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Thiol-dependent reduction of the triester and triamide derivatives of Finland trityl radical triggers O₂-dependent superoxide production

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**Thiol-dependent reduction of the triester and triamide
derivatives of Finland trityl radical triggers O₂-dependent
superoxide production**

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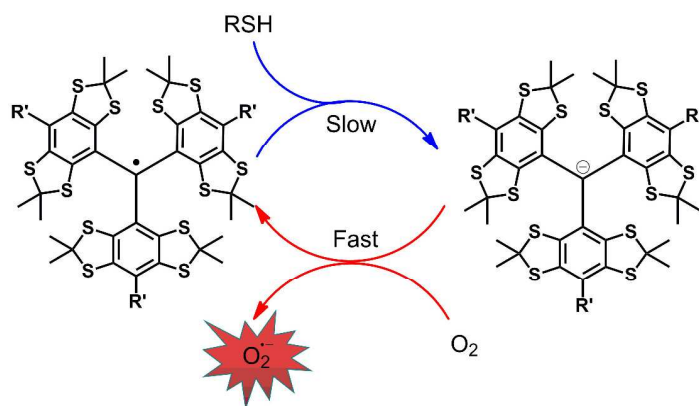
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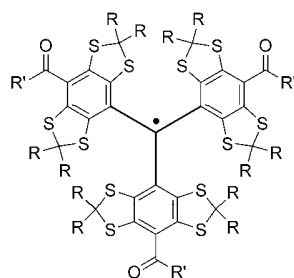
Abstract

Tetrathiatriaylmethyl (trityl) radicals have found wide biomedical applications as magnetic resonance probes. Trityl radicals and their derivatives are generally stable towards biological reducing agents such as glutathione (GSH) and ascorbate. We demonstrate that the triester (ET-03) and triamide (AT-03) derivatives of Finland trityl radical exhibit unique reduction by thiols such as GSH and cysteine (Cys) to generate the corresponding trityl carbanions as evidenced by the loss of EPR signal and appearance of characteristic UV-Vis absorbance at 644 nm under anaerobic conditions. The trityl carbanions can be quickly converted back to the original trityl radicals by oxygen (O_2) in air, thus rendering the reaction between the trityl derivative and biothiol undetectable under aerobic conditions. The reduction product of O_2 by the trityl carbanions was shown to be superoxide radical ($O_2^{\bullet-}$) by EPR spin-trapping. Kinetic studies showed that the reaction rate constants (k) depend on types of both trityl radicals and thiols with the order of $k_{ET-03/Cys}$ ($0.336\text{ M}^{-1}\text{ s}^{-1}$) $>$ $k_{ET-03/GSH}$ ($0.070\text{ M}^{-1}\text{ s}^{-1}$) $>$ $k_{AT-03/Cys}$ ($0.032\text{ M}^{-1}\text{ s}^{-1}$) $>$ $k_{AT-03/GSH}$ ($0.027\text{ M}^{-1}\text{ s}^{-1}$). The reactivity of trityl radicals with thiols is closely related to the para-substituents of trityl radicals as well as the pKa of the thiols and is further reflected by the rate of $O_2^{\bullet-}$ production and consumptions of O_2 and thiols. This novel reaction represents a new metabolic process of trityl derivatives and should be considered in design and application of new trityl radical probes.

Key words: trityl radical; superoxide; electron paramagnetic resonance (EPR); spin trapping; thiol; glutathione; cysteine

Introduction

Tetrathiatriarylmethyl (trityl, TAM) radicals such as CT-03 (also named as Finland trityl radical, Chart 1) and Ox063 have recently received much attention because of their unique properties of high biostability, water solubility, very sharp electron paramagnetic resonance (EPR) single line as well as long relaxation times.^{1,2} Due to these outstanding properties, trityl radicals have found wide applications in EPR spectroscopy and imaging,^{3,4} dynamic nuclear polarization (DNP)^{1,5,6} as well as Overhauser- enhanced MRI (OMRI).^{7,8} The great interest in trityl radicals has also stimulated effort to develop efficient approaches for their synthesis and derivatization, and the resulting compounds with improved properties enable new applications. The trityl derivatives are mostly based on CT-03 owing to its well-established synthetic procedures.^{2,9,10} For example, the esterification^{9,11-14} and amidation¹⁵⁻¹⁷ of the carboxylic acid(s) in CT-03 endow them with high stability, intracellular targeting and enhanced oxygen sensitivity. Using similar strategies, trityl spin labels for proteins^{18,19} and nucleic acids²⁰ as well as trityl-nitroxide biradicals as redox probes²¹ and efficient polarizing agents for high-field dynamic nuclear polarization²² were also developed.



CT-03 R = CH₃, R' = OH

OX063 R = CH₂CH₂OH, R' = OH

ET-03 R = CH₃, R' = OCH₂C(O)OH

AT-03 R = CH₃, R' = NHCH₂C(O)OH

Chart 1. Molecular structure of various trityl radicals.

It has been long established that CT-03 and OX063 exhibit extraordinary stability towards biological oxidoreductants such as glutathione (GSH), ascorbate, hydrogen peroxide and hydroxyl radical,^{16,23} except for their specific reactivity with superoxide radical ($O_2^{\bullet-}$). Owing to this unique reactivity, these trityl radicals have been used to detect $O_2^{\bullet-}$ in various systems.²³⁻²⁵ Recently, a series of important studies from Boucher's group^{13, 26-28} has demonstrated that these two radicals and their ester derivatives can undergo enzyme-mediated oxidation and reduction to the corresponding quinone methide and triarylmethane, respectively. However, to date there has been no report on direct reaction of trityl radicals with small-molecule biothiols such as GSH. Glutathione has been accepted to be one of the most critical substrates in the processes of nonenzymatic metabolisms of most drugs.

In the present study, we demonstrate for the first time that the triester (ET-03, Chart 1) and triamide (AT-03) derivatives of CT-03 can readily react with GSH and cysteine to generate the corresponding carbanions which further reduce O_2 under aerobic conditions to $O_2^{\bullet-}$, accompanying with recovery of the trityl derivatives. In contrast, CT-03 is inert to these biothiols. The reaction rate constants of ET-03 and AT-03 with the biothiols were determined under anaerobic conditions. Redox potentials of ET-03 and AT-03 were measured by cyclic voltammetry. Moreover, effects of types of both trityl radicals and thiols on the generation of $O_2^{\bullet-}$, O_2 and thiol consumptions were also investigated. Based on the results obtained, the reaction

mechanism between the trityl derivatives and biothiols under aerobic conditions was proposed. This new reaction mechanism should be considered in biomedical applications of trityl derivatives and may account for their potential toxicity in biological systems. Therefore, there is a great need to develop new strategies for derivatization of trityl radicals which prevent the generation of $O_2^{\cdot -}$ but without compromising their excellent properties.

Material and methods

Reagents

Glutathione (GSH), cysteine (Cys), dimethyl sulfoxide (DMSO), diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD) from bovine erythrocytes and 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Dojindo Molecular Technologies. Trityl radicals CT-03⁹ and ET-03¹² were synthesized as previously described. 5-Tert-butoxycarbonyl-5-methyl-1-pyrroline N-oxide (BMPO) was synthesized using the previous reported method.²⁹ The concentration of trityl radicals used in this study was determined by EPR using the purified CT-03 as standard. Phosphate buffer (PB, 200 mM, pH 7.4) was prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate in the presence of DTPA (400 μ M). Unless otherwise indicated, PB containing DTPA was used throughout our study. Stock solutions (1 mM) of trityl radicals as carboxylate sodium forms were prepared in phosphate buffer (PB) and stored at -20 °C, whereas the solutions of GSH

(10 mM) and Cys (10 mM) in PB were freshly prepared each day. All other chemicals were commercially available unless otherwise indicated.

Synthesis of ET-03

To the solution of CT-03 (50 mg, 0.05 mmol), in dry DMF (5 ml) was added *N,N*-diisopropylethylamine (DIPEA, 40 μ L, 0.21 mmol) under N_2 . The reaction mixture was stirred at room temperature for 2 min and then excess of *tert*-butyl bromoacetate (30 μ L, 0.21 mmol) was added. The resulting mixture was continuously stirred for 12 h at room temperature. Citric acid solution (6%, 30 mL) and ethyl acetate (30 mL) were added to quench the reaction. The organic layer was washed with brine (3 \times 30 mL), dried on anhydrous Na_2SO_4 and concentrated under vacuum. The *tert*-butyl ester form of ET-03 was purified by column chromatography on silica gel using hexane:ethyl acetate = 100 : 5 to 100 : 10 as eluents. The resulting brown solid was further treated with TFA overnight. The solvent was then removed under vacuum, and the residue was dissolved in PB (0.2 M, pH 7.4) and purified by reverse phase C-18 column chromatography using water followed by 0-20% methanol in water as eluents to give ET-03 as a brown-yellow solid (47 mg, 75%). Purity: 95% by HPLC with a retention time of 8.48 min (See Figure S1 in Supplementary Information). HRMS (High resolution mass spectrum, $[M+H_2O]^+$, m/z): 1190.9882 (measured, Figure S2), 1190.9713(calculated).

Synthesis of AT-03

To the solution of CT-03 (200 mg, 0.2 mmol), 1-hydroxybenzotriazole (HOBt, 162 mg, 1.2 mmol) and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluoro-phosphate (BOP, 530 mg, 1.2 mmol) in dry DMF (20 ml) was added DIPEA (200 μ L) under N₂. The reaction mixture was stirred at room temperature for 20 min and then excess of glycine *tert*-butyl ester hydrochloride (150 mg, 1.2 mmol) was added as solid. The resulting mixture was continuously stirred for 18 h at room temperature. Citric acid solution (6%, 30 mL) and ethyl acetate (30 mL) were added to quench the reaction. The organic layer was washed with brine (3 \times 30 mL), dried on anhydrous Na₂SO₄ and concentrated under vacuum. The *tert*-butyl ester form of AT-03 was purified by column chromatography on silica gel using hexane:ethyl acetate = 100 : 5 to 100 : 10 as eluents. The resulting brown solid was further treated with TFA overnight. The solvent was then removed under vacuum, and the residue was dissolved in PB (0.2 M, pH 7.4) and purified by reverse phase C-18 column chromatography using water followed by 0-20% methanol in water as eluents to give AT-03 as a brown-green solid (178 mg, 72%). Purity: 96% by HPLC with a retention time of 9.59 min (See Figure S1 in Supplementary Information). HRMS ([M-H]⁻, *m/z*): 1168.9875 (measured, Figure S2), 1168.9966 (calculated).

EPR spectroscopy

EPR measurements were carried out on Bruker EMX-plus X-band spectrometer at room temperature. General instrumental settings were as follows: modulation frequency, 30-100 kHz; microwave power, 0.05-10 mW; modulation amplitude,

0.01-0.5 G. Measurements were performed in 50 μ L capillary tubes. Spectral simulation was performed by the program developed by professor Rockenbauer.³⁰ The reported hyperfine splitting constants of the spin adducts of BMPO were used as initial parameters for spectral simulations (see Table S1). The hyperfine splitting constants obtained by the simulation were well consistent with the reported values.²⁹

As described previously,⁹ EPR measurements under anaerobic conditions were carried out using a gas-permeable Teflon tube (i.d. = 0.8 mm). Briefly, the reaction solution was transferred to the tube which was then sealed at both ends. The sealed sample was placed inside a quartz EPR tube with open ends. Argon gas was allowed to bleed into the EPR tube and then EPR spectrum was recorded.

Cyclic Voltammetry

Cyclic voltammetry was performed on a potentiostat and computer-controlled electroanalytical system. Electrochemical measurements were carried out in a 10 mL cell equipped with a glassy carbon working electrode (7.07 mm²), a platinum-wire auxiliary electrode and a Ag/AgCl reference electrode. Solutions of trityl radicals (0.5 mM) were degassed by bubbling with the nitrogen gas before the detection. The redox potentials were calculated according to the relation $E = (E_p^a + E_p^c)/2$.

Oxygen consumption

The O₂ consumption was measured by EPR as previously described.²⁴ The solution containing trityl radical and thiol was transferred to a 50 μ L capillary tube which was

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2
3 immediately sealed at both ends with corks to avoid any incorporation of bubble
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6 inside. Then, EPR spectra were continuously recorded with a period of up to 12 hours.
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9 The O₂ sensitivity of trityl radicals was obtained according to their peak-to-peak
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11 linewidths under aerobic and anaerobic conditions as previously described.⁹ Then, the
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13 O₂ concentration at each time point was calculated based on the measured
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15 peak-to-peak linewidth and O₂ sensitivity of trityl radical (See details in Figure S4).
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18 All the experiments were repeated three times and the average rates of O₂
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20 consumption in the reaction system of trityl radicals with thiols were shown in Table
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29 *Detection of the trityl carbanion of ET-03 by UV-Vis spectroscopy*

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31 Reactions were monitored at room temperature using a U-3900 UV-Vis
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33 spectrophotometer in 1-cm path length quartz cuvette which was pre-purged with
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35 argon and stoppered with a rubber septum. A solution of ET-03 (50 μM) in degassed
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37 PB (50 mM, pH 7.4) was mixed with GSH (500 μM) or sodium dithionite (100 μM)
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39 under anaerobic conditions and UV-Vis spectra were collected immediately to
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41 monitor the generation of the trityl carbanion.¹³ To recover the trityl radical from its
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43 carbanion, air was bubbled to the above anaerobic solution for 3 min. Of note is that
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45 ET-03 was only partially reduced under these conditions. Complete reduction of
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47 ET-03 (50 μM) was achieved in the presence of high concentration of sodium
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49 dithionite (500 μM) under anaerobic conditions (Figure S9).
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Detection of $O_2^{\bullet-}$ by EPR-spin trapping technique

The production of $O_2^{\bullet-}$ was determined by EPR spin-trapping method. GSH (2 mM) was added to the solution containing ET-03 (50 μ M) or AT-03 (50 μ M) and the spin trap BMPO (50 mM) in PB and then EPR spectra were recorded 105s later. While SOD (200 U/mL) was used to confirmed the production of $O_2^{\bullet-}$ in the reaction system, DMSO (2%, v/v) was used as an efficient HO^{\bullet} scavenger to check if HO^{\bullet} was involved in the system since it can effectively scavenge HO^{\bullet} to generate the secondary methyl radical which can be further trapped by BMPO to result in the methyl spin adduct.

Measurement of the relative $O_2^{\bullet-}$ levels by spin trapping

The well characterized nitron spin trap DMPO was used.³¹ Thiol (2 mM) was added to the solution containing trityl radical (50 μ M) and DMPO (100 mM) in PB and then EPR spectra were recorded after 2-min incubation. The double integration of the low-field peak of each EPR signal was used to measure the relative $O_2^{\bullet-}$ levels (Figure 4). In addition, the relative $O_2^{\bullet-}$ levels were also determined by EPR simulation (see Table S2). Very similar results were obtained for both methods.

Kinetic studies of the reaction of trityl radicals with thiols under anaerobic conditions

Trityl radical (50 μ M) was mixed with various concentrations of thiols (0.5 mM, 1mM, 2 mM) in PB and the resulting solution was immediately transferred to a gas-permeable Teflon tube with both ends sealed. The sealed sample was then placed

inside a quartz EPR tube with open ends. Argon gas was allowed to bleed into the EPR tube. After 4-min equilibrium incremental EPR spectra were recorded over 25 min. Since the thiol concentration used was in excess relative to the concentration of trityl radical (50 μM), the reaction of trityl radical with the thiol compound follows pseudo first-order kinetics. Due to the relatively slow reaction, the initial decay rate of trityl radical was determined within 5-15 min. Data was plotted using the following equation:

$$\ln[T] = \ln[T]_0 - k_{obs}t$$

where $[T]_0$ is the initial concentration of trityl radical, $[T]$ the concentration of trityl radical at each time point, and k_{obs} the apparent first-order rate constant. Second-order rate constants (k) of trityl radicals with thiols were eventually determined by plotting k_{obs} with the thiol concentrations (Table 1) (See details in Figure S5).

Determination of thiol concentrations by UV-Vis spectroscopy

The Ellman's method was used to measure the concentration of thiols.³² Thiol (2 mM) was mixed with the solution of trityl radical (80 μM) in PB at room temperature. After 1 hour, excess of the Ellman's agent DTNB (6 eq) was added to the solution and UV-vis spectrum was recorded after appropriate dilutions with PB. The concentration of thiol left in the system was calculated according to the absorbance at 412 nm using the reported molar absorption coefficient ($\epsilon = 14.15 \text{ mM}^{-1} \text{ cm}^{-1}$).

Results

Reduction of trityl radicals by GSH

As shown in Figure 1, ET-03 had a single sharp EPR line with a linewidth of 308 mG under aerobic conditions and this signal did not show any decay after 30-min incubation with GSH under aerobic conditions (Figure 1A). It seems that no reaction occurs between ET-03 and GSH under this condition. However, when the capillary containing ET-03 and GSH in PB was sealed at both ends, the EPR line of ET-03 was gradually narrowed with the peak-to-peak linewidths decreasing from 308 to 255 mG after 11-hour incubation (Figure 1B). Correspondingly, the O₂ concentration was reduced from 260 to 98 μM (see details for measurement of O₂ concentrations in the section of Material and methods), indicating that O₂ consumption occurred in the sealed capillary. The double integration of the EPR signals indicates ~25% decay of ET-03 after 11-hour incubation in the sealed capillary (Figure 1C). Therefore, limiting access of O₂ to the reaction system leads to the decay of ET-03 in the presence of GSH (500 μM). To further verify the role of O₂, we carried out the same reaction under anaerobic conditions. As shown in Figure 1D, complete removal of O₂ from the reaction system induced a much faster decay of ET-03 with ~23% decrease (from 50 to 38.3 μM) of its signal only in 2 hours. Further increase of the incubation time (4.5 hours) led to further decay of ET-03 (~53%) (Figure S6). Kinetic studies showed that ET-03 had a half-life of 4.83 ± 0.08 h under anaerobic conditions in the presence of GSH (500 μM), compared with 21.49 ± 0.03 h under aerobic conditions (Figure S7). Interestingly, reoxygenation of the above anaerobic solution by air resulted in quick recovery of ET-03 (48.5 μM, 97%). These results consistently demonstrate that ET-03

can react with GSH to generate a relatively stable diamagnetic species which can be recovered back to ET-03 by O_2 . Similarly, AT-03 (50 μM) can also react with GSH (500 μM) but has much weaker reactivity with a slight loss of AT-03 (2.9 μM) as compared to 11.7 μM for ET-03 under the similar condition (Figure 1D). The weaker reactivity of AT-03 towards GSH than ET-03 was further verified by comparing their half-life times with the values of 9.56 ± 0.27 h for AT-03 and 4.83 ± 0.08 h for ET-03 under anaerobic conditions. Surprisingly, CT-03 did not show any reactivity to GSH under the same condition (Figure 1D). The introduction of GSH (2 mM) did not result in any decay of CT-03 either (Data not shown).

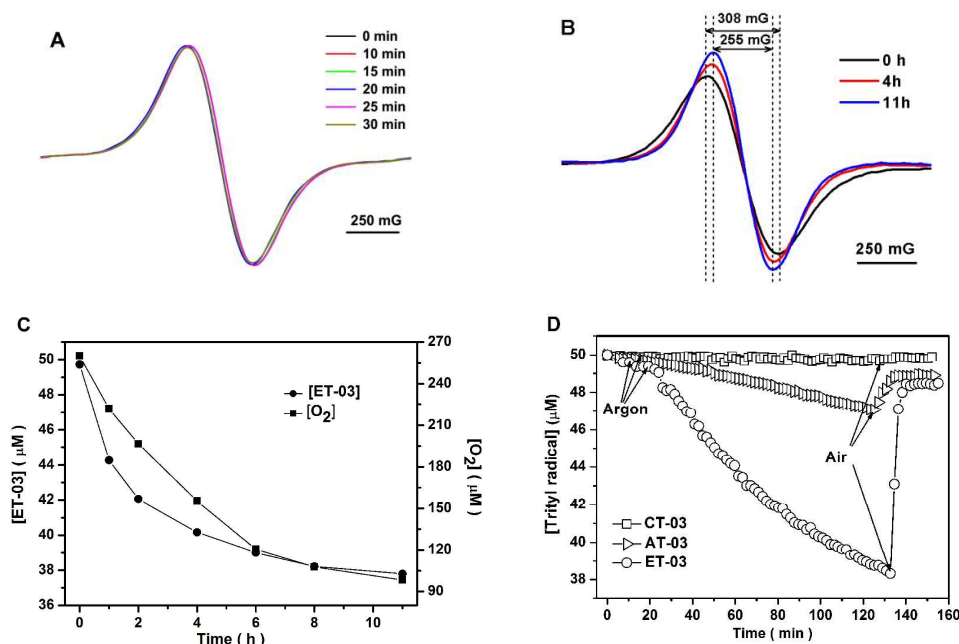


Figure 1 (A) EPR spectra of ET-03 (50 μM) after incubation with GSH (500 μM) in PB (50 mM, pH 7.4) for 0 - 30 min under aerobic conditions. (B) EPR spectra recorded from the solution of ET-03 (50 μM) and GSH (500 μM) in PB (50 mM, pH 7.4) in a sealed capillary at the incubation time points of 0, 4, and 11 hours. (C) Plot of the concentrations of ET-03 (circle) and O_2 (square) as a function of time in a sealed capillary containing ET-03 (50 μM) and GSH (500 μM) in PB (50

mM, pH 7.4). (D) Plot of concentrations of ET-03 (circle), AT-03 (triangle) and CT-03 (square) as a function of time after the reactions of each trityl radical (50 μ M) with GSH (500 μ M) in a gas-permeable tube which was passed through argon and then air.

Redox potentials of trityl radicals

Redox potentials of ET-03 and AT-03 were measured by cyclic voltammetry and compared with CT-03 to interpret their thermodynamic feasibility for reactions with GSH. As shown in Figure S8, both trityl radicals can undergo one-electron quasi-reversible reduction to the corresponding trityl carbanion and one-electron reversible oxidation to the carbocation form. Table 1 summarizes the oxidation and reduction potentials of trityl radicals. ET-03 (-0.309 V vs. Ag/AgCl) has much higher reduction potential than AT-03 (-0.459 V vs. Ag/AgCl) and CT-03 (-0.624 V vs. Ag/AgCl).³³ As for the oxidation potentials, the opposite tendency was observed for these trityl radicals (Table 1). Replacement of the negatively charged carboxylates in CT-03 with the electron-withdrawing ester or amide groups led to higher reduction potentials for ET-03 and AT-03 than CT-03. Similar reduction and oxidation potentials were also reported for other ester and amide derivatives of trityl radicals.^{13, 17} Thus, from the thermodynamic point of view, the reduction of either ET-03 or AT-03 by GSH is more favorable than CT-03.

Table 1 Redox potentials of trityl radicals relative to Ag/AgCl and their reaction rate constants with GSH (k_{GSH}) and cysteine (k_{cys}) under anaerobic conditions, and $O_2^{\bullet -}$ (k_{sp}) in PB (50 mM, pH

7.4). See more details in the section of Material and methods.

Radicals	$E_{\text{red}}(\text{V})$	$E_{\text{ox}}(\text{V})$	$k_{\text{GSH}}(\text{M}^{-1}\text{s}^{-1})$	$k_{\text{cys}}(\text{M}^{-1}\text{s}^{-1})$	$k_{\text{sp}}(\text{M}^{-1}\text{s}^{-1})$
CT-03	-0.624	0.456	No reaction	No reaction	3.1×10^3
AT-03	-0.459	0.643	0.027 ± 0.002	0.032 ± 0.004	No reaction
ET-03	-0.309	0.733	0.070 ± 0.002	0.336 ± 0.004	No reaction

Note: k_{sp} of CT-03 ($3.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) was reported previously in the reference.²⁴

Detection of the trityl carbanion of ET-03 by UV-Vis spectroscopy

GSH is a relatively strong reducing agent which is a key component of cellular redox homeostasis.³⁴ The relatively high reduction potentials of ET-03 and AT-03 imply that they may be reduced by GSH to the corresponding carbanions. To verify this hypothesis, we used UV-Vis spectroscopy to detect the trityl carbanion of ET-03. As illustrated in Figure 2, the reaction of ET-03 with GSH under anaerobic conditions led to appearance of a new absorbance at 644 nm. In the previous study,¹³ an almost identical UV-vis absorbance ($\lambda_{\text{max}} = 644 \text{ nm}$) was also observed when the other trityl ester derivatives were reduced by sodium dithionate. One of the reduction products was determined by mass spectroscopy to be the corresponding trityl carbanion.¹³ Using sodium dithionite as a reducing agent, we also observed the absorbance at 644 nm from ET-03 but with higher intensity than that observed in the case of GSH (Figure 2). This implied that the reduction of ET-03 by either GSH or sodium dithionite led to the same product which was most likely assigned to its trityl carbanion. Increasing the concentration of sodium dithionite led to complete transformation of ET-03 into its carbanion with the strong absorbance at 644 nm and

complete disappearance of the absorbance of ET-03 at 491 nm (Figure S9). Consistent with the above EPR results, aerating the anaerobic solution containing the reduction product of ET-03 effectively recovered ET-03 as evidenced by the increase of the absorption intensity at 491 nm, accompanying with disappearance of the absorbance at 644 nm (Figure 2). As noted earlier,¹³ the trityl carbanion of ET-03 could be detected by mass spectrometry. However, our attempt was not successful due to leakage of air to the anaerobic solution of the carbanion, as seen from the change of the color of the solution from blue to light brown before its injection into the spectrometry.

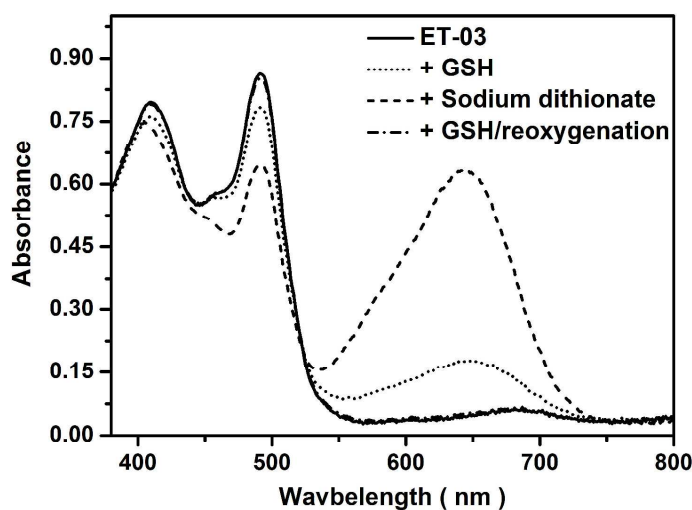


Figure 2 UV-Vis spectra of ET-03 (50 μ M) in aqueous solutions in the absence (solid line) or presence of GSH (500 μ M, dotted line) or sodium dithionate (100 μ M, dashed line) under anaerobic conditions as well as the spectrum (dash-dotted line which was overlapped with the solid line) obtained by aerating the solution which was pre-incubated for 30 min under anaerobic conditions.

Detection of $O_2^{\cdot -}$ by EPR-spin trapping technique

As mentioned above, ET-03 and AT-03 can be effectively recovered from their carbanions by transferring one electron to O_2 . As such, we performed EPR-spin trapping experiments using BMPO as a spin trap to determine the reduction product of O_2 . Figure 3A shows the vertically expanded EPR spectrum obtained from the solution of BMPO, ET-03 and GSH in PB (50 mM, pH 7.4). Due to the presence of the strong signal of ET-03, the signals of other paramagnetic species were not clearly seen in the non-expanded spectrum (Figure S10 in SI). EPR simulation (Figure 3B) indicates that this spectrum consists of three components which can be assigned to the superoxide ($BMPO/\cdot OOH$) and hydroxyl ($BMPO/\cdot OH$) spin adducts of BMPO as well as ET-03. Assignments of $BMPO/\cdot OOH$ and $BMPO/\cdot OH$ were based on the good agreement of their hyperfine splitting constants with the previously reported values (see Table S1 in SI).²⁹ The signals of $BMPO/\cdot OOH$ and $BMPO/\cdot OH$ were completely suppressed by SOD (200 U/ml, Figure 3C) with only the signal of ET-03 remaining, indicating that the signals of both spin adducts originate from $O_2^{\cdot -}$. In addition, neither $BMPO/\cdot OOH$ nor $BMPO/\cdot OH$ was observed in control experiments without any one of three agents (data not shown). The EPR signal of $BMPO/\cdot OH$ could arise from direct trapping of $\cdot OH$ by BMPO or from the decomposition of $BMPO/\cdot OOH$ in the presence of GSH.³⁵ To further verify the origin of $BMPO/\cdot OH$, we used dimethyl sulfoxide (DMSO) as a highly efficient $\cdot OH$ scavenger. Hydroxyl radical, if any, can be detected by spin trapping of the secondary methyl radical formed in the reaction of $\cdot OH$ with DMSO. Almost identical EPR spectra were observed in the presence

(Figure 3D) or absence (Figure 3A) of DMSO, indicating that $\text{BMPO}/^{\bullet}\text{OH}$ is most likely due to the decomposition of $\text{BMPO}/^{\bullet}\text{OOH}$.³⁵ Similarly, both spin adducts of BMPO were also observed from the solution containing AT-03, GSH and BMPO (Figure S11). Thus, the reduction of O_2 by the trityl carbanions results in the production of $\text{O}_2^{\bullet-}$.

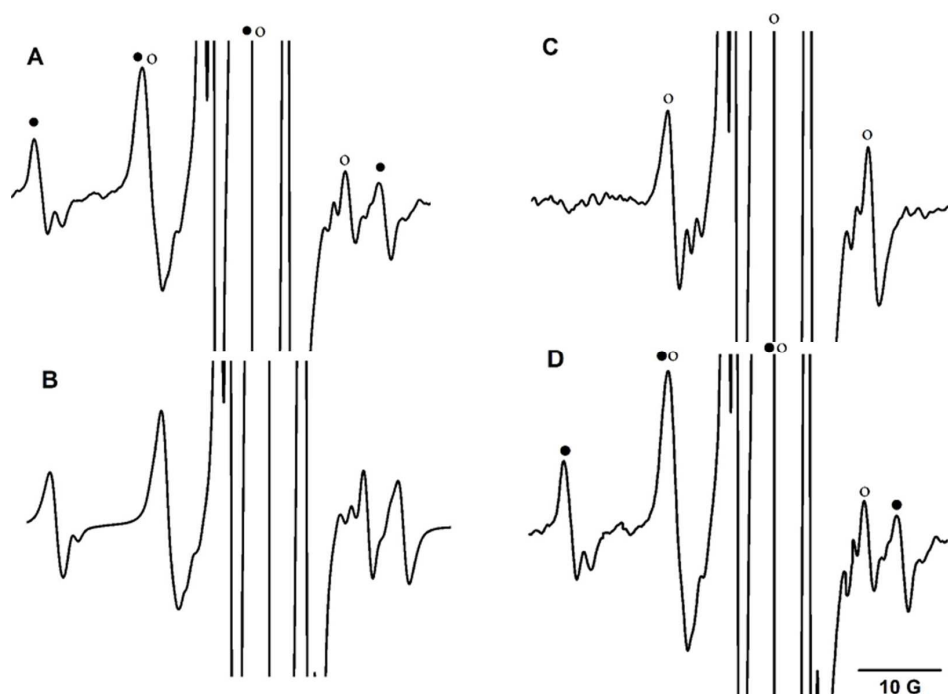


Figure 3 (A) EPR spectrum (15-fold enlargement as compared to the original spectra) obtained by addition of BMPO (50mM) to the solutions of ET-03 (50 μM) and GSH (2 mM) in PB (50 mM, pH 7.4) in air. *Note:* (filled circle) the overlapped signals of superoxide ($\text{BMPO}/^{\bullet}\text{OOH}$) and hydroxyl ($\text{BMPO}/^{\bullet}\text{OH}$) spin adducts of BMPO; (unfilled circle) the signal of ET-03. (B) Computer simulation of the spectrum A. Hyperfine splitting constants used for EPR simulation were shown in Table S1 in SI. (C) Same as (A) but EPR spectrum was recorded in the presence of SOD (200 U/mL). The weak signals at both sides were attributed to the ^{13}C hyperfine splitting of the central

carbon (23.5 G). (D) Same as (A) but EPR spectrum was recorded in the presence of DMSO (2%, v/v).

Reactivity of trityl radicals with thiols

In order to quantitatively describe the reactivity of trityl radicals with thiols, their second-order rate constants were measured by EPR under anaerobic conditions where the O₂-induced recovery of trityl radicals from the trityl carbanions was completely inhibited. As shown in Table 1, ET-03 has the higher second-order rate constant ($0.070 \pm 0.002 \text{ M}^{-1}\text{s}^{-1}$) with GSH than AT-03 ($0.027 \pm 0.002 \text{ M}^{-1}\text{s}^{-1}$), whereas CT-03 is inert to GSH. The replacement of GSH by cysteine leads to 4.8- and 1.2-fold increases of the rate constants for ET-03 ($0.336 \pm 0.004 \text{ M}^{-1}\text{s}^{-1}$) and AT-03 ($0.032 \pm 0.004 \text{ M}^{-1}\text{s}^{-1}$), respectively. High reactivity of ET-03 with thiols is closely associated with the strong electron-withdrawing character of the ester groups. Meanwhile, since thiolate is the reactive form of thiol in redox reactions, higher reactivity of cysteine than GSH can be attributable to lower pK_a (8.3) of the thiol group in cysteine than the latter (8.8).³⁶

Effect of trityl radicals and thiols on the generation of O₂^{•-}

Our above results showed that both ET-03 and AT-03 can be reduced by GSH into the corresponding trityl carbanions which further reduce O₂ to O₂^{•-}. It can be thus expected that the relative levels of O₂^{•-} generated from these reaction systems are associated with the reactivity of trityl radicals with thiols. However, it has been shown

that trityl radicals including CT-03 and OX063 can also react with $O_2^{\bullet-}$.^{37, 24} Thus, evaluating the reactivity of both ET-03 and AT-03 with $O_2^{\bullet-}$ is prerequisite to measure the $O_2^{\bullet-}$ levels generated in their reactions with biothiol. As shown in Figure S13, the characteristic UV-vis absorbance intensities of ET-03 at 484 nm or AT-03 at 462 nm remained to be almost identical before and after the introduction of $O_2^{\bullet-}$ in the system. Moreover, the introduction of either ET-03 or AT-03 into the solution containing DMPO, xanthine and xanthine oxidase did not change the EPR signals (Figure S14). Thus, neither ET-03 nor AT-03 reacted with $O_2^{\bullet-}$. In other words, these two trityl derivatives do not consume $O_2^{\bullet-}$ generated in their reactions with thiols.

Thereafter, the relative levels of $O_2^{\bullet-}$ generated from the reactions of both ET-03 and AT-03 with thiols were measured by the EPR spin-trapping technique. The $O_2^{\bullet-}$ spin adduct of DMPO has a relatively short half-life time ($t_{1/2} \sim 1$ min) and is gradually decomposed to the $\cdot OH$ spin adduct. Thus, the double integration of the low-field multiple peaks from both $O_2^{\bullet-}$ and $\cdot OH$ spin adducts was used to measure the relative levels of $O_2^{\bullet-}$ (Figure S15). As shown in Figure 4, the order of the relative EPR double integrations is ET-03/Cys (1.0) > ET-03/GSH (0.57) > AT-03/Cys (0.46) > AT-03/GSH (0.31), consistent with the order of their rate constants (Table 1). Additionally, EPR simulation was carried out to quantitate the levels of $O_2^{\bullet-}$ and similar results were obtained (Table S2).

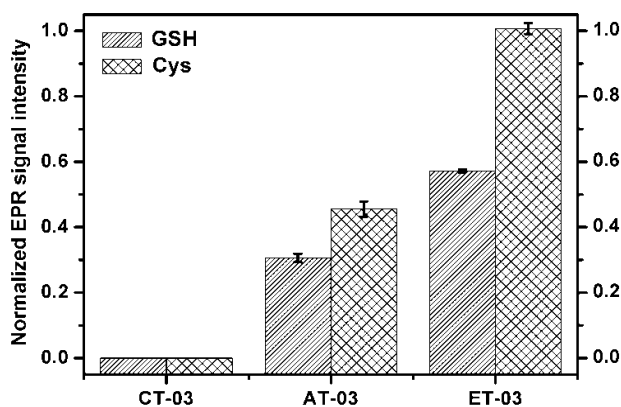


Figure 4 The relative EPR signal intensity of DMPO spin adducts which were generated by adding DMPO (100 mM) to the solution of trityl radical (50 μ M) and thiol (2 mM). The relative double integration of the low-field multiple peaks of each EPR signal was used.

Effect of trityl radicals and thiols on the O₂ consumption

Since the EPR line width of trityl radicals exhibits good sensitivity to O₂, the O₂ consumption can be determined by monitoring the change in the line widths of trityl radicals. Figure 5 shows a plot of the consumed O₂ concentrations versus time. A rapid O₂ consumption was observed in the ET-03/Cys system with complete O₂ consumption only in one and a half hours. Conversely, the relatively low O₂ consumption was observed in the AT-03/GSH system in which the O₂ consumption was not complete even after 12 hours. According to the data in Figure 5, the initial O₂ consumption rate for each reaction system (Table 2) was calculated with the order of ET-03/Cys (209.8 \pm 8.6 μ M O₂ /h) > ET-03/GSH (70.5 \pm 4.2 μ M O₂/h) > AT-03/Cys (43.2 \pm 3.1 μ M O₂/h) > AT-03/GSH (23.8 \pm 2.2 μ M O₂/h).

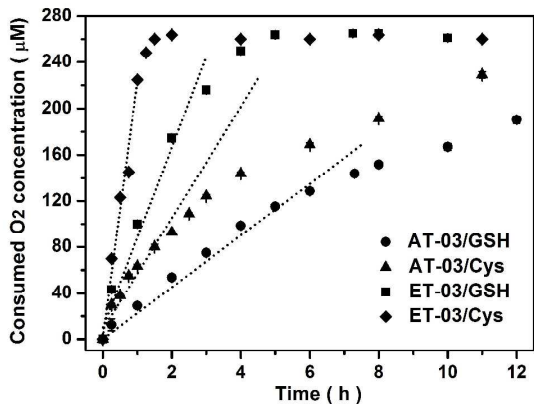


Figure 5 Plot of the consumed O₂ concentration as a function of time in the sealed capillary containing trityl radical (80 μM) and thiol (2 mM) in PB (pH 7.4, 50 mM); AT-03/GSH (circle), AT-03/Cysteine (triangle), ET-03/GSH (square), ET-03/Cysteine (rhombus). All the experiments were repeated three times and error bars are small in some cases and within the symbols.

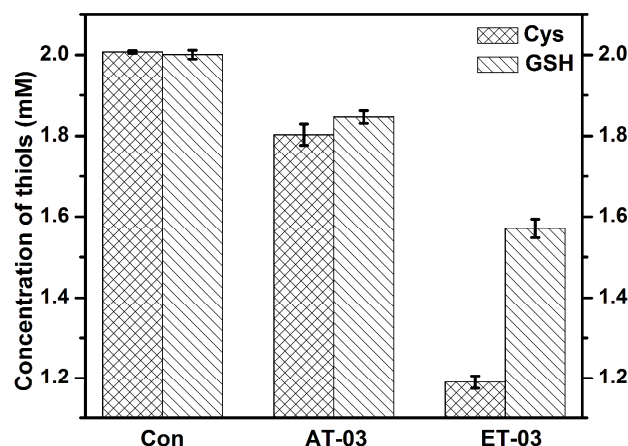
Table 2 O₂ consumption rates (μM O₂/h) in the sealed capillary containing trityl radical (80 μM) with thiols (2 mM) in PB (pH 7.4, 50 mM).

