# Invention of the First Azo Compound To Serve as a Superoxide Thermal Source under Physiological Conditions: Concept, Synthesis, and Chemical Properties[1]

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Received August 18, 1997

In quantitative terms, the superoxide radical anion ($O_2^{\bullet-}$) is the most important radical formed in aerobic organisms.[3] Indeed, a significant fraction (1−4%) of the oxygen metabolized in the mitochondrial respiratory chain escapes complete 4-electron reduction to water after accepting the initial electron. Superoxide is also formed *in vivo* as a direct (or side) consequence of various enzymatic processes[4,5] and by the autoxidation of a number of biologically significant compounds.[6] Although superoxide can inactivate certain enzymes,[7] it is very unreactive in typical free radical reactions such as hydrogen atom abstraction. However, its conjugate acid, the hydroperoxyl radical ($pK_a \approx 4.7$), has a hydrogen-atom-abstracting ability comparable to that of the alkylperoxyl radicals which are responsible for lipid peroxidation.[11] Superoxide and the reactive
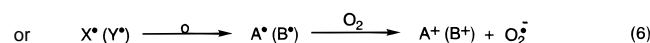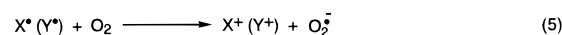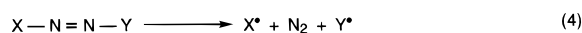
$$O_2 + e^- \longrightarrow O_2^{\bullet-} \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} HOO^{\bullet} \qquad (1)$$
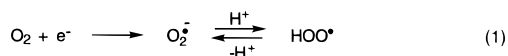
oxygen species derived therefrom ($HOO^{\bullet}$, H_2O_2, and $HO^{\bullet}$) are believed to be responsible for a host of pathological processes ranging from simple inflammation, through atherosclerosis and DNA damage, all the way to the very aging process itself.[12,13] Indeed, aerobic life would not be possible without Nature's defenses against superoxide.[14] These defenses are provided by enzymes known as superoxide dismutases (SODs) which catalyze the dismutation of superoxide to oxygen and hydrogen peroxide (which is then dismutated by catalase, CAT, to water and oxygen).

$$2\,O_2^{\bullet-} + 2\,H^+ \longrightarrow O_2 + H_2O_2 \qquad (2)$$

$$2\,H_2O_2 \xrightarrow{\text{CAT}} 2\,H_2O + O_2 \qquad (3)$$
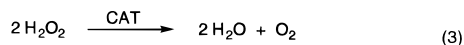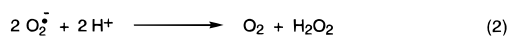
Various methods have been developed for generating superoxide for *in vitro* and *ex vivo* studies. These methods either produce superoxide "instantaneously"[15] or over relatively short

periods of time.[16] Unfortunately, such methods do not mimic the *in vivo* situation where superoxide is produced very slowly but continuously over the entire lifetime of a cell, and it should be remembered that some cells (e.g., neurons) are as old as the organism itself. Thus, current methods generate superoxide in relatively high concentrations which will favor reaction 2, the uncatalyzed bimolecular dismutation. The biological damage due to superoxide itself may therefore be partly or wholly disguised by damage due to H_2O_2 and the highly reactive $HO^{\bullet}$ radical.[16]

To overcome the potential problems inherent in earlier methods, we have invented a procedure for generating superoxide at a known, slow and well-defined rate by the thermal (or, if desired, photochemical) decomposition of a suitable precursor. These superoxide thermal sources (SOTSs) are azo compounds which decompose to yield, *either directly or indirectly,* electron-rich, carbon-centered radicals, many of which are known to react with dioxygen to yield carbocations and superoxide.[19]

$$X-N=N-Y \longrightarrow X^{\bullet} + N_2 + Y^{\bullet} \qquad (4)$$

$$X^{\bullet}\,(Y^{\bullet}) + O_2 \longrightarrow X^+\,(Y^+) + O_2^{\bullet-} \qquad (5)$$

$$\text{or} \quad X^{\bullet}\,(Y^{\bullet}) \xrightarrow{\;o\;} A^{\bullet}\,(B^{\bullet}) \xrightarrow{O_2} A^+\,(B^+) + O_2^{\bullet-} \qquad (6)$$

The first of a novel family of compounds which meets these stringent requirements is di(4-carboxybenzyl)hyponitrite, **SOTS-1**, which was synthesized from α-bromotoluic acid by esterification, reaction with silver hyponitrite[20] and de-esterification.[21] The decomposition of **SOTS-1** in water at physiological temperature (37 °C) and pH 7 (where **SOTS-1** is ionized) occurs by the mechanism shown in Scheme 1.

