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Perchlorotrityl radical-fluorophore conjugates as dual fluorescence and EPR probes for superoxide radical anion

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ABSTRACT

Perchlorotrityl radicals, mono-substituted with a fluorophore using an amide linker of varying chain length, were synthesized and characterized. Electron paramagnetic resonance (EPR) spectroscopic study indicated free-electron coupling with the aromatic hydrogen nuclei and long-range coupling with the methylene hydrogens of the linker group. Reactivity of the fluorophore-conjugated trityls with superoxide radical anion showed quenching of EPR signal and enhancement of fluorescence emission spectrum. This work presents the first example of a perchlorotrityl-fluorophore conjugate that can potentially be employed as a dual probe for the detection of superoxide under oxidative stress-mediated conditions in biological systems.

1. Introduction

Superoxide radical anion ($O_2^{\cdot-}$), a reactive free radical species formed by one-electron reduction of molecular oxygen, has been implicated in a variety of disease conditions such as ischemia-reperfusion injury, diabetes, atherosclerosis, chronic inflammation, viral infection, and malignant diseases.[1] Superoxide produced by mammalian cells serves as a signaling molecule that can modulate events such as enzyme phosphorylation, cell growth, hypertrophy and apoptosis,[2,3] but increased $O_2^{\cdot-}$ production can lead to cellular dysfunction. Therefore, there is a need for improved techniques for the detection and quantification of $O_2^{\cdot-}$ in order to gain knowledge on its role in the progression of oxidative stress-related diseases and pathophysiological conditions.

A variety of analytical techniques have been developed to detect and quantify $O_2^{\cdot-}$ radicals in biological systems. These include spectrophotometric methods that use reduction of ferricytochrome *c* or nitroblue tetrazolium (NBT) by $O_2^{\cdot-}$, chemiluminescence, fluorescence-based techniques and electron paramagnetic resonance (EPR) spectroscopy using spin-trapping techniques.[4–7] However, these methods have certain limitations for measuring $O_2^{\cdot-}$. In the lucigenin-based luminescence detection of $O_2^{\cdot-}$, it has been demonstrated that the probe itself can redox-cycle, resulting in artifactual production of $O_2^{\cdot-}$, whereas the cytochrome c reduction assay is affected by the presence of oxidoreductases in cellular systems.[8,9] The EPR method requires the use of high concentrations (10–200 mM) of spin trap that may perturb the redox balance in the biological system.

Superoxide requires a probe that is sensitive enough to efficiently compete with intracellular components for its detection. This is due to a relatively short half-life of the molecule and the presence of endogenous intracellular antioxidant enzymes that efficiently scavenge $O_2^{\cdot-}$. Additionally, these probes must have target specificity to sub-cellular compartments to reflect radical production at their site of formation. One of the most promising approaches to monitor the redox status or the detection of free radicals in cells is the use of bi-functional fluorogenic spin probes that can be detected by fluorescence and/or EPR spectroscopy. In the past, such dual probes containing a fluorescent chromophore and a paramagnetic nitroxide radical have been reported.[10–13] In these molecules, the paramagnetic nitroxide moiety acts as a quencher of the fluorescence of the chromophore fragment. Reduction of the nitroxide by free radicals results in the formation of diamagnetic moiety and hence can cause decay of the EPR signal and in turn, will potentiate fluorescence. Hence, the conjugate allows all the nitroxide spin probe features to be retained, but also may gain new advantages by being able to monitor redox and radical production processes by a very sensitive and simpler fluorescence technique. However, the chemical property of nitroxide limits its

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application for intracellular radical detection due to its susceptibility to be metabolically reduced in the cell. Thus, a highly EPR sensitive (narrow/single line) probe with good biostability against cellular oxidoreductants and high specificity for $O_2^{\cdot-}$ is desirable for radical detection in biological systems.

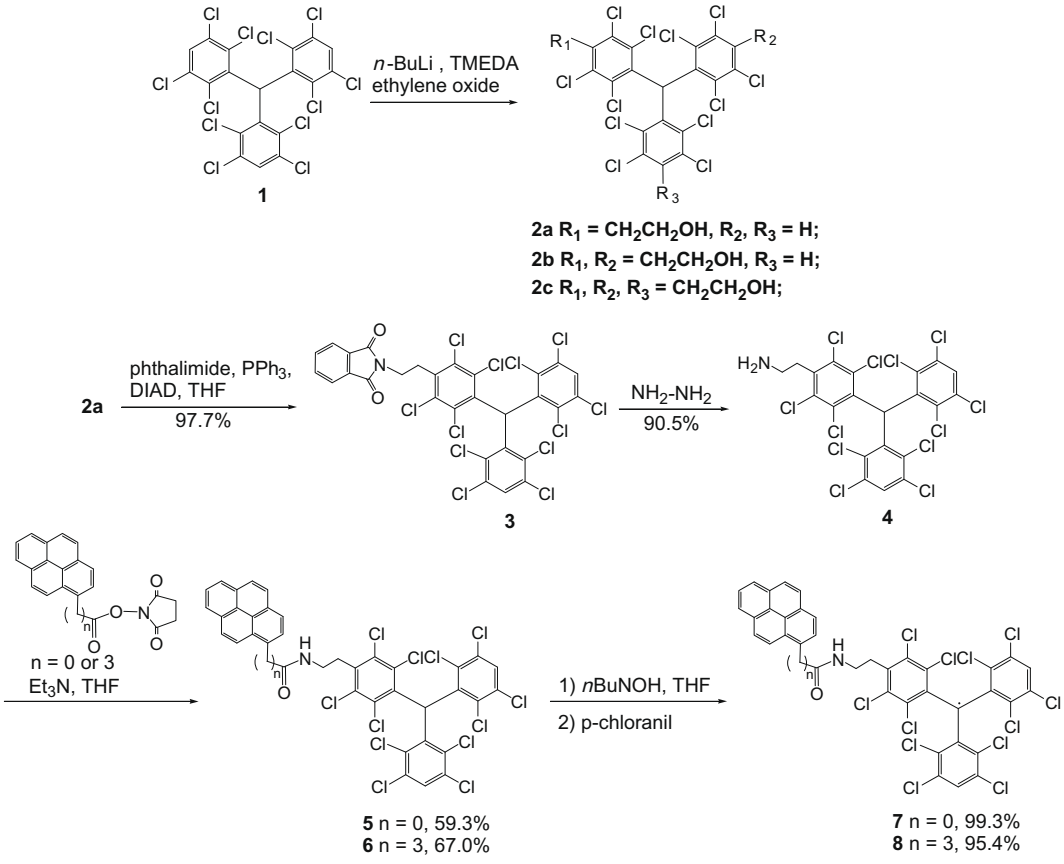
Recently, triarylmethyl (trityl) radicals have been reported as probes for detecting $O_2^{\cdot-}$ in biological systems.[14,15] Perchlorotriphenylmethyl radicals are a novel class of trityls whose structures are based on perchlorotriphenyl methyl radical (PTM). Due to the full steric blockage of the central carbon, the PTM radical and its derivatives are highly stable against a variety of reactive chemical agents, and hence are called 'inert free radicals'.[16,17] The ability of two derivatives, tris(2,3,5,6-tetrachloro-4-carboxylate-phenyl) methyl radical (PTM-TC) and tris(2,3,5,6-tetrachloro-4-ethyloxycarbonylphenyl) methyl radical (PTM-TE), to trap $O_2^{\cdot-}$ have been tested.[18,19] These species demonstrated enhanced stability in biological systems, excellent water solubility (in the cases of carboxylic acid), and a narrow, single EPR signal. Therefore, the development of trityl-fluorophore conjugate is proposed which may offer improved $O_2^{\cdot-}$ detection by using EPR and/or fluorescence techniques.

