Nitro-Triarylmethyl Radical as Dual Oxygen and Superoxide Probe

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Abstract

Superoxide radical is involved in numerous physiological and pathophysiological processes. Tetrathiatriarylmethyl (TAM) radicals are knows to react with superoxide allowing measurement of superoxide production in biological media. We report the synthesis of a Nitro conjugated TAM radical showing a rate constant of $7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ which is two order of magnitude higher than other TAMs allowing high sensitivity measurement of superoxide

Superoxide radical formed by one electron reduction of molecular oxygen is one of the most biologically significant reactive oxygen species (ROS) involved in numerous physiological and pathophysiological processes. Trityl radicals have been recognized for their ability to react with superoxide allowing the assessment of superoxide production by electron paramagnetic resonance (EPR) or spectrophotometry in a biological media.[1] The chemical mechanism involved depends on the family of trityl radical used. Superoxide radical can add to a tetrathiatriarylmethyl radical (TAM) like **CT-03** or **Ox063** (Figure 1) in its para-position triggering the decarboxylation and leading to an EPR silent quinone-methide (QM) product. [2] This reaction occurs with a decay of the EPR signal. The apparent second order rate constants for **Ox063** and **CT-03** were reported to be about $3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ at physiological pH (7.4).[1, 3] The **CT-02H** (see Figure 1) derivative of TAM family where a carboxylic acid have been substituted by an hydrogen atom shows an increased reactivity toward superoxide with the rate constant equals to $1.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$.[3] In this molecule the superoxide radical reacts at the para-position substituted by the hydrogen. The increase reactivity can be explained by a lower steric hindrance and a lower electrostatic repulsion between the superoxide and the carboxylate anion.

The reactivity of superoxide with a perchlorinated trityl (**PCT**) involved a reduction of the radical by superoxide leading to an EPR silent perchlorotriarylmethane. This reaction have

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been reported to be selective to superoxide as the radical exhibits high stability toward other reducing agents such as ascorbic acid. The selectivity could be explain by the steric hindrance of the paramagnetic center only accessible to small reducing agent. The bimolecular rate constant of this reaction was measured to be equal to $8.3 \times 10^8 \, M^{-1}s^{-1}$ making the superoxide measurement by EPR using **PCT** highly sensitive.

However, the intrinsic linewidth of a **PCT** radical is 500 mG[4] while it is less than 100 mG for a TAM radicals and less than 50 mG when the hydrogen nuclei of the methyl groups are substituted with deuterium nuclei.[5] Narrow linewidth provides a higher sensitivity of detection (the intensity is inversely proportional to the square of the linewidth) and is of primary importance for *in vivo* EPR oximetry.[6, 7] Moreover, no *in vivo* application of water soluble **PCT** radicals have been reported yet while TAM radicals have been extensively used *in vivo* and are considered to be remarkably nontoxic. In this work we explore an opportunity to combine the redox properties of the **PCT** radical with the narrow linewidth of TAM radical by introducing electron-withdrawing group in TAM structure which should increase the reduction potential and make a tetrathiatriarylmethyl radical reducible by the superoxide.

Materials and methods

Chemicals

Hypoxanthine (HX), xanthine oxidase (XO), diethylenetriaminepentaacetic acid (DTPA), Cu-Zn superoxide dismutase (SOD) and all solvents were purchased from Sigma-Aldrich. Potassium hexachloroiridate (IV) was purchased from Combi-Block. ^{15}N-labeled sodium nitrite was purchased from Cambridge isotope. HyperSep C18 columns were purchased from Thermo Scientific.

Synthesis

CT-03 was synthesized according to previously reported procedure[8]. Nitro-TAM (see Scheme 1) was synthesized by dissolving **CT03** sodium salt (100 mg, 0.09 mmol, 1eq) in 50 mL of water, K_2IrCl_6 (46.3 mg, 0.09mmol, 1eq) was added and the solution was stirred for 10 min. $NaNO_2$ (62 mg, 0.9 mmol, 10 eq) was added in one portion. The solution turned red and was stirred for 1h after that, aqueous HCl was added (10 mL, 1M) and compound extracted with ethyl acetate (3×10 mL). The combined organic layers were dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude residue was purified on HyperSep C18 column (10g/75 mL) using a gradient of elution from water:acetonitrile (20:80) containing 0.1 % of trifluoroacetic acid (TFA) to acetonitrile containing 0.1 % of TFA. The Nitro-TAM was isolated in 31 % yield. 42 % of **CT-03** was recovered. ^{15}N labeled mono-nitro TAM (**nTAM**) was synthesized using the same procedure using ^{15}N-labeled sodium nitrite.

Cyclic Voltammetry

The cyclic voltammograms was recorded using a computer controlled Metrohm Autolab PGSTAT128N Potentiostat. 5 mL of 500 μM of **nTAM** in 0.1M phosphate buffer (PB), pH = 7.4, was place in the electroanalytical cell. Oxygen was removed by bubbling argon for 10

min. A glassy carbon electrode was used as working electrode, a platinum wire as counter electrode and an Ag/AgCl 3M NaCl as reference electrode. Scan rate was 0.2V/s. Starting potential 0 V, lower potential −0.4 V, upper potential +0.8V.

HPLC

All chromatograms were recorded on a Waters Alliance e2695 system equipped with a 2998 PDA detector using an XBridge 4.6 mm × 50 mm, 2.5 μM. Solvent A was water, solvent B acetonitrile and Solvent C water containing 1% of TFA. The condition of elution were as following: flow rate = 1.5 mL/min, column temperature = 40°C, t=0min 90%A/10%B/ 10%C, t=5min 0%A/90%B,10%C, t=6min 0%A/100%B/0%C. Run time = 8 min, UV detection from 200 nm to 800 nm.

EPR spectroscopy

All spectra were recorded on a Bruker X-Band EMX EPR spectrometer, the samples were loaded in glass capillaries 50 μL. For spectra recorded in deoxygenated solution, the samples were bubbled with argon gas for 15 min before loading into capillaries. Typical EPR parameters were as following: sweep width, 2.5 G; power, 0.2mW; modulation frequency, 10 KHz; modulation amplitude, 0.05G; conversion time, 20.48 ms; time constant, 40.96 ms; resolution, 1024 pt.

UV spectroscopy

UV-Vis spectra were recorded on Cary 50 UV-Vis spectrometer.

Determination of rate constant with superoxide

Estimation of bimolecular rate constant of ^{15}N-substituted Nitro-TAM (**nTAM**) reduction by superoxide was determined by standard competition approach using superoxide dismutase as competitor. In this experiment superoxide is generated by xanthine oxidase with the rate V(XO) and is consumed in the competing reactions of superoxide ($O_2^{\cdot-}$) with **nTAM** and superoxide dismutase (bimolecular rate constants, k and k_{SOD}, correspondingly):

$$\frac{dO_2^{\cdot-}}{dt} = V(XO) - k \cdot [nTAM] \cdot [O_2^{\cdot-}] - k_{SOD} \cdot [SOD] \cdot [O_2^{\cdot-}] \tag{1}$$

In the steady-state conditions, the rate of superoxide-induced nTAM reduction measured in the absence of SOD, V(no SOD), is equal to the rate of superoxide generation, V(XO), therefore:

$$V(no\,SOD) = V(XO) \tag{2}$$

$$[O_2^{\cdot-}] = \frac{V(no\,SOD)}{k \cdot [nTAM] + k_{SOD} \cdot [SOD]} \tag{3}$$

$$\frac{V(no\,SOD)}{V(SOD)} - 1 = \frac{k_{SOD} \cdot [SOD]}{k \cdot [nTAM]} \quad (4)$$

where V(SOD) – the rate of **nTAM** reduction at given SOD concentration.

The linear fitting of the experimental data on inhibition of superoxide-induced **nTAM** reduction by SOD using equation (4) and assuming the value of k_{SOD} of $2 \cdot 10^9$ $M^{-1}s^{-1}$ [9]yields the value of bimolecular constant, k.

