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Toward Stable Electron Paramagnetic Resonance Oximetry Probes: Synthesis, Characterization, and Metabolic Evaluation of New Ester Derivatives of a Tris-(*para*-carboxyltetrathiaaryl)methyl (TAM) Radical

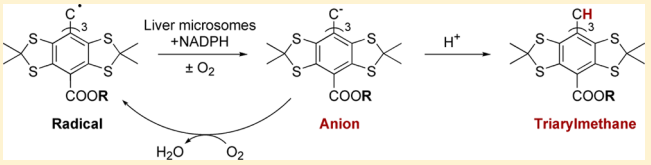
Christophe Decroos,[†] Véronique Balland,[‡] Jean-Luc Boucher,*[,†] Gildas Bertho,[†] Yun Xu-Li,[†,§] and Daniel Mansuy[†]

[†]Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601 CNRS, Université Paris Descartes, Sorbonne Paris Cité, 45 rue des Saints-Pères, 75270 Paris, France

[‡]Laboratoire d'Electrochimie Moléculaire, UMR CNRS 7591, Université Paris Diderot, Sorbonne Paris Cité, 15 rue J.-A. de Baïf, 75205 Paris, France

Supporting Information

ABSTRACT: Tris(*p*-carboxyltetrathiaaryl)methyl (TAM) radicals, such as **1a** ("Finland" radical), are useful EPR probes for oximetry. However, they are rapidly metabolized by liver microsomes in the presence of NADPH, with the formation of diamagnetic quinone-methide metabolites resulting from an oxidative decarboxylation of one of their carboxylate substituents. In an effort to obtain TAM derivatives potentially more metabolically stable *in vivo*, we have synthesized four new TAM radicals in which the carboxylate substituents of **1a** have been replaced with esters groups bearing various alkyl chains designed to render them water-soluble. The new compounds were completely characterized by UV−vis and EPR spectroscopies, high resolution mass spectrometry (HRMS), and electrochemistry. Two of them were water-soluble enough to undergo detailed microsomal metabolic studies in comparison with **1a**. They were found to be stable in the presence of the esterases present in rat liver microsomes and cytosol, and, contrary to **1a**, stable to oxidation in the presence of NADPH-supplemented microsomes. A careful study of their possible microsomal reduction under anaerobic or aerobic conditions showed that they were more easily reduced than **1a**, in agreement with their higher reduction potentials. They were reduced into the corresponding anions not only under anaerobic conditions but also in the presence of dioxygen. These anions were much more stable than that of **1a** and could be characterized by UV−vis spectroscopy, MS, and at the level of their protonated product. However, they were oxidized by O_2, giving back to the starting ester radicals and catalyzing a futile cycle of O_2 reduction. Such reactions should be considered in the design of future stable EPR probes for oximetry *in vivo*.



INTRODUCTION

The dioxygen partial pressure (pO_2) is an important parameter in the metabolic processes of living organisms. Measurement of dioxygen concentration *in vitro* and *in vivo* is of crucial importance in the study of both physiological and pathological situations including cancers and ischemia-reperfusion.[1,2] Various methods have been developed to detect and monitor pO_2.[3−10] Among them, electron paramagnetic resonance spectroscopy measures the O_2-dependent linewidths of exogenous paramagnetic contrast agents such as nitroxides and triarylmethyl radicals, as well as particulate-based probes such as chars and lithium phthalocyanines.[11−13] Spin−spin interaction between the paramagnetic spin probes and dioxygen, also a paramagnetic molecule, modifies the relaxation time of the probe and its peak-to-peak linewidth.[3,14] For these spectroscopic and imaging modalities, useful spin probes should display one single sharp line in their EPR spectra with a high O_2-dependent line width. Furthermore, it should possess good stability in the presence of biological systems, low toxicity, and good water solubility.

Triarylmethyl (TAM) radicals **1a** and **1b** (called Oxo63) (Figure 1) are stable carbon-centered radicals that have been developed for use as contrast agents in NMR imaging and as probes for EPR spectroscopy and EPR imaging (EPRI).[9,14−18] They have been demonstrated to be very effective spin probes for measuring pO_2 *in vitro* and *in vivo* through line-broadening analysis because their EPR spectrum displays one narrow single line whose linewidth linearly depends upon the O_2 concentration.[3,14−19] Oxo63, **1b**, is highly soluble in water and weakly toxic, and its pharmacokinetics in mice has been followed by EPR spectroscopy and imaging.[19] *In vitro*, **1b** is stable in the presence of oxidoreductants such as ascorbate, glutathione, NADPH, hydrogen peroxide, and hydroxyl radical.[20−22] However, several studies have shown that TAMs **1a−b** readily

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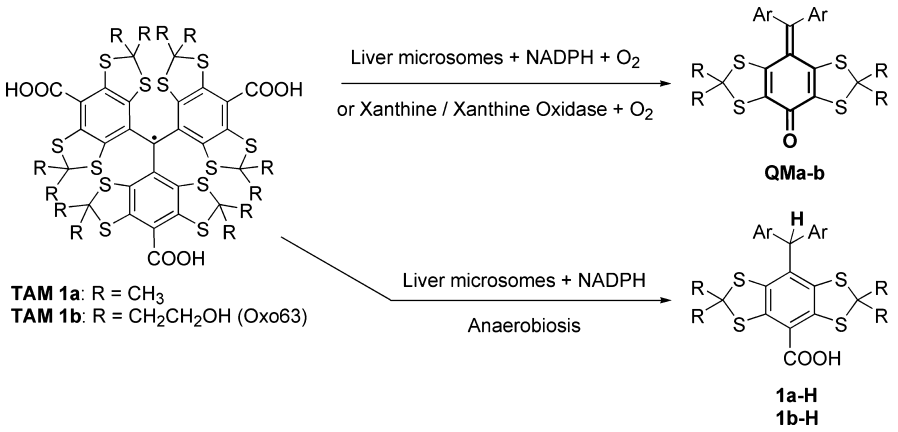

Figure 1. Structure and metabolism of TAM **1a** and **1b** by liver microsomes. Adapted from ref 24. Copyright 2009 American Chemical Society.

react with superoxide[20−22] with the formation of the corresponding diamagnetic quinone-methides **QMa-b**[22] (Figure 1), and some TAM radicals may be used for the specific measurement of superoxide, $O_2^{\bullet-}$, in cell-free and cellular systems.[20,21,23]

