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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 4062–4065

Synthesis and characterization of a perchlorotriphenylmethyl (trityl) triester radical: A potential sensor for superoxide and oxygen in biological systems

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Received 6 March 2007; revised 21 April 2007; accepted 23 April 2007
Available online 27 April 2007

Abstract—Synthesis and characterization of an inert perchlorotriphenylmethyl triester radical, PTM-TE, are reported. PTM-TE was prepared by a facile 3-step synthesis using Friedel-Crafts reaction of tetrachlorobenzene with chloroform followed by ethoxycarbonylation and subsequent oxidation. PTM-TE is paramagnetic and exhibits a single sharp EPR spectrum. In solution, the EPR linewidth of PTM-TE is highly sensitive to the dissolved oxygen content, thus enabling accurate measurement of oxygen concentration (oximetry). In addition, the radical also shows high reactivity towards superoxide. The ester radical has the potential for use as a high-sensitive probe for determination of oxygen concentration and superoxide in biological systems.

In 1900, Moses Gomberg reported the existence of a stable, trivalent organic free radical which challenged the prevailing belief that carbon could only have four chemical bonds.[1] As this report provided new possibilities to carbon chemistry, research aimed at understanding and developing these radicals continued to thrive and permeated various fields of science including medicine and industrial applications. Most notably, Gomberg's work paved the way for future research on using persistent trityl radicals in clinical applications.[2–4] However, it is known that some radicals and diradicals are short-lived and very reactive – the half-life of triphenylmethyl radicals in aerated solution may be as low as a fraction of a second. Studies have also shown that these reactive radicals become stable and chemically inert upon perchlorination.[5–8] In light of this endeavor, highly chlorinated mono-, di-, and triarylmethanes were developed and since then they have become the most valuable chemical precursors of inert free radicals with estimated half-lives in the order of 100 years.[6] Together, these studies have been regarded as both promising and significant.[5] As a result, the synthesis of perchlorinated trityl radical has become an immense undertaking with promising implications.

The role of reactive oxygen species (ROS) such as superoxide, hydroxyl, and alkylperoxyl radicals has been linked to a variety of pathophysiological processes.[9] Since an increased production of ROS, especially superoxide, leads to oxidative stress, accurate determination of these free radicals can help us to gain a better understanding of oxidative stress-related diseases. Of the many techniques currently available for the detection of superoxide in biological systems, electron paramagnetic resonance (EPR) spectroscopy has the distinct capability for direct and real-time detection. However, the EPR technique requires the use of high concentrations (10–100 mM) of spin-trap molecules to maximize the efficiency of trapping, which may alter the redox environment of the system.[10,11] The poor efficiency of the spin-trap molecules for superoxide detection is attributed to small bimolecular reaction rate constants (typically about 10^2 M^{-1} s^{-1}). Thus, there is a great need to develop probes with orders of magnitude increased

Keywords: Perchlorotriphenylmethyl radical; Perchlorotriphenylmethyl triester; Superoxide; Free radicals; EPR; Oximetry; Spectrophotometry.
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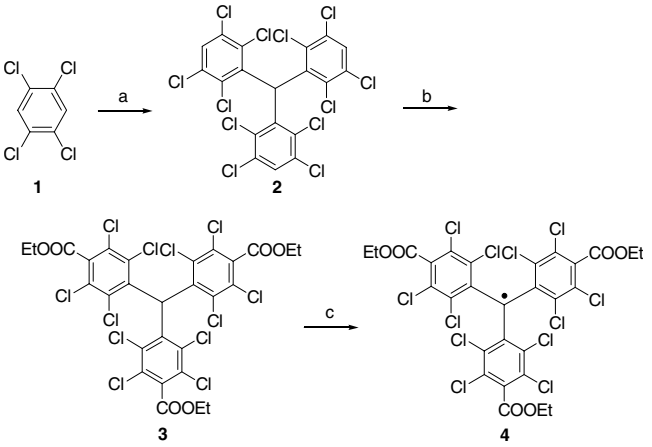
doi:10.1016/j.bmcl.2007.04.070

reactivity with superoxide. To this end, we have developed a perchlorotriphenylmethyl triester radical (PTM-TE) that can overcome this limitation. PTM-TE has a high detection-sensitivity due to sharp single-line EPR spectrum. In addition, PTM-TE is an attractive candidate for measurement of oxygen concentration (oximetry). Furthermore, the ester nature of the PTM-TE radical is expected to facilitate its penetration into cells, followed by hydrolysis by cellular enzymes such as esterases into carboxylate radical and thus can be trapped in the cells. This will enable the detection of superoxide radicals produced inside the cells. As an oxygen probe, PTM-TE can be utilized for measurement as well as mapping (imaging) of oxygen distribution in tissues.[12] In this communication, we report the synthesis, characterization, and preliminary evaluation of PTM-TE for oximetry and detection of superoxide.