Results and discussion

Synthesis

Recently Decross et al. reported an elegant way to synthesize asymmetrical TAM radicals through the substitution of one carboxylic acid of **CT-03** by various –N, -S and –P nucleophiles.[10] This reaction involved the formation of the **CT-03** cation by a water soluble iridium (IV) complex. The addition of the nucleophile triggers the decarboxylation leading to the mono-functionalized TAM. However, when sodium nitrite was used, a mixture of mono-, di- and tri-nitro TAM was observed by HPLC-MS (Scheme 1).[10] Using an excess of oxidant, the non-water soluble tri-nitro compound was isolated. We hypothesized that the mono-nitro TAM should be an excellent candidate for being efficient superoxide trap due to the expected increase of the reduction potential in the presence of highly electron- withdrawing group and high aqueous solublity provided by the presence of two carboxyl groups in the structure

The **CT-03** was synthesized as previously reported and subsequently reacted with one equivalent of K_2IrCl_6 to procure quantitatively the trityl cation in water. Then, an excess of sodium nitrite was added.

The reverse phase HPLC chromatogram of the reaction crude showed a mixture of **CT-03**, mono-, di- and tri-nitro TAM in agreement with the literature (Figure 2, b). Purification on C18 column allowed isolation of the mono nitro-TAM (**Nitro-TAM**) in 31% yield. The purity was evaluated to 95% by HPLC (Figure 2, a).

Physicochemical characterization

The radical was stable in phosphate buffer pH = 7.4 as no degradation was observed after 24h at room temperature. The X-Band EPR spectrum was recorded in phosphate buffer (0.1 M, pH = 7.4). According to the chemical structure, the spectrum exhibits a triplet (1:1:1) pattern as a result of the interaction of the odd electron with the ^{14}N nucleus of the nitro moiety ($S_{N14}=1$) With the hyperfine splitting constant (hfc), a_N, equals to 440 mG (Figure 3a). In order to simplify the spectrum and increase the signal to noise ratio the ^{15}N labeled mono-nitro TAM (**nTAM**) was synthesized using the same procedure. As the ^{15}N nucleus possesses a spin of $S_{N15}=1/2$, the spectrum of **nTAM** exhibits a well resolved doublet with $a_N =610$ mG. The ratio of hfs for ^{15}N- and ^{14}N-substituted mono-nitro-TAMs of 1.4 is in a good agreement with the ratio of the gyromagnetic ratios of the two nitrogen isotopes.

The EPR spectrum of **nTAM** recorded under deoxygenated solution shows a linewidth of 120 mG. The linewidth increases linearly with the concentration of oxygen due to Heisenberg spin exchange-induced line broadening to reach 200 mG under normal air condition (pO_2=160 mmHg) allowing the measurement of dissolved molecular oxygen with a sensitivity of about 1 mmHg (80 mG broadening per 160 mmHg of pO_2). Note that **PCT** radical shows only 40 mG line broadening when compared in deoxygenated and normal air conditions. This could be explained by a larger contribution of an oxygen-insensitive Gaussian component in the EPR line shape of the **PCT** radical compared with that for **nTAM.**

The effect of electron-withdrawing nitro group on the redox potential was measured using cyclic votammetry (Figure 4). The cyclic votrammogram shows a reduction of the **nTAM** presumably to the trityl anion at a half-wave potential of −0.147 V. This results supports an expected effect of the nitro group on redox properties of TAM as it increases the reduction potential by 0.495 V compared with the **CT-03**. The **PCT** shows a redox half-wave a-0.320 V (Table 1). This comparison shows that the nTAM can be reduced more easily than the **PCT** and therefore should be reduced by superoxide anion (reduction potential, −0.523 versus Ag/AgCl). On positive potentials, the **nTAM** is oxidized to the cation with a half wave of +0.615 V. As expected the nitro group makes the oxidation of the radical more difficult. Redox potentials of **nTAM**, **CT-03** and **PCT** are summarized in the Table 1.

Reactivity with superoxide

In order to assess the reactivity of the mono-nitro-TAM derivatives toward superoxide, **nTAM** was incubated in hypoxanthine / xanthine oxidase superoxide generating system. The reaction was followed by EPR and spectrophotometrically. Upon the reaction, appearance of a peak around 610 nm in the UV-Vis spectrum was observed, (Figure 5), which might result from the formation of an **nTAM** anion (Scheme 2). No UV-Vis spectral change was observed after 2h at room temperature An absorption peak at 640 nm was previously reported for the TAM anion.[12]

EPR measurements in HX/XO superoxide generating system (Figure 6) show decay of the signal indicating that that **nTAM** is converted to an EPR-silent compound.

The second order rate constant measured using superoxide dismutase as competitor (see material and method for the details) has been found to be equal to 7×10^5 $M^{-1}s^{-1}$which is two order of magnitude higher than previously reported rate constants for the TAM radicals (Figure 7)

The high rate constant of the reaction with the superoxide and high aqueous solubility makes the **nTAM** useful probe for superoxide detection in biological systems.

Acknowledgments

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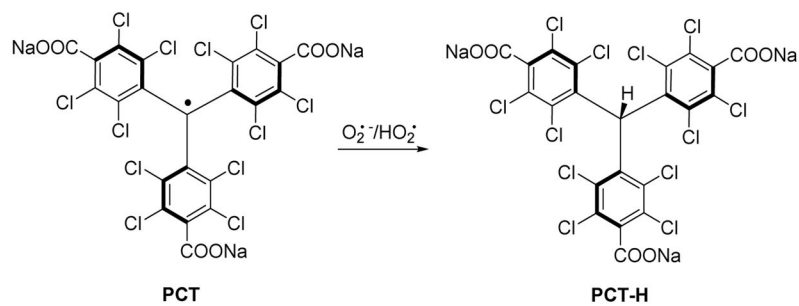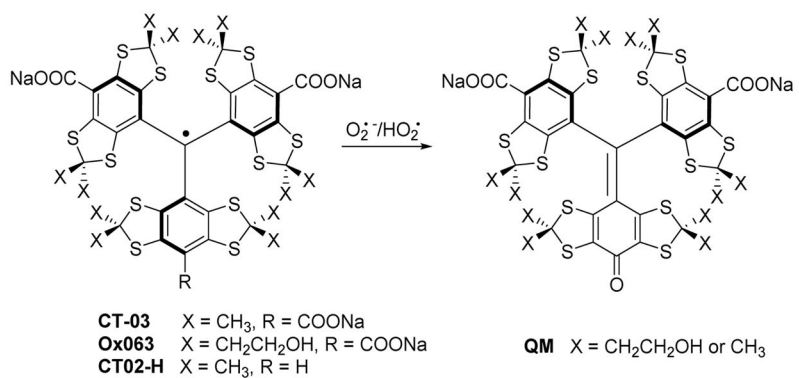
Figure 1.
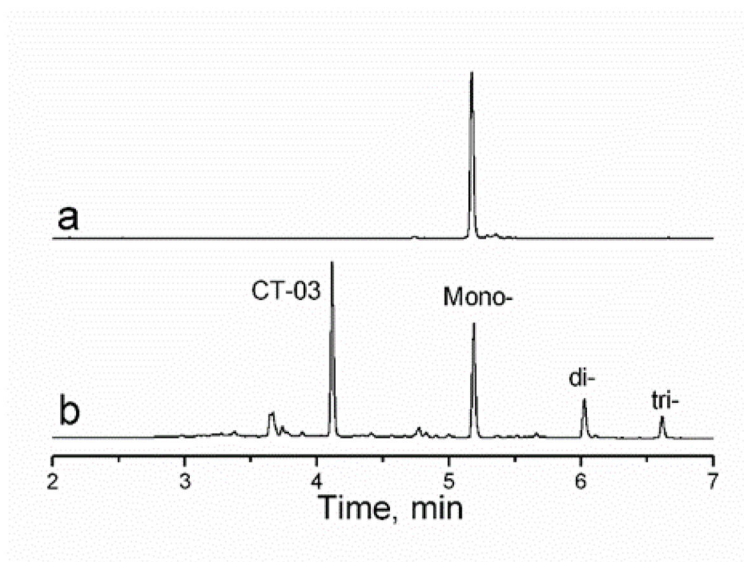Structure and reactivity of trityl radicals with superoxide.