We have recently demonstrated that **1a−b** undergo several metabolic transformations.[24,25] Their aerobic incubations in the presence of rat, human, and pig liver microsomes and NADPH led to the quinone-methides **QMa-b** that result from the oxidative decarboxylation of **1a−b** by superoxide[24] that is known to be generated by cytochromes P450 and P450 reductase under decoupling conditions.[26,27] Under anaerobic conditions, **1a−b** are reduced by NADPH to the diamagnetic triarylmethane derivatives **1a-b-H** (Figure 1) in reactions also catalyzed by P450s and P450 reductase.[24] Finally, we have also shown that hemeproteins (hemoglobin and myoglobin) and peroxidases (lactoperoxidase, prostaglandin hydroperoxide synthase, and horseradish peroxidase) efficiently catalyze the oxidative decarboxylation of **1a−b** by hydrogen peroxide or hydroperoxides, leading eventually to the quinone-methides **QMa-b**, via the corresponding cations **1a−b**$^+$.[25]

These data indicated that decarboxylation of **1a−b** was mainly at the origin of the metabolic instability of these compounds. In an effort to improve the metabolic stability of these EPR probes, we have synthesized new compounds in which the carboxylate groups of **1a−b** were replaced with ester functions. In order to obtain probes with sharp EPR signals, we excluded amide functions since some TAM radicals bearing an amide group have been synthesized and found to display large linewidths due to hyperfine coupling with the N-atoms.[28,29] Some ester derivatives of **1a−b** have been synthesized, and EPR studies have shown that they display intermediate linewidths since the H-atoms of the ester alkyl chain also induce hyperfine coupling, but this was smaller than that observed with amide groups.[30−34]

This article describes the synthesis and UV−vis, HRMS, EPR, and electrochemical characteristics of four new TAM esters bearing either positive or negative charges or polyether chains that should make them water-soluble enough. It also compares the metabolic stability of the two most water-soluble new TAM esters in the presence of liver microsomes, with or without NADPH, with that previously reported for **1a−b**.

EXPERIMENTAL PROCEDURES

Chemicals. Tris-(8-carboxyl-2,2,6,6-tetramethylbenzo-[1,2-*d*;4,5-*d*′]bis[1,3]dithiol-4-yl)methyl sodium salt (**1a**) was synthesized according to a previously described method.[35] Cytochrome *c* was purchased from Sigma-Aldrich (St. Quentin Fallavier, France). All other chemicals and solvents were of the highest grade commercially available.

Spectroscopic Methods. All NMR experiments (^{1}H, ^{13}C, heteronuclear single quantum correlation, and heteronuclear multiple bond correlation) were carried out at room temperature on a Bruker Biospin Advance II 500 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane and coupling constants (J) in Hz. UV−vis spectra were recorded on a Cary 300 spectrometer (Varian, Les Ulis, France). Mass spectra were obtained on an LCQ Advantage ion trap spectrometer (Thermo Scientific, Courtaboeuf, France) by electrospray ionization in both positive (ESI$^+$) and negative (ESI$^-$) ionization detection modes under the following conditions: sheath gas, 40; auxiliary gas, 10; spray voltage, 5 kV; capillary temperature, 300 °C; capillary voltage, 7 V; and scanning in full scan mode (m/z from 200 to 1500). Data were recorded and analyzed with the XCalibur acquisition system. High-resolution mass spectra (HRMS) were obtained by ESI in time-of-flight detection mode on a LCT (Waters-Micromass, Guyancourt, France) spectrometer at ICSN (Gif sur Yvette, France). EPR spectra were recorded at 21 °C using a Bruker Elexsys 500 EPR spectrometer (Bruker, Wissenheim, France) operating at X-band (9.85 GHz) and an AquaX quartz cell (Bruker) fitted in a TM 110 cavity under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.03 G; time constant, 40.96 ms; conversion time, 40.96 ms; and microwave power, 1 mW. Data acquisition and processing were performed using Bruker Xepr software.

Synthesis of Esters 2−5. *Tris(8-[3-trimethylammonium]-propyloxycarboxyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d′]bis[1,3]-dithiol-4-yl)methyl Radical, Bromide, 2.* Cesium carbonate (65.0 mg, 200 μmol) was added to a solution of **1a** (50.0 mg, 50.0 μmol) in 1 mL of freshly distilled *N*,*N*-dimethylformamide (DMF). After 30 min at room temperature, (3-bromopropyl)trimethylammonium bromide (196.0 mg, 750 μmol) and KI (2.0 mg, 12.0 μmol) were added, and the mixture was stirred under argon for 15 h. The solvent was evaporated under high vacuum, and the crude product was purified by chromatography on neutral alumina with CH_2Cl_2/EtOH gradients to afford compound **2** as a green-orange solid (70.0 mg, 91%). R_f = 0.11 (Al_2O_3, CH_2Cl_2/ethanol/water 50:45:5 v/v/v). HRMS (ESI$^+$) calcd for $C_{58}H_{81}N_3O_6S_{12}$, 1299.2774; found, 1299.2757.

Tris(8-[3-sulfonic acid]propyloxycarboxyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d′]bis[1,3]dithiol-4-yl)methyl Radical, Sodium Salt, 3. Cesium carbonate (32.6 mg, 100 μmol) was added to a solution of **1a** (25.0 mg, 25.0 μmol) in 1.5 mL of freshly distilled DMF. After 30 min at room temperature, sodium 3-bromopropanesulfonate (84.4 mg, 375 μmol) and KI (1.0 mg, 6.0 μmol) were added. The mixture was stirred under argon for 24 h, and the solvent was evaporated under high vacuum. Five milliliters of ethanol were added, and the solution was filtered. Ethanol was evaporated, and the crude product was purified by flash-chromatography on a

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preconditionned C18 column (AIT, Houilles, France) using water/CH_3CN gradients to afford compound **3** as a green-orange solid (32.0 mg, 89%). R_f = 0.37 (SiO_2, CH_2Cl_2/methanol 2:1 v/v + 0.1% acetic acid). HRMS (ESI^-) calcd for $C_{49}H_{54}O_{15}S_{15}$, 1361.9273; found, 1361.9226.

*Tris(8-[2-[2-(2-methoxyethoxy)ethoxy]ethoxycarboxyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d′]bis[1,3]dithiol-4-yl)methyl Radical, **4**.* Cesium carbonate (19.5 mg, 60.0 μmol) was added to a solution of **1a** (15.0 mg, 15.0 μmol) in 1.0 mL of freshly distilled DMF. After 30 min at room temperature, 1-bromo-2-[2-(2-methoxyethoxy)ethoxy]ethane (obtained from 2-[2-(2-methoxyethoxy) ethoxy]ethanol using CBr_4 and triphenylphosphine in THF, following a described protocol)[36] (52.0 mg, 229 μmol) and KI (1.0 mg, 6.0 μmol) were added. The mixture was stirred under argon for 36 h, and the solvent was evaporated under high vacuum. The crude product was purified by chromatography on SiO_2 with diethyl ether/cyclohexane gradients to afford compound **4** as a brown solid (18.0 mg, 82%). R_f = 0.85 (SiO_2, CH_2Cl_2/methanol 9:1 v/v + 0.1% acetic acid). HRMS for **4** chelating Na^+(ESI^+): calcd for $C_{61}H_{81}NaO_{15}S_{12}$, 1460.2122 ; found, 1460.2122.