The critical step in the overall reaction involves an unusual 1,2-H-atom shift[22] (reaction 9) which converts the primary alkoxyl radical formed from the hyponitrite into the desired electron-rich carbon-centered radical, $^-O_2CC_6H_4\dot{C}HOH$. This

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(3) The first report on superoxide in biology involved the demonstration that the aerobic xanthine/xanthine oxidase (X/XO) system produced $O_2^{\bullet-}$.[4] A 1995 review by Fridovich cites 54 (!) earlier reviews on the biology of $O_2^{\bullet-}$ and the superoxide dismutase enzymes which remove it.[5]

(4) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1968**, *243*, 5753−5760.

(5) Fridovich, I. *Ann. Rev. Biochem.* **1995**, *64*, 97−112.

(6) E.g., ascorbate, thiols, sugars, ubiquinol, etc.

(7) E.g., 6-phosphogluconate dehydratase,[8] aconitase,[9] and ribonucleotide reductase[10] (the rate-limiting enzyme for DNA synthesis).

(8) Gardner, P. R.; Fridovich, I. *J. Biol. Chem.* **1991**, *266*, 1478−1483.

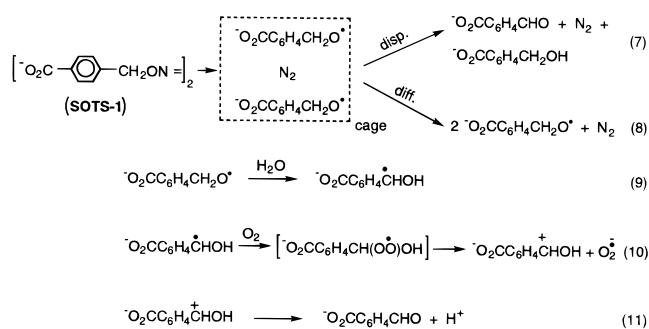(9) Gardner, P. R.; Fridovich, I. *J. Biol. Chem.* **1991**, *266*, 19328−19333. Flint, D. H.; Tuminello, J. F.; Emptage, M. H. *J. Biol. Chem.* **1993**, *268*, 22369−22376. Hausladen, A.; Fridovich, I. *J. Biol. Chem.* **1994**, *269*, 29405−29408.

(10) Gaudu, P.; Nivière, V.; Pétillot, Y.; Kaupi, B.; Fontecave, M. *FEBS Lett.* **1996**, *387*, 137−140.

(11) Zaikov, G. E.; Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1969**, *47*, 3017−3029. Korcek, S.; Chenier, J. H. B.; Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1972**, *50*, 2285−2297.

(12) See references cited in ref 5.

(13) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 2nd ed.; Clarendon Press: Oxford, U.K. 1989. *Oxidative Stress*; Sies, H., Ed.; Academic: London, U.K. 1985. *Oxidative Stress, Oxidants and Antioxidants*; Sies, H., Ed.; Academic: London, U.K. 1991.

(14) See, e.g., ref 5 and Longo, V. D.; Gralla, E. B.; Valentine, J. S. *J. Biol. Chem.* **1996**, *271*, 12275−12280.

(15) E.g., by addition of organic solutions of KO_2 and a crown ether to the aqueous medium or by pulse radiolysis of formate solutions.

(16) The method most favored by biochemists is the traditional aerobic X/XO system[3,4] (or aerobic acetaldehyde (AA)/XO).[17] However, at high XO concentrations, the reaction is soon over because of a relatively rapid depletion of the substrate. In some systems there may also be a deactivation of the XO. In addition, commercial XO may be contaminated with iron which can initiate hydroxyl radical chemistry by reaction with the H_2O_2 formed in reaction 2. As a consequence, any biological effects of $O_2^{\bullet-}$ can be masked by effects due to the much more aggressive $HO^{\bullet}$ radical. Heroic efforts to remove XO-associated iron and to chelate any remaining iron have been reported,[17,18] but its complete absence in a particular XO preparation is, of course, impossible to prove.

(17) Dix, T. A.; Hess, K. M.; Medina, M. A.; Sullivan, R. W.; Tilly, S. L.; Webb, T. L. *Biochemistry* **1996**, *35*, 4578−4583.

(18) Lloyd, R. V.; Mason, R. P. *J. Biol. Chem.* **1990**, *265*, 16733−16736. Britigan, B. E.; Pou, S.; Rosen, G. M.; Lilleg, D. M.; Buettner, G. R. *J. Biol. Chem.* **1990**, *265*, 17533−17538.

(19) von Sonntag, C.; Schuchmann, H.-P. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1229−1253.

(20) Ogle, C. A.; Martin, S. W.; Dziobak, M. P.; Urban, M. W.; Mendenhall, G. D. *J. Org. Chem.* **1983**, *48*, 3728−3733.

(21) Following standard procedures, α-bromotoluic acid was converted to its acid chloride which was reacted with benzyl alcohol to produce the benzyl ester which was then converted to the hyponitrite. Ester cleavage was performed under aqueous basic conditions at 0 °C to yield **SOTS-1**: ^{1}H NMR (600 MHz, Na phosphate buffer in D_2O, 278 K, acetone as internal standard) δ 7.90−7.86 (2H, m (AB system), arom H), 7.47−7.43 (2H, m (AB system), arom H), 5.35 (2H, s, CH_2); ^{13}C NMR (150 MHz, Na phosphate buffer in D_2O, 278 K, acetone as internal standard) δ 176.3 (*C*OOH), 139.5 and 137.1 (arom *C*−C), 130.0 and 129.3 (arom *C*−H), 75.7 (CH_2). Full details of the synthesis will be published later.

(22) Gilbert, B. C.; Holmes, R. G. G.; Laue, H. A. H.; Norman, R. O. C. *J. Chem. Soc., Perkin Trans. 2* **1976**, 1047−1052.

Scheme 1

$$^-O_2CC_6H_4CH_2O^\bullet \xrightarrow{disp.} {}^-O_2CC_6H_4CHO + N_2 + {}^-O_2CC_6H_4CH_2OH \quad (7)$$

$$\xrightarrow{diff.}\ 2\ ^-O_2CC_6H_4CH_2O^\bullet + N_2 \quad (8)$$

$$^-O_2CC_6H_4CH_2O^\bullet \xrightarrow{H_2O} {}^-O_2CC_6H_4\overset{\bullet}{C}HOH \quad (9)$$

$$^-O_2CC_6H_4\overset{\bullet}{C}HOH \xrightarrow{O_2} \left[^-O_2CC_6H_4CH(O\overset{\bullet}{O})OH\right] \longrightarrow {}^-O_2CC_6H_4\overset{+}{C}HOH + O_2^{\bullet-} \quad (10)$$

$$^-O_2CC_6H_4\overset{+}{C}HOH \longrightarrow {}^-O_2CC_6H_4CHO + H^+ \quad (11)$$

class of radical rearrangements only occurs in the presence of water (or alcohols).

The solubility of **SOTS-1** in water at pH 7 is ca. 1.5 mM at room temperature. It decays with first-order kinetics, and its half-life in water at physiological pH (6.5−8) and temperature (37 °C) was found to ca. 4900 s by three independent procedures:[23] (i) ^{1}H NMR (in D_2O in the presence of O_2, the *only* products detected were the expected (Scheme 1) alcohol and aldehyde which were formed in yields of roughly 40 and 60%, respectively); (ii) spectrophotometry at 350 nm using tetranitromethane;[19,25,26a] (iii) spectrophotometry at 550 nm using Fe^{III} cytochrome c.[26a] Methods ii and iii depend on the formation of $O_2^{\bullet-}$ and have a simple 1:1 stoichiometry, reactions 12 and 13.

$$O_2^{\bullet-} + C(NO_2)_4 \longrightarrow O_2 + NO_2 + {}^-C(NO_2)_3 \quad (12)$$

$$O_2^{\bullet-} + Fe^{III}\ Cyt\ c \longrightarrow O_2 + Fe^{II}\ Cyt\ c \quad (13)$$
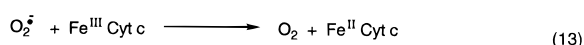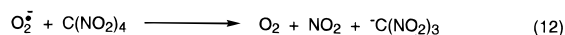
These two reactions therefore provide a method for determining the molar efficiency, $2e$,[27] of superoxide formation from **SOTS-1** (i.e., $2e$ = moles of $O_2^{\bullet-}$/mole of **SOTS-1**) since the extinction coefficients of the absorbing species are known, viz., $\epsilon_{350} = 15\,000$ M^{-1} cm^{-1} for $^-C(NO_2)_3$[25b] and ϵ_{550} (reduced−oxidized) = 21 000 M^{-1} cm^{-1} for cytochrome c.[28] Comparison of the initial rates (first 5−10 min) of growth of these absorptions with the measured initial rate of **SOTS-1** decomposition gave $2e = 0.46 \pm 0.10$ and 0.32 ± 0.06 for $^-C(NO_2)_3$ and cytochrome c, respectively, while the maximum absorbance reached after ca. 6 h gave $2e = 0.42 \pm 0.006$ and 0.44 ± 0.02, respectively. We conclude that $2e = 0.41$ which is ca. 20% of its theoretical maximum value (in the absence of cage effects) of 2.0.

(23) The $O_2^{\bullet-}$-induced absorption at 530 nm using nitro blue tetrazolium[24] was useful only in a diagnostic sense. With both **SOTS-1** and XO/AA superoxide sources, this absorption grows in for only a few minutes, perhaps because the absorbing species is insoluble.

(24) Beauchamp, C.; Fridovich, I. *Anal. Biochem.* **1971**, *44*, 276−287. Bielski, B. H. J.; Shiue, G. G.; Bajuk, S. *J. Phys. Chem.* **1980**, *84*, 830−833.

(25) (a) Rabani, J.; Mulac, W. A.; Matheson, M. S. *J. Phys. Chem.* **1965**, *69*, 53−70. (b) Bielski, B. H. J.; Allen, A. O. *J. Phys. Chem.* **1967**, *71*, 4544−4549.

(26) (a) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049−6055. (b) In ref 26a, a solution of $O_2^{\bullet-}$ in dimethylformamide was infused into aqueous $C(NO_2)_4$ at 25 °C, and the growth of the 350 nm absorption was shown to be retarded by SOD. The critical difference between the McCord and Fridovich experiment with $C(NO_2)_4$/SOD and our own using both **SOTS-1** and the XO/AA couple has not yet been explored. However, Professor Fridovich has kindly suggested that it relates to the fact that we were unable in our experiments to add sufficient SOD to compete with the very fast $O_2^{\bullet-}$/$C(NO_2)_4$ reaction. This problem does not occur in the cytochrome c experiments because its reaction with $O_2^{\bullet-}$ is very much slower.

(27) The efficiency of escape of the initial radical pair from the solvent cage is e $(=k_8/(k_7 + k_8))$.

(28) Massey, V. *Biochim. Biophys. Acta* **1959**, *34*, 255−256.

Further confirmation of the chemistry shown in Scheme 1 was obtained by 266 nm laser flash photolysis (LFP). Esters (ethyl, *tert*-butyl) of **SOTS-1** in acetonitrile subjected to LFP showed the expected[29] absorption in the visible at 460 nm which is not quenched by O_2 and is due to the benzyloxyl radicals, $ROC(O)C_6H_4CH_2O^\bullet$. By way of contrast, in the absence of O_2 LFP of **SOTS-1** in phosphate buffer, pH 7.6 (and in acetonitrile/water) showed no sign of the benzyloxyl radical but instead gave an instantaneous absorption at 320 nm with a shoulder at 360 nm and broad, weaker absorption centered at 515 nm. The 320 nm absorption disappears upon LFP at pH 12 in the absence of O_2 leaving behind the 360 and 515 nm absorptions (ratio of O.D.'s, 4:1). The 320 nm absorption is obviously due to the ketyl radical, $^-O_2CC_6H_4\overset{\bullet}{C}HOH$, and the 360 and 515 nm absorptions are due to the corresponding anion radical, $^-O_2CC_6H_4\overset{\bullet}{C}HO^-$. We estimate that these two species are present in approximately equal concentrations at pH 7.6. All three of these absorptions are quenched by O_2, implying that they do, indeed, arise from carbon-centered radicals.

Seeking final confirmation that **SOTS-1** did indeed generate superoxide, we examined the effect of (Cu/Zn) SOD and catalase on reaction 12. As would be expected, the initial rate of growth of the 350 nm absorption due to $^-C(NO_2)_3$ at 37 °C was unaffected by catalase (cf., reaction 3). However, and to our dismay in view of pioneering studies of McCord and Fridovich on $O_2^{\bullet-}$/$C(NO_2)_4$/SOD,[26] the initial rate of growth of this absorption was completely unaffected by the addition of SOD (cf., reaction 2). Our immediate presumption was that **SOTS-1** did not yield superoxide. This presumption was eventually demonstrated to be unfounded because the AA/XO induced growth of the $^-C(NO_2)_3$ absorption was also unaffected by SOD[26b] (or catalase). Furthermore, the **SOTS-1** induced growth of the reduced cytochrome c absorption (reaction 13) was, indeed, inhibited by SOD in a dose-dependent manner[26b] (and was unaffected by catalase).

In summary, the invention of **SOTS-1** provides a unique and novel tool for investigating superoxide-dependent oxidative stress under well-defined, metal ion free, biomimetic conditions. We are currently comparing the effect of equal fluxes of superoxide from **SOTS-1** and the aerobic XO/AA couple on strand scission of *Escherichia coli* plasmid DNA (both super-coiled and a short linear restriction fragment). We are also investigating the effect of superoxide on certain cell lines in culture by adding **SOTS-1** to the culture medium, by injecting it directly into the cells and (hopefully) by having it irreversibly transported into the cells by adding it as its methyl ester in DMSO to the medium. The ability of **SOTS-1** to initiate the peroxidation of lipids, including lipoproteins, dispersed in water is also under study. We believe that **SOTS-1** (and other **SOTS**) will provide new insights into the role of superoxide in biological systems.

(29) Avila, D. V.; Ingold, K. U.; Di Nardo, A. A.; Zerbetto, F.; Zgierski, M. Z.; Lusztyk, J. *J. Am. Chem. Soc.* **1995**, *117*, 2711−2718.