The synthesis of PTM-TE radical **4** (Scheme 1) used tris(2,3,5,6-tetrachlorophenyl)methane generated from the Friedel-Crafts reaction of 1,2,4,5-tetrachlorobenzene and chloroform.[13] Reaction of compound **2** with slight excess of butyllithium and TMEDA in THF at low temperature yielded the corresponding tri-anion. This tri-anion was reacted with ethylchloroformate in situ to yield compound **3**.[14] Compound **3** was then converted into **4** by a two-step process: conversion into the triphenylcarbanion with NaOH in a mixture solution of DMSO and Et_2O, followed by oxidation with I_2.[15] Since compound **3** has the ethoxycarbonyl functional group, it can be easily modified to afford more variants for future research.

PTM-TE was characterized by EPR spectroscopy (Fig. 1). The solid showed a broad EPR signal with a peak-to-peak width (linewidth, ΔB_{pp}) of 5.650 G in room air. However, a solution of PTM-TE (100 μM) in dimethylsulfoxide (DMSO) at ambient conditions showed a single sharp peak with a linewidth of 0.540 G. No self-induced spin-spin broadening was observed for up to 12 mM concentration of PTM-TE. The linewidth of PTM-TE in DMSO, however, was



Scheme 1. Synthesis of PTM-TE radical. Reagents and conditions: (a) $CHCl_3$, $AlCl_3$; (b) *n*-BuLi, TMEDA, ethyl chloroformate, 77%; (c) 1—NaOH, DMSO/Et_2O, 2—I_2, Et_2O, 93%.

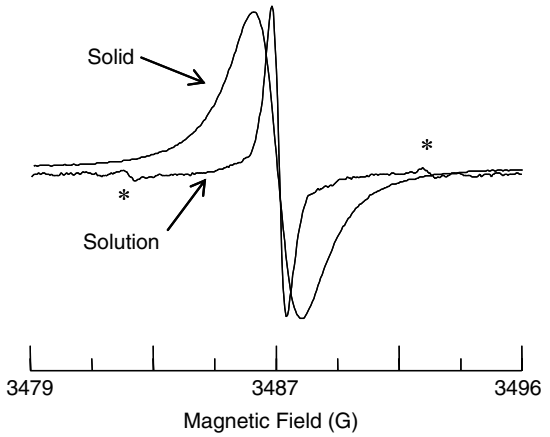


Figure 1. EPR spectra of PTM-TE. The spectra were measured from a sample of crystalline powder and in a solution of PTM-TE (100 μM) in DMSO at ambient conditions using an X-band EPR spectrometer. The EPR acquisition settings were: modulation amplitude, 160 mG; microwave power, 1 mW; time constant, 40 ms; scan time, 15 s. The first derivative peak-to-peak widths were 5.650 G for solid and 0.540 G for solution. The small satellite peaks indicated with * on both sides of the main peak are due to hyperfine coupling with ^{13}C nuclei (natural abundance).

dependent on the concentration of dissolved molecular oxygen in solution. We measured the linewidth of PTM-TE (100 μM) in DMSO as well as in hexafluorobenzene (HFB) as a function of partial pressure of equilibrating oxygen (pO_2). As shown in Figure 2, the linewidth increased proportionally to pO_2. The oxygen-induced broadening is attributed to the paramagnetic nature of molecular oxygen, which upon collision with PTM-TE molecule can undergo Heisenberg spin exchange interaction resulting in broadening of the observed EPR signal. It should be noted that this exchange interaction does not chemically alter the two molecules involved. Furthermore, the oxygen-induced broadening has no effect on the EPR signal intensity (double-inte-