2. Results and discussion

2.1. Synthesis

Due to the steric hindrance and high stability of tris(2,3,5,6-tetrachlorophenyl)methane, **1**, it is difficult to directly derivatize the compound with a chromophore. Instead, our initial efforts involved adding a linker with appropriate functional group between the trityl core structure and the chromophore. Treatment of **1** with *n*-butyl lithium at −78 °C followed by nucleophilic addition of the trityl to ethylene oxide, afforded compound **2** with spacer(s) incorporated (Scheme 1). By controlling the molar ratios of **1**, ethylene oxide and base, compounds **2a** or **2b** can be isolated as major products. However, only small amounts of compound **2c** were formed even when six equivalents of ethylene oxide and *n*-butyl lithium were added. It was found that compounds **2a** and **2b** have good solubility in THF and dichloromethane, while compound **2c** was insoluble in any commonly-used solvents. The solubility differences in compounds **2a**–**2c** can control the equilibrium concentrations of these three compounds in solution. The primary amine, **4**, was synthesized via Mitsunobu reaction using **2a** and phthalimide followed by hydrazinolysis, with sufficiently high purity for use in the succeeding step according to previously establish procedure.[20] Two different linker distances were introduced to compounds **4** in order to shed insights into how these distances can affect the chromophore-radical interaction. Amidation reaction of **4** with both 1-pyrenecarboxylic acid *N*-hydroxysuccinimide ester and pyrenebutyric acid *N*-hydroxysuccinimide ester was carried on to form fluorescence chromophore-conjugated compounds **5** and **6**, respectively.[21] Finally, radicals **7** and **8** were obtained by treating compounds **5** or **6** with excess of $n\mathrm{Bu_4N^+OH^-}$ and subsequent oxidation of the resulting anions with *p*-chloranil in a one-pot reaction with near quantitative yield.[22]

Two methods were employed for the synthesis of **10** (Scheme 2). In Method A, treatment of **1** with 2.4 equiv of *n*-BuLi and TMEDA followed by 2.4 equiv of ethyl chloroformate afforded di-ethoxycarbonyl trityl **9** in 46.7% yield as well as mono-ethoxycarbonyl trityl, which can be reused.[19] However, reaction of **9** with ethylene oxide gave poor yield and a complex mixture that was difficult to characterize. The unsuccessful synthesis of **10** using Method A could be due to the susceptibility of ethoxycarbonyl functionality to nucleophilic addition by ethylene oxide under strong basic condition.



Scheme 1. Synthesis of compounds **7** and **8**.

Therefore, an alternative method (Method B) was carried out in which **1** was reacted with ethylene oxide first followed by ethyl chloroformate. Although one more hydrolysis step was required, the overall yield increased twofold. Following the earlier procedure, radical **15** was synthesized from intermediate **10** (Scheme 3) which was slightly soluble in water, making radical **15** possible to be characterized in aqueous solution.

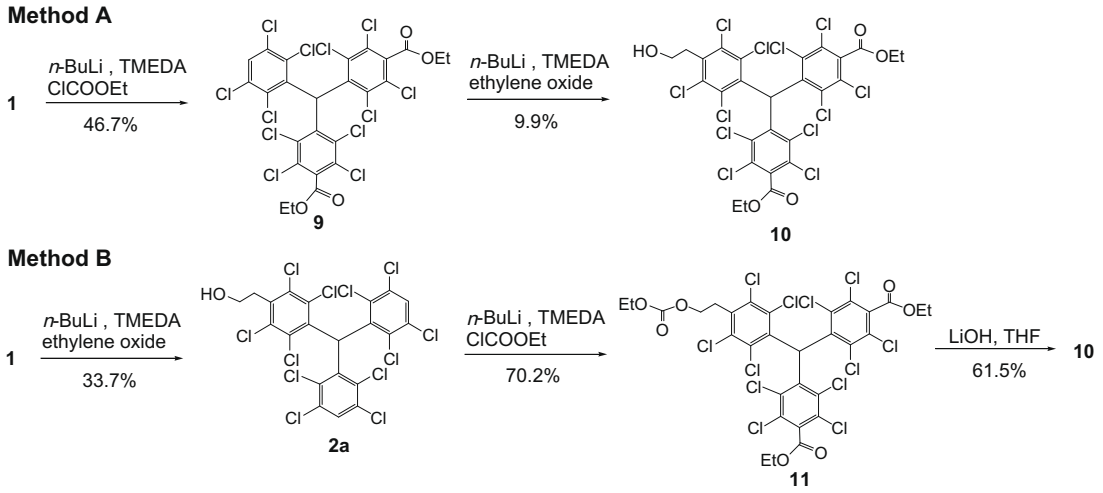
2.2. EPR Studies

Figure 1 shows the solid state EPR spectra of fluorophore-trityls **7**, **8** and **15**. The peak-to-peak linewidth (ΔB_{pp}) for **15** is the broadest compared to **7** and **8**. In spite of the absence of aromatic protons in **15**, this suggests that the line-broadening mechanism is caused mostly by spin–spin exchange interaction. It can be assumed that the packing interaction for **15** is more efficient due, perhaps, to the short linker group that allows a more ordered lattice structure. Figure 2 shows the EPR of spectra **7**, **8** and **15** in DMSO under anaerobic conditions. Both **7** and **8** exhibited a multiplet (7-line) EPR spectrum arising from the hyperfine coupling of two aromatic and two methylene hydrogen atoms from the linker group, suggesting that the unpaired electron is delocalized over the whole PTM moiety (see Table 1 for the simulated EPR parameters). However, trityl **15** exhibited a triplet spectrum with 1:2:1 ratio due to the methylene protons from the linker group. The observed differences in hyperfine coupling constants of the two aromatic and methylene protons are due to the asymmetry of the molecule as shown in Figure 3.

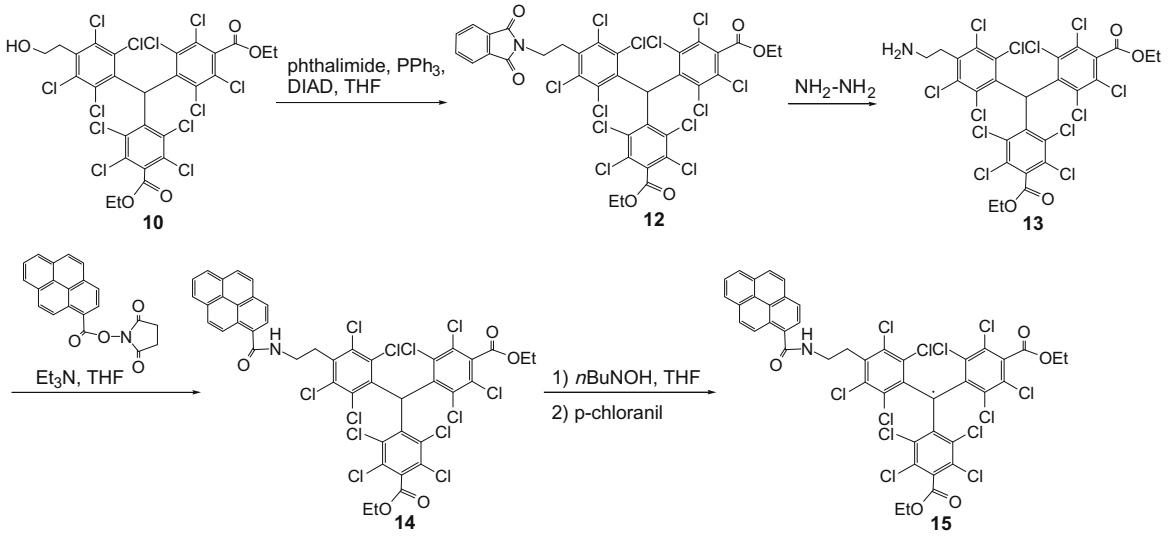
2.3. Reactivity with superoxide radical anion

2.3.1. EPR Spectroscopy

The addition of KO_2 DMSO solution to trityls **7**, **8** or **15** resulted in the disappearance of their EPR signal (Fig. 4) suggesting the formation of a diamagnetic product. Using the analogue perchorotritylmethyl-tricarboxylate, PTM-TC, we postulated that the nature of PTM reactivity to $O_2^{\cdot-}$ is a redox reaction resulting in the formation of the reduced product, perchlorotriphenylmethane and oxidation of $O_2^{\cdot-}$ to molecular oxygen as analyzed by electrochemical and mass spectrometric methods.[18] Competitive kinetics experiments revealed that PTM-TC reacts with $O_2^{\cdot-}$ with an apparent second-order rate constant of $8.3 \times 10^8\ M^{-1}\ s^{-1}$. The high reactivity of PTM-



Scheme 2. Methodologies for the synthesis of compound **10**.



Scheme 3. Synthesis of compound **15**.

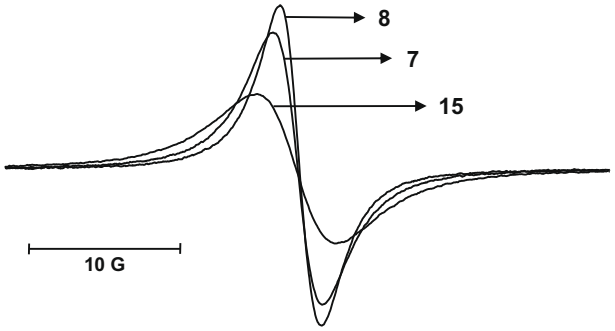


Figure 1. X-band EPR spectra of fluorophore-trityls **7**, **8** and **15** as crystalline powders in air as measured at room temperature. The acquisition settings were: microwave frequency, 9.786 GHz (X-band), modulation amplitude, 1 G; microwave power, 2 mW; time constant, 40 ms; scan time, 15 s. The first derivative peak-to-peak widths of **7**, **8**, and **15** were 3.26 G, 2.76 G, and 5.14 G, respectively.

TC with $O_2^{\cdot-}$ may offer a potential opportunity for measurement of superoxide in biological systems. It can, therefore, be assumed that based on these preliminary evidences that $O_2^{\cdot-}$ reactivity with **7**, **8** or **15** can parallel that of PTM-TC. Further, we have previously shown that the perchloro-class of trityl radicals, for example PTM-TC, is not reactive or has no specificity of reaction toward hydroxyl, peroxyl, or nitric oxide radicals in aqueous solutions.[18]

2.3.2. Fluorescence spectroscopy

The excitation and emission profiles for compounds **7**, **8** and **15** are shown in Figure 5. The spectrum showed two emission bands, at 377 nm and 397 nm, corresponding to excitations at 330 and 346 nm, respectively. Enhanced fluorescence spectra can be observed for all compounds upon addition of $O_2^{\cdot-}$, however, this effect is more pronounced for compounds **7** and **15**. This enhanced effect of $O_2^{\cdot-}$ on the emission spectra is consistent with the trityl radical quenching as demonstrated by the EPR studies mentioned above. This study further demonstrates the potential use of fluorophore-trityl conjugate as both an EPR and fluorescent probe for the detection of $O_2^{\cdot-}$.

3. Summary and conclusions

Perchlorotrityl-fluorophore conjugates were synthesized and characterized, and EPR spectra show long-range electronic coupling with the methylene protons of the linker group. Reactivity with $O_2^{\cdot-}$ was investigated using EPR and fluorescence spectroscopy and the results show disappearance of the EPR signal of the trityl with a concomitant increase in the emission intensity, respectively, suggesting a strong, perhaps a through-space interaction, between the unpaired spin of the trityl and the excited electronic state of the fluorophore. This work suggests potential application of perchlorotrityl-flurophore conjugates as dual EPR and fluorescence probe for $O_2^{\cdot-}$ detection.

The new class of bimodal probes, with higher sensitivity when compared to existing probes, may enable accurate and reliable quantitative determination of superoxide concentration in biological systems. Superoxide determination in cells in vitro would require cellular internalization of the probe, while in vivo measurements need further considerations including mode of administration of the probe, detection sensitivity, and other complexities in the living system. A further difficulty may be the limitation of the methods for deep tissue measurements. In the case of experimental animal models, one can use excised tissue biopsies following intravenous administration of the probe. The tissue samples could then be immediately flash frozen, preserved, and sectioned for EPR or fluorescence microscopy imaging. However, in

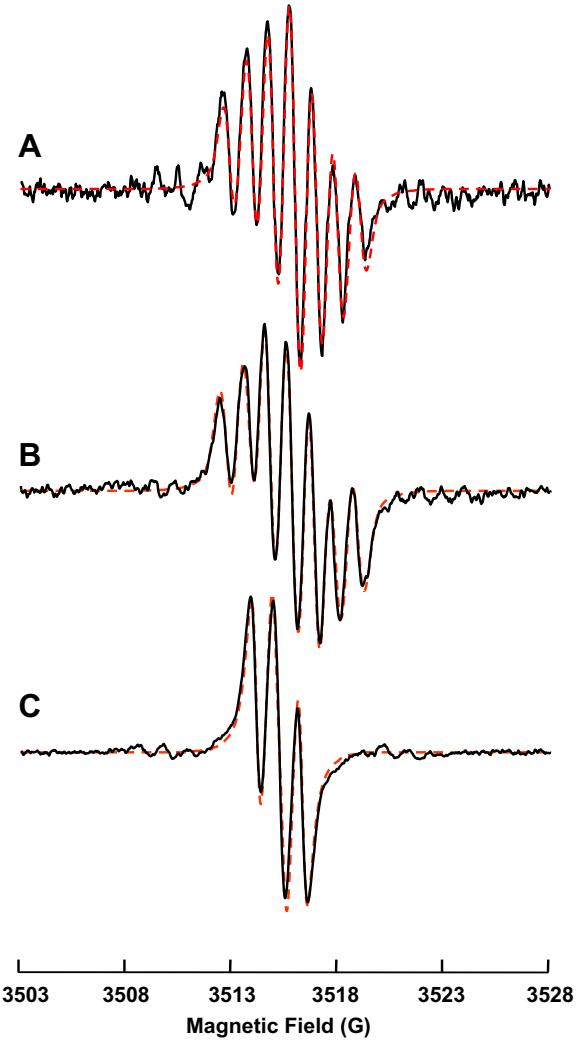
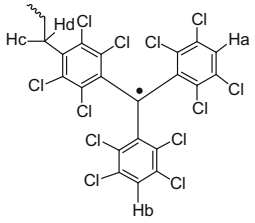


Figure 2. X-band EPR spectra of fluorophore-trityls in anaerobic solution. The EPR spectra of compounds (A) **8**; (B) **7**; and (C) **15** in DMSO (100 μM) measured at room temperature (see Section 4 for instrument settings). The broken red-colored trace superimposed on each spectrum was simulated using the parameters shown in Table 1.

Table 1
Experimental EPR hyperfine splitting constants (a_{Hx}) and anaerobic linewidths (ΔB_{pp}) in G for trityls **7**, **8** and **15** in DMSO at 298 K



Compound	Hyperfine splitting constants a_H (G)		ΔB_{pp} (G)
	Aromatic H's (H_a and H_b)	Methylene H's (H_c and H_d)	
7	2.09, 1.91	1.24, 0.95	0.70
8	2.05, 1.96	1.14, 1.02	0.62
15	n/a	1.26, 0.94	0.66

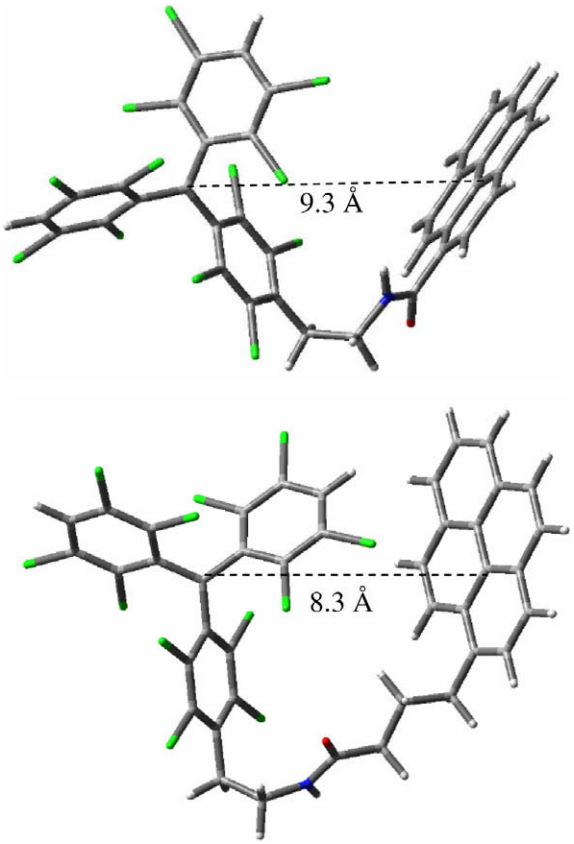


Figure 3. Preferred conformations for trityls **7** (top) and **8** (bottom) using MMFF MonteCarlo search and geometry optimization at the B3LYP/6-31G* level of theory showing the intramolecular distance between the trityl and fluorophore moieties.