*Tris(8-[4-triphenylphosphonium]butyloxycarboxyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d′]bis[1,3]dithiol-4-yl)methyl Radical, Bromide, **5**.* Cesium carbonate (78.0 mg, 240 μmol) was added to a solution of **1a** (60.0 mg, 60.0 μmol) in 3 mL of freshly distilled DMF. After 30 min at room temperature, (4-bromobutyl)-triphenylphosphonium bromide (431.0 mg, 900 μmol) and KI (2.5 mg, 15.0 μmol) were added, and the mixture was stirred under argon for 24 h. The solvent was evaporated under high vacuum, and the crude product was purified by chromatography on neutral alumina with CH_2Cl_2/ethanol gradients to afford compound **5** as a green-orange solid (115.0 mg, 87%). R_f = 0.69 (Al_2O_3, CH_2Cl_2/ethanol/water 50:45:5 v/v/v). HRMS (ESI^+) calcd for $C_{106}H_{105}O_6P_3S_{12}$, 1950.3773; found, 1950.3771.

Synthesis of Triarylmethanes 2-H and 3-H. *Tris(8-[3-trimethylammonium]propyloxycarboxyl-2,2,6,6-tetramethylbenzo-[1,2-d;4,5-d′]bis[1,3]dithiol-4-yl)methane, Bromide, **2-H**.* Radical **2** (10.0 mg, 6.5 μmol) was dissolved in 10 mL of 0.01 M deoxygenated HCl, and $Na_2S_2O_4$ (2.3 mg, 13.2 μmol) was added. After 15 min at room temperature under argon, water was evaporated under vacuum, and the crude product was dissolved in 1 mL of CH_3CN. The solution was filtered and concentrated under vacuum to afford triarylmethane **2-H** (10.0 mg, 100%) as a yellow solid. ^1H NMR (CD_3OD) δ 5.52 (s, 1H), 4.44–4.52 (m, 6H), 3.60 (t, 6H, J = 8.5), 3.21 (s, 27 H), 2.34–2.40 (m, 6H), 1.80 (s, 9H), 1.79 (s, 9H), 1.75 (s, 9H), 1.68 (s, 9H). ^{13}C NMR (CD_3OD) δ 23.95, 28.82, 28.94, 33.87, 34.99, 54.01, 63.48, 63.92, 64.16, 64.52, 65.49, 121.26, 131.43, 140.73, 141.46, 142.05, 142.97, 167.02. HRMS (ESI^+) calcd for $C_{58}H_{82}N_3O_6S_{12}$, 1300.2852; found, 1300.2816.

*Tris(8-[3-sulfonic acid]propyloxycarboxyl-2,2,6,6-tetramethylbenzo[1,2-d;4,5-d′]bis[1,3]dithiol-4-yl)methane, Sodium Salt, **3-H**.* Radical **3** (10.0 mg, 7.0 μmol) was dissolved in 10 mL of 0.01 M deoxygenated HCl, and $Na_2S_2O_4$ (2.4 mg, 13.8 μmol) was added. After 15 min at room temperature under an argon atmosphere, water was evaporated under vacuum, and the crude product was dissolved in 1 mL of methanol. The solution was filtered and concentrated under vacuum to afford triarylmethane **3-H** (10.0 mg, 100%) as a yellow solid. ^1H NMR (CD_3OD) δ 5.46 (s, 1H), 4.44–4.55 (m, 6H), 3.07 (t, 6H, J = 7.5), 2.27–2.33 (m, 6H), 1.80 (s, 9H), 1.77 (s, 9H), 1.74 (s, 9H), 1.67 (s, 9H). ^{13}C NMR (CD_3OD) δ 25.93, 29.14, 29.36, 33.51, 34.62, 49.75, 63.42, 63.64, 64.26, 66.51, 121.44, 131.29, 140.57, 141.40, 141.91, 142.96, 167.38. HRMS (ESI^-) calcd for $C_{49}H_{55}O_{15}S_{15}$, 1362.9352; found, 1362.9318.

Cyclic Voltammetry. Cyclic voltammetry was carried out with a PST20 Autolab potensiostat (Metrohm Autolab BV, Utrecht, The Netherlands) interfaced with a PC computer. Experiments were performed in a water-jacketed electrochemical cell maintained at 20 °C with a circulating water bath, using a platinum-wire auxiliary electrode, and a saturated calomel reference electrode (+ 0.242 V vs standard hydrogen electrode). The working electrode was a glassy carbon electrode (0.071 cm^2). Measurements were carried out in 0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA for radicals **1a-3** and in methanol containing 0.05 M tetrabutylammonium hexafluoroborate for radicals **1a-5**. Solutions were carefully degassed by the bubbling of nitrogen before each measurement. The redox potentials were calculated according to the equation $E = (E_{p,a} + E_{p,c})/2$.

HPLC Analysis. Analysis of products was performed at room temperature on a Super ODS (TSK gel reverse-phase, 50 mm × 4.6 mm, 2 μm, Interchim, Montluçon, France) using a Spectra Physics HPLC system. The mobile phase was a mixture of solvent A (10 mM ammonium acetate, pH 6.5) and solvent B (acetonitrile) with the following gradient: 0–2 min, isocratic elution with 5% B; 2–22 min, linear increase from 5 to 90% B; 22–25 min, isocratic elution with 95% B; 25–27 min, linear decrease to 5% B; and 27–35 min, re-equilibration at 5% B. The flow rate was 1 $mL{\cdot}min^{-1}$, and the absorbance was monitored at 270 nm using a Borwin data acquisition software. Under these conditions, the retention times for **1a** and **1a-H**, **QMa**, **2**, and **2-H**, and **3** and **3-H** were 7.3, 10.7, 16.5, and 9.6 min, respectively. HPLC-MS studies were performed on a Surveyor HPLC instrument coupled to the LCQ Advantage ion trap spectrometer (Thermo Scientific, Courtaboeuf, France) under the above-described conditions, except that the flow rate was 500 $\mu L{.}min^{-1}$.

Preparation of Rat Liver Microsomes and Cytosols. Male Sprague–Dawley rats (200–250 g) were provided laboratory chow and water ad libitum. After 7 days of adaptation, animals were treated with phenobarbital (80 $mg{\cdot}kg^{-1}$, in 0.9% saline, i.p. for 4 days). Liver cytosols and microsomes were prepared by differential centrifugation as previously reported and stored at −80 °C until use.[37] Protein concentrations were determined by the Bradford assay with bovine serum albumin as standard.[38] Cytochrome P450 contents were determined by the method of Omura and Sato.[39]

*Hydrolytic Stability of Radicals **2** and **3** in the Presence of Rat Liver Cytosol or Microsomes.* The hydrolytic stability of esters **2** and **3** (100 μM) was tested at 37 °C in the presence of 0.10 $mg{\cdot}mL^{-1}$ rat liver cytosolic or 1.0 $mg{\cdot}mL^{-1}$ microsomal proteins in 0.1 M phosphate buffer containing 0.1 mM EDTA. The reactions were stopped by the addition of cold acetonitrile followed by centrifugation for 15 min at 13,000 rpm, and the supernatants were analyzed by UV–vis spectroscopy. The final concentrations of radicals were estimated using $\varepsilon_{491\ nm}$ = 14,500 $M^{-1}{\cdot}cm^{-1}$ and 15,000 $M^{-1}{\cdot}cm^{-1}$ for **2** and **3**, respectively. The supernatants were also analyzed by HPLC as described above. The esterase activity of rat liver cytosol and microsomes toward *para*-nitrophenol acetate was determined following a previously described colorimetric method.[40] The reactions were performed at 37 °C in 0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA, 0.10 $mg{\cdot}mL^{-1}$ cytosolic, or 1.0 $mg{\cdot}mL^{-1}$ microsomal proteins and were initiated by the addition of 1 mM *para*-nitrophenol acetate (from a fresh 0.1 M solution in Me_2SO). The formation of *para*-nitrophenol was determined by following changes in absorbance at 405 nm for 3 min and quantitated using $\Delta\varepsilon_{405\ nm}$ = 13,000 $M^{-1}{\cdot}cm^{-1}$.

*Incubations of Radicals **2** and **3** in the Presence of Rat Liver Microsomes and NADPH under Aerobic Conditions.* Incubations were performed at 37 °C in Eppendorf tubes. Typical aerobic incubation mixtures (final volume 150 μL) contained 100 μM radicals **2** or **3** in 0.1 M phosphate buffer at pH 7.4, 0.1 mM EDTA and 0.5–0.8 $mg{\cdot}mL^{-1}$ microsomal proteins (1.0 μM P450). After equilibration for 5 min at 37 °C, the reactions were started by the addition of NADPH (1 mM, final concentration). After 30 min, the reaction mixtures were quenched by the addition of cold acetonitrile (same volume) and centrifuged for 10 min at 13,000 rpm, and aliquots were analyzed by HPLC as described above. The final concentrations of radicals were estimated using $\varepsilon_{491\ nm}$ = 14,500 $M^{-1}{\cdot}cm^{-1}$ and 15,000 $M^{-1}{\cdot}cm^{-1}$ for **2** and **3**, respectively.

*Metabolism of **2** and **3** by Rat Liver Microsomes in the Presence of NADPH under Anaerobic Conditions.* Anaerobic incubations were performed at 37 °C in 1-cm path length quartz cuvettes previously purged with argon and stopped with a rubber septum. Liver microsomes were gently degassed by argon flowing at the surface of the sample for 5 min at 4 °C. Meanwhile, the buffer was also degassed by argon bubbling for at least 30 min at 4 °C before being added to degassed liver microsomes. Typical anaerobic incubation mixtures

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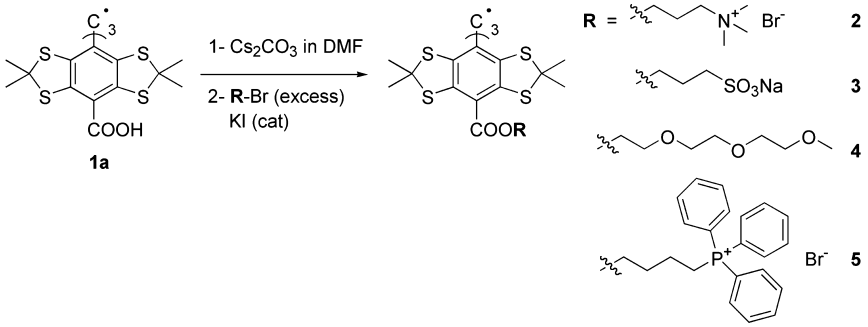
Scheme 1. Synthesis of Radicals 2−5 from TAM 1a



(final volume 150 μL) contained 100 μM radicals **2** or **3** in 0.1 M phosphate buffer at pH 7.4 containing 100 μM EDTA and 0.005−0.008 mg·mL^{-1} microsomal proteins (∼10 nM P450). After equilibration for 5 min at 37 °C, the reactions were started by the addition of NADPH (1 mM final concentration) and monitored by UV−vis at 37 °C either by repetitive scanning between 380 and 880 nm or by following the increase in absorbance at 644 nm to estimate the rate of formation of anions $\mathbf{2}^-$ and $\mathbf{3}^-$ with $\varepsilon_{644\ nm}$ values of 31,800 and 36,300 M^{-1}·cm^{-1}, respectively. Protonation of $\mathbf{2}^-$ and $\mathbf{3}^-$ was neglected on the time scale of the experiments (3 min).

Reaction of $\mathbf{2}^-$ with O_2. Reactions were performed at room temperature in 1-cm path length quartz cuvettes previously purged with argon and stopped with a rubber septum. A solution of 100 μM radical **2** in degassed 0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA was treated with a substoichiometric amount of sodium dithionite (65 μM) to generate the anion $\mathbf{2}^-$. The effects of successive additions of aerated buffer solution (containing ∼260 μM O_2) were monitored by UV−vis spectroscopy either by repetitive scanning between 380 and 880 nm or by following the decrease in absorbance at 644 nm (maximal absorption of $\mathbf{2}^-$).

RESULTS AND DISCUSSION

Synthesis and Characterization of TAM Esters 2−5. Esters **2−5** were obtained in good yields (80−90%) by the reaction of **1a** with an excess of the corresponding bromides in the presence of Cs_2CO_3 and catalytic amounts of KI (see Experimental Procedures) (Scheme 1). They were characterized by UV−vis, MS, HRMS, and EPR spectroscopy (see Experimental Procedures and Table 1). Esters **2** and **3** that bear a trimethylammonium or sulfonate group at the end of their alkyl-chains were soluble in water, which allowed us to study their metabolic stability in phosphate buffer at pH 7.4. By contrast, esters **4** and **5**, bearing a neutral group or a triphenylphosphonium moiety, were not soluble enough in water to permit such metabolic studies. However, their spectroscopic and electrochemical characteristics were determined in organic solvents such as methanol or acetonitrile.

