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Biomimetic Synthesis of the Putative Cytotoxin Peroxynitrite, ONOO⁻, and Its Characterization as a Tetramethylammonium Salt

D. Scott Bohle,* Bernhard Hansert, Scott C. Paulson, and Bryan D. Smith

Department of Chemistry, University of Wyoming
Laramie, Wyoming 82071-3838

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Nitric oxide and the superoxide anion are important components of the cytotoxic flux produced by cytokine-activated macrophages in inflamed tissues.[1] The contemporaneous high local concentrations of these two reactive molecules have led to the hypothesis that their reaction product, peroxynitrite, $ONOO^-$, may also be a significant cytotoxin.[2] Although the recently redetermined rate constant for this reaction ($k = (6.7 \pm 0.9) \times 10^9$ L mol^{-1} s^{-1}) is near the diffusion limit,[3] approximately 3-fold faster than the rate of superoxide dismutation by Cu–Zn SOD,[4] there is only circumstantial evidence for the presence of peroxynitrite in these tissues.[5] This is due to the pronounced reactivity of pernitrous acid ($pK_a = 6.5 \pm 0.1$), which rapidly decomposes under physiological conditions ($t_{1/2} \sim 1$ s at pH = 7.4),[6] and to a lack of information concerning the physical properties and reactivity of peroxynitrogen compounds in general. Although our understanding of the kinetics of pernitrous acid decomposition is well established,[6] attempts to determine the free-radical yields from this reaction have been hampered by both the reactions of the peroxynitrite anion itself and the use of peroxynitrite solutions which contain high concentrations of the other oxoanions of nitrogen.[4] Reported syntheses of peroxynitrite include a variety of aqueous techniques, *cf.* the reaction of nitrite and hydrogen peroxide with acid followed by a base quench,[7,8] hydrogen peroxide and nitrite ester in base,[9] and a solid-state preparation by UV photolysis of potassium nitrate to give 30 μmol/g of ONOOK as a solid mixture of peroxynitrite and nitrate.[10] Prior attempts to isolate salts of this anion have failed,[8] and the reported spectroscopic data are rather limited.[11] Herein we describe (1) a biomimetic synthesis of peroxynitrite from nitric oxide and superoxide, (2) the first isolation of the peroxynitrite anion as a tetramethylammonium salt which is soluble in a wide range of solvents, (3) the solvatochromatism for the UV absorption band of this molecule, (4) the IR, Raman, and ^{15}N NMR spectroscopic results for this species, (5) *ab initio* calculations of the optimized ground-state geometries and frequencies for the *trans* and *cis* conformations, and (6) differential scanning calorimetric results for the isomerization of peroxynitrite to nitrate.

The reaction between superoxide and nitric oxide in liquid ammonia results in the rapid formation of deep orange-yellow solutions of peroxynitrite. When tetramethylammonium superoxide and a slight excess of nitric oxide are used, eq 1, it is possible

$$[Me_4N][O_2] + NO \xrightarrow{NH_3(l)} [Me_4N][ONOO] \qquad (1)$$

to isolate tetramethylammonium peroxynitrite as a hygroscopic microcrystalline yellow solid by a nitrogen purge followed by removal of the ammonia *in vacuo*.[12] There is no detectable contamination of this product with nitrite or nitrate by Raman, IR, or ^{15}N NMR spectroscopy (see below). An analytically pure sample of this salt is obtained by fractional crystallization from liquid ammonia, the main impurities being less soluble salts such as tetramethylammonium hydroxide. When prepared rigorously dry and maintained under nitrogen, these salts have been stored for several months with little detectable decomposition. Attempts to isolate the potassium salt of peroxynitrite, prepared with the conditions shown in eq 1 but from potassium superoxide, result in rapid decoloration of the bright yellow solid on drying. Furthermore, the direct reaction of solid potassium superoxide and nitric oxide is reported to give a mixture of potassium nitrite and nitrate.[14]

Peroxynitrite can exist in either *trans* or *cis* configurations: the vibrational spectroscopic results, collected in Table 1, are consistent with the presence of both conformations in the solid state. In particular, the Raman spectrum for solid-phase tetramethylammonium peroxynitrite, shown in Figure 1, has bands at 813 and 645 cm^{-1} which shift 10 cm^{-1} upon incorporation of ^{15}NO. The vibrational spectrum of pernitrous acid has been measured in inert gas matrices, and the $\nu(N{=}O)$ and $\nu(O{-}O)$ modes were assigned to bands at 1701.4 and 960.5 cm^{-1}, respectively,[16] *ca.* 10 cm^{-1} *higher* than the energies predicted by MP2/6-31G* *ab initio* calculations.[17] As is found for the other

