR spectra were recorded on a Bruker ESP 300 E spectrometer equipped with a Bruker nitrogen flow cryostat. Spectra were treated using the WINEPR software and simulated using the Bruker SIMFONIA software.

**Electrochemistry**: Electrochemical measurements were carried out using a PAR model 273 potentiostat equipped with a Kipp-Zonen *x-y* recorder. Experiments were performed in a standard three-electrode cell under argon atmosphere. An $Ag/AgNO_3$ (0.01 M) reference electrode was used. All the potentials given in the text are referred to the regular $Fc/Fc^+$ redox couple used as an internal reference (+87 mV versus $Ag/AgNO_3$). A glassy carbon disc electrode (5 mm diameter), which was polished with 1 μm diamond paste, was used as the working electrode. The electrochemical behavior of the ligands and complexes was studied by cyclic voltammetry (CV) and with a rotating disc electrode (RDE) in acetonitrile solutions containing 0.1 M tetrabutylammonium perchlorate (TBAP) as supporting electrolyte. Electrolysis was performed at 233 K using the same apparatus and a carbon felt working electrode.

**Preparation of the ligands**

**2,4-Dimethoxyphenol**: 2,4-Dimethoxybenzaldehyde (3.32 g, 20 mmol) was dissolved in methanol (30 mL). $H_2O_2$ (2.6 g, 20 mmol) and concentrated $H_2SO_4$ (0.4 mL) were added dropwise at 0 °C with stirring. The solution was stirred for 24 h at room temperature and then poured into cold water (100 mL), and the resulting mixture was extracted with $CH_2Cl_2$ (2 × 50 mL). The organic layer was dried ($Na_2SO_4$) and concentrated. 2,4-Dimethoxyphenol was purified by column chromatography on silica gel with $CH_2Cl_2$ as eluent. 2,4-Dimethoxyphenol (2.46 g, 80 %) was obtained as a yellow oil. $^1$H NMR (200 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 3.76 (s, 3 H), 3.86 (s, 1 H), 6.39 (dd, $^3J$(H,H) = 8.6 Hz, $^3J$(H,H) = 2.8 Hz, 1 H), 6.49 (d, $^3J$(H,H) = 2.8 Hz, 1 H), 6.82 (d, $^3J$(H,H) = 8.6 Hz, 1 H); $^{13}$C NMR (75 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 56.2 (q), 99.3 (d), 104.7 (d),

114.5 (d), 140.2 (s), 147.5 (s), 153.9 (s); MS (DCI, $NH_3$ isobutane): *m*/*z*: 154 (2,4-dimethoxyphenol); elemental analysis calcd (%) for $C_8H_{10}O_3$: C 62.33, H 6.54; found: C 62.34, H 7.04.

**HL$^o$OMe**: 2,4-Dimethoxyphenol (462 mg, 3 mmol), bis(2-pyridylmethyl)-amine (537 mg, 3 mmol), and formaldehyde (1.11 mL of a 37% aqueous solution) in EtOH/$H_2O$ (4:6, 10 mL) were heated for 24 h at 80°C. The reaction mixture was then extracted with $CH_2Cl_2$ (2×50 mL) and the organic layer was dried ($Na_2SO_4$) and concentrated. The ligand was purified by column chromatography on neutral alumina (+3% isopropylamine) with $CH_2Cl_2$/ethyl acetate (1:1) as eluent. HL$^o$OMe (439 mg, 40%) was obtained as a beige solid. $^1$H NMR (200 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 3.76 (s, 2H), 3.87 (s, 4H), 3.88 (s, 3H), 6.25 (d, $^3J$(H,H) = 2.7 Hz, 1H), 6.46 (d, $^3J$(H,H) = 2.7 Hz, 1H), 7.15 (m, 2H), 7.37 (d, $^3J$(H,H) = 12 Hz, 2H), 7.61 (m, 2H), 8.55 (d, $^3J$(H,H) = 3.9 Hz, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 53.9 (q), 55.1 (t), 57.2 (t), 97.6 (d), 103.7 (d), 120.3 (d), 120.9 (s), 121.3 (d), 135.0 (d), 138.9 (s), 147.0 (d), 150.3 (s), 156.3 (s); MS (DCI, $NH_3$ isobutane): *m*/*z*: 365 (HL$^o$OMe); elemental analysis calcd (%) for $C_{21}H_{23}N_3O_3$: C 69.02, H 6.34, N 11.50; found: C 68.89, H 6.31, N 11.46.