Table 1. UV−Vis and EPR Characteristics of Radicals 1a and 2−5 in Phosphate Buffer or Acetonitrile and in the Absence or Presence of O_2

radical	solvent	λ_{max} (nm)	linewidth of $-O_2$[b]	the EPR signal[a] $+O_2$[c]
1a	phosphate buffer	374, 469, 641	90 ± 2	180 ± 2
2	phosphate buffer	408, 491, 682	290 ± 2	381 ± 2
3	phosphate buffer	410, 491, 684	290 ± 2	380 ± 2
2	acetonitrile	408, 490, 677	350 ± 5	1430 ± 20
3	acetonitrile	406, 487, 673	370 ± 5	1470 ± 20
4	acetonitrile	405, 487, 676	350 ± 5	1430 ± 20
5	acetonitrile	407, 489, 664	340 ± 5	1450 ± 20

[a]Linewidths (in mG) measured as the differences between the two highest lines of the septuplet. [b]Phosphate buffer (0.1 M, pH 7.4) and acetonitrile were deoxygenated by bubbling with argon for 1 h. [c]Phosphate buffer (0.1 M, pH 7.4) and acetonitrile were exposed to normal atmosphere (260 μM O_2 at 25 °C for buffer).

In acetonitrile, all radicals **2−5** displayed two intense UV−vis absorption bands around 410 and 490 nm, and a broad band around 680 nm. In phosphate buffer at pH 7.4, radicals **2** and **3** exhibited very similar spectra with bands that were about 30 nm red-shifted when compared to those of **1a** (Table 1). In oxygenated phosphate buffer, radicals **2** and **3** displayed almost identical EPR spectra that were characterized by a single signal centered at g = 2.0050 with a linewidth of 380 ± 2 mG (Figure 2A). This linewidth was sensitive to the concentration of dioxygen and decreased to 290 ± 2 mG in anaerobic buffer (Table 1). Because of the presence of six protons in their ester chains, the EPR signal of radicals **2** and **3** consisted of a septuplet with a hyperfine coupling constant a_H = 0.11 G (Figure 2B−C).[32] In order to compare the EPR spectra of **2−5** under identical conditions, further experiments were performed in a solvent where all compounds were soluble. The effects of spin exchange between radicals **2−5** and O_2 on the linewidth broadening of the signal were thus measured in acetonitrile. All new radicals displayed an identical behavior with a 4-fold increase in their signal linewidth when passing from anaerobic to aerobic conditions (Table 1).

The redox behavior of esters **2−5** was investigated by cyclic voltammetry in methanol, a solvent where all compounds are soluble, and compared to that of **1a**. The cyclic voltammograms recorded under argon at 0.1 V·s^{-1} are given as Supporting Information (Figure S1). For all compounds, two electrochemical processes were identified corresponding either to the reduction of the radical to the corresponding anion or to the oxidation of the radical to the corresponding cation. For compound **1a**, the electrochemical reduction process showed a nonideal, only quasi-reversible behavior, which most likely arose from the presence of water traces in methanol that led to a partial protonation of the anion of **1a** to triarylmethane **1a-H** (see below). For compounds **3** and **4**, both electrochemical processes were fully reversible, allowing the unambiguous determination of the corresponding standard potentials. At the same scan rate, the waves associated with the oxidation of **2** and **5** were poorly defined because of their overlap with the very intense oxidation wave of the associated bromide counterions. This interpretation was confirmed by the electrochemical study of tetrabutylammonium bromide that showed an oxidation wave at the same potential (data not shown). Accordingly, the standard potential of the oxidation process was better estimated

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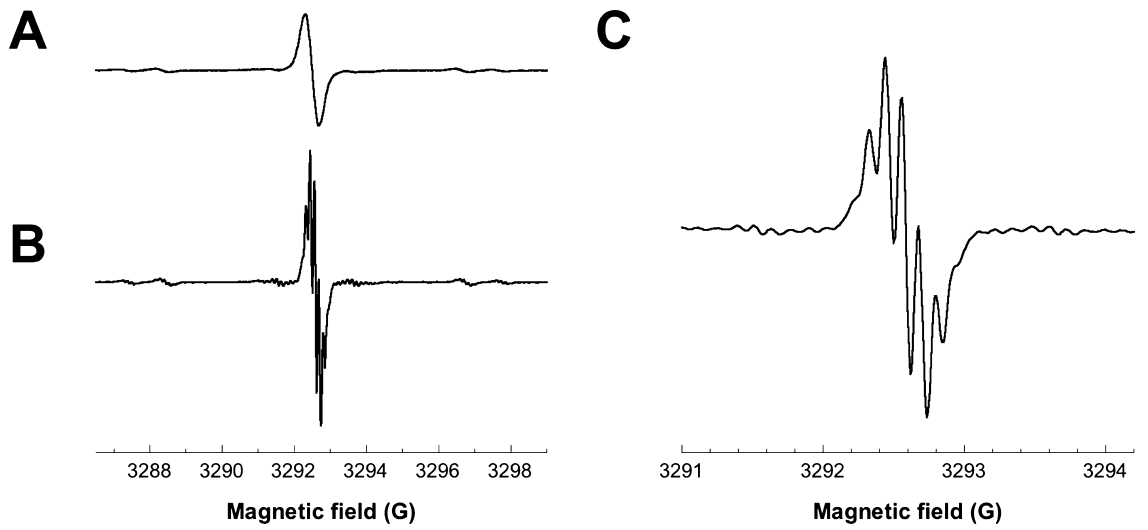

Figure 2. EPR spectrum of radical **3** in phosphate buffer (0.1 M, pH 7.4) in the presence of O_2 (A) or in the absence of O_2 (B). Enlargement of the spectrum recorded under anaerobic conditions to observe the hyperfine structure (C).

from the cyclic voltammograms recorded at higher scan rates (5 $V{\cdot}s^{-1}$, Figure S1, Supporting Information). For compound **2**, the shape of the reduction wave associated with the oxidation process indicated some adsorption of the product on the electrode, leading to a less precise estimation of the standard potential associated with the reduction process. A similar adsorption process also occurred for the reduction of compound **5**.