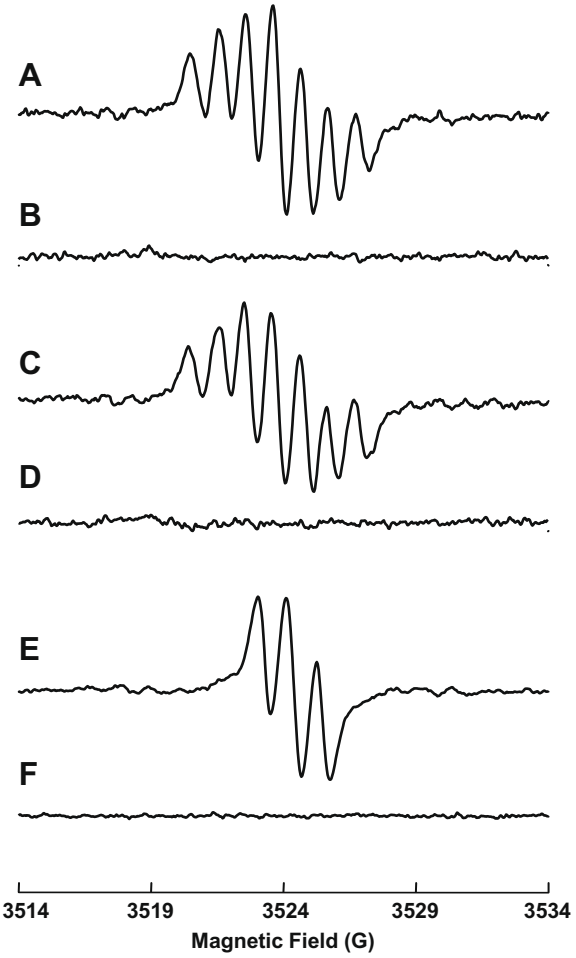


Figure 4. EPR spectra of (A) **8**, (C) **7** and (D) **15**; and (B), (D) and (F), respectively, are the resulting spectra upon addition of KO_2 in DMSO. The acquisition settings were: microwave frequency, 9.786 GHz, modulation amplitude, 1 G; microwave power, 5 mW; time constant, 40 ms; scan time, 21 s. The broken red-colored trace superimposed on each spectrum was simulated using the parameters shown in Table 1. The small satellite peaks indicated with * on both sides of the main peaks are due to hyperfine coupling with ^{13}C nuclei (natural abundance).

the case of clinical situations this may not be readily feasible. Thus the near-term usefulness of the probes is in non-clinical research applications both in vitro using cell culture models and in vivo using animal models.

4. Experimental section

4.1. Bis[2,3,5,6-tetrachlorophenyl]-[4-(2-hydroxyetheyl)-2,3,5,6-tetrachlorophenyl] methane (2a),bis[4-(2-hydroxyetheyl)-2,3,5,6-tetrachlorophenyl]-[2,3,5,6-tetrachloro-phenyl] methane (2b), tri[4-(2-hydroxyetheyl)-2,3,5,6-tetrachlorophenyl] methane (2c)

To a solution of **1** (1.6 g, 2.46 mmol) and TMEDA (0.44 ml, 2.95 mmol) in anhydrous THF (30 ml) at −78 °C, *n*-BuLi (1.84 ml, 1.6 M solution in THF) was added and stirred for 1 h. Ethylene oxide was added (2.95 ml, 1.0 M solution in Et_2O) and the reaction mixture was warmed to room temperature and stirred overnight. The reaction was quenched by addition of 1.0 ml water. The solvent was removed and the residue was dissolved with CH_2Cl_2, washed with water and brine, and dried with Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/EtOAc = 5:1 to 1: 1.5) afforded the product **2a** (0.58 g, 0.48 mmol, 33.7%) **2b** (0.36 g, 0.48 mmol, 19.6%), and **2c** (0.12 g, 0.15 mmol, 6.2%). Compound **2a**: ^1H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 2H), 6.97 (s, 1H), 3.87 (t, J = 7.2 Hz, 2H), 3.37 (t, J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 138.7, 137.0, 136.4, 134.8, 134.6, 134.4, 134.4, 133.8, 133.6 (2 carbons), 133.5, 133.3 (2 carbons), 132.4, 132.4, 130.3 (2 carbons), 60.2, 56.3, 37.4; HRMS $(M+Na)^+$ calcd for $C_{21}H_8Cl_{12}ONa$ 724.6644, found 724.6649. Compound **2b**: ^1H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H), 6.97 (s, 1H), 3.87 (t, J = 7.2 Hz, 4H), 3.37 (t, J = 7.2 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.9, 136.9, 136.9, 136.6, 136.5, 134.8, 134.8, 134.6 (2 carbons), 134.4, 133.8, 133.7, 133.5, 133.4, 133.4, 133.3, 132.3, 130.2, 60.2, 56.4, 37.4. HRMS $(M+Na)^+$ calcd for $C_{23}H_{12}Cl_{12}O_2Na$ 768.6909, found 768.6929. Compound **2c**: ^1H NMR (400 MHz, $CDCl_3$) δ 7.02 (s, 1H), 3.90 (m, 6H), 3.40 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.8 (6 carbons), 134.8 (3 carbons), 134.5 (3 carbons), 133.8 (3 carbons), 133.4 (3 carbons), 60.3(3 carbons), 56.7, 37.4 (3 carbons). HRMS $(M+Na)^+$ calcd for $C_{25}H_{16}Cl_{12}O_3Na$ 812.7171, found 812.7204.

4.2. Bis(2,3,5,6-tetrachlorophenyl)-[4-(2-phthalimideetheyl)-2,3,5,6-tetrachlorophenyl] methane (3)

To a solution of **2a** (0.32 g, 0.46 mmol), phthalimide (0.13 g, 0.91 mmol), triphenylphosphine (0.24 g, 0.91 mmol) in anhydrous THF (10 ml), DIAD (0.18 L, 0.91 mmol) was added. The reaction mixture was stirred overnight at room temperature. The solvent was removed in vacuo and the residue was dissolved in EtOAc, washed with water and brine, and dried with Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/EtOAc = 1:0 to 10:1) afforded the product **3** (0.37 g, 0.45 mmol, 97.7%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.81 (dd, J = 5.4 Hz, 3.0 Hz, 2H), 7.68 (dd, J = 5.4 Hz, 3.0 Hz, 2H), 7.62 (s, 1H), 7.61 (s, 1H), 6.93 (s, 1H), 4.10 (m, 1H), 4.01 (m, 1H), 3.52 (m, 1H), 3.44 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.9 (2 carbons), 138.8, 138.6,

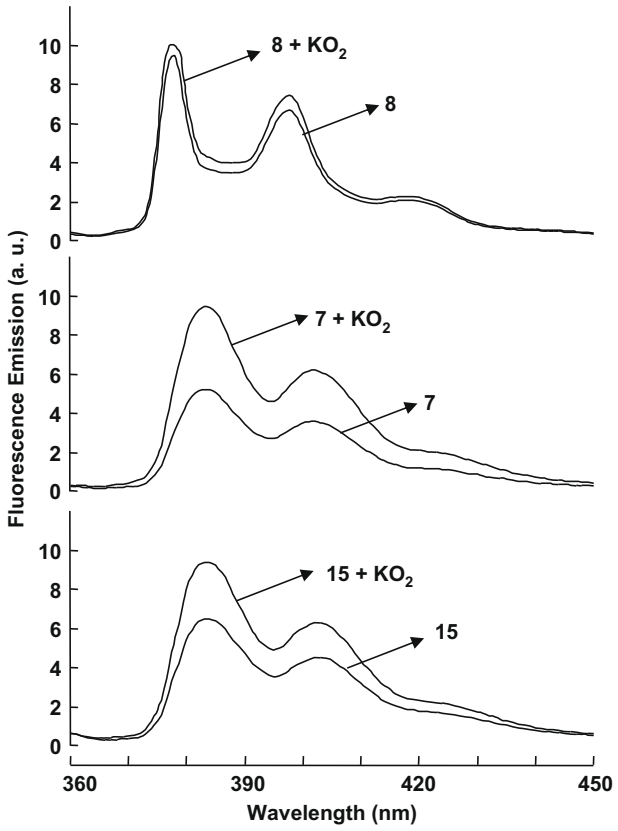


Figure 5. Excitation and emission profiles of **8**, **7**, and **15** in the presence and absence of KO_2 in DMSO. Data show two emission bands, at 377 nm and 397 nm, corresponding to excitations at 330 and 346 nm, respectively.

136.9, 136.7, 134.8, 134.6, 134.5, 134.3, 134.0 (2 carbons), 133.7, 133.6, 133.5, 133.5, 133.4, 133.2, 132.4, 132.3, 131.9 (2 carbons), 130.2, 130.2, 123.3 (2 carbons), 56.3, 34.7, 33.1; HRMS $(M+Na)^+$ calcd for $C_{29}H_{11}Cl_{12}NO_2Na$ 851.6888, found 851.6903.

4.3. Bis(2,3,5,6-tetrachlorophenyl)-[4-(2-aminoetheyl)-2,3,5,6-tetrachlorophenyl] methane (4)

To a solution of **3** (0.25 g, 0.30 mmol) in absolute EtOH (5 ml) was added hydrazine hydrate. The reaction mixture was refluxed for 4 h. The mixture was then cooled, diluted with water, and extracted with CH_2Cl_2 (2×). The organic extracts were washed with brine and dried over $MgSO_4$. Removal of the solvent afforded the crude product **4** (0.14 g, 0.20 mmol), which was used in the next step without purification. HRMS $(M+H)^+$ calcd for $C_{21}H_{10}Cl_{12}N$ 701.6987, found 701.6931.

4.4. Bis(2,3,5,6-tetrachlorophenyl)-{4-[2-(1-pyrenecarbonyl)aminoetheyl]-2,3,5,6-tetrachlorophenyl} methane (5)

To a solution of **4** (0.14 g, 0.20 mmol), 1-pyrenecarboxyl *N*-hydroxysuccinimide ester (0.08 g, 0.24 mmol) in anhydrous THF (10 ml), Et_3N (0.10 ml, 0.72 mmol) was added. The reaction mixture was stirred at 50 °C for 2 h. The solvent was removed and the residue was purified by column chromatography (hexanes = 100% to hexanes/EtOAc/CH_2Cl_2 = 15:4:10) afforded the product **5** (0.11 g, 0.12 mmol, 59.3%).^{1}H NMR (500 MHz, $CDCl_3$) δ 8.44 (d, J = 10.0 Hz, 1H), 8.16 (d, J = 7.5 Hz, 1H), 8.13 (d, J = 7.5 Hz, 1H), 7.9–8.1 (m, 6H), 7.64 (s, 1H), 7.63 (s, 1H), 7.05 (s, 1H), 6.48 (t, J = 5.9 Hz, 1H), 3.8–4.0 (m, 2H), 3.54 (t, J = 6.9 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.1, 138.8, 138.7, 137.8, 136.7, 135.0, 134.7, 134.44, 134.39, 133.9, 133.6 (2 carbons), 133.4, 133.3, 132.5, 132.45 (2 carbons), 131.1, 130.6, 130.5, 130.3 (2 carbons), 128.62 (2 carbons), 128.56, 127.0, 126.3, 125.8, 125.7, 124.6, 124.5, 124.35, 124.28, 124.17, 56.5, 38.2, 34.3; HRMS $(M+Na)^+$ calcd for $C_{38}H_{17}Cl_{12}NONa$ 951.7384, found 951.7368.

4.5. Bis(2,3,5,6-tetrachlorophenyl)-{4-[2-(1-pyrenebutylcarboxy)aminoethyl]-2,3,5,6-tetrachlorophenyl} methane (6)

To a solution of **4** (0.14 g, 0.20 mmol), 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (0.09 g, 0.24 mmol) in anhydrous THF (10 ml), Et_3N (0.10 ml, 0.72 mmol) was added. The reaction mixture was stirred at 50 °C for 2 h. The solvent was removed and the residue was purified by column chromatography (hexanes = 100% to hexanes/EtOAc/CH_2Cl_2 = 1:0.33:1) afforded the product **6** (0.13 g, 0.13 mmol, 67.0%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 10.0 Hz, 1H), 8.13 (d, J = 7.5 Hz, 2H), 8.0–8.1 (m, 2H), 7.9–8.0 (m, 3H), 7.79 (d, J = 7.8 Hz, 1H), 7.59 (s, 1H), 7.58 (s, 1H), 6.94 (s, 1H), 5.58 (t, J = 5.9 Hz, 1H), 3.4–3.6 (m, 2H), 3.33 (t, J = 7.0 Hz, 2H), 3.23 (t, J = 7.0 Hz, 2H), 2.1–2.2 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.7, 140.67, 140.65, 139.6, 138.5, 137.8, 136.8, 136.5, 136.4, 135.8, 135.5, 135.4, 135.3, 134.4, 133.4, 132.9, 132.3, 132.0, 130.8, 129.5, 129.4, 128.8, 127.9, 127.2, 127.0, 126.96, 126.8, 125.4, 58.3, 39.3, 37.9, 36.2, 34.8, 29.2; HRMS $(M+Na)^+$ calcd for $C_{41}H_{23}Cl_{12}NONa$ 993.7851, found 993.7851.