**2-Bromo-4-*tert*-butylphenol**: 4-*tert*-Butylphenol (5 g, 27.7 mmol) and tetra-*n*-butylammonium tribromide (16 g, 27.7 mmol) were dissolved in $CH_2Cl_2$/methanol (9:6; 150 mL) and the solution was stirred for 30 min at room temperature. The solvents were then evaporated and the concentrated reaction mixture was added to water (50 mL) and extracted with diethyl ether (2××50 mL). The combined organic layers were washed with NaCl, dried ($Na_2SO_4$), and concentrated. The solvent was evaporated and 2-bromo-4-*tert*-butylphenol (5.71 g, 90%) was obtained as a colorless oil. $^1$H NMR (300 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 1.27 (s, 9H), 6.94 (d, $^3J$(H,H) = 6.4 Hz, 1H), 7.22 (dd, $^4J$(H,H) = 2.3 Hz, 1H), 7.43 (d, $^3J$(H,H) = 2.3 Hz, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 31.6 (q), 34.6 (s), 110.3 (s), 115.9 (d), 126.6 (d), 129.2 (d), 145.5 (s), 150.3 (s); MS (DCI, $NH_3$ isobutane): *m*/*z*: 228 (2-bromo-4-*tert*-butylphenol); elemental analysis calcd (%) for $C_{10}H_{13}BrO$: C 52.42, H 5.72, Br 34.88; found: C 51.30, H 5.77, Br 33.72.

**4-*tert*-Butyl-2-methoxyphenol**: 2-Bromo-4-*tert*-butylphenol (5 g, 21.8 mmol), anhydrous $CuCl_2$ (1.5 g, 11.2 mmol), and sodium methoxide (12 g, 0.218 mol) were dissolved in DMF (150 mL). The mixture was heated under reflux for 1.5 h, filtered, and the solvent was evaporated. The solution was poured into water and HCl was added until neutralization. The mixture was extracted with $CH_2Cl_2$ and the combined organic layers were washed with NaCl, dried ($Na_2SO_4$), and concentrated. Column chromatography on silica gel ($CH_2Cl_2$ as eluent) yielded 4-*tert*-butyl-2-methoxyphenol (3.14 g, 80%) as an orange oil. $^1$H NMR (200 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 1.30 (s, 9H), 3.89 (s, 3H), 6.80–6.94 (m, 3H); $^{13}$C NMR (75 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 31.9 (s), 34.8 (q), 56.3 (q), 114.2 (d), 118.3 (d), 120.6 (d), 143.6 (s), 143.8 (s), 147.3 (s); MS (DCI, $NH_3$ isobutane): *m*/*z*: 180 (4-*tert*-butyl-2-methoxyphenol); elemental analysis calcd (%) for $C_{11}H_{16}O_2$: C 73.30, H 8.95; found: C 72.58, H 8.69.

**5-*tert*-Butyl-2-hydroxy-3-methoxybenzaldehyde**: 4-*tert*-Butyl-2-methoxyphenol (1 g, 5.55 mmol) and hexamethylenediamine (0.8 g, 6.9 mmol) were dissolved in trifluoroacetic acid (10 mL). The mixture was heated under reflux for 2.5 h, hydrolyzed with 4 M hydrochloric acid (10 mL) for 12 h at room temperature, and extracted with $CH_2Cl_2$. The combined organic layers were washed with 4 M hydrochloric acid and brine, dried ($Na_2SO_4$), and concentrated. Column chromatography on silica gel (diethyl ether/pentane (1:7) as eluent) yielded 5-*tert*-butyl-2-hydroxy-3-methoxybenzaldehyde (0.93 g, 80%) as a yellow solid. $^1$H NMR (300 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 1.33 (s, 9H; *t*Bu), 3.93 (s; OMe), 7.16 (dd, $^4J$(H,H) = 2.0 Hz, 2H), 9.90 (s, 1H; CHO); $^{13}$C NMR (75 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 31.9 (q), 34.8 (s), 56.8 (q), 116.9 (d), 120.5 (s), 121.0 (d), 143.2 (s), 148.2 (s), 150 (s), 197.2 (s); MS (DCI, $NH_3$ isobutane): *m*/*z*: 208 (5-*tert*-butyl-2-hydroxy-3-methoxybenzaldehyde); elemental analysis calcd (%) for $C_{12}H_{16}O_3$: C 69.21, H 7.74; found: C 69.29, H 7.79.

**HL$^o$*t*Bu**: 5-*tert*-Butyl-2-hydroxy-3-methoxybenzaldehyde (656 mg, 3.15 mmol), bis(2-pyridylmethyl)amine (627 mg, 3.15 mmol), ten drops of acetic acid, and sodium cyanoborohydride (198 mg, 3.15 mmol) in MeOH (50 mL) were stirred for three days at room temperature. The reaction mixture was extracted with $CH_2Cl_2$ and the combined extracts were washed with brine, dried ($Na_2SO_4$), and concentrated. The ligand was purified by column chromatography on silica gel, eluting first with methanol/ethyl acetate (6:1) and then with methanol. HL$^o$*t*Bu (740 mg, 60%) was obtained as a white solid. $^1$H NMR (300 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 1.28 (s, 9H), 3.79 (s, 2H), 3.87 (s, 4H), 3.90 (s, 3H), 6.66 (d, $^4J$(H,H) = 2.3 Hz, 1H), 6.85 (d, $^4J$(H,H) = 2.3 Hz, 1H), 7.14 (m, 2H), 7.39 (d, $^3J$(H,H) = 9 Hz, 2H), 7.61 (m, 2H), 8.56 (d, $^3J$(H,H) = 6 Hz, 2H); $^{13}$C NMR (75 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 53.9 (q), 55.1 (t), 57.2 (t), 97.6 (d), 103.7 (d), 120.3 (d), 120.9 (s), 121.3 (d), 135.0 (d), 138.9 (s), 147.0 (d), 150.3 (s), 156.3 (s); MS (DCI, $NH_3$ isobutane): *m*/*z*: 365 (HL$^o$*t*Bu); elemental analysis calcd (%) for $C_{24}H_{29}N_3O_2$: C 73.63, H 7.47, N 10.73; found: C 74.06, H 7.75, N 10.34.


[1] a) D. J. Kosman, M. J. Ettinger, R. E. Weiner, E. J. Massaro, *Arch. Biochem. Biophys.* **1974**, *165*, 456; b) M. M. Whittaker, J. W. Whittaker, *J. Biol. Chem.* **1988**, *263*, 6074; c) M. M. Whittaker, J. W. Whittaker, *J. Biol. Chem.* **1990**, *265*, 9610; d) N. Ito, S. E. V. Phillips, C. Stevens, Z. B. Ogel, M. J. McPherson, J. N. Keen, K. D. S. Yadav, P. F. Knowles, *Nature* **1991**, *350*, 87; e) G. T. Babcock, M. K. El-Deeb, P. O. Sandusky, M. M. Whittaker, J. W. Whittaker, *J. Am. Chem. Soc.* **1992**, *114*, 3727; f) N. Ito, S. E. V. Phillips, K. D. S. Yadav, P. F. Knowles, *J. Mol. Biol.* **1994**, *238*, 794; g) R. Sayle, E. F. Milner-White, *Trends Biochem. Sci.* **1995**, *20*, 374; h) J. W. Whittaker, in *Metal Ions in Biological Systems*, *Vol. 30* (Eds.: H. Sigel, A. Sigel), Marcel Dekker, New York, **1994**, pp. 315–360; i) J. W. Whittaker, "Copper containing proteins" in *Advances in Protein Chemistry*, *Vol. 60* (Eds.: J. S. Valentine, E. B. Gralla), Academic Press, Elsevier, **2002**, pp. 1–49; j) J. W. Whittaker, *Chem. Rev.* **2003**, *103*, 2347.

[2] a) J. P. Klinman, *Chem. Rev.* **1996**, *96*, 2541; b) K. E. Silva, T. E. Elgren, L. Que, M. T. Stankovich, *Biochemistry* **1995**, *34*, 14093.

[3] a) M. S. Rogers, A. J. Baron, M. J. McPherson, P. F. Knowles, D. M. Dooley, *J. Am. Chem. Soc.* **2000**, *122*, 990; b) M. S. Rogers, D. M. Dooley, *Curr. Opin. Chem. Biol.* **2003**, *7*, 189; c) S. J. Firbank, M. S. Rogers, C. M. Wilmot, D. M. Dooley, M. A. Halcrow, P. F. Knowles, M. J. McPherson, S. E. V. Phillips, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12932.

[4] M. M. Whittaker, J. W. Whittaker, *J. Biol. Chem.* **2003**, *278*, 22090.