The standard potentials obtained for compounds **2**–**5** are given in Table 2 and compared to those of **1a**. Replacement of the carboxylate substituents with esters resulted in a large increase (about 500 mV) of the oxidation and reduction potentials.[31] Easier reduction and more difficult oxidation of the esters should be related to the greater electron-withdrawing effect of the ester substituents relative to carboxylates. Within this ester series, one must note the effects of their global charge on their redox potentials. Thus, the negatively charged compound **3** was the most difficult to reduce and the easiest to oxidize.

Table 2. Redox Potentials of TAM Radicals 1a and 2–5 Determined by Cyclic Voltammetry in Phosphate Buffer or Methanol

radicals	solvent	E_{red} [a]	E_{ox} [a]
1a	phosphate buffer[b]	−660 (−70)[c]	415 (−410)[c]
2	phosphate buffer	−230	[d]
3	phosphate buffer	−325	[d]
1a	methanol	−920	390
2	methanol	−350	890[e]
3	methanol	−435	820
4	methanol	−415	850
5	methanol	−350[e]	890

[a]Redox potentials (mV/saturated calomel electrode) (SCE) of TAM radicals **1a** and **2**–**5** calculated as the half sum of the anodic and the cathodic potentials for each redox process. [b]0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA. [c]Data from ref 29 recorded in 0.1 M phosphate buffer at pH 7.4 containing 0.1 M NaCl; redox potentials were recalculated vs SCE. [d]Not determined. [e]Estimated value due to adsorption at the electrode.

Electrochemical characterization of the water-soluble esters **2** and **3** was also performed in phosphate buffer (100 mM, pH 7) and compared to that of **1a**. The corresponding cyclic voltammograms are given Figure 3. For compound **1a**, the

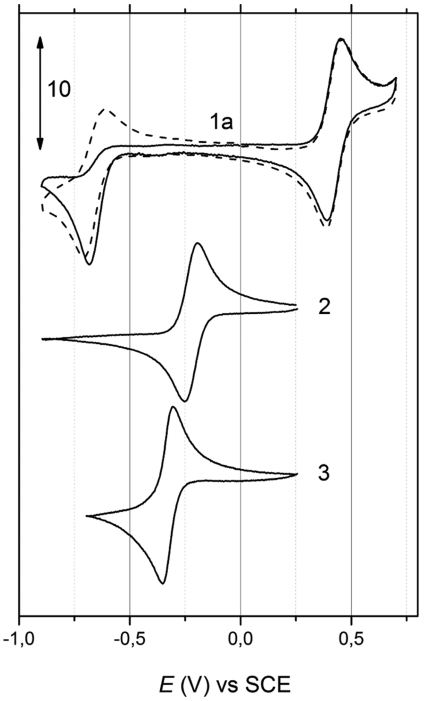

Figure 3. Cyclic voltammograms of radicals **1a**, **2**, and **3** (400 μM) in phosphate buffer (0.1 M, pH 7.4, containing 0.1 mM EDTA) at 0.02 $V{\cdot}s^{-1}$ (solid line) and 0.5 $V{\cdot}s^{-1}$ (dashed line). Intensities (ordinate, in μA) were divided by the square root of the scan rate to allow direct comparisons.

reduction process was irreversible at a scan rate of 0.02 $V{\cdot}s^{-1}$ because of the fast protonation of the anion formed at the electrode in aqueous solvent. Full reversibility could be recovered by increasing the scan rate to 0.5 $V{\cdot}s^{-1}$. Simulation of the cyclic voltammograms recorded at various scan rates according to an EC mechanism, where the electrochemical

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process is coupled to a quasi-irreversible chemical reaction, allowed us to determine the pseudo-first-order kinetic rate of the protonation process that was estimated to be 1.2 s^{-1} under our experimental conditions (see Figure S2, Supporting Information). Assuming that the proton arises from the phosphate buffer, the second order kinetic rate was estimated to be 24 $mol^{-1}\cdot L\cdot s^{-1}$. For compounds **2** and **3**, the reduction processes were fully reversible at all scan rates. The standard potentials associated with the reduction process are given in Table 2. As in methanol, one observed a large increase of the values reported for **2** and **3** as compared to those of **1a**. Moreover, the lack of reactivity of anions $\mathbf{2}^-$ and $\mathbf{3}^-$ is most likely related to their pK_a value, which should be lower than that of $\mathbf{1a}^-$, if one takes into account the greater electron-withdrawing effect of the ester substituents relative to carboxylates.

Stability of TAM Ester Radicals 2 and 3 in the Presence of Rat Liver Microsomes and Cytosol. In a first series of experiments, radicals **2** and **3** were incubated for 30 min at 37 °C in the presence of rat liver cytosol or rat liver microsomes, in the absence of NADPH. UV−vis and HPLC analyses of the incubation mixtures did not show any transformation of **2** and **3** that were fully recovered unchanged. Similar incubations performed in the presence of *para*-nitrophenyl acetate, a common substrate of microsomal and cytosolic esterases,[40] showed that these esterases were active, 375 ± 30 and 5500 ± 155 $nmol\cdot min^{-1}\cdot mg\ protein^{-1}$ of *para*-nitrophenol being formed with microsomes and cytosol, respectively. These data showed that compounds **2** and **3** were stable toward microsomal and cytosolic esterases under our conditions. A similar resistance to pig liver esterase was previously reported for the methyl- and *tert*-butyl esters of **1a**.[32,33] This should be due to the great steric hindrance around the ester group directly bound to the aryl substituents of these helical TAM radicals.[32]

In a second series of experiments, similar incubations of **2** and **3** were performed in the presence of rat liver microsomes and NADPH to study their possible reductase- and/or P450-dependent metabolism. After 30 min of incubation under these conditions, a UV−vis study of the reaction mixture showed that **2** and **3** were mostly recovered unchanged (96 ± 3 and 94 ± 3%, respectively) and that no new product could be detected using HPLC. So, as expected for compounds having no *para*-carboxyl aromatic substituents, **2** and **3** were much more stable toward microsomal oxidation than **1a**.

From these data, it appeared that esters **2** and **3** were stable toward esterases and much more resistant toward microsomal metabolism than **1a**. However, as the small part of **2** and **3** (about 5%) consumed upon microsomal incubation in the presence of NADPH could have come from their reduction into triarylmethanes whose retention times were identical to those of **2** and **3**, we have more deeply investigated their possible reductive metabolism.