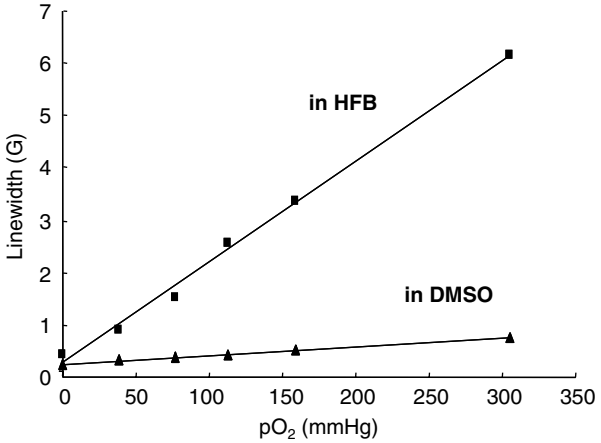


Figure 2. Effect of oxygen on the EPR linewidth of PTM-TE in solution. Measurements were made by equilibrating a solution of PTM-TE (100 μM) in DMSO or HFB with pre-calibrated gas mixtures of known O_2 concentration. The data show a linear dependence of linewidth with pO_2. The oxygen-sensitivity was 17.71 mG/mmHg for HFB and 1.6 mG/mmHg for DMSO.

gral of the EPR spectrum). Hence, the width of the EPR signal can be used to measure the concentration of O_2 in the system. The calibration curves of EPR linewidth versus pO_2 can be generated for any solvent by equilibrating it with known concentrations of oxygen.

The results shown in Figure 2 indicate a significant contrast between the slopes of the calibration curves of PTM-TE in DMSO (1.6 mG/mmHg) and HFB (17.71 mG/mmHg). The higher sensitivity in HFB is largely attributed to higher solubility of oxygen in HFB. For example, the solubility of oxygen at atmospheric pressure (21% of oxygen) at 25 °C is 4400 μM in HFB as compared to 478 μM in DMSO.[16] In addition, viscosity and polarity of solvent molecule may also contribute to the observed difference.

The reactivity of PTM-TE to superoxide was determined. PTM-TE in DMSO (0.8 mM) was treated with a solution of potassium superoxide (KO_2) in DMSO (2.4 mM). As shown in Figure 3, the EPR spectrum of PTM-TE was completely quenched by superoxide suggesting that PTM-TE radical reacts with superoxide to form a diamagnetic product. Although the mechanism by which PTM-TE reacts with superoxide is unclear, previous reports suggested that the neutral protonated form of superoxide, $^{\cdot}OOH$, rather than $O_2^{\cdot-}$ is involved in the reaction, as illustrated in the following equation:

$$[R_3C^{\cdot}] + {}^{\cdot}OOH \rightarrow [R_3C\text{–}OOH]$$

It is likely that the superoxide anion or $^{\cdot}OOH$ radical interacts with PTM-TE to form a covalent bond between the methyl radical epicenter and the oxygen atom of $^{\cdot}OOH$.[10] The resulting adduct is diamagnetic and hence, EPR silent. This observation is confirmed by ^1H NMR spectroscopy where only the triplet and quartet splittings for the ethoxycarbonyl substituent were identified. The absence of a singlet peak at δ 7 (ppm) corresponding to the epicenter methine proton (data not shown) indicates that the resulting compound appears most likely to be the proposed adduct, $[R_3C\text{–}OOH]$.

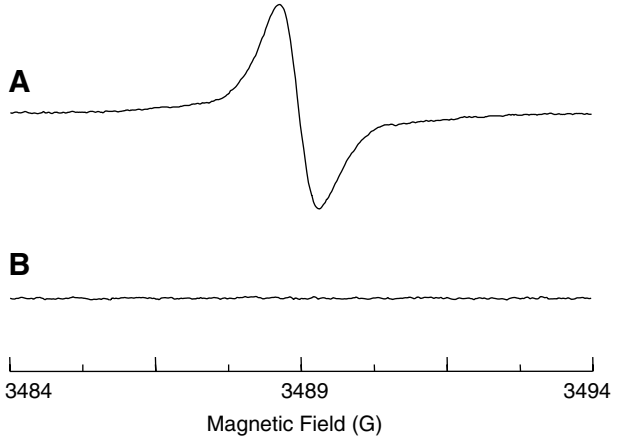


Figure 3. The effect of superoxide on the EPR spectrum of PTM-TE radical in DMSO. (A) EPR spectrum of PTM-TE in DMSO (0.8 mM). (B) EPR spectrum of PTM-TE in DMSO following addition of solid KO_2 (2.4 mM). The PTM-TE signal is quenched by $O_2^{\cdot-}$.