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(1) (a) Hibbs, J. B., Jr.; Taintor, R. R.; Vavrin, Z.; Rachlin, E. M. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 87. (b) Marletta, M. A.; Yoon, P. S.; Iyengar, R.; Leaf, C. D.; Wishnok, J. S. *Biochemistry* **1988**, *27*, 8706. (c) Stuehr, D. J.; Nathan, C. F. *J. Exp. Med.* **1989**, *169*, 1543. (d) Johnson, K. J.; Ward, P. A. In *Superoxide Dismutase, Vol. III*; Oberley, L. W., Ed.; CRC Press: Boca Raton, FL, 1985; p 129. (e) Wang, J.-F.; Komarov, P.; Sies, H.; De Groot, H. *Biochem. J.* **1991**, *279*, 311. (f) Ding, A. H.; Nathan, C. F.; Stuehr, D. J. *J. Immunol.* **1988**, *141*, 2407.

(2) (a) Beckman, J. S. *Nature* **1990**, *345*, 27. (b) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620.

(3) Huie, R. E.; Padmaja, S. *Free Rad. Res. Commun.* **1993**, *18*, 195.

(4) Yang, G.; Candy, T. E. G.; Boaro, M.; Wilkin, H. E.; Jones, P.; Nazhat, N. B.; Saadalla-Nazhat, R. A.; Blake, D. R. *Free Rad. Biol. Med.* **1992**, *12*, 327.

(5) (a) Hogg, N.; Darley-Usmar, V. M.; Wilson, M. T.; Moncada, S. *Biochem. J.* **1992**, *281*, 419. (b) Gryglewski, R. J.; Palmer, R. M. J.; Moncada, S. *Nature* **1986**, *320*, 454.

(6) See: Løgager, T.; Sehested, K. *J. Phys. Chem.* **1993**, *97*, 6664 and references therein.

(7) Reed, J. W.; Ho, H. H.; Jolly, W. L. *J. Am. Chem. Soc.* **1974**, *96*, 1248.

(8) Hughes, M. N.; Nicklin, H. G. *J. Chem. Soc. (A)* **1968**, 450.

(9) Leis, J. R.; Peña, M. E.; Ríos, A. *J. Chem. Soc., Chem. Commun.* **1993**, 1298.

(10) King, P. A.; Anderson, V. E.; Edwards, J. O.; Gustafson, G.; Plumb, R. C.; Suggs, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 5430.

(11) For a recent review of the chemistry of peroxynitrites, see: Edwards, J. O.; Plumb, R. C. *Prog. Inorg. Chem.* **1994**, *41*, 599.

(12) Peroxides are potentially hazardous, and appropriate safety precautions should be taken. NMe_4O_2[13] (50 mg, 0.47 mmol) was transferred under a nitrogen atmosphere into a 100-mL Schlenk flask. After the NMe_4O_2 was cooled to −78 °C, about 20 mL of ammonia was condensed into the flask, and dry nitric oxide was bubbled very slowly for 25 min through the solution, which turned deep orange. A slight flow of nitrogen was used to prevent the development of subatmospheric pressures within the system. Excess nitric oxide and about half of the ammonia was removed using a stream of nitrogen, and the solution was then filtered to remove a small amount of insoluble material. The product was isolated as a bright yellow-orange lyophilized powder by freezing the solution with liquid nitrogen and then removing the ammonia *in vacuo*. Yield of $[NMe_4][ONOO]$, 62 mg (97%). Small crystals of analytically pure peroxynitrite can be obtained by slowly cooling a saturated ammonia solution from −40 °C to −78 °C over a period of 2 days. Anal. Calcd for $C_4H_{12}N_2O_3$: C, 35.29; H, 8.88; N, 20.58. Found: C, 35.77; H, 9.33; N, 21.21. UV–vis: λ_{max} in water (pH = 14), 302 nm ($\epsilon = 1705 \pm 10$ M^{-1} cm^{-1}); in ethanol, 304 nm; in liquid ammonia (at −76 °C), 346 nm; and in dichloromethane, 340 nm.

(13) Prepared from the pentahydrate of tetramethylammonium hydroxide by the method described in the following: Sawyer, D. T.; Calderwood, T. S.; Yamaguchi, K.; Angelis, C. T.; *Inorg. Chem.* **1983**, *22*, 2577. ***Caution!*** When predried tetramethylammonium hydroxide was employed as described in a modified preparation by these authors (Yamaguchi, K.; Calderwood, T. S.; Sawyer, D.T. *Inorg.Chem.* **1986**, *25*, 1289), a serious explosion occurred during the metathesis of tetramethylammonium hydroxide with potassium superoxide. Although there is an increased consumption of potassium superoxide when the pentahydrate is used, there is little difficulty in separating the potassium hydroxide byproduct in the subsequent liquid ammonia extraction step. It is therefore recommended that the pentahydrate be used in these metatheses.

(14) Firsova, T. P.; Filatov, E. Ya. *Izv. Akad. Nauk SSSR, Ser. Khim.* **1974**, 1416; *Chem. Abstr.* **1974**, *81*, 141485w.

(15) Frisch, M. J.; Trucks, G. W.; Head-Gordon, M.; Gill, P. M. W.; Wong, M. W.; Foresman, J. B.; Johnson, B. G.; Schlegel, H. B.; Robb, M. A.; Replogle, E. S.; Gomperts, R.; Andres, K.; Raghavachari, K.; Binkley, J. S.; Gonzales, C.; Martin, R. L.; Fox, D. J.; DeFrees, D. J.; Baker, J.; Stewart, J. J. P.; Pople, J. A. *GAUSSIAN 92*; Gaussian Inc.: Pittsburgh, PA, 1992.

(16) Cheng, B.-M; Lee, J.-W.; Lee, Y.-P. *J. Phys. Chem.* **1991**, *95*, 2814.

(17) McGrath, M. P.; Francl, M. M.; Rowland, F. S.; Hehre, W. J. *J. Phys. Chem.* **1988**, *92*, 5352.

0002-7863/94/1516-7423$04.50/0

Table 1. Observed and Calculated Vibrational Modes for Peroxynitrite (cm^{-1})[a]

		observed		calculated[d]	
mode	isomer	IR[b]	Raman[c]	$O^{14}NOO^-$	$O^{15}NOO^-$
ν(N=O)	*trans*	1496 (*f*)[e]	1483 (1448)	1488	1460
ν(N—O)	*trans*	1224 (1199)	*f*	1221	1198
ν(OO)	*trans*	932 (927)	955 (945)	947	947
δ(ONOO)	*cis*	809 (797)	813 (803)	847	846
δ(ONOO)	*trans*	*f*	645 (635)	663	661

[a] Bands in parentheses are for the ^{15}N-labeled anion. [b] Measured either as a mull in Nujol or in Kel-F between sodium chloride plates. [c] Measured as a fine powder under nitrogen with 514.5-nm excitation radiation. [d] Calculated by *ab initio* techniques at the Møller–Plesset level (MP2) with a 6-311G* basis set.[15] All listed bands are in plane modes with *A′* symmetry. [e] Measured as a fluorocarbon mull. [f] Not observed or obscured by bands due to the tetramethylammonium cation.

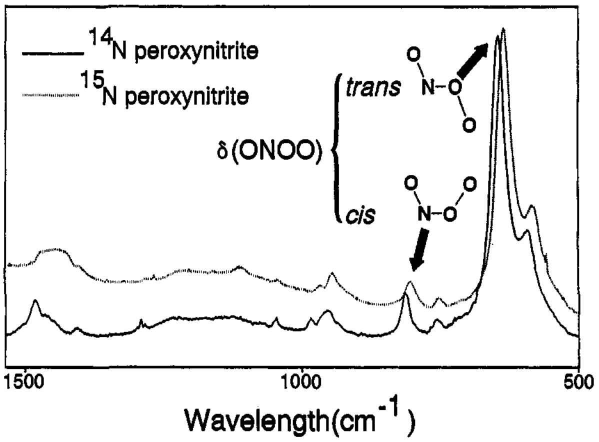

Figure 1. Raman spectra for solid-phase tetramethylammonium nitrite with 514.5-nm excitation.

oxoanions of nitrogen, protonation of peroxynitrite increases the ν(N=O) band by *ca.* 200 cm^{-1}, a result of the greater energy separation in the two modes associated with the nitrogen–oxygen stretches. Significantly, the ν(O—O) bands for the base and its conjugate acid are very similar and are typical of peroxo compounds.[18]

In order to help assign the IR and Raman spectra, we have performed *ab initio* calculations at the MP2(FU)/6-311G* level of theory.[15] The *trans* geometry is the global minimum, 2.9 kcal mol^{-1} more stable than the *cis* configuration.[19] In contrast, the structural, spectroscopic, and theoretical results for the thio analogues of peroxynitrite, $SNSS^-$ and $ONSS^-$, indicate a *cis* ground-state geometry.[21a–c] The computed vibrational frequencies for the MP2 level of theory are contrasted with the observed values for tetramethylammonium peroxynitrite in Table 1.