4.6. Bis(2,3,5,6-tetrachlorophenyl)-{4-[2-(1-pyrenecarbonylamino)etheyl]-2,3,5,6-tetrachlorophenyl} methyl radical ($PTMH_2$-PyC) (7)

An aqueous solution of tetra *n*-butyl ammonium hydroxide (84.0 mg, 0.105 mmol) was added to a solution of **5** (65.0 mg, 0.070 mmol) in dry tetrahydrofuran (5 ml) at room temperature. The resulting red solution was stirred overnight, and then *p*-chloranil (51.7 mg, 0.21 mmol) was added. The resulting intensely brown solution was stirred for 1 h. After this time, the solution was evaporated to dryness and the resulting product was purified by chromatography (hexanes = 100% to hexanes/EtOAc/CH_2Cl_2 = 9:1:2) and afforded the product **7** (64.6 mg, 0.070 mmol, 99.3%). HRMS $(M+Na)^+$ calcd for $C_{38}H_{16}Cl_{12}NONa$ 950.7305, found 950.7291.

4.7. Bis(2,3,5,6-tetrachlorophenyl)-{4-[2-(1-pyrenebutylcarboxy)aminoetheyl]-2,3,5,6-tetrachlorophenyl} methyl radical ($PTMH_2$-PyB) (8)

Aqueous solution of tetra *n*-butyl ammonium hydroxide (73.6 mg, 0.092 mmol) was added to a solution of **6** (60.0 mg, 0.062 mmol) in dry tetrahydrofuran (5 ml) at room temperature. The resulting red solution was stirred overnight and then *p*-chloranil (46.0 mg, 0.186 mmol) was added. The resulting intensely brown solution was stirred for 1 h. After this time, the solution was evaporated to dryness and the resulting product was purified by chromatography (hexanes = 100% to hexanes/EtOAc/CH_2Cl_2 = 1:0.33:1) afforded the product **8** (57.2 mg, 0.059 mmol, 95.4%). HRMS $(M+Na)^+$ calcd for $C_{41}H_{22}Cl_{12}NONa$ 992.7776, found 992.7775.

4.8. Bis[2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl]-(2,3,5,6-tetrachlorophenyl) methane (9)

To a solution of **1** (1.15 g, 1.76 mmol) and TMEDA (0.64 ml, 4.22 mmol) in anhydrous THF (80 ml) at −78 °C, *n*-BuLi (2.65 ml, 1.6 M solution in THF) was added and stirred for 1 h. Ethyl chloroformate was added (0.41 ml, 4.2 mmol) and the reaction mixture was allowed to warm up to room temperature and stirred overnight. The reaction was quenched by addition of 1 M HCl to pH 2. The solvent was removed and the residue was dissolved with

CH_2Cl_2, washed with brine, and dried with Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/CH_2Cl_2/EtOAc = 100:1:1) afforded the product **9** (0.66 g, 0.82 mmol, 46.7%) and mono-substituted compound (0.43 g, 0.59 mmol, 33.4%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (s, 1H), 6.98 (s, 1H), 4.46 (q, J = 7.1 Hz, 4H), 1.40 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.2, 138.8 (2 carbons), 138.0, 135.4 (2 carbons), 135.02, 134.99, 134.4, 134.0, 133.9, 133.7, 133.3, 132.6, 130.6, 130.5, 130.4, 129.43, 129.40, 63.1, 56.3, 14.0; HRMS (M+Na)$^+$ calcd for $C_{25}H_{21}Cl_{12}O_4Na$ 824.6805, found 824.6819.

4.9. Bis[2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl]-[4-(2-hydroxyetheyl)-2,3,5,6-tetrachloro-phenyl] methane (10)

Method A: To a solution of **9** (0.48 g, 0.60 mmol) and TMEDA (0.23 ml, 1.50 mmol) in anhydrous THF (30 ml) at −78 °C, *n*-BuLi (0.94 ml, 1.6 M solution in THF) was added and stirred for 1 h. Ethylene oxide was added (1.5 ml, 1.0 M solution in Et_2O) and the reaction mixture was stirred allowing the reaction to warm to room temperature. The reaction was quenched by addition of 1 M HCl to pH 2. The solvent was removed and the residue was dissolved with CH_2Cl_2, washed with brine, and dried with Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/EtOAc/CH_2Cl_2 = 6:1:1) afforded the product **10** (0.05 g, 0.059 mmol, 9.9%). *Method B*: To a solution of **11** (0.67 g, 0.73 mmol) in THF/H_2O (1:1, 10 ml) at room temperature, LiOH (0.07 g, 2.92 mmol) was added and stirred overnight. After the reaction completed, the pH of the reaction mixture was adjusted to 2 by addition of 1 M HCl. The reaction mixture was extracted by dichloromethane three times. Removal of the solvent followed by column chromatography (hexanes/EtOAc/CH_2Cl_2 = 6:1:1) afforded the product **10** (0.38 g, 0.45 mmol, 61.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (s, 1H), 4.46 (q, J = 7.1 Hz, 4H), 3.87 (t, J = 7.2 Hz, 2H), 3.37 (t, J = 7.2 Hz, 2H), 1.40 (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.3, 139.0, 138.9, 137.4, 135.7, 135.34, 135.31, 135.02, 134.99, 134.75, 134.69, 133.98, 133.92, 133.74, 133.64, 130.43, 130.39, 129.38, 129.36, 63.1, 60.2, 56.5, 37.4, 14.0; HRMS (M+Na)$^+$ calcd for $C_{27}H_{16}Cl_{12}O_5Na$ 868.7068, found 868.7065.

4.10. Bis[2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl]-[4-(2-ethoxycarbonyloxy-ethyl)-2,3,5,6-tetrachloro-phenyl] methane (11)

To a solution of **2a** (0.73 g, 1.04 mmol) and TMEDA (0.73 ml, 4.84 mmol) in anhydrous THF (60 ml) at −78 °C, *n*-BuLi (3.03 ml, 1.6 M solution in THF) was added and stirred, for 1 h. Ethyl chloroformate (0.86 ml, 8.96 mmol) was added and the reaction mixture was stirred, allowing the reaction to warm up to −20 °C. The reaction was quenched by addition of 1 M HCl to pH 2. The solvent was removed and the residue was dissolved with CH_2Cl_2, washed with brine, and dried with Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/CH_2Cl_2/EtOAc = 20:1:1) afforded the product **11** (0.67 g, 0.73 mmol, 70.2%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.00 (s, 1H), 4.46 (q, J = 7.1 Hz, 4H), 4.35 (m, 2H), 4.18 (q, J = 7.1 Hz, 2H), 3.48 (t, J = 7.0 Hz, 2H), 1.40 (t, J = 7.1 Hz, 6H), 1.28 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.2, 155.0, 138.92, 138.85, 136.3, 136.2, 135.38, 135.35, 135.01, 134.98, 134.85, 134.83, 133.96, 133.92, 133.81, 133.77, 130.44, 130.40, 129.39, 129.37, 64.2, 63.1, 56.5, 36.6, 33.6, 14.3, 14.0; HRMS (M+Na)$^+$ calcd for $C_{30}H_{20}Cl_{12}O_7Na$ 940.7281, found 940.7281.

