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Triarylmethyl-based biradical as a superoxide probe

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Abstract

Superoxide radical represents one of the most biologically relevant reactive oxygen species involved in numerous physiological and pathophysiological processes. Superoxide measurement through the decay of an EPR signal of a triarylmethyl (TAM) radical possesses the advantage of a high selectivity and relatively high rate constant of TAM reaction with the superoxide. Hereby we report a straightforward synthesis and characterization of a TAM-TAM biradical showing a high reactivity with superoxide (second-order rate constant, $(6.7\pm0.2)\cdot10^3$ M^{-1} s^{-1}) enabling the measurement of superoxide radical by following the increase of a sharp EPR signal associated with the formation of a TAM-quinone methide monoradical product.

Keywords

Electron paramagnetic resonance (EPR); superoxide; atropoisomerism; biradical; trityl; reactive oxygen species (ROS)

Introduction

Among the reactive oxygen species (ROS), superoxide radical ($O_2^{\cdot-}$) formed by a one-electron reduction of molecular oxygen, is probably one of the most biologically relevant radicals, being involved in multiple physiological and pathophysiological mechanisms.[1, 2, 3, 4, 5, 6] For this reason, numerous methods, such as the use of 2-hydroxyethidium[7], cytochrome *c*[8], organic cyclic hydroxylamines[9] or lucigenin[10] assays have been developed for its measurement. The use of a nitrone spin-trap is a very popular method for the assay of superoxide, as it gives rise to a nitroxide adduct with a specific electron paramagnetic resonance (EPR) spectrum, but it suffers from a low chemical stability of the adduct and low rate constants, typically of the order of 10 M^{-1} s^{-1}. Therefore, this method requires the use of high concentrations of spin trap in the range from 10 to 200 mM[11, 12], which could perturb the redox balance.

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The measurement of superoxide using triarylmethyl (TAM, trityl) radicals involves the chemical addition of superoxide to the para-position of the TAM (Scheme 1). The resulting intermediate spontaneously decarboxylates. This reaction leads to the irreversible formation of an EPR-silent quinone-methide (**QM**) compound.[13] The reaction rate for this process has been reported to be about 10^3-10^4 M^{-1} s^{-1} at pH close to 7 depending on the particular structure [14, 15, 16]. The measurement of superoxide is carried out by following the decay of the EPR signal of the TAM radical. It is noteworthy that TAM radicals react with superoxide and peroxyl radicals to give the QM products. By contrast, the EPR signal is not altered upon incubation with other biological oxidants and reductants, such as ascorbic acid, glutathione (GSH), hydrogen peroxide, nitric oxide, peroxynitrite, hydroxyl radical[13, 14, 16].

However, for a high sensitivity of detection, an increase of signal is preferred, in particular in vivo it allows to discriminate between the reaction with the superoxide and probe clearance. In this article, we report the synthesis and physicochemical characterization of a biradical TAM-TAM probe as a new superoxide sensor that combines the advantages of the specificity and high reactivity of TAM radicals with superoxide with an increase of the EPR signal occurring during the reaction. The spin exchange between two TAM radical fragments increases the EPR linewidth [17, 18, 19] therefore drastically decreasing the peak intensity of the signal. The reaction of one of the TAM radical fragments with superoxide results in the formation of a diamagnetic quinone methide moiety and the corresponding appearance of a strong singlet EPR line of unaffected monoradical TAM fragment. A TAM-TAM biradical, where the two radical fragments are linked through a disulfide bond, was previously reported. [19] Note that the potential reactivity of disulfide TAM-TAM to superoxide can be affected by its reaction with thiol-containing redox sensitive molecules.

Material and methods

Chemicals

(−)-1,4-Di-O-tosyl-2,3-O-isopropylidene-L-threitol, sodium carbonate, hypoxanthine, xanthine oxidase, cytochrome *c*, diethylenetriaminepentaacetic acid (DTPA), ammonium iron (II) sulfate hexahydrate (99%), glutathione (98%) and sodium ascorbate (98%) were purchased from Sigma-Aldrich. Potassium iodide and trifluoroacetic acid (99.5%) were purchased from Acros Organics. Hydrochloric acid (99.999%) and hydrogen peroxide (30%) were purchased from Fisher Scientific. All solvents were purchased from Fisher Scientific. DETA NONOate was purchased from Cayman Chemical. Synthesis of TAM-TAM biradical was carried out in dry DMF and inside flame-dried glassware and maintained under argon during reaction time. All commercially available reagents were used as received without further purification.

HPLC

Analytical chromatography was performed on a Waters Alliance e2695 system, equipped with a 2998 PDA detector. The columns used were Waters XBridge BEH C18 4.6 mm × 50 mm, 2.5 μm or Waters Symmetry C18 4.6 mm × 250 mm, 3.5 μm. Solvent A was water, solvent B acetonitrile, and solvent C water containing 1% of trifluoroacetic acid. The Waters

XBridge column was used under gradient conditions as follows: flow rate, 1.5 mL/min; column temperature, 40°C; *t*=0 min 80% A/10% B/10% C; *t*=5 min 0% A/90% B/10% C; *t*= 6 min 0%A/100% B/0% C; run time 8 min; UV detection from 200 to 800 nm. The Waters Symmetry column was used under isocratic conditions as follows: flow rate 1.5 mL/min; column temperature, 35°C; *t*=0 min 35% A/55% B/10% C.