[5] M. Taki, H. Kumei, S. Nagatomo, T. Kitagawa, S. Itoh, S. Fukuzumi, *Inorg. Chim. Acta* **2000**, *300–302*, 622; b) A. Sokolowski, H. Leutbecher, T. Weyhermüller, R. Schnepf, E. Bothe, E. Bill, P. Hildenbrandt, K. Wieghardt, *J. Biol. Inorg. Chem.* **1997**, *2*, 444; c) C. Ochs, F. E. Hahn, R. Fröhlich, *Eur. J. Inorg. Chem.* **2001**, 2427; d) Y. Wang, J. L. DuBois, B. Hedman, K. O. Hodgson, T. D. P. Stack, *Science* **1998**, *279*, 537; e) Y. Wang, T. D. P. Stack, *J. Am. Chem. Soc.* **1996**, *118*, 13097; f) P. Chaudhuri, M. Hess, J. Müller, K. Hildenbrandt, E. Bill, T. Weyhermüller, K. Wieghardt, *J. Am. Chem. Soc.* **1999**, *121*, 9599; g) P. Chaudhuri, M. Hess, T. Weyhermüller, K. Wieghardt, *Angew. Chem.* **1999**, *111*, 1165; *Angew. Chem. Int. Ed.* **1999**, *38*, 1095; h) D. Zurita, I. Gautier-Luneau, S. Ménage, J.-L. Pierre, E. Saint-Aman, *J. Biol. Inorg. Chem.* **1997**, *2*, 46; i) S. Itoh, M. Taki, S. Takayama, S. Nagatomo, T. Kitagawa, N. Sakurada, R. Arakawa, S. Fukuzumi, *Angew. Chem.* **1999**, *111*, 2944; *Angew. Chem. Int. Ed.* **1999**, *38*, 2774; j) M. Vaidyanathan, M. Palaniandavar, R. S. Gopalan, *Inorg. Chim. Acta* **2001**, 241; k) M. Vaidyanathan, R. Viswanathan, M. Palaniandavar, T. Balasubramanian, P. Prabhaharan, P. T. Muthiah, *Inorg. Chem.* **1998**, *37*, 6418; l) J. A. Halfen, B. A. Jazdzewski, S. Mahapatra, L. M. Berreau, E. C. Wilkinson, L. Que Jr., W. B. Tolman, *J. Am. Chem. Soc.* **1997**, *119*, 8217.

[6] F. Thomas, G. Gellon, I. Gautier-Luneau, E. Saint-Aman, J.-L. Pierre, *Angew. Chem.* **2002**, *114*, 3173; *Angew. Chem. Int. Ed.* **2002**, *41*, 3047.

[7] A. Philibert, F. Thomas, C. Philouze, S. Hamman, E. Saint-Aman, J.-L. Pierre, *Chem. Eur. J.* **2003**, *9*, 3803.

[8] S. Ménage, G. Gellon, J.-L. Pierre, D. Zurita, E. Saint-Aman, *Bull. Soc. Chim. Fr.* **1997**, *134*, 785.

[9] F. Thomas, O. Jarjayes, H. Jamet, S. Hamman, E. Saint-Aman, C. Duboc, J.-L. Pierre, *Angew. Chem.* **2004**, *116*, 604; *Angew. Chem. Int. Ed.* **2004**, *43*, 594; b) T. Maki, Y. Araki, Y. Ishida, O. Onomura, Y. Matsumura, *J. Am. Chem. Soc.* **2001**, *123*, 3371; c) L. Benisvy, A. J. Blake, D. Collison, E. S. Davies, C. D. Garner, E. J. L. McInnes, J. McMaster, G. G. Whittaker, C. Wilson, *Chem. Commun.* **2001**,

1824; d) P. Chaudhuri, K. Wieghardt, *Prog. Inorg. Chem.* **2001**, *50*, 151.

[10] A mixed titration of HLOMe with 0 to 2 molar equivalents of metal was performed: copper(II) was added up to one molar equivalent and then the titration was continued with zinc(II). Similarly, zinc(II) was used first and then replaced by copper(II) after one molar equivalent had been added. Formation of a phenoxyl radical species was observed only in the second experiment, confirming that copper(II) is the oxidizing agent.

[11] Excess copper(II) does not oxidize the unprotonated $[Cu^{II}(LOMe)(CH_3CN)]^+$ complex formed at 0.5 molar equivalents of copper(II) added. This may be interpreted either in terms of copper(II) complexation, which occurs more rapidly than oxidation, or in terms of a comproportionation reaction that consumes the radical species as soon as it is formed.

[12] Excess copper(II) catalyzes the degradation of the radical species. The highest concentration of radical species was obtained at 1.3 molar equivalents of copper(II) added, corresponding to an 8% conversion. The UV/Vis spectrum recorded after the addition of two molar equivalents of copper(II) exhibits only a shoulder at 420 nm, indicating that the concentration of the radical species is less than 1% of the total complex concentration.

