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Authors: Pierre Moenne-Loccoz, Kenneth D. Karlin, Jeffery J. Liu, and Maxime A. Siegler

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Direct Resonance Raman Characterization of a Peroxynitrito Copper Complex Generated from O₂ and NO and Mechanistic Insights into Metal Mediated Peroxynitrite Decomposition

Jeffrey J. Liu,^[a] Maxime A. Siegler,^[a] Kenneth D. Karlin,^{*[a]} and Pierre Moënne-Loccoz^{*[b]}

Abstract: We report the formation of a new copper peroxynitrite (**PN**) complex $[Cu^{II}(TMG_3tren)(\kappa^1-OONO)]^+$ (**PN1**) from reaction of $[Cu^{II}(TMG_3tren)(O_2^-)]^+$ (**1**) with NO[•]_(g) at -125 °C. The first resonance Raman spectroscopic characterization of such a metal bound **PN** moiety supports a *cis* κ^1 -OONO⁻ geometry. **PN1** transforms thermally to an isomeric form (**PN2**) with κ^2 -O,O'-(⁻OONO) coordination, which undergoes O–O bond homolysis to generate a putative cupryl (LCu^{II}– O') intermediate and NO₂⁺. These transient species do not recombine to give a nitrato (NO₃⁻) product but instead proceed to effect oxidative chemistry and formation of a Cu(II)-nitrito (NO₂⁻) complex (**2**).

Nitric oxide (NO') is an essential molecule in biology due to its role as a signaling agent,^[1-3] its utilization in the human immune response,[4-6] and as an obligatory intermediate in the geobiochemical nitrogen cycle.^[7,8] At elevated concentrations (μ M) NO[•] is toxic^[4,9–12] and its downstream derivatives can lead to oxidation and nitration of proteins^[13-15], lipids^[16,17] and nucleic acids.^[18,19] Phagocytes leverage the toxicity of nitric oxide in combination with other reactive nitrogen and oxygen species (such as O_2^{-} , H_2O_2 and NO_2^{-}) as tools to combat invading microorganisms.^[5,6] Phagocytes also employ metal toxicity, and in particular copper, as a bactericide, but the synergy between destructive reactive nitrogen and oxygen species and copper burst in the macrophage remains largely unknown.^[6,20-22] A key intermediate postulated to be responsible for nitric oxide's toxicity is peroxynitrite (-OONO, PN), which may be derived from NO' and O2⁻ (Scheme 1).^[23-25] Peroxynitrite can exist in two isomers, *cis*, or trans, and upon protonation, or in the presence of CO₂, yield either nitrate, or freely diffusing radicals such as NO2, HO, and CO₃^{-.[10,26]} Studies of aqueous peroxynitrite have deduced that the predominant decay pathway is isomerization to nitrate.^[27,28]

Metal coordinated peroxynitrites are of interest due to the intimate role metals play in the regulation and processing of both NO' and $O_2^{-,[3,29-32]}$ which may implicate metals as important factors in the physiological formation and transformations of peroxynitrite (Scheme 1). While progress has been made to implicate metal ions in the decay of peroxynitrite, a discrete peroxynitrite coordination complex is rarely observed.^[33-42] Examples of stable peroxynitrite complexes are scarce^[43,44], and

[a]	J. J. Liu, M. A. Siegler, Prof. K. D. Karlin
	Department of Chemistry
	Johns Hopkins University
	Baltimore, MD 21218 (USA)
	E-mail: karlin@jhu.edu
[b]	Prof. P. Moënne-Loccoz
	Department of Biochemistry and Molecular Biology
	Oregon Health & Science University
	Portland, OR 97239 (USA)
	E-mail: moennelo@ohsu.edu

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those containing vibrational (i.e., IR spectroscopic) information about the peroxynitrite ligand even more so. $^{[45-48]}$



Scheme 1. Formation of Peroxynitrite from Superoxide and Nitric Oxide, and Mechanisms of Peroxynitrite Decomposition through Metal Ion Coordination.