Semi-preparative chromatography was performed on a Waters Autopurification system composed of a Binary Gradient Module 2545 pump, a 996 PDA detector and a 2767 Sample Manager. Solvent A was water with 0.1% TFA, solvent B acetonitrile with 0.1% TFA. The column used was a Waters XBridge OBD C18 10 mm × 100 mm, 5 μm and the conditions were as follows: *t*=0 min 55% A/ 45%B; *t*= 5 min 55% A/45%B; *t*= 5.5 min 50% A/50% B; *t*=12 min 50% A/50% B; t=12.5 min 45%A/55%B; *t*=17 min 45% A/55%B; *t*=19 min 0% A/100% B; run time 25 min; UV detection from 200 to 800 nm.

Synthesis

CT-03 trityl radical (Scheme 1) was synthesized according to a previously reported procedure [20, 21]. TAM-TAM biradical **3** (Scheme 2) was synthesized by dissolving **CT-03** (200 mg, 0.2 mmol, 4 eq.) in 4 mL of N,N′-dimethylformamide (DMF), potassium iodide (16 mg, 0.1 mmol, 2 eq.), Na_2CO_3 (83 mg, 0.8 mmol, 16 eq.) and (−)-1,4-Di-O-tosyl-2,3-O-isopropylidene-L-threitol 1 (23 mg, 0.05 mmol, 1 eq.) were added. The solution was stirred for 1h at 120°C. After cooling, 1M HCl (40 mL) was added and the compound was extracted with ethyl acetate (3 × 20 mL). The organic layer was collected, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was dissolved in 50 mL acetonitrile and then 2 mL of 37% HCl were added at 0°C. The mixture was stirred at room temperature for 30 min, then diluted with deionized water and freeze-dried. The biradical 3 was purified by semi-preparative HPLC and isolated in 24% yield (29 mg). HRMS (MALDI-TOF) calcd for $[C_{84}H_{84}O_{14}S_{24}^{\bullet}+H_3O]^+$ 2102.9342 m/z, found 2102.9270 m/z.

TAM-QM monoradical **4** was produced by reaction of TAM-TAM biradical **3** with superoxide, generated using hypoxanthine (100 μM)/xanthine oxidase (5 mU) system prepared in Na-Phosphate buffer (100mM, pH 7.0, 500 mL) in the presence of DTPA (0.1 mM) and purified by semi-preparative HPLC using the same conditions as for **3**. HRMS (MALDI-TOF) calcd for $[C_{83}H_{83}O_{13}S_{24}^{\bullet}+H]^+$ 2055.9209 m/z, found 2055.9099.

EPR

Measurements were performed on an X-band ELEXSYS E 580 EPR spectrometer (Bruker, Germany). Gas composition and temperature was controlled by a gas-temperature controller (Noxygen, Germany) using Teflon tubes with a diameter of 1.14 mm and wall thickness of 60 μm (Zeus, Inc., USA). EPR acquisition parameters were as follows: microwave power, 1.5 mW; modulation amplitude, 0.5 G; modulation frequency, 100 kHz; sweep width, 20 G; sweep time, 20.48 s; conversion time, 20.00 ms, number of points, 1024.

Determination of Rate Constant with Superoxide and Selectivity Experiments

Superoxide was generated using hypoxanthine (100 μM)/ xanthine oxidase (5 mU) system prepared in Na-Phosphate buffer (100 mM, pH 7.0) in the presence of DTPA (0.1 mM). The

rate constant of superoxide reaction with TAM-TAM biradical was measured by competition with superoxide self-dismutation reaction. The rate of superoxide generation was measured by Cytochrome C assay[8]. Superoxide generated in hypoxanthine/xanthine oxidase system decays via reactions with the TAM-TAM biradical and self-dismutation:

$$TAM{-}TAM + O_2^{\cdot\,-} + 2H^+ \xrightarrow{k} TAM - QM + H_2O + CO_2 \quad (1)$$

$$O_2^{\cdot\,-} + O_2^{\cdot\,-} + 2H^+ \xrightarrow{k_d} O_2 + H_2O_2 \quad (2)$$

This can be written using the following system of differential equations:

$$\frac{d[TAM - QM]}{dt} = k \cdot [TAM - TAM] \cdot [O_2^{\cdot\,-}] = V \quad \text{(i)}$$

$$\frac{d[O_2^{\cdot\,-}]}{dt} = V_0 - k \cdot [TAM - TAM] \cdot [O_2^{\cdot\,-}] - 2 \cdot k_d \cdot [O_2^{\cdot\,-}]^2 \quad \text{(ii)}$$

where V_0 is the rate of superoxide production and V is the rate of superoxide consumption by TAM-TAM biradical.