Figure 2.
Reverse phase HPLC chromatograms. a) Purified mono-nitro-TAM (**Nitro-TAM**).. b)
Reaction crude showing the mixture of **CT-03**, mono-, di- and tri-nitro substituted TAM
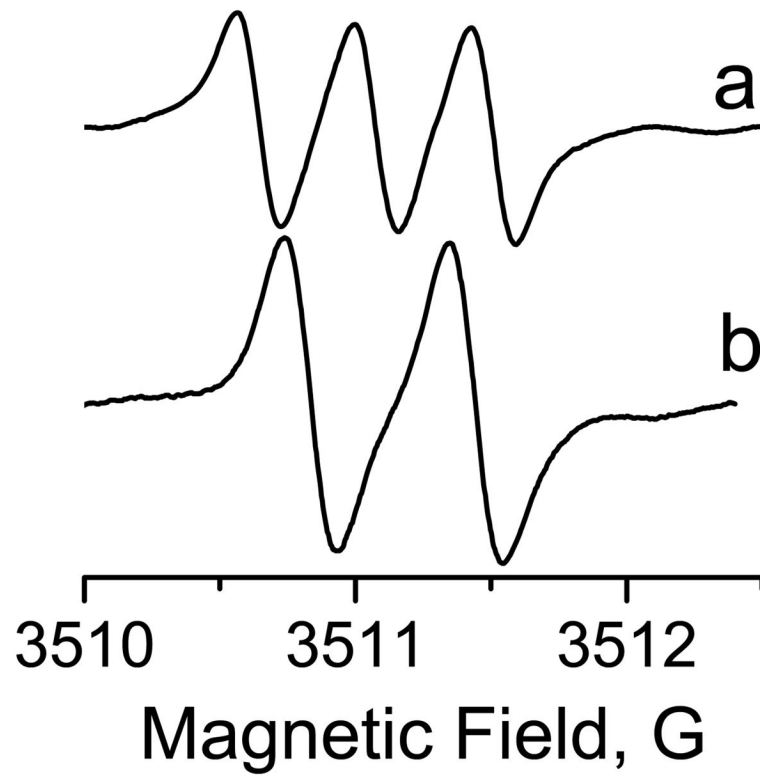
Figure 3.
X-band EPR spectra of ^{14}N (a) and ^{15}N (b) labeled **Nitro-TAMs** (100 μM) in 0.1 M phosphate buffer, pH 7.4, under normal air pressure.
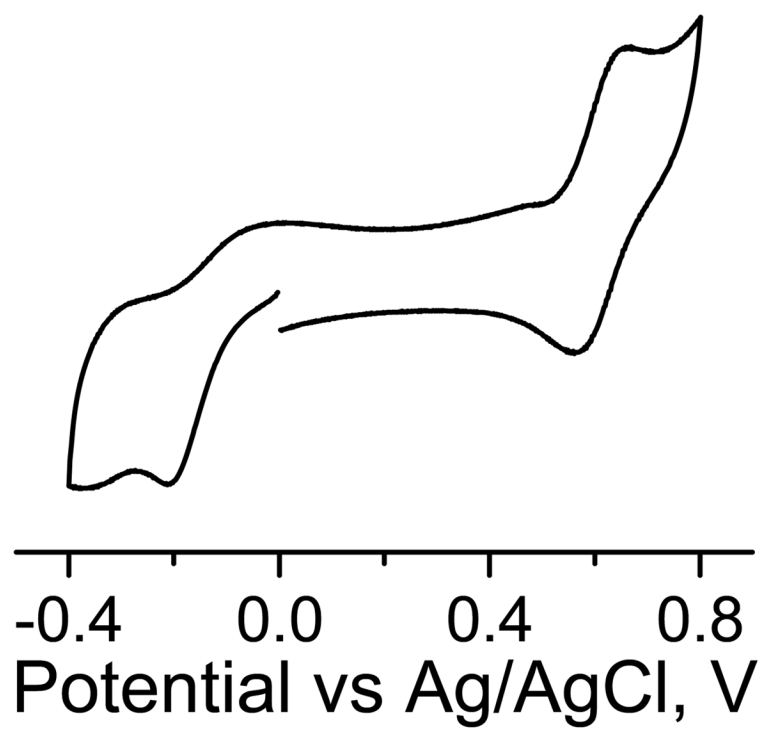
Figure 4.
Cyclic voltammogram of 1mM **nTAM** in phosphate buffer, pH = 7.4, scan rate = 0.2 V/s.
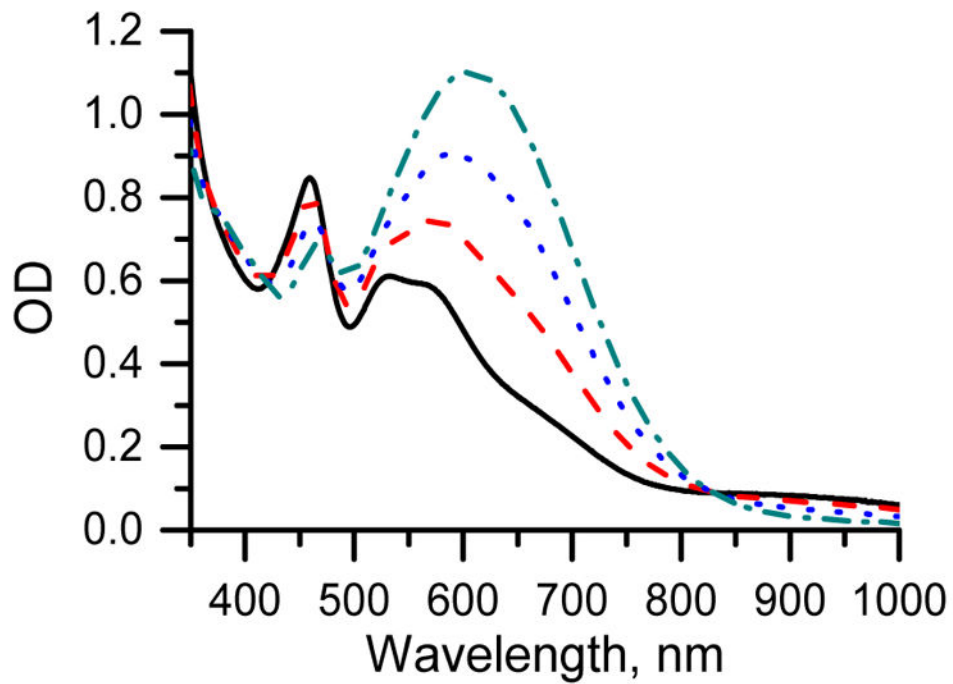
Figure 5.
The UV-Vis spectra of 100 μM **nTAM** in the presence of HX/XO superoxide generating system ([HX] =0.25 mM, [XO] =10 mU/ml) in 0.1 M phosphate buffer solution, pH = 7.4 (dotted lines corresponds to different incubation times of 6, 13 and 25 minutes). Solid line represents the UV-Vis spectrum of **nTAM** in the absence of HX/XO.
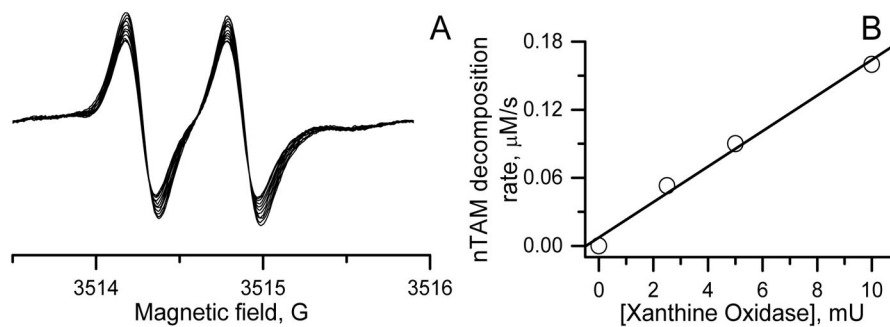
Figure 6.
A. The time evolution of the EPR spectra of 100 μM **nTAM** in superoxide generating system that contains 0.25 mM of hypoxanthine and 10 mU/ml of xanthine oxidase in 0.1 M phosphate buffer. **B.** The dependence of rate of **nTAM** EPR signal decay on concentration of xanthine oxidase.
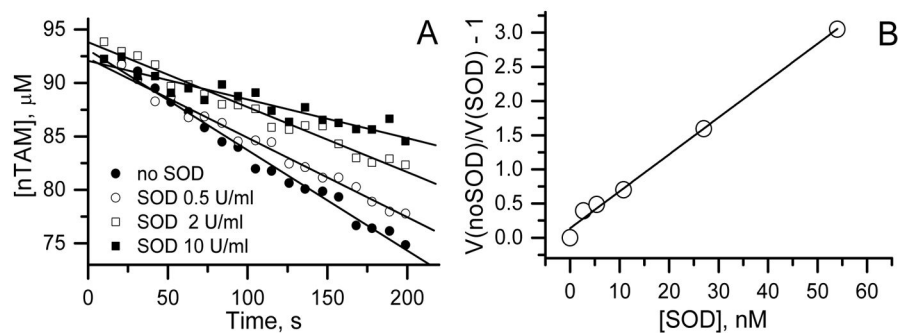
Figure 7.
A. The kinetics of EPR signal decay of **nTAM** radical upon superoxide generation in HX/XO system ([HX] =0.25 mM, [XO] =10 mU/ml) in the presence of different SOD concentrations. **B.** The dependence of relative efficiency of SOD-induced inhibition of **nTAM** reduction by superoxide on SOD concentration. Linear fit yields the bimolecular rate constant of **nTAM** reduction by superoxide equal to 7×10^5 M^{-1}s^{-1}. See Materials and Method Section for the details.
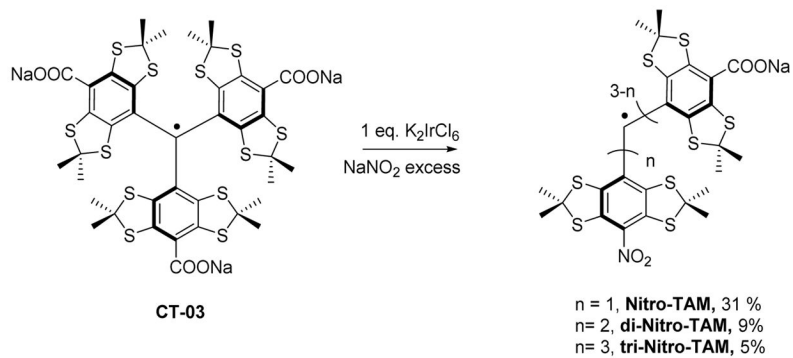
Scheme 1.
Synthesis of Nitro-TAM
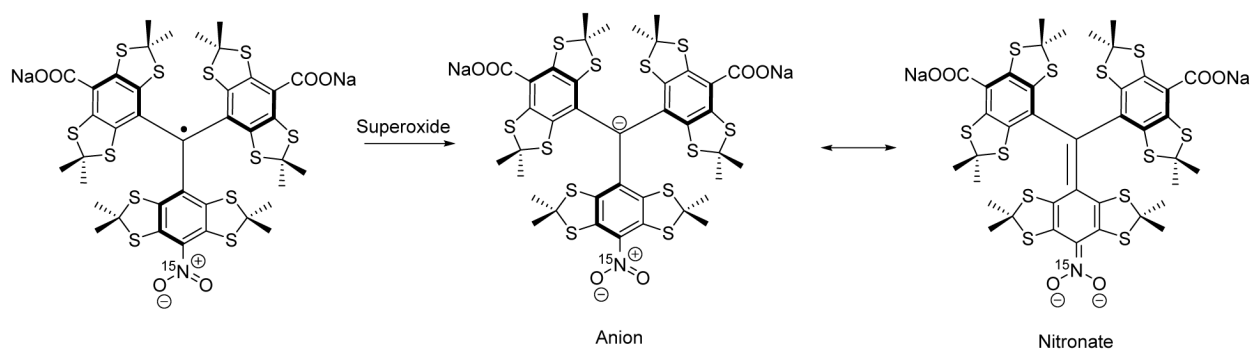
Scheme 2.
Postulated formation of the trityl anion in resonance with its nitronate form.

Table 1

Half-wave redox potential for **CT-03**, **PCT** and **nTAM**

Compound	Solvent	$E_{1/2}$ ox[a]	$E_{1/2}$ red[a]
CT-03[11]	PBS, pH=7.4	+0.434	−0.642
PCT[4]	PBS, pH=7.4	/	−0.320
nTAM	PB, pH = 7.4	+0.615	−0.147

[a] Calculated according to $E_{1/2} = (E_{pa} + E_{pc})/2$