PTM-TE was specifically designed with the ethoxycarbonyl substituent attached to each of the phenyl rings for a specific purpose, that is, cellular internalization. The hydrophobic character of the molecule permits it to easily diffuse across the membrane lipid bilayers. Then, by the nonspecific action of intracellular esterases, the molecule is expected to be cleaved to yield the corresponding tricarboxylate radical, PTM-TC. The resulting PTM-TC is another stable 'inert' radical which also gives a single sharp EPR peak in aqueous solutions.[17] Additionally, PTM-TC gives a characteristic UV–vis absorption at 380 nm. Our recent study has shown that PTM-TC, on reaction with superoxide, induces a decrease in EPR signal or optical absorption at 380 nm with high specificity and sensitivity.[18] Thus, PTM-TE can be used to monitor and quantify the production of intracellular superoxide.

In summary, we have developed an efficient synthetic route for the synthesis of PTM-TE. Additionally, we have demonstrated the effect of molecular oxygen and superoxide on the EPR spectrum of PTM-TE in solution. Unlike molecular oxygen, superoxide induces a signal loss. Thus, PTM-TE potentially can be used to determine oxygen and/or superoxide generation in biological applications.

Acknowledgment

This study was supported by NIH Grant EB004031.

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14. Tris(2,3,5,6-tetrachlorophenyl)methane (486 mg, 0.74 mmol) and TMEDA (377 μl, 2.4 mmol) were dissolved in dry THF (50 ml) under dry nitrogen atmosphere and cooled to −78 °C. A solution of 1.6 M butyllithium in hexanes (1.6 ml, 2.5 mmol) was added in one portion and the

mixture was stirred at low temperature for 1 h. The reaction was quenched with ethyl chloroformate and allowed to reach room temperature overnight. Solvent was evaporated and the residue dissolved in dichloromethane. The organic layer was washed with water and dried over sodium sulfate. Solvent was evaporated under vacuum. Silica gel chromatography (hexanes/AcOEt:12/1) yielded 500 mg (77%) of tris(2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl)methane **3** as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.00 (s, 1H); 4.47 (q, J = 7.1 Hz, 6H); 1.41(t, J = 7.1 Hz, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.2, 138.5, 135.5, 135.0, 134.0, 130.5, 129.5, 63.1, 56.3, 14.0. UV–vis (hexanes): $\lambda_{max}(\varepsilon)$ 292 (2220), 302 (2480). IR: ν 2982, 2936, 1743, 1556, 1465, 1446, 1371, 1341, 1329, 1299, 1263, 1225, 1208, 1121, 1096, 1019, 859 cm^{-1}. HRMS $[M+Na]^+$ calcd for $C_{28}H_{16}Cl_{12}O_6Na$ 896.7018, found 896.7041.

15. Tris(2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl)methane (1.49 g, 1.78 mmol) was dissolved in the mixture of DMSO/Et_2O (75/425). To the solution, powdered NaOH was added and the mixture was stirred in the dark for 24 h. The mixture was filtered through a dry funnel into a solution of iodine (1.9 g) in Et_2O (100 ml). The solution was left undisturbed in the dark for 24 h. The resulting solution was then washed with concentrated $NaHCO_3$ (39%, 50 ml), brine, and then water (50 ml × 2). Solvent was dried over Na_2SO_4 and evaporated. The residue was purified by silica gel chromatography (Hexane/AcOEt: 80/20) to give a red solid (1.378 g, 92.5%). EPR (solid, 298 K): single peak, ΔB_{pp} = 5.650 G. UV–vis (hexane): $\lambda_{max}(\varepsilon)$ 289 (5010), 378 (8830). IR: ν 2938, 1740, 1536, 1445, 1380, 1329, 1284, 1222, 1136, 1007, 858, 720 cm^{-1}. HRMS $[M+Na]^+$ calculated for $C_{28}H_{15}Cl_{12}O_6Na$ 895.694, found 895.692.
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