Electronic Supplementary Material (ESI) for RSC Advances.

Electronic Supplementary Information

Large-Scale Synthesis of a Monophosphonated Tetrathiatriarylmethyl Spin Probe for concurrent *in vivo* measurement of pO_2, pH and inorganic phosphate by EPR

Teresa D. Gluth[ab] , Martin Poncelet[ab] , Stephen DeVience[bc], Marieta Gencheva[bc], Emily H. Hoblitzell[bd], Valery V. Khramtsov[bc], Timothy D. Eubank[bd] and Benoit Driesschaert[ab*]

[a]Department of Pharmaceutical Sciences, West Virginia University, School of Pharmacy, Morgantown, WV, 26506, USA.

[b]In Vivo Multifunctional Magnetic Resonance center, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV, 26506, USA.

[c]Department of Biochemistry, West Virginia University, School of Medicine, Morgantown, WV, 26506, USA.

[d]Department of Microbiology, Immunology, and Cell Biology, West Virginia University, School of Medicine, Morgantown, WV, 26506, USA.

These authors contributed equally.

Email: benoit.driesschaert@hsc.wvu.edu

Table of Contents

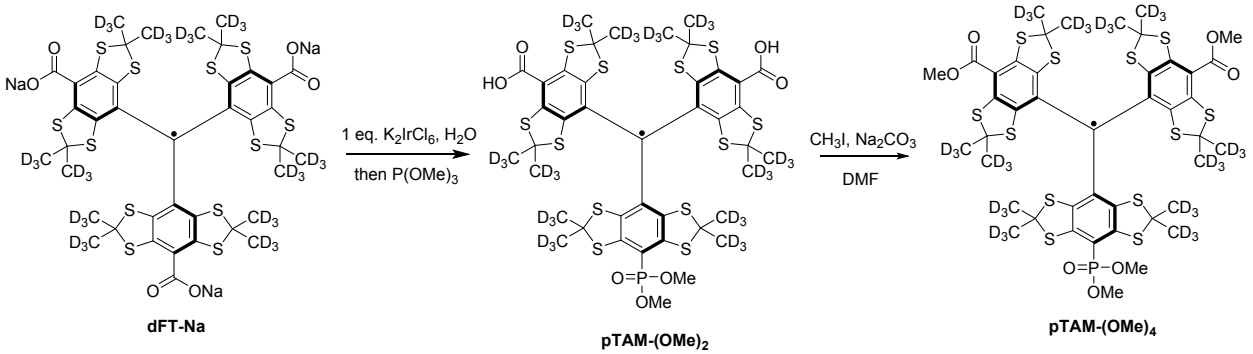
1. General Information

HRMS data were collected on a Thermo Scientific Q Exactive Mass Spectrometer with an Electron Spray Ionization (ESI) source. All purifications were performed on a Teledyne Combiflash Rf+ purificator. X-band EPR spectra (9.5 GHz) were performed on an X-band ELEXSYS E580 EPR spectrometer. L-band EPR spectra (1.2 GHz) were performed on a Magnettech spectrometer. All solvents were purchased from Fisher Scientific. All commercially available reagents were purchased from Sigma-Aldrich and used as received without further purification. HPLC analyses were carried out on a Waters Alliance e2695 separation module equipped with an SQD2 Mass Detector and a 2998 PDA detector. The separations were performed on a Waters XBridge BEH C18 4.6 mm x 50 mm, 2.5 µm column. Gradient conditions were as follows: column temperature, 40°C; UV detection from 210 to 800 nm, flow rate 1.5 mL/min.

Time (min)	Water (%)	Acetonitrile (%)	Water with 1% trifluoroacetic acid (%)
0	80	10	10
5	0	90	10
6	0	100	0
10	0	100	0

2. Synthetic procedures

2.1 Synthesis of pTAM-(OMe)$_4$



Scheme S1. Synthesis of **pTAM-(OMe)$_4$**.