In a previous report we have described the formation of a cupric-peroxynitrite complex from the addition of NO[•] to $[Cu^{II}(TMG_3tren)(O_2^{--})]^+$ (1) at -80 °C to generate the cupric-peroxynitrito complex $[Cu^{II}(TMG_3tren)(\kappa^2-O,O'-OONO)]^+$ (PN2) (Scheme 2).^[49] Direct vibrational evidence of this peroxynitrite complex was unable to be obtained, and information concerning the structural identity was derived from EPR spectroscopy and density functional theory (DFT) calculations. Herein we report the formation of a new peroxynitrite complex, $[Cu^{II}(TMG_3tren)(\kappa^1-OONO)]^+$ (PN1) formed prior to PN2, and the resonance Raman (RR) characterization of a metal-peroxynitrite species.



Scheme 2. Generation of PN1 from addition of NO to complex 1. Thermal transformation of PN1 leads to formation of PN2.

The complex **PN1** is generated from the addition of NO[•] solubilized in 2-methyltetrahydrofuran (NO_(MeTHF), see SI), to **1** at -125 °C (Scheme 2). Monitoring the reaction by UV-vis spectroscopy reveals the gradual decrease of the cupric-superoxide features at 448, 679 and 791 nm and accompanied by

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the appearance of a new intense electronic transition at 310 nm (7300 M^{-1} cm⁻¹) and a weak d-d transition at 1000 nm (~410 M^{-1} cm⁻¹) (Figure 1). The intense high-energy feature near 310 nm has been has been reported for alkali and ammonium salts^[50,51] and in metal coordination complexes of peroxynitrite.^[43,45]



Figure 1. Addition of NO_{(MeTHF)} (7 equiv) to 1 (0.15 mM) in MeTHF at -125 °C (black); full conversion to PN1 was observed within 30 minutes (red).

Formation of **PN1** is easily monitored via EPR spectroscopy. Addition of NO_(MeTHF) to the EPR silent **1** leads to the generation of an active EPR signal (Figure 2), consistent with the radical coupling of **1** (S = 1)^[52] with NO' (S = 1/2) to give a S = 1/2 complex. A quantitative analysis of the EPR data through a Job's plot shows maximal **PN1** formation at a 1:1 mole ratio of Cu to NO', confirming the 1:1 cupric-superoxide to NO stoichiometry (Figure S1).



Figure 2. X-band EPR spectra of 1 (black, EPR silent), PN1 (red, 0.41 mM) and fitted PN1 spectra (purple; $g_{1:3}$ = 1.9874, 2.1945, 2.2040; $A_{1:2}$ = 77.2 G, 111.3 G)

The RR spectra of PN1 reveal multiple isotopically sensitive features assigned to stretching and bending modes of the cupricperoxynitrite complex (Figure 3). The feature with the highest intensity is at 658 cm⁻¹ and shows a 30 cm⁻¹ downshift with full labeling of the peroxynitrite ligand (i.e., ¹⁸O¹⁸O¹⁵N¹⁸O). This RR signal is unusually broad with a 29 cm⁻¹ full width at half maximum (FWHM), and is reminiscent of the broad 642 cm⁻¹ band observed in the Raman spectrum of the *cis* peroxynitrite anion in aqueous solution. $^{[53,54]}$ Labeling with $^{18}\text{O}_2$ or $^{15}\text{N}^{18}\text{O}$ shifts the 658 cm 1 band -26 and -9 cm⁻¹ respectively, supporting significant O-N stretching character to this mode which was otherwise assigned to an out of plane deformation in the free ion (Table 1 and Figure S2). Indeed, the observed shifts match well calculated shifts based on Hooke's law for an O-N oscillator (-29.5 cm⁻¹ with ¹⁸O- ^{15}N , -17 cm 1 with $^{18}O-N$, and -12 cm 1 with O- ^{15}N). The DFT vibrational analysis also supports a mode description that combines O-N stretch with O-ON=O bending (see below).

A much weaker and sharper band at 1545 cm⁻¹ band is readily assigned to the peroxynitrite v(N=O) stretch based on its frequency and large downshift with ¹⁵N¹⁸O (-65 cm⁻¹) (Figure 3). This assignment is further strengthened by the limited sensitivity of this mode to the labeling of O₂ ($\Delta^{18}O_2 = -2 \text{ cm}^{-1}$) (Table 1 and Figure S3).

A band at 929 cm⁻¹ strongly impacted by O₂ labeling ($\Delta^{18}O_2 = -29 \text{ cm}^{-1}$) as well as NO labeling ($\Delta^{15}N^{18}O = -17 \text{ cm}^{-1}$) is assigned to an O–O stretch mixed with an O-N=O bend (Table 1 and Figure S4). A very weak band at 812 cm⁻¹, which can be extracted from the MeTHF Raman spectrum background only after labeling of both O₂ and NO, is tentatively assigned to another mixed vibrational mode that combines O–O stretch with in plane OO-N=O deformation. Finally, a broad signal at 1282 cm⁻¹ that shifts -60 cm⁻¹ after full labeling of the peroxynitrito group and that shows a moderate intensity relative to the fundamental mode at 658 cm⁻¹ is likely to correspond to its first overtone.^[55]



Figure 3. RR spectra of PN1 (407 nm excitation) generated from O_2 and $^{14}N^{16}O$ (black) or $^{18}O_2$, $^{15}N^{18}O$ (red). Difference spectrum (unlabeled – labeled) is shown in green.

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Table 1. Summary of Isotopically Sensitive Vibrations of PN1; observed RR frequencies in cm⁻¹ (isotope shift, cm⁻¹), and calculated frequencies for *cis* and *trans* conformers (italicized numbers).

Head 1	OONO	¹⁸ O ₂ ¹⁵ N ¹⁸ O	¹⁸ O ₂ NO	00 ¹⁵ N ¹⁸ 0
ν(O–N) +δ(NO ₂) cis trans	658 708 845	628 (-30) (-35) (-45)	637 (–21) (–26) (–35)	647 (-11) (-9) (-12)
ν(O–O) +δ(NO ₂) cis trans	812 833 630	772 (–40) (–45) (–35)	(-37) (-23)	(1 <i>4</i>) (-1 <i>5</i>)
$v(O-O) + \delta(NO_2)$ cis trans v(N=O)	929 970 1002	881 (-48) (-53) (-51)	900 (-29) (-35) (-35) 1543 (-2)	912 (-17) (-16) (-15) 1482 (-43)
cis trans	1601 1627	(-68) (-74)	(-2) (-3)	(-68) (-72)

To aid interpretation of the RR data, predicted structures for PN1 were obtained via DFT calculations. The reverse axial copper EPR signal (Figure 2) is indicative of a trigonal bipyramidal copper geometry. Based on that constraint two possible structures were calculated, where the peroxynitrite ligand in either a cis or trans confirmation (Figures S5 & S6). The cis cupricperoxynitrite isomer is predicted to be the more stable isomer by 4.1 kcal/mol, in line with previously reported calculations of the anion where the *cis* isomer is more stable by 3 kcal/mol.^[53] The calculated Raman spectra provide satisfactory match with the experimental data, with the most highly Raman-active vibration observed in the mid-frequency range with significant O-N stretching character (Figures S7-S9). Calculated frequencies for this mode are 708 cm⁻¹ in the *cis*-PN and 845 cm⁻¹ in the *trans*-PN conformer, the former matching better with the 658 cm⁻¹ observed frequency. Similarly, the calculated frequencies for modes dominated by v(O–O) and v(N=O) for the cis-PN are better match than that of the trans-PN (Table 1). In fact, the four fundamental Raman frequencies observed in PN1 are reproduced in the calculated spectrum of cis-PN with overestimations ranging from 0.02 to 0.08 typical of DFT calculations. Thus, the RR data support a cis geometry for the peroxynitrite ligand in PN1, as depicted in Scheme 2.

When NO' is added to 1 at -80 °C a chelating peroxynitrite intermediate [Cu^{II}(TMG₃tren)(κ^2 -O,O'-OONO)]⁺ (**PN2**) is formed, giving an axial EPR spectrum;^[49] as described above, **PN1** is formed at -125 °C exhibiting a reverse axial copper geometry (Figure S10). If NO' is added at an intermediate temperature (-98 °C) **PN1** is observed as the initial species followed by conversion to **PN2**. The conversion from reverse axial to axial EPR signal (Figure 4) reflects a change in the copper center geometry from trigonal bipyramidal to square pyramidal. Such a change results in the deligation one of the tetramethylguanidine (TMG) arms which allows for the adaptation of a square pyramidal geometry.^[49] Careful analysis of the EPR spectra after 60 minutes reveal the presence of two axial Cu signals (Figure 4) which is assigned to the partial decomposition of **PN2** to the final Cu-nitrito product (see below). Attempts to obtain RR spectra from **PN2** were inconclusive. Gradual photo-bleaching of **PN1** was observed during the acquisition of the Raman spectra and presumably **PN2** exhibits a greater photosensitivity.