4.11. Bis[2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl]-[4-(2-phthalimide-etheyl]-2,3,5,6-tetrachlorophenyl] methane (12)

To a solution of **10** (110 mg, 0.13 mmol), phthalimide (38 mg, 0.26 mmol), triphenylphosphine (68.4 mg, 0.26 mmol) in anhydrous THF (5 ml), DIAD (51 μL, 0.26 mmol) was added. The reaction mixture was stirred overnight at room temperature. The solvent was removed and the residue was dissolved with EtOAc, washed with water and brine, and dried with Na_2SO_4. Removal of the solvent was followed by column chromatography (hexanes/EtOAc = 8:1 to 3:1) which afforded the product **12** (66.7 mg, 0.068 mmol, 52.3%) with minimal impurity. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 5.4 Hz, 3.1 Hz, 2H), 7.67 (d, J = 5.4 Hz, 3.1 Hz, 2H), 6.94 (s, 1H), 4.46 (q, J = 6.9 Hz, 4H), 4.0–4.1 (m, 2H), 3.4–3.6 (m, 2H), 1.40 (t, J = 6.9 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.9, 163.2, 139.0, 138.8, 137.3, 136.1, 135.30, 135.27, 135.1, 134.94, 134.89, 134.6, 134.0, 133.96, 133.85, 133.76, 133.67, 131.9, 130.4, 130.3, 129.34, 129.26, 123.3, 63.0, 56.5, 35.2, 33.1, 14.0; HRMS (M+Na)$^+$ calcd for $C_{35}H_{19}Cl_{12}NO_6Na$ 997.7286, found 997.7291.

4.12. Bis[2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl]-[4-(2-aminoetheyl)-2,3,5,6-tetrachlorophenyl] methane (13)

To a solution of **12** (50 mg, 0.0513 mmol) in absolute EtOH (5 ml) was added hydrazine hydrate. The reaction mixture was heated and refluxed for 2 h. The mixture was cooled while precipitate appeared. The reaction mixture was diluted with water, and extracted with CH_2Cl_2 (2×). The organic extracts were washed with brine, dried over $MgSO_4$. Removal of the solvent afforded the crude product **13** (45 mg, 0.053 mmol), which was used in the next step without purification. HRMS (M+H)$^+$ calcd for $C_{27}H_{18}Cl_{12}NO_4$ 845.7410, found 845.7401.

4.13. Pyrene-1-carboxylic acid (2-{4-[bis-(2,3,5,6-tetrachloro-4-ethoxycarbonyl-phenyl)- methyl]-2,3,5,6-tetrachloro-phenyl} -ethyl)-amide (14)

To a solution of **13** (45 mg, 0.20 mmol), 1-pyrenecarboxyl *N*-hydroxysuccinimide ester (0.08 g, 0.053 mmol) in anhydrous THF (5 ml), Et_3N (0.033 ml, 0.24 mmol) was added. The reaction mixture was stirred at 50 °C for 2 h. The solvent was removed and the residue was purified by column chromatography (hexanes = 100% to hexanes/EtOAc/CH_2Cl_2 = 4.5:0.8:2) afforded the product **14** (30 mg, 0.03 mmol, 52.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, J = 10.0 Hz, 1H), 8.19 (d, J = 8.5 Hz, 2H), 7.9–8.1 (m, 6H), 7.05 (s, 1H), 6.37 (m, 1H), 4.48 (m, 4H), 3.8–4.1 (m, 2H), 3.56 (m, 2H), 1.41 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 163.3, 139.0, 138.9, 138.2, 136.1, 135.39, 135.37, 135.1, 135.0, 134.9, 134.8, 134.97 (2 carbons), 133.92, 133.7, 132.6, 131.1, 130.7, 130.6, 130.5, 130.4, 129.42, 129.40, 128.7 (2 carbons), 128.6, 127.1, 126.3, 125.8 (2 carbons), 124.8, 124.44, 124.39, 124.35, 124.26, 63.0, 56.5, 38.2, 34.3, 14.0; HRMS (M+Na)$^+$ calcd for $C_{44}H_{25}Cl_{12}NO_5Na$ 1095.7809, found 1095.7797.

4.14. Bis(2,3,5,6-tetrachlorophenyl)-{4-[2-(1-pyrenecarbonyl)aminoetheyl]-2,3,5,6-tetrach-lorophenyl} methyl radical (15)

An aqueous solution of tetra *n*-butyl ammonium hydroxide (22.6 μL, 0.028 mmol) was added to a solution of **14** (20.0 mg, 18.6 μmol) in dry tetrahydrofuran (5 ml) at room temperature. The resulting red solution was stirred overnight, and then *p*-chloranil (13.7 mg, 0.056 mmol) was added. The resulting intensely brown solution was stirred for 1 h. After this time, the solution was evaporated to dryness and the resulting product was purified by chromatography (hexanes = 100% to hexanes/EtOAc/CH_2Cl_2 = 6:2:1) afforded the product **15** (19.8 mg, 18.5 μmol, 99.0%); HRMS (M+Na)$^+$ calcd for $C_{44}H_{24}Cl_{12}NO_5Na$ 1094.7731, found 1094.7732.

4.15. EPR measurements

EPR measurements were performed at room temperature using a Bruker EMX spectrometer equipped with a high-sensitivity

resonator. Solutions of the compounds in DMSO were drawn into a 50-ml capillary tube and used for EPR measurements. The acquisition settings were microwave frequency, 9.786 GHz, modulation amplitude, 1 G; microwave power, 5 mW; time constant, 40 ms; scan time, 21 s. Spectral simulations were carried out using a custom-developed software (EPRDAP).

4.16. Fluorescence measurements

The fluorescence scan measurements were performed on the compounds in DMSO at room temperature using an Aminco-Bowman Series 2 spectrofluorometer (Aminco Spectronic Instruments, New York, USA).

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