dFT as a trisodium salt synthesized as previously reported[1-2] (20 g, 18.1 mmol) was dissolved in 4 L deionized water (dFT concentration was 4.5 mM), then potassium hexachloroiridate (IV) (8.76 g 18.1 mmol, 1 eq.) was added, and the reaction was stirred at room temperature for 5 min. Trimethyl phosphite (21.4 mL, 0.18 mol, 10 eq.) was added, and the reaction was stirred overnight at room temperature. The mixture was acidified to pH 2 with concentrated hydrochloric acid. The trityl radicals were extracted with 12 L of ethyl acetate, then the organic phase was dried over $MgSO_4$, filtered and

evaporated under reduced pressure. The crude was dissolved in 200 mL of dry DMF, then anhydrous Na_2CO_3 (5 g, excess) and iodomethane (11.3 mL, 0.18 mol, 10 eq.) was added, and the reaction was stirred at 50°C overnight. 1L of brine was added to initiate precipitation, then the suspension was filtered, the solid was washed with water (3x), and then dried under reduced pressure. The remaining water was eliminated by azeotropic distillation with toluene (3X1L). The compound was purified by flash chromatography using 0-100% ethyl acetate in hexane, affording 7.3 g of pure compound **pTAM-(OMe)$_4$** (35% yield). The trimethyl ester of dFT (**dFT-(OMe)$_3$**), 12.4 g was also recovered (63%). **HRMS (ESI)** calcd. for $[C_{43}H_{12}D_{36}O_7PS_{12}]^+$ m/z=1127.2046, found m/z=1127.1976.