Microsomal Reduction of 2 and 3 under Anaerobic Conditions. UV−vis studies of incubations of **2** or **3** with rat liver microsomes in the presence of NADPH under anaerobic conditions showed a fast disappearance of the bands of the starting compounds and the appearance of an intense band at 644 nm (Figure 4). The corresponding species have been prepared by a reaction of **2** or **3** with sodium dithionite and characterized by MS. Thus, the direct injection into the mass spectrometer of a solution of **2** just after its reduction by dithionite led to a molecular ion at m/z = 649.7 that well

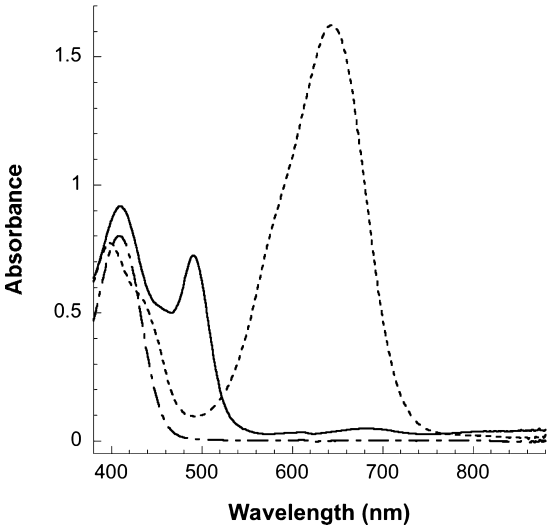

Figure 4. UV−vis spectra of radical **2** (solid line), its anion $\mathbf{2}^-$ (dotted line), and triarylmethane **2-H** (dashed line). Trityl anion $\mathbf{2}^-$ was formed by the reduction of radical **2** (50 μM **2** in 0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA) with an excess of sodium dithionite.

corresponded to the anion $\mathbf{2}^-$ resulting from a one-electron reduction of **2** (m/z calcd for $C_{58}H_{81}N_3O_6S_{12}^{2+}$ = 649.6). Protonation of these anions derived from **2** and **3** in anaerobic phosphate buffer at pH 7.4 was very slow (6 and 10% after 30 min for $\mathbf{2}^-$ and $\mathbf{3}^-$, respectively). It was much faster at lower pHs and, at pH 2, one observed a complete disappearance of the 644 nm absorbing anion in less than 5 min with the formation of a new species absorbing at 410 nm (Figure 4). Further HPLC-MS analyses of the resulting solution showed that only one compound exhibiting MS characteristics expected for the triarylmethane **2-H** or **3-H** was formed in these experiments. After extraction and purification, triarylmethanes **2-H** and **3-H** were completely identified by HRMS, and ^1H and ^{13}C NMR spectroscopy (see Experimental Procedures and Tables S1 and S2 of Supporting Information). Their ^1H and ^{13}C NMR spectra are very similar to those previously described for TAM **1a-H** and indicate that these three reduced metabolites have a C-3 symmetry axis and noncoplanar aromatic rings.[24]

Thus, anaerobic reduction of radicals **1a**, **2**, and **3** either by NADPH in the presence of rat liver microsomes or by dithionite led to the same final TAM-H metabolites, via the same TAM^- anions. However, the stabilities of the intermediate anions were quite different, $\mathbf{1a}^-$ being hardly detectable during the formation of **1a-H**,[24] whereas $\mathbf{2}^-$ and $\mathbf{3}^-$ were stable for at least 30 min in phosphate buffer at pH 7.4. Another main difference between **1a** and **2** or **3** was the much greater rate of anaerobic microsomal reduction of **2** and **3** in the presence of NADPH, when compared to **1a**. Table 3 shows that the initial rates of microsomal reduction of 100 μM **2** and **3** by 1 mM NADPH were comparable to the reduction rate of cytochrome *c* by these microsomes under identical conditions (586 ± 53 $nmol\cdot min^{-1}\cdot mg\ protein^{-1}$). By contrast, the initial rate of **1a** reduction into **1a-H** under these conditions was very much smaller (29 ± 2 nmol **1a-H**$\cdot min^{-1}\cdot mg\ protein^{-1}$). These greater rates of reduction of **2** and **3** and greater stability of their anions when compared to those of **1a** were in agreement with their greater reduction potentials (Table 2).

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Table 3. Comparison of the Anaerobic Reduction Rates of 2 and 3 to the Anions 2^- and 3^- Catalyzed by Rat Liver Microsomes

radicals	reduction rates[a]
2	510 ± 40
3	450 ± 30

[a]The initial rates of anaerobic reduction of radicals **2** and **3** were determined by UV−vis spectroscopy by monitoring the absorbance at 644 nm and quantified using $\Delta\varepsilon$ values 31,800 and 36,300 $M^{-1}\cdot cm^{-1}$ for 2^- and 3^-, respectively. The protonation of the anions has been neglected on the time scale of the experiments (3 min). Incubations were performed at 37 °C in deoxygenated 0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA, 100 μM **2** or **3**, 0.005−0.008 mg microsomal protein·mL^{-1}, and 1 mM NADPH. Rates are expressed in nmol radical reduced·min^{-1}·mg $prot^{-1}$ and are the means ± SD from 3 experiments.

Microsomal Reduction of 2 and 3 under Aerobic Conditions. Careful examination of the UV−vis spectra of aerobic incubations of **2** and **3** with rat liver microsomes in the presence of NADPH revealed the formation of the 644 nm absorbing species corresponding to anions 2^- and 3^-. However, as indicated above, **2** and **3** were found in great part unchanged after 30 min of reaction. A possible explanation for these results could be that 2^- and 3^- would be reoxidized by O_2 into **2** and **3**, respectively. Figure 5A shows that progressive additions of phosphate buffer at pH 7.4 containing 260 μM O_2 to an anaerobic solution of 2^- previously prepared by reduction of 100 μM **2** with substoichiometric amounts of dithionite (65 μM) led to the progressive disappearance of the 644 nm peak of 2^- and appearance of the characteristic peak of **2** at 491 nm. Figure 5B shows that 0.25 mol of O_2 was sufficient to reoxidize 1 mol of 2^- into **2**, which means that 1 mol of O_2 was able to oxidize 4 mol of 2^-.

These data indicated that radicals **1a**, **2**, and **3** underwent a one-electron reduction into the corresponding anions by NADPH-containing liver microsomes under anaerobic conditions. Anions 2^- and 3^- were stable in the incubation medium (phosphate buffer at pH 7.4), whereas $\mathbf{1a}^-$ would be quite rapidly protonated with the formation of **1a-H**. It has been reported that such microsomal reduction of **1a** was catalyzed by the P450/P450 reductase system,[24] and it is likely that this system was also involved in the reduction of **2** and **3**.