^{15}N-labeled peroxynitrite is readily prepared by the method in eq 1, and the product has a single peak in the ^{15}N NMR spectrum at 203.3 ppm in liquid ammonia at −60 °C and 191.4 ppm in basic water at room temperature.[22] These shifts are similar to those of the nitrite anion in these media (251.0 and 244.8 ppm, respectively) and indicate that the deshielded nitrogen has a bent geometry. That only a single peak is present in these spectra also indicates that either the *cis* and *trans* conformers interconvert rapidly on the NMR time scale or one of the two conformers is markedly stablized by solvation.

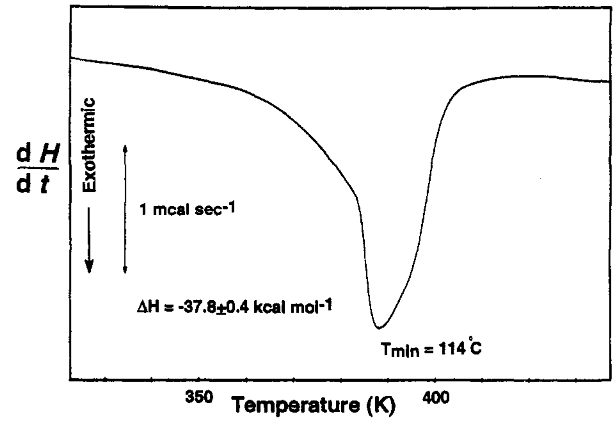

Figure 2. Differential scanning calorimeter trace for the exothermic decomposition of tetramethylammonium peroxynitrite.

As measured by differential scanning calorimetry, Figure 2, tetramethylammonium peroxynitrite exothermically decomposes at *ca.* 110 °C, with $\Delta H = -37.8 \pm 0.4$ kcal mol^{-1}.[23] When this reaction is followed by thermal gravimetric analysis, less than 0.04% of the sample is lost as gas. Raman and IR spectroscopies of the residual colorless powder are consistent with the sole product being tetramethylammonium nitrate; there is no detectable nitrite present. This enthalpy is similar to one measured for the solution-phase decomposition of pernitrous acid, $\Delta H = -38.8 \pm 2$ kcal mol^{-1},[24] and lends support to a recent thermodynamic analysis of this reaction.[25] Experiments are currently underway to determine the mechanism and product distribution of this isomerization as a function of pH and metal ion and will be described elsewhere.

Note Added in Proof: The Raman spectrum of peroxynitrite in water has recently been described.[26]

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(18) Silbert, L. S. In *Organic Peroxides*; Swern, D., Ed.; Wiley-Interscience: New York, 1971; Vol. II, p 637.

(19) Optimized geometries for the *trans* and *cis* (in braces) geometries: O=N 1.242{1.236} Å; N—O 1.314{1.335} Å; O—O 1.406{1.360} Å; O–N–O 111.1 {115.5}°; N–O–O 112.7 {118.1}°. Calculated absolute energies *E* (in au) for MP2/6-311G* calculations: *trans*-$ONOO^-$, −278.880 533 775; *cis*-$ONOO^-$, −278.875 932 897. The optimized geometries and relative energies of the two conformers have trends similar to those reported by Schaefer *et al.* for Hartree–Fock SCF calculations with STO-3G, DZ, and DZP basis sets.[20]

(20) Shen, M.; Xie, Y.; Schaefer, H. F., III; Deakyne, C. A *J. Chem. Phys.* **1990**, *93*, 3379.

(21) (a) Bojes, J.; Chivers, T.; Laidlaw, W. G.; Trsic, M. *J. Am. Chem. Soc.* **1982**, *104*, 4837. (b) Seel, F.; Kuhn, R.; Simon, G.; Wagner, M.; Krebs, B.; Dartmann, M. *Z. Naturforsch. B* **1985**, *40b*, 1607. (c) Seel, F.; Wagner, M. *Z. Naturforsch.* **1985**, *40b*, 762.

(22) Referenced to 40% $H^{15}NO_3$ as an external standard.

(23) Differential scanning calorimetry data are for lyophilized, moisture-free, solid samples of tetramethylammonium peroxynitrite sealed in aluminum capsules and heated at 5 deg min^{-1} on a Perkin-Elmer DSC-2 calibrated against indium.

(24) Ray, J. D. *J. Inorg. Nucl. Chem.* **1962**, *24*, 1159.

(25) Koppenol, W. H.; Moreno, J. J.; Pryor, W. A.; Ischiropoulos, H.; Beckman, J. S. *Chem. Res. Toxicol.* **1992**, *5*, 834.

(26) Tsai, J.-H. M.; Harrison, J. G.; Martin, J. C.; Hamilton, T. P.; van der Woerd, M.; Jablonsky, M. J.; Beckman, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 4115.