Upon further warming to -60 °C, **PN2** decays to a chelating copper nitrito species [Cu^{II}(TMG₃tren)(κ^2 -O,O'-NO₂)]⁺ (2) confirmed by ESI–MS (Figure S11). EPR spectroscopy (Figure S12) indicates the copper maintains a tetragonal coordination geometry. UV-vis quantification versus a closely related square pyramidal Cu^{II}-NO₂ analog (Figure S13 & 14) puts the yield of **2** at 95 ± 4% (Figure S15), and the presence of nitrite was independently quantified via a Griess assay was found to be in good yield (75 ± 2 %) (Figure S16 & Table S1). The kinetics of **PN2** transformation to Cu^{II}-NO₂ species **2** indicated overall first order in Cu behavior (Figure S17) consistent with unimolecular decay, most likely though cleavage of the O–O bond. This result rules against higher order copper adducts participating in peroxynitrite decay as suggested in the aqueous H⁺ and CO₂ mediated decay pathways.^[27,28]



Figure 4. (Top) Conversion of PN1 (red) to PN2 (blue) at -98 °C (see Figure S10 for fitted EPR parameters of PN2). (Bottom) Speciation of PN1 and PN2 (calculated via EasySpin, Figure S27) as a function of time and fit to a single exponential. Conversion of 90% of PN1 to PN2 occurs within 30 minutes.

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The detection and characterization (see SI) of a high yield cupric-nitrite (NO2⁻) product from PN2 thermal transformation is consistent with the formation of cupryl [Cull(TMG₃tren)(O')]⁺ and NO2[•] intermediates following peroxynitrite homolytic O-O bond cleavage; the most common recombination of such radicals to give a Cu-nitrate product does not occur (Scheme 1). In the absence of substrate we postulate the putative cupryl intermediate goes on to hydroxylate the solvent via a radical rebound pathway to give LCu^I and R-OH (R = solvent) before LCu^I reacts with NO₂⁻ to yield LCu^{II}-(NO₂⁻) as the final product (Scheme S1).^[36,44,46,56,57] To verify the generation of these radical species, addition of >500 equiv excess of 2,4-di-tert-butylphenol (DTBP) results in the nearly quantitative formation of both 2 and the coupled bis-phenol product, 3,3',5,5'-tetra-tert-butyl-[1,1'biphenyl]-2,2'-diol (Figures S18 & S19). The coupling of phenoxyl species to give the observed diol can be rationalized by successive hydrogen atom abstractions by LCu^{II}-O' and NO₂' (a strong oxidant^[58,59]) to give LCu^{II}-OH and HNO₂ before ligand exchange to give LCu^{II}-NO₂ and H₂O (Scheme 3). A concerted O-O bond cleavage / O-H bond activation pathway is also plausible; further mechanistic studies will be required to differentiate between stepwise and concerted substrate oxidation. Additional experiments using 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 500 equiv), a known radical trapping agent used to probe O-O bond scission reactions,[60,61] resulted in detection of LCu^{II}(O-DMPO) by ESI-MS (Figure S20) following warming of the reaction mixture. These results are consistent with the generation of two equivalents of oxidant per equivalent of copperperoxynitrite, where one of the oxidizing equivalents is oxygenderived.



Scheme 3. Proposed mechanism for 2,4-di-*tert*-butylphenol oxidation and Cu^{II} -NO₂⁻ production via **PN2** thermal transformation.

In summary, we have described the synthesis and preparation of the first transition metal-peroxynitrite complex characterized by resonance Raman spectroscopy. Comparison with Raman data from alkylammonium *cis*-peroxynitrite, combined with analytical Raman calculations have suggested the peroxynitrito ligand is coordinated to copper in a *cis*-geometry. Thermal transformation of this peroxynitrite complex leads to O–O homolysis and the generation of LCu–O[•] and NO₂[•] which go on to perform further oxidative chemistry. These results further underscore the importance of understanding the biological role transition metal ions play in biochemical oxidative and nitrative damage.

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