Trityl radical	GSH	Cys
AT-03	23.8±2.2	43.2±3.1
ET-03	70.5±4.2	209.8±8.6

Effect of trityl radicals and thiols on the thiol consumption

Figure 6 shows the thiol concentrations measured after the incubation of thiol with ET-03 or AT-03 for 1 hour in PB (pH 7.4, 50mM). No significant consumption of thiols was observed in the absence of the trityl radicals. Addition of the trityl radical to the solution of either GSH or cysteine in PB induced the thiol consumption. The concentrations of thiols remaining after 1-h incubation with the trityl radicals are

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4 closely related to types of trityl radicals and thiols: 1.85 ± 0.02 mM (AT-03/GSH) >
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6 1.80 ± 0.03 mM (AT-03/Cys) > 1.57 ± 0.02 mM (ET-03/GSH) > 1.19 ± 0.02 mM
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8 (ET-03/Cys). The fast thiol consumption was observed for the system of ET-03/Cys
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11 with 40% of cysteine consumed during the period of 1 hour.



31 **Figure 6** Thiol concentrations remaining after 1-h incubation with trityl radicals. Thiol (2 mM)
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33 was mixed with the solution of trityl radical (80 μ M) in PB (pH 7.4, 50 mM) at ambient
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35 atmosphere. After one hour, the thiol concentrations in different systems were determined using
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37 the Ellman's method.
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40 Discussion

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46 High stability of trityl radicals is the key feature accounting for their wide
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48 applications in magnetic resonance-related fields. It was previously shown that these
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50 radicals and their derivatives exhibit reactivity only towards some oxidants such as
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52 $O_2^{\bullet-}$, alkylperoxyl radicals or peroxides in the presence of redox enzymes.^{16, 23, 27, 37}
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56 However, the direct reduction of trityl radicals by thiols was not previously reported,
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4 although the enzyme-mediated reduction of trityl radicals was observed in rat liver
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6 microsomes in the presence of NADPH.^{13, 26} In this study, we demonstrate that the
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8 trityl derivatives ET-03 and AT-03 can be reduced by biothiols such as GSH and
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10 cysteine to form the corresponding trityl carbanions which can rapidly react with O₂
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12 in air to generate O₂^{•-} with the recovery of the trityl derivatives.
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16 Oxygen plays an important role in the reactions of both ET-03 and AT-03 with
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18 thiols. Under aerobic conditions, the reaction of ET-03 (50 μM) with GSH (500 μM)
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20 did not cause any significant change in its EPR signal intensity in half an hour (Figure
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22 1A). However, limiting access of O₂ to the reaction mixture in the sealed capillary
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24 leads to the gradual decay of ET-03 (Figure 1C). Complete removal of O₂ from the
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26 reaction mixture (Figure 1D) induced a relatively faster decay of ET-03 with t_{1/2} =
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28 4.83 h as compared to t_{1/2} = 21.49 h under aerobic conditions (Figure S7). The direct
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30 evidence for the involvement of O₂ stems from the O₂ consumption in the reactions of
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32 the trityl derivatives with thiols (Figure 1C and 5, Table 2). Therefore, O₂ is the
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34 “mask” to conceal the reactivity of trityl derivatives with thiols.
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41 Interestingly, the reactivity with thiols is closely related to the substituents of the
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43 trityl radicals as well as pK_a's of the thiol groups (Table 1). The order of their reaction
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45 rate constants is as follows: $k_{\text{ET-03/Cys}} (0.336 \text{ M}^{-1} \text{ s}^{-1}) > k_{\text{ET-03/GSH}} (0.07 \text{ M}^{-1} \text{ s}^{-1}) >$
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47 $k_{\text{AT-03/Cys}} (0.032 \text{ M}^{-1} \text{ s}^{-1}) > k_{\text{AT-03/GSH}} (0.027 \text{ M}^{-1} \text{ s}^{-1})$. While both ET-03 and AT-03
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49 exhibit reactivity with thiols, CT-03 is inert to thiols under the similar condition.
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51 Cysteine with low pK_a (8.3) has higher k values with ET-03 and AT-03 than GSH
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53 (Table 1), implying that the thiolate is the reactive form of thiol in their oxidation
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4 reactions. In order to check if other reducing agents can also initiate the reduction of
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6 the trityl radicals, we also tested the reactivity of ET-03 (50 μM) under anaerobic
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8 conditions with ascorbic acid (10 mM) under anaerobic conditions which is a crucial
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10 reducing agent for the intracellular reduction of nitroxide radicals. However, no
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12 reaction was observed between them under our experimental conditions (Figure S17).
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14 Certainly, our present results do not exclude the susceptibility of trityl radicals to
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16 other reducing agents. In the previous studies, the reductive metabolism of trityl
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18 radicals (e.g., CT-03) and their ester derivatives under anaerobic conditions was
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20 observed in the presence of NADPH in rat liver microsomes. The direct reduction of
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22 the trityl radicals by NADPH may be also involved in this system.
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29 The oxidation of the trityl carbanions by O_2 resulted in the recovery of the
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31 corresponding trityl radicals (Figure 1D). However, it was reported previously that the
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33 trityl carbanions were also able to undergo protonation to the corresponding
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35 diamagnetic triarylmethane in the presence of water or other proton sources.^{13, 26} Thus,
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37 the protonation of the trityl carbanions may compete with their oxidation by O_2 in
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39 aqueous solution. Careful examination of the result in Figure 1D shows that both
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41 ET-03 and AT-03 have relatively high recovery efficiencies from their carbanions by
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43 O_2 with the percentages of 87% [(48.5-38.3) μM /(50-38.3) μM] and 62% [(48.9-47.1)
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45 μM /(50-47.1) μM], respectively, during the same period (~ 2 hours). Relatively
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47 efficient recovery of trityl radicals demonstrates that the oxidation reactions of both
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49 trityl carbanions by O_2 instead of their protonations are predominant under aerobic
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51 conditions. That the trityl carbanions have low tendency for the protonation can be
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3 rationalized by the strong electron-withdrawing character of the ester/amide
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5 substituents which stabilizes the carbanions by efficient delocalization of the negative
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7 charges and further lowers pKa's of the corresponding triarylmethanes. As expected,
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9 the protonation of these trityl carbanions would be more significant in tumor or
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11 ischemic tissues where there are pH and O₂ concentrations.³⁸ On the other hand,
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13 considering that the trityl carbanions can be oxidized by O₂ back to trityl radicals, the
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15 triarylmethanes may also react with O₂ to result in the trityl radicals, provided that the
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17 triarylmethanes can be deprotonated to the corresponding carbanions in the presence
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19 of appropriate bases.
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26 As mentioned above, the one-electron transfer of these trityl carbanions to O₂ is
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28 the dominant route responsible for their decomposition. Thus, the O₂ consumption
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30 rate (Table 2) and subsequent production of O₂^{•-} (Figure 4) in the reaction systems
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32 should be correlated with the second-order rate constants of trityl radicals with thiols
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34 (Table 1). Considering that the electron transfer between the trityl carbanions and O₂
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36 is much faster than the reaction of trityl radical with thiols (Figure 1D), the production
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38 of the trityl carbanions is the rate-limiting step for the O₂ consumption. However, the
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40 O₂ consumption rate (~ 35 μM/h at the first 2 hours, Figure 1C) in the reaction system
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42 of ET-03 with GSH is 6-fold higher than the decay rate of ET-03 (~ 6 μM/h, Figure
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44 1D), thus indicating that additional pathway(s) may be involved in the O₂
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46 consumption in this system. It has been shown that the cycle reaction among O₂, O₂^{•-}
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48 and GSH greatly promotes the O₂ consumption.³⁶ Moreover, the fact that a large
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50 amount of thiols were consumed after incubation with the trityl radicals (Figure 6)
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3 further confirms that the thiol-involved cycle reaction exists in the trityl radical/thiol
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6 systems and the trityl radicals catalyze the consumption of the thiols. Therefore, trityl
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9 radical-catalyzed thiol and O₂ consumptions as well as subsequent O₂^{•-} production
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11 may arouse oxidative damage to biological systems and this adverse effect should be
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13 considered in their biomedical applications.
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16 In summary, our results show that the ester and amide derivatives of CT-03 (i.e.,
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18 ET-03 and AT-03) can be reduced by biothiols such as GSH and cysteine with the
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20 consumption of these biothiols, resulting in the production of the corresponding trityl
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22 cabanions which are relatively stable at neutral pH under anaerobic conditions but can
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24 be quickly recovered back by O₂ in air to the trityl derivatives accompanied by the
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26 generation of O₂^{•-} (Figure 7). An alternative mechanism may also involve O₂ addition
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28 to the *para* position of one aromatic group to form the corresponding trityl peroxy
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30 radical³⁹ that is further be reduced by thiol to the trityl hydroperoxide, followed by the
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32 release of [•]OH and the original trityl radical via homolytic cleavage of the C-O bond.
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34 Since the carboxylic groups in CT-03 are the only modification sites, both
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36 esterification and amidation have already been its preferred derivatization strategies.<sup>9,
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11-17 Although our observation was based on CT-03 and its derivatives, similar results
would also be suitable for its hydrophilic analogue OX063 which has similar redox
properties with CT-03. Our present study should be of significant importance at the
following aspects: (1) this study demonstrates a new metabolic mechanism for these
trityl derivatives and provides a warning for their in vivo applications due to the
production of the potentially toxic O₂^{•-} and the consumption of the thiol antioxidants.

(2) The production of $O_2^{\bullet-}$ and further reaction with thiols may partially deplete cysteines in proteins and thus reduce the labeling efficiency of proteins when using esterified trityl spin labels.⁴⁰ (3) The thiol-dependent generation of $O_2^{\bullet-}$ from the trityl derivatives may find applications in the treatment of cancers in which radical generation can be exploited to kill cancer cells. (4) This reaction also has to be considered for design of new trityl radicals as $O_2^{\bullet-}$ probes since the generation of $O_2^{\bullet-}$ from trityl radicals themselves could interfere with the detection of this reactive species in biological systems.²³⁻²⁵ The covalent dendritic encapsulation of trityl radicals would be a feasible way to prevent their reactions with thiols.^{14, 41} Development of new dendritic trityl probes with high selectivity to $O_2^{\bullet-}$ is underway.

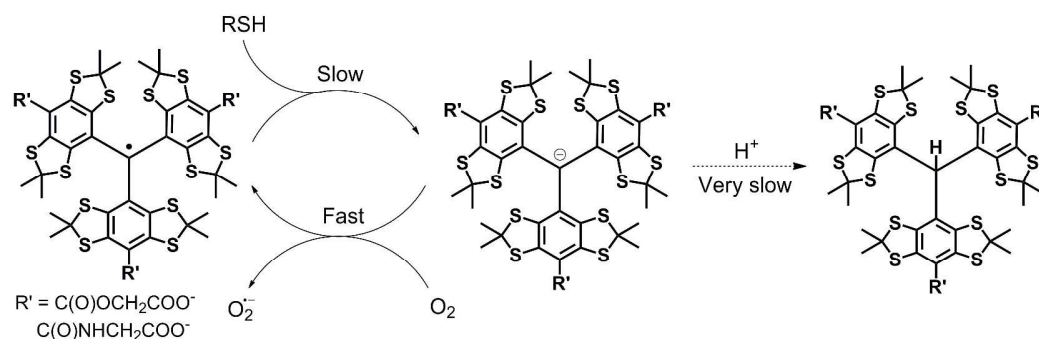


Figure 7 Reaction mechanism of trityl radical with thiol (RSH) under aerobic condition.

Supporting Information

HPLC chromatograms and high resolution mass spectra of ET-03 and AT-03; EPR spectra and the peak-to-peak linewidths of ET-03 and AT-03 under anaerobic and aerobic conditions; kinetic studies for the reactions of trityl radicals with thiols under anaerobic conditions; cyclic voltammograms of ET-03 and AT-03; UV-Vis analysis for

the stability of ET-03 and AT-03 towards superoxide radicals; hyperfine splitting constants of the spin adducts of BMPO. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

DMF, N,N-dimethylformamide; EPRI, electron paramagnetic resonance imaging; ESI, electrospray ionization; HRMS, highresolution mass spectrometry; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; HOBt, 1-hydroxybenzotriazole; SOD, superoxide dismutase; DTNB, 5,5'-Dithiobis-(2-nitrobenzoic acid); GSH, Glutathione; Cys, cysteine; DMPO, 5,5-Dimethyl-1-pyrroline N-oxide; BMPO, 5-tert-Butoxycarbonyl-5-methyl-1-pyrroline N-oxide; TAM, triarylmethyl radical. $O_2^{\cdot-}$, superoxide radical; $\cdot OH$, hydroxyl radical; PB, phosphate buffer; DNP, dynamic nuclear polarization.

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