[13] An LMCT transition was no longer observed in the visible spectrum of the degradation products of $[Cu^{II}(LOMe^{\bullet})(CH_3CN)]^{2+}$ and $[Cu^{II}(L\mathit{t}Bu^{\bullet})(CH_3CN)]^{2+}$.

[14] No oxidation of free $Cu^{I}$ occurs in the absence of 0.5–1 molar equivalent of HLOMe.

[15] Analogously, evidence for a thermal equilibrium between $Ni^{3+}$-phenolate and $Ni^{2+}$-phenoxyl complexes has recently come to light (see Y. Shimazaki, F. Tani, K. Fukui, Y. Naruta, O. Yamauchi, *J. Am. Chem. Soc.* **2003**, *125*, 10512).

[16] We have previously isolated, in the presence of triethylamine, the copper(II) complex of $H_2L'(OMe)_2$ (in the non-radical form) as the dimeric species $[Cu^{II}_2\{L'(OMe)_2\}_2]$ (ref. [7]). Its structure was confirmed by X-ray diffraction analysis. Such a dimeric structure is preserved in acetonitrile, as evidenced by UV/Vis and EPR spectroscopy; the latter reveals an antiferromagnetic coupling between the two copper(II) atoms mediated by two μ-phenolato bridges (ref. [7]). Gradual addition of one molar equivalent (relative to the ligand concentration) of copper(II) to $[Cu^{II}_2\{L'(OMe)_2\}_2]$ results in the progressive disappearance of the LMCT, while absorptions appear at 410, 530, and 850 nm in the visible spectrum ($\varepsilon$ = 3450 $M^{-1}cm^{-1}$, 1900 $M^{-1}cm^{-1}$, and 1040 $M^{-1}cm^{-1}$, respectively, based on the ligand concentration). The UV/Vis feature of the final species is identical to that obtained after the addition of two molar equivalents of copper(II) to $H_2L'(OMe)_2$ in the presence of $NEt_3$.

[17] Y. Shimazaki, S. Huth, S. Hirota, O. Yamauchi, *Inorg. Chim. Acta* **2002**, *331*, 168.

[18] P. Chaudhuri, M. Hess, U. Flörke, K. Wieghardt, *Angew. Chem.* **1998**, *110*, 2340; *Angew. Chem. Int. Ed.* **1998**, *37*, 2217.

[19] Y. Shimazaki, S. Huth, A. Odani, O. Yamauchi, *Angew. Chem.* **2000**, *112*, 1732; *Angew. Chem. Int. Ed.* **2000**, *39*, 1666.

[20] X. Ribas, D. A. Jackson, B. Donnadieu, J. Mahia, T. Parella, R. Xifra, B. Hedman, K. O. Hodgson, A. Llobet, T. D. P. Stack, *Angew. Chem.* **2002**, *114*, 3117; *Angew. Chem. Int. Ed.* **2002**, *41*, 2991.

[21] Since the behavior upon addition of copper(II) to $N_2O_2$ ligands is quite different to that seen with $N_3O$ ligands, we have attempted to study the kinetics of the reactions by the stopped-flow technique. Unfortunately, even at 15 °C, both the copper complexation and oxidation occur during the dead time of the apparatus (3 ms, $k_{obs} > 300\ s^{-1}$), precluding any detailed kinetic analysis.

[22] $^1$H NMR data of the copper(I) complex of HLOMe prepared in a glove box in the presence of base (200 MHz, $CDCl_3$, 298 K, TMS): $\delta$ = 1.31 (s, 9H), 3.66 (s, 3H), 3.84 (s, 3H), 3.89 (s, 2H), 6.68 (s, 2H), 7.28–7.34 (m, 4H), 7.74–7.79 (m, 2H), 8.53 (s, 2H).

[23] Since our results were obtained in acetonitrile, and thus under non-biologically relevant conditions, in a last set of experiments acetonitrile was replaced by ethanol, containing 10–50 molar equivalents of pyridine (relative to the ligand) in order to mimic potential N-containing ligands (water could not be used due to the limited solubility of $H_2L'(OMe)_2$). Under these conditions, all of the ligands were converted into the $Cu^{II}$-phenoxyl radical form when copper(II) was added. However, this conversion was not quantitative, presumably due to a competition between the excess pyridine and the ligand to coordinate the copper (the copper is not entirely chelated by the ligand, as reflected in the case of $N_3O$ ligands by a low intensity of the LMCT band at one molar equivalent of copper(II) added).

Received: February 2, 2004
Published online: July 21, 2004