Applying a steady state approximation to the system of equations ($\frac{d[O_2^{\cdot\,-}]}{dt} = 0$), the steady state concentration of superoxide can be found from the second equation:

$$[O_2^{\cdot\,-}] = \frac{\sqrt{\left(1 + 4 \cdot k_d \cdot \frac{V_0}{(k \cdot [TAM - TAM])^2}\right)} - 1}{\frac{2 \cdot k_d}{k \cdot [TAM - TAM]}} \quad \text{(iii)}$$

Substitution of the steady state superoxide concentration in the first equation allows to find the function of the rate of TAM-TAM conversion to TAM-QM on TAM-TAM concentration:

$$V = \frac{\sqrt{\left(1 + 4 \cdot k_d \cdot \frac{V_0}{(k \cdot [TAM - TAM])^2}\right)} - 1}{\frac{2 \cdot k_d}{(k \cdot [TAM - TAM])^2}} \quad \text{(iv)}$$

Fitting this equation to experimentally measured dependence of V on [TAM-TAM] (see Figure 5) yields k = (6.7±0.2)·10^3 $M^{-1}s^{-1}$; using k_d = 3·10^5 $M^{-1}s^{-1}$ (at pH = 7.0)[22] and V_0 = 26 nM/s (measured by Cytochrome C assay).

The reactivity of the TAM-TAM biradical towards various biologically relevant oxidizing and reducing agents was assessed by incubating 20 μM of TAM-TAM in Na-Phosphate buffer (100 mM, pH 7.4) with H_2O_2 (1 mM), GSH (1 mM), sodium ascorbate (1 mM), hydroxyl radical generated with the Fenton system (1 mM H_2O_2 and 0.1 mM ammonium iron sulfate), nitric oxide generated from DETA NONOate (2.5 mM) and the EPR signal was measured after 30 min.

Cyclic Voltammetry

Cyclic voltammograms were recorded using a Metrohm Autolab PGSTAT128N potenstiostat, a gold electrode was used as the working electrode, platinum wire as the counter electrode and Ag/AgCl 3M KCl as reference electrode. 1 mL of 2 mM **CT-03** or 1 mM TAM-TAM biradical **3** in phosphate buffer was placed in the electroanalytical cell. Oxygen was removed by bubbling nitrogen for 10 min. Scan rate was 0.1 V/sec, starting potential 0 V, lower potential -0.85 V, and upper potential +0.8 V.

Mass spectroscopy

Mass spectra were recorded on a Thermofisher Scientific Q Exactive Mass Spec.

Results and discussion

Synthesis

Synthesis of TAM-TAM biradical **3** was achieved in a straightforward two-step sequence, starting from **CT-03** (Scheme 2). On one hand, the synthetic strategy was designed in order to bring the radical centers close enough to facilitate spin exchange and efficiently broaden the EPR signal. On the other hand, this strategy provides the compound with a reasonable hydrophilicity allowing for its use in aqueous media. For this purpose, the commercially available protected ditosyl threitol **1** was selected as a short hydrophilic linker. An excess of **CT-03** underwent reaction with **1** in DMF in the presence of potassium iodide, leading to the desired biradical **2**. The excess of **CT-03** was used in order to avoid the formation of multimers as in principle the three carboxylic acids of the **CT-03** can react. After deprotection of the acetonide moiety, semi-preparative HPLC allowed for the isolation of pure deprotected biradical **3** and also the recovery of **CT-03** using the same elution conditions (see material and method).

HPLC

The purity of the TAM-TAM biradical **3** was assessed by reverse-phase analytical HPLC (Figure 1) and evaluated to 98%. While fast gradient conditions using a 50 mm long C18 column show a single peak, two peaks with identical UV spectra were obtained under isocratic conditions using a 250 mm C18 column. It was previously reported that the triarylmethyl motif adopts two enantiomeric helicoidal conformations, namely a right-handed (P) and a left-handed (M) propeller-shaped conformation. The bulky aromatic moiety prevents the two conformations from isomerization at room temperature.[23, 24] The TAM covalent linker is stereogenic and gives (P)-(S,S)-(P), (M)-(S,S)-(M) and the identical (P)-(S,S)-(M) = (M)-(SS)-(P) system. HPLC under isocratic conditions shows two peaks with a ratio of 1:3 as a result of a partial resolution of these stereoisomers. In our hand, it was not possible to further improve this resolution.

X-Band EPR spectroscopy

The X-band (9.5 GHz) EPR spectrum of TAM-TAM biradical **3** was recorded under nitrogen at room temperature and exhibits a broad single line (Figure 2). The measured linewidth of 930 mG is significantly higher than the intrinsic linewidth of a mono TAM radical ($\Delta H_{pp} \approx 70$ mG). This is the result of spin-spin interactions between the two paramagnetic centers. Note that similar linewidths have been previously reported for TAM biradicals.[18, 19] By increasing temperature, the linewidth was decreased as a result of an increase of the rate of spin exchange between the two radical fragments (at 42 °C, $\Delta H_{pp} \approx$ 390 mG). Similar effect of the temperature increase on the linewidth of the TAM biradicals has been previously reported.[17]

The biradical show an excellent stability as no change of EPR signal was observed upon incubation in aerated phosphate buffer (pH 7.4, 0.1 M) at room temperature overnight.

Reactivity with Superoxide and Selectivity

The reactivity of the TAM-TAM biradical with superoxide was assessed by EPR in the hypoxanthine/xanthine oxidase superoxide generation system. The EPR spectrum (Figure 3) shows two components, a broad line corresponding to the biradical ($\Delta H_{pp} \approx 930$ mG), and a more narrow line ($\Delta H_{pp} \approx 390$ mG), with a linewidth characteristic of a TAM monoradical. The kinetics shows an increase of the narrow component of the EPR spectrum apparently reflecting an accumulation of TAM-QM monoradical.

The reaction mixture was immediately analyzed by HPLC and shows the appearance of a new product with a retention time of 5.4 min (see Figure 4) with the characteristic λmax of the trityl group (488 nm) and the quinone-methide (513 nm). This reaction was performed on a preparative scale, allowing the isolation by HPLC of 2.5 mg of this newly formed compound. High resolution mass spectrometry confirmed the identity of the postulated structure of TAM-QM (see Scheme 3 for the proposed reaction). The reaction rate constant of TAM-TAM with superoxide was assessed using the reaction of superoxide self-dismutation as a competition reaction (see Materials and Methods). Figure 5 represents the rate of formation of the TAM-QM monoradical on TAM-TAM concentration, allowing the determination of the observed bimolecular reaction rate constant of TAM-TAM biradical at

pH 7.0, k = (6.7±0.2)·10^3 $M^{-1}s^{-1}$. The obtained rate constant is similar to previously reported for TAM radicals.[15]

TAM radicals show an extraordinary stability towards various other biological oxidants and reductants.[13, 14, 16]. In order to confirm that our biradical keeps this selectivity, TAM-TAM was incubated with an excess of biologically relevant oxidizing and reducing agents (see Material and methods). No significant decay of the EPR biradical signal (more than 5%) was observed upon incubation with ascorbic acid, GSH, hydrogen peroxide, nitric oxide and hydroxyl radical, showing that the coupling of the two TAM radicals using an ester linker does not have a significant effect on the reactivity of the trityl radical.

Cyclic voltammetry

TAM radicals show a high stability towards biological oxidants and reductants. To gain insight on the effect of the esterification on the redox properties of the radical center, cyclic voltammetry of the TAM-TAM biradical was carried out and compared with **CT-03**. In aqueous solution, **CT-03** shows a reduction of the trityl radical to the trityl anion at a potential of -0.633 V vs Ag/AgCl (see Table 1 and Figure 6), while oxidation of the trityl radical to the cation occurs at a potential of +0.467 V. Compared to **CT-03**, both radical centers of the TAM-TAM biradical **3** bear an ester group, which has a stronger electron-withdrawing effect than carboxylate functions. This is expected to increase both the reduction and oxidation potentials, as the anion would be more stabilized by a withdrawing group and the cation would be destabilized. The cyclic voltammogram of the TAM-TAM biradical **3** shows only one reduction wave at a potential of -0.511 V, which is 0.122 V higher than **CT-03**. Note that only one wave is observed, which implies that both radicals are reduced at a similar potential. The same effect is observed for the oxidation, with an oxidation of TAM-TAM occurring at a potential of 84 mV higher compared to **CT-03** (See Table 1).

Trityl radicals bearing a strong electron-withdrawing groups has been reported in the literature, such as nitro derivatives[25] (**nTAM**) (reduced at -0.147 V vs Ag/AgCl) or perchlorinated trityl[26] (**PCT**), reduced at -0.320 V vs Ag/AgCl. These trityls have been reported to be reduced by the superoxide radical.

Conclusion

In conclusion, we have reported the synthesis of a TAM-TAM biradical showing a reactivity with a superoxide with a rate constant of (6.7±0.2)·10^3 $M^{-1}s^{-1}$ similar to previously reported TAM monoradicals[14, 15]. The measurement of superoxide can be performed by following the increase of the EPR signal of the TAM-quinone methide monoradical, TAM-QM, which has been identified as the product of the reaction.

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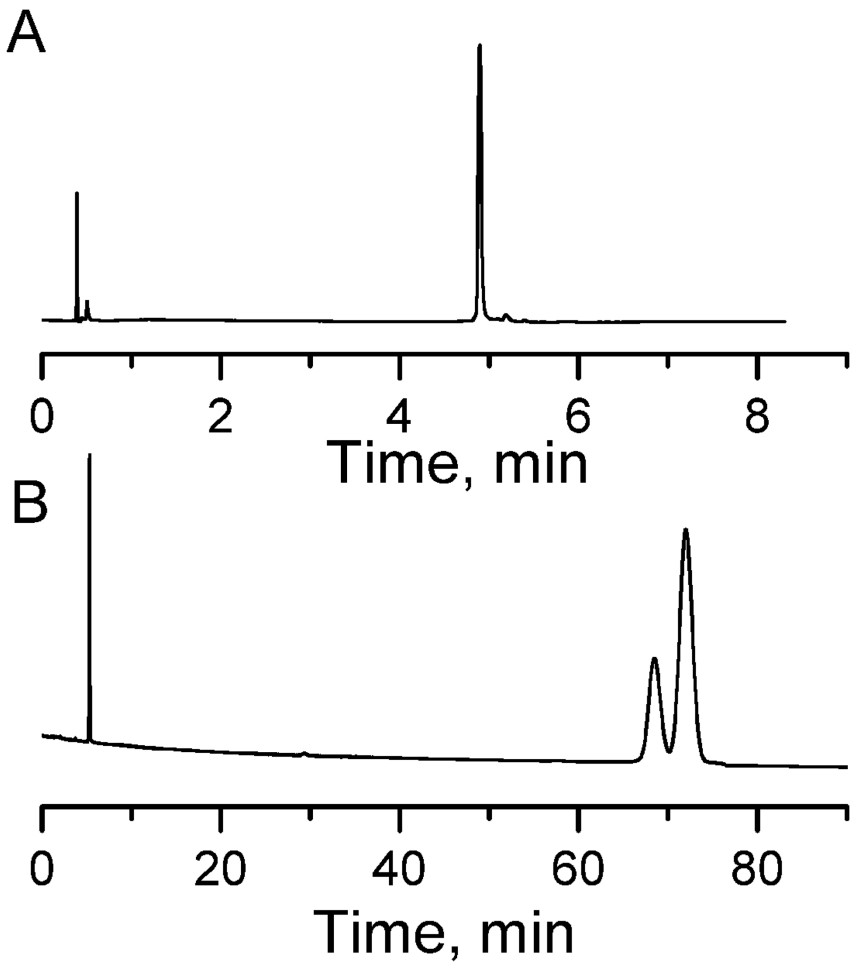


Figure 1.
HPLC chromatogram of deprotected TAM-TAM biradical (A) under gradient conditions using an XBridge 4.6 × 50 mm, (B) under isocratic conditions on a 4.6 × 250 mm Symmetry column.

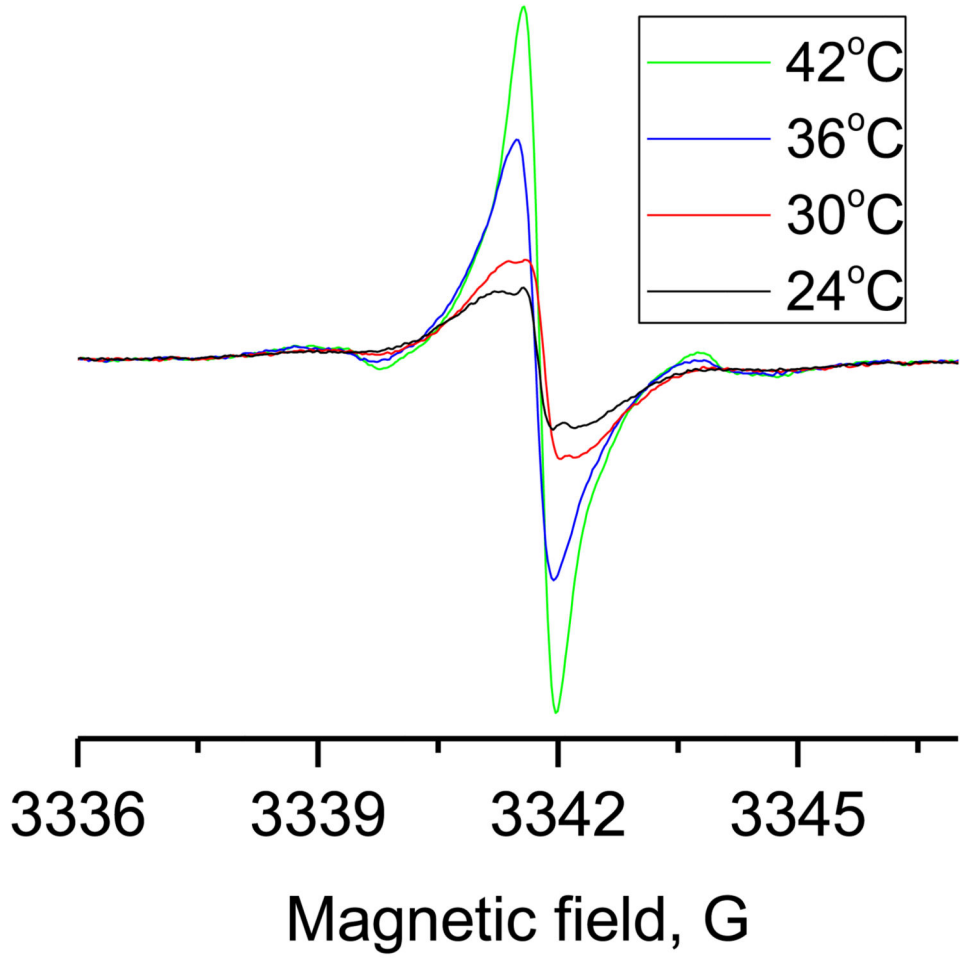


Figure 2.
X-Band EPR spectra of TAM-TAM biradical acquired under nitrogen atmosphere at various temperatures.

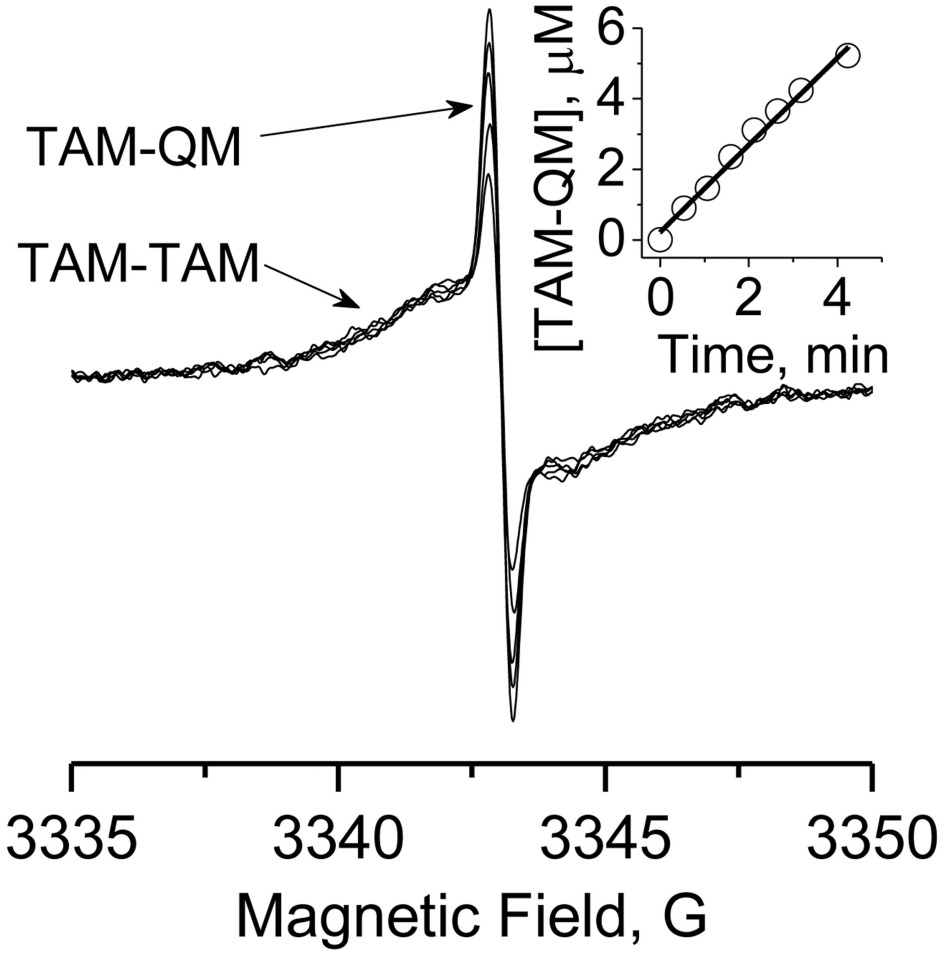


Figure 3.

EPR spectra showing the conversion of TAM-TAM biradical (40 μM) into TAM-QM monoradical upon reaction with superoxide. Insert: Increase of TAM-QM radical concentration upon reaction of TAM-TAM biradical with superoxide. The line shows the linear fit of the increase of TAM-QM radical concentration at a rate of 20.6 nM/s.

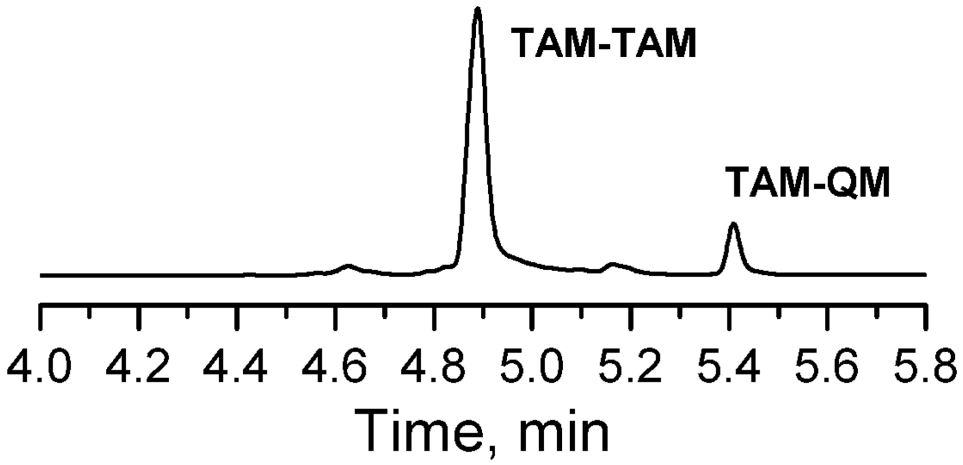


Figure 4.
HPLC chromatogram showing the reaction mixture resulting from the reaction between TAM-TAM biradical and superoxide. The peak at 4.8 min is the biradical and the peak at 5.4 min is the new compound formed from this reaction.

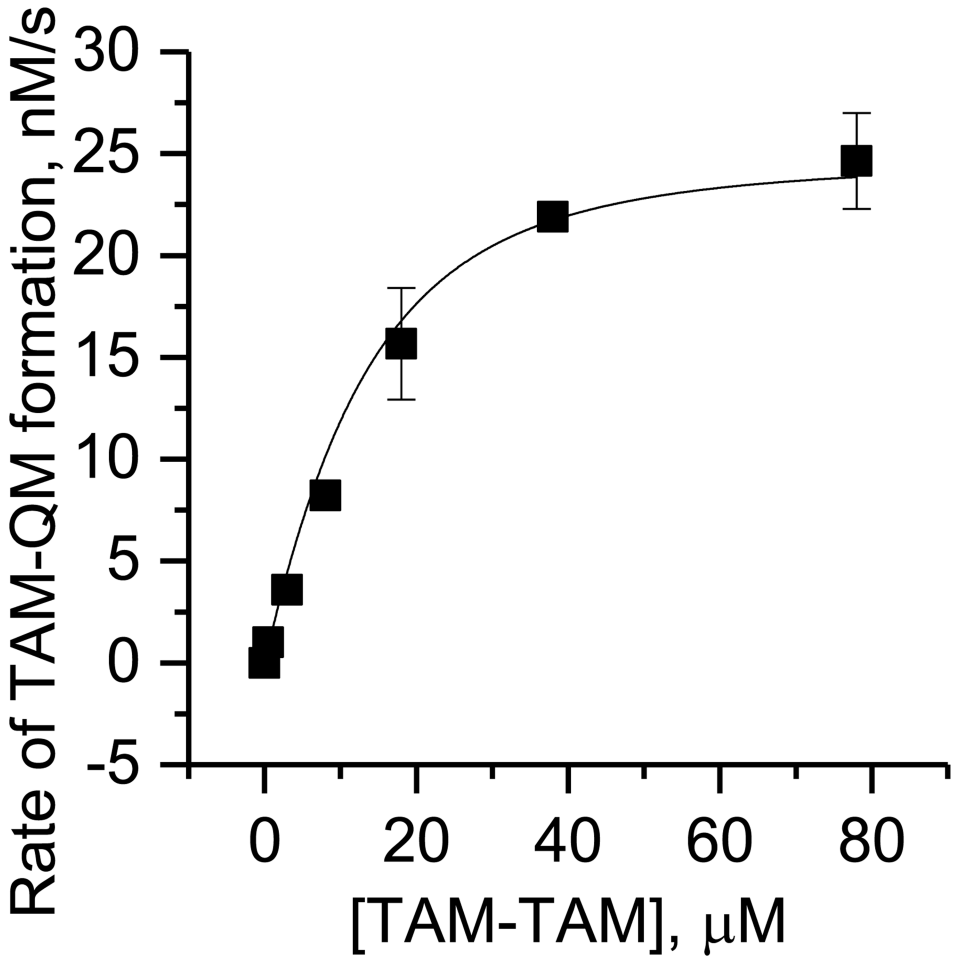


Figure 5.
The dependence of the rate of TAM-QM radical formation on TAM-TAM biradical concentration during superoxide generation.

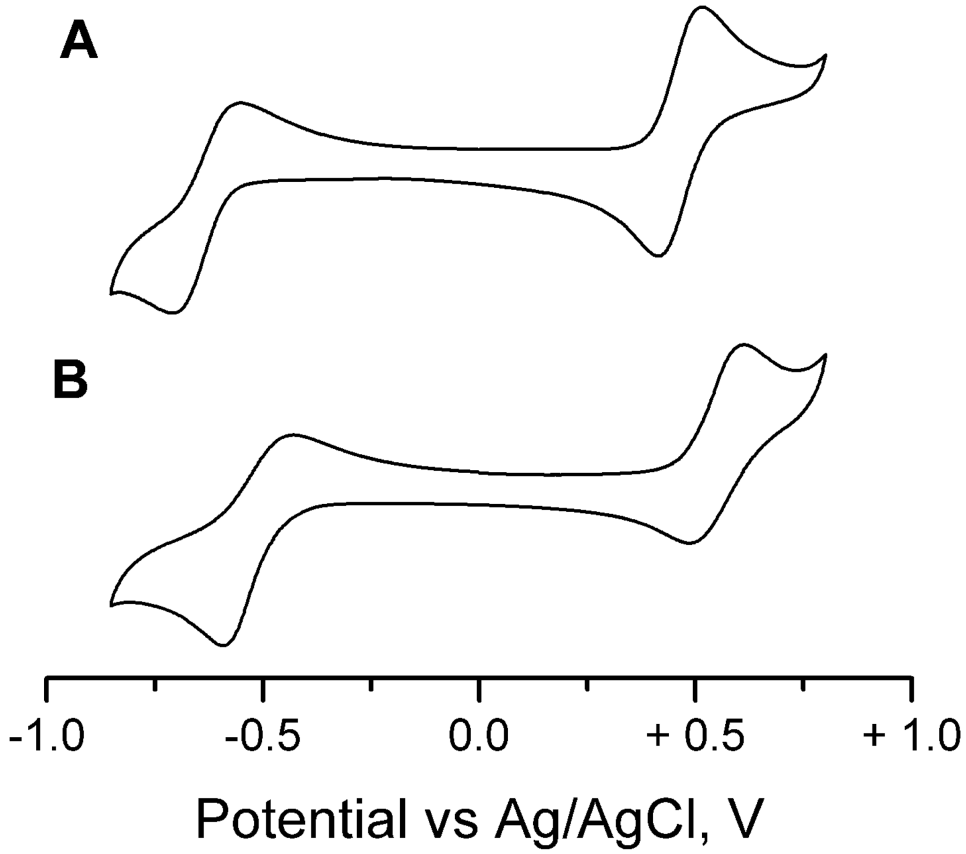
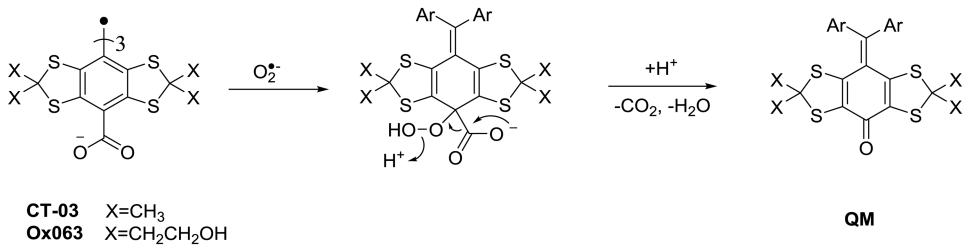
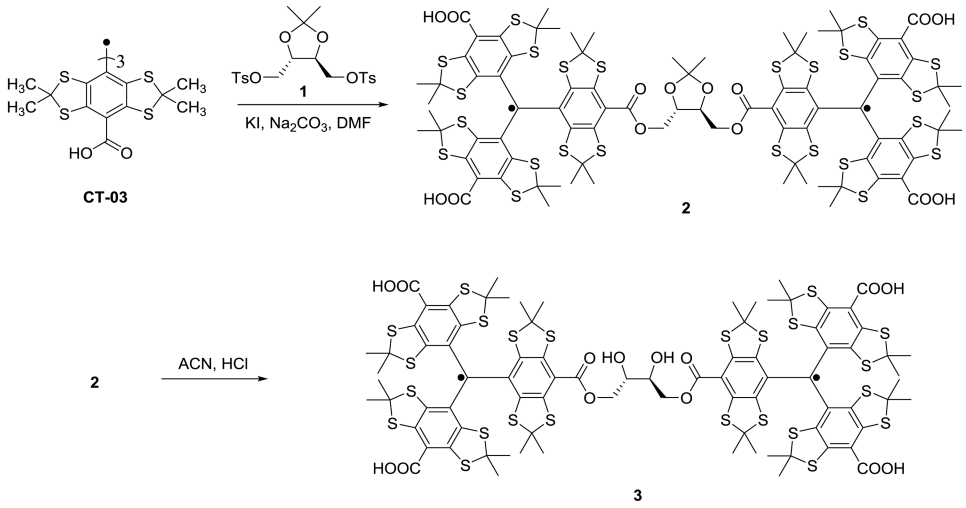


Figure 6.

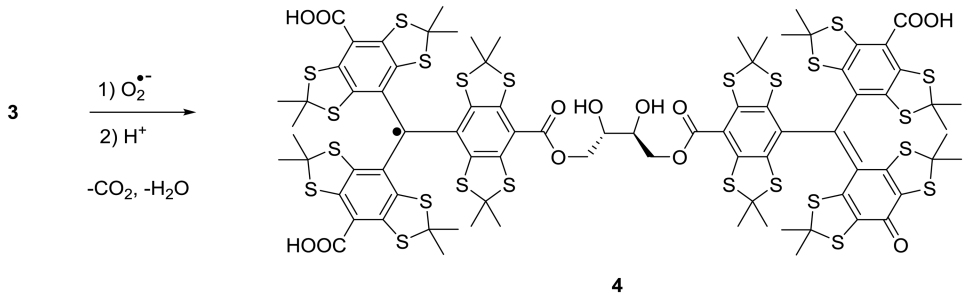
Cyclic voltammograms of (A) 2 mM **CT-03** in phosphate buffer saline, pH = 7.4, scan rate = 0.1 V/s. (B) 1 mM **TAM-TAM** biradical in phosphate buffer, pH 7.4, scan rate = 0.1 V/sec.



Scheme 1.
Reaction mechanism of TAM radicals with superoxide.



Scheme 2.
Synthesis of TAM biradical **3** (TAM-TAM).



Scheme 3.

The proposed reaction of superoxide to TAM-TAM biradical **3**, leading to a TAM-QM monoradical **4**.

Table 1

Half-waves redox potentials for various trityl radicals.

Compound	Solvent	$E_{1/2}$ ox[a,b]	$E_{1/2}$ red[a,b]
CT-03	PBS, pH=7.4	+0.467 V	-0.633 V
TAM-TAM	PB, pH=7.4	+0.551 V	-0.511 V
nTAM[25]	PB, pH = 7.4	+0.615 V	-0.147 V
PCT[26]	PBS, pH = 7.4	/	-0.320 V

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

[b] versus Ag/AgCl reference electrode