Under aerobic conditions, **1a** cannot compete with dioxygen for the electrons coming from the P450/P450 reductase system, and it was not reduced under these conditions.[24] By contrast, **2** and **3**, which have higher reduction potentials (Table 2), successively compete with O_2 to receive electrons with the formation of 2^- and 3^-. However, these anions are rapidly oxidized by O_2 leading back to the starting radicals **2** and **3**. The latter radicals thus catalyzed a futile cycle of O_2 reduction by NADPH-containing microsomes (Scheme 2) and were almost not consumed in those reactions. This could explain why they appear globally stable after aerobic microsomal incubation (see above). The small amounts of **2** and **3** (about 5%) lost after 30 min of aerobic incubation in the presence of rat liver microsomes and NADPH could be due to the formation of **2-H** and **3-H** upon protonation of 2^- and 3^-, which is slow at pH 7.4.

■ CONCLUSIONS

TAM radicals **1a** and **1b** are useful EPR probes for oximetry; however, they are rapidly metabolized by liver microsomes in the presence of NADPH with the formation of diamagnetic QM metabolites resulting from oxidative decarboxylation. In an effort to obtain TAM derivatives potentially more metabolically stable *in vivo*, we have synthesized four new TAM radicals, **2**−**5**, in which the carboxylate sustituents of **1a** were replaced with esters bearing various alkyl chains that were designed to render them water-soluble. They were characterized by UV−vis, EPR spectroscopy, HRMS, and electrochemistry. Two of them, **2** and **3**, were water soluble enough to undergo detailed microsomal metabolic studies in comparison with **1a**. They were found to be metabolically stable toward the esterases present in rat liver microsomes and cytosol. Radicals **2** and **3** were also found to be oxidation-resistant and globally stable in the presence of aerobic NADPH-supplemented microsomes, contrary to **1a**. However, a careful study of their possible microsomal reduction under anaerobic or aerobic conditions

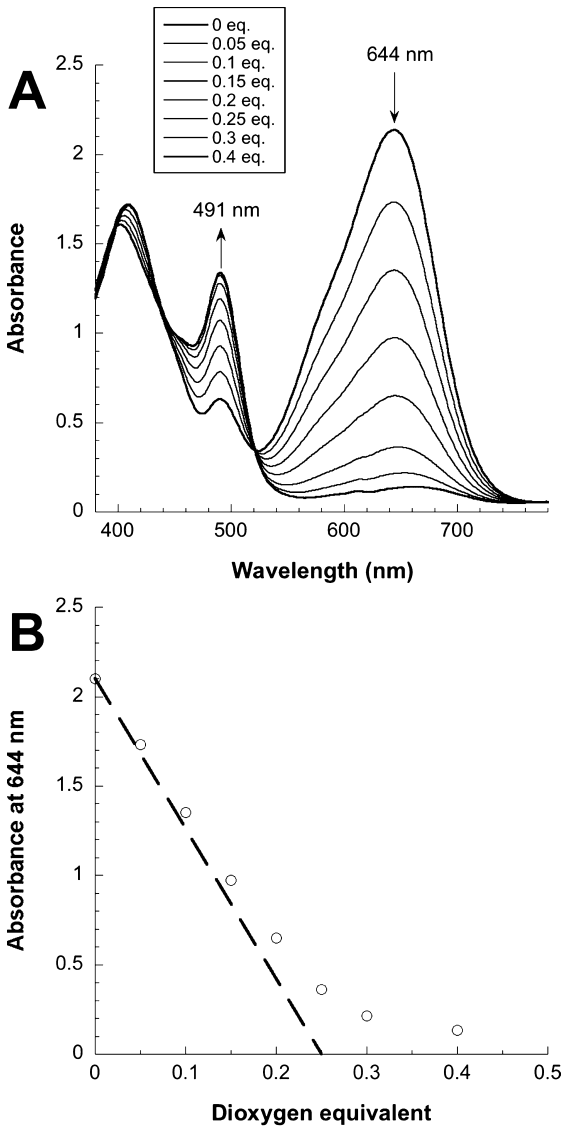

Figure 5. Reaction of anion 2^- with O_2. (A) UV−vis spectra showing the effects of the stepwise additions of a buffer solution containing 260 μM O_2 to a solution of the anion formed upon the reduction of radical **2** (100 μM in 0.1 M phosphate buffer at pH 7.4 containing 0.1 mM EDTA) by substoichiometric amounts of sodium dithionite (65 μM). (B) Plot of the absorbance at 644 nm as a function of the O_2 equivalents added to 2^-.

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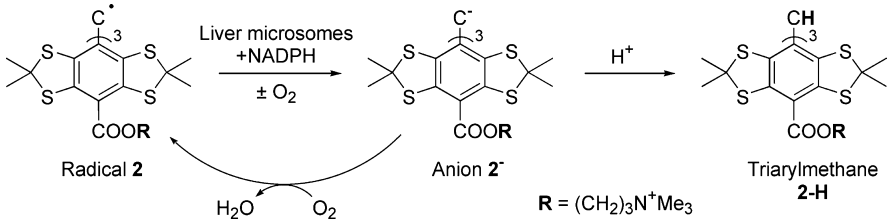
Scheme 2. Fates of Radical 2 in the Presence of Rat Liver Microsomes and NADPH[a]



[a]Radical **2** was reduced to the corresponding anion 2^- that was either protonated to triarylmethane derivative **2-H** in the absence of O_2 or oxidized by O_2 to starting radical **2** under aerobic conditions.

showed that they were more easily reduced than **1a**, in agreement with their higher reduction potentials. They were reduced into the corresponding anions not only under anaerobic conditions but also in the presence of dioxygen. These anions were much more stable than that of **1a** and have been characterized by UV−vis spectroscopy, MS, and at the level of their protonated diamagnetic products. However, they were oxidized by O_2, giving back the starting radicals **2** or **3** and catalyzing a futile cycle of O_2 reduction. Such reactions should be considered in the design of future metabolically stable TAM EPR probes for oximetry *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

Assignment of ^1H and ^{13}C NMR signals of triarylmethanes **2-H** and **3-H**; cyclic voltammograms of **1a** and **2**−**5** recorded in methanol; and experimental cyclic voltammograms of **1a** in phosphate buffer and simulations obtained with the DigiElch program. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 33 1 42 86 21 91. Fax: 33 1 42 86 83 87. E-mail:jean-luc.boucher@parisdescartes.fr.

Present Address

[§]Y.X.-L.: Laboratoire de Chimie des Processus Biologiques, Collège de France, 11 Place M. Berthelot, 75005 Paris, France.

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Notes

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■ ABBREVIATIONS

DMF, *N*,*N*-dimethylformamide; EPRI, electron paramagnetic resonance imaging; ESI, electrospray ionization; HRMS, high-resolution mass spectrometry; pO_2, dioxygen partial pressure; QM, quinone-methide; SCE, saturated calomel electrode; TAM, triarylmethyl radical

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