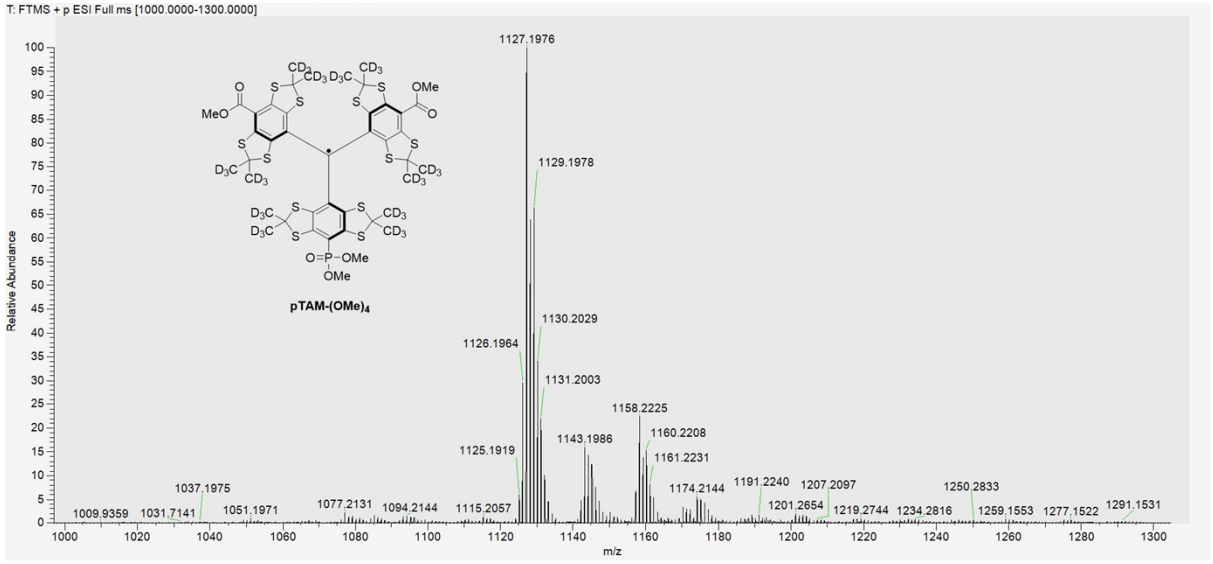


Figure S1. HRMS (ESI) of **pTAM-(OMe)$_4$**

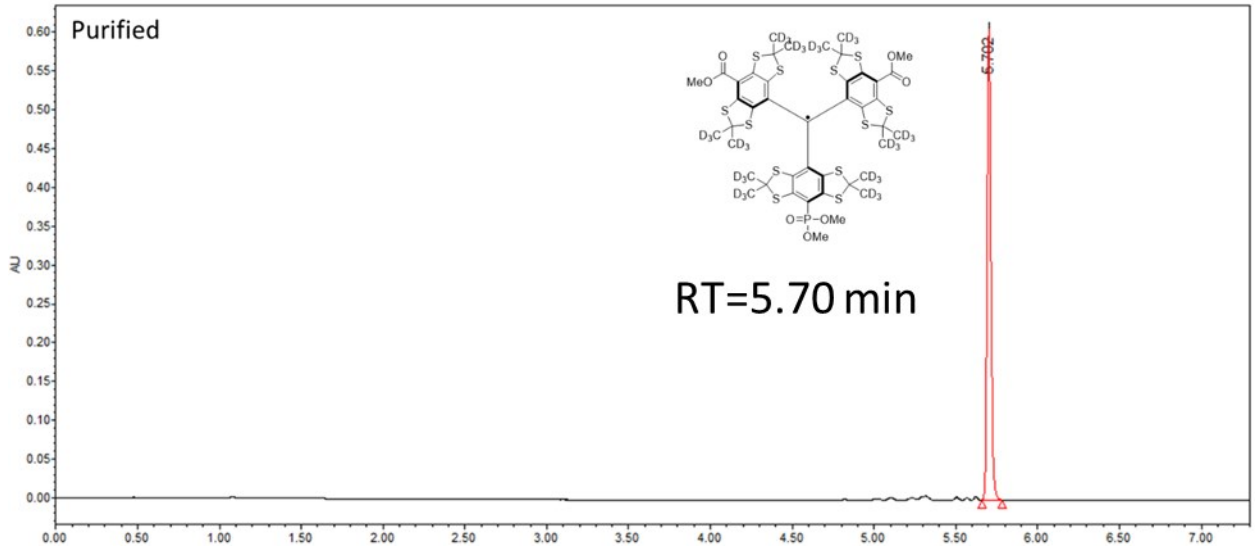
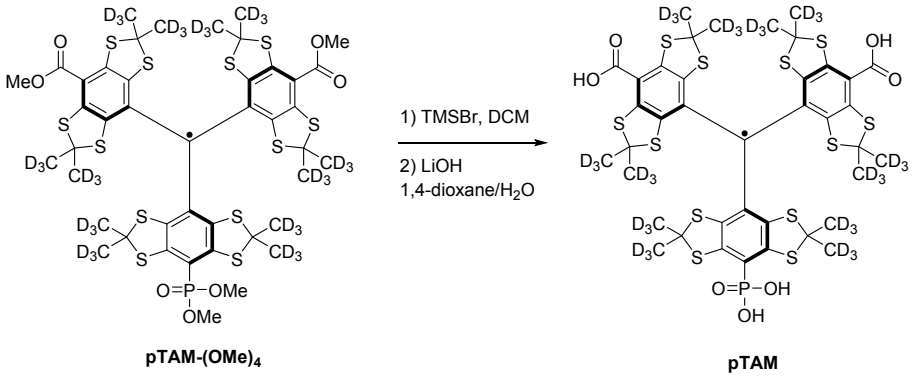


Figure S2. HPLC chromatogram of **pTAM-(OMe)$_4$**

2.2 Synthesis of pTAM



Scheme S2. Synthesis of **pTAM**.

pTAM-(OMe)$_4$ (7.3 g, 6.47 mmol) was dissolved in 2 L of dichloromethane and cooled down to 0°C. Bromotrimethylsilane (3.5 mL, 25.9 mmol, 4 eq.) was added at 0°C, then stirred overnight at room temperature. The solvent was evaporated under reduced pressure, then 500 mL methanol were added, stirred 15 min at room temperature, and then evaporated. 200 mL of 1,4-dioxane were added, followed by 100 mL of a 2.5M solution of LiOH (6 g LiOH in 100 mL deionized water). The mixture was stirred at 80°C for 3h. After cooling, the pH was adjusted to pH 2 using concentrated hydrochloric acid, then **pTAM** was extracted with 3 L of ethyl acetate. The organic phase was dried on magnesium sulfate, filtered, and then dried under reduced pressure. The final compound was purified by chromatography, using a C18 column. The column was eluted with 30% acetonitrile in water containing 0.1% TFA to afford 6.6 g of pure **pTAM** (95% yield) after evaporation of the acetonitrile and then freeze-drying. Then, **pTAM** acidic form was suspended in water (1.5 L) and titrated to pH=7 with NaOH and then freeze-dried again. **HRMS (ESI)** calcd. for $[C_{39}H_3D_{36}O_7PS_{12}]^-$ m/z=1070.1347, found m/z=1070.1383.

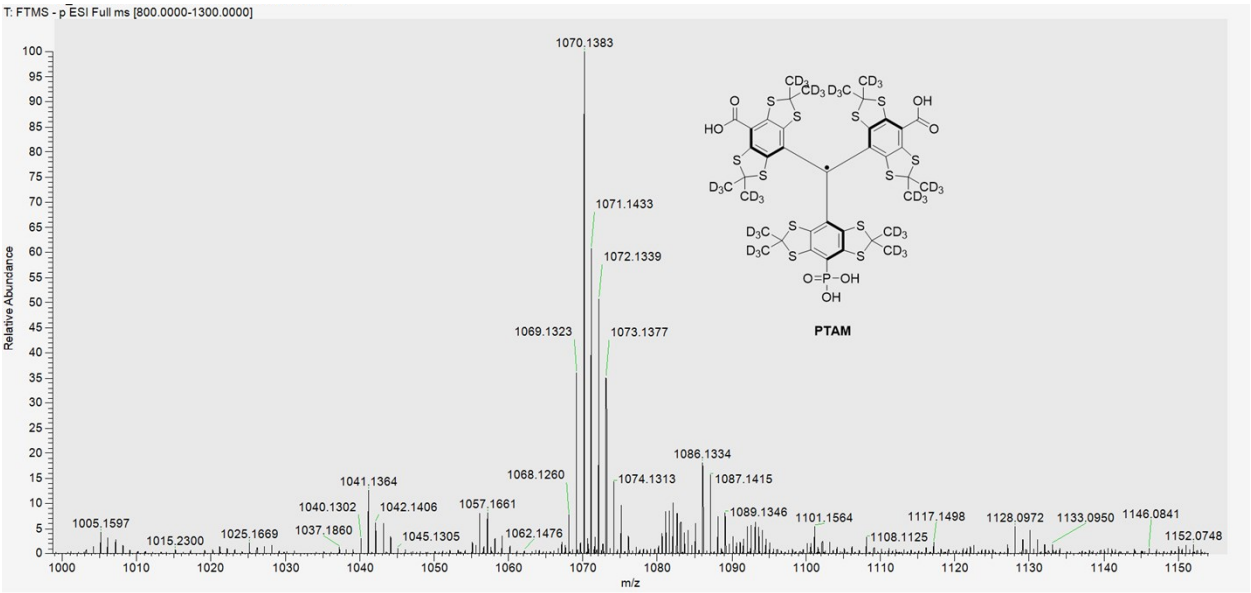


Figure S3. HRMS (ESI) of **pTAM**

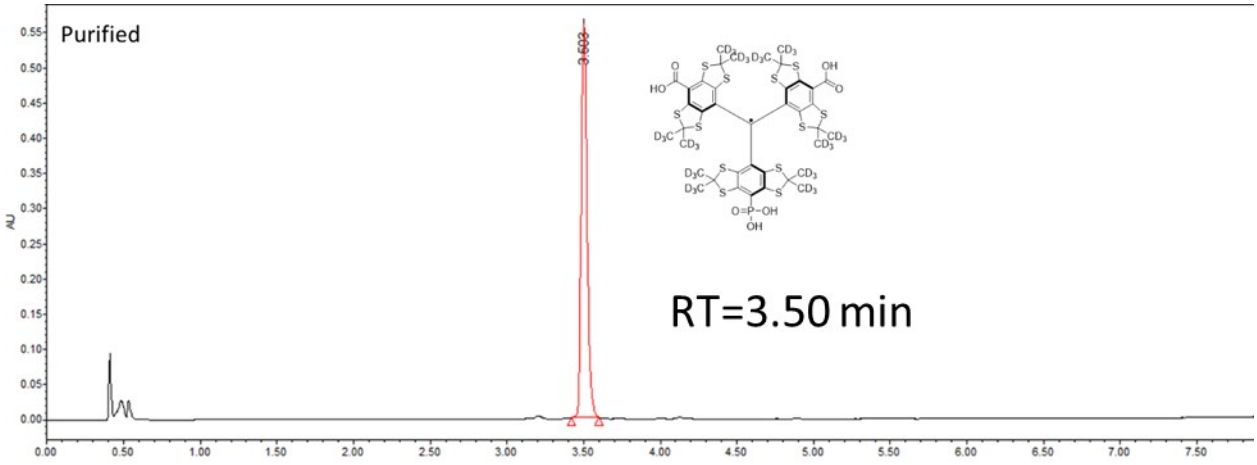
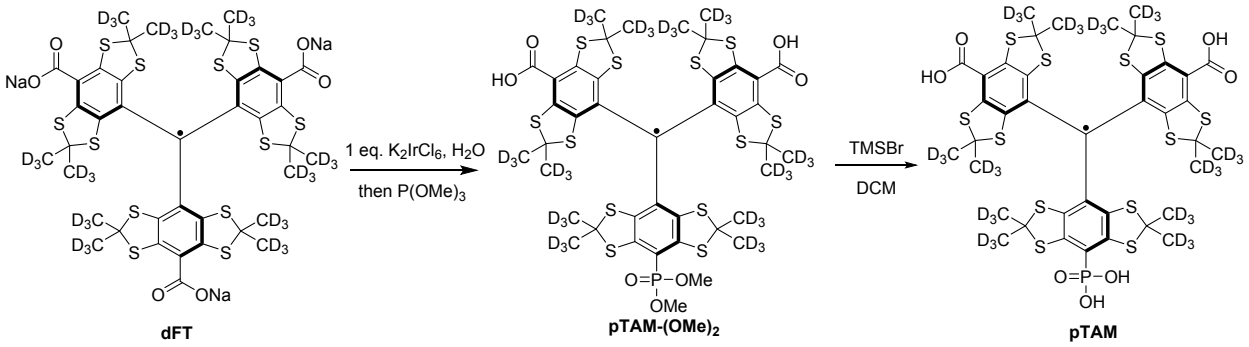


Figure S4. HPLC chromatogram of **pTAM**

2.3 Alternate protocol of the synthesis of pTAM on a small scale.



Scheme S3. Alternative synthesis of **pTAM**.

dFT as a trisodium salt (500 mg, 0.45 mmol) was dissolved in 100 mL deionized water, then potassium hexachloroiridate (IV) (220 mg 0.45 mmol, 1 eq.) was added, and the reaction was stirred at room for temperature for 5 min. Trimethyl phosphite (0.6 mL, 4.5 mmol, 10 eq.) was added, and the reaction was stirred overnight at room temperature. The mixture was acidified at pH 2 with concentrated hydrochloric acid. The trityl radicals were extracted with 300 mL of ethyl acetate, then the organic phase was dried on $MgSO_4$, filtered and evaporated under reduced pressure. The crude mixture was purified on a C18 column with a gradient water/acetonitrile containing 0.1% TFA from 0 to 100% ACN to afford 280 mg (60%) of **dFT** and 150 mg (30%) of **pTAM-(OMe)$_2$** .**HRMS (ESI)** calcd. for $[C_{41}H_8D_{36}O_7PS_{12}]^+$ m/z= 1099.1733, found m/z=1099.1729.

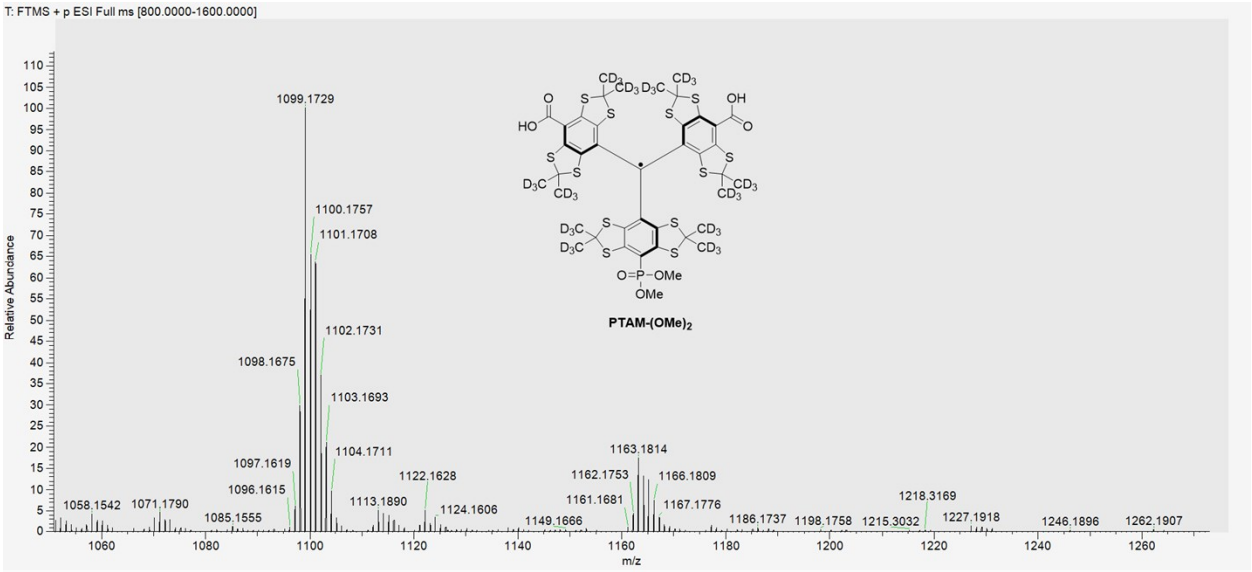


Figure S5. HRMS (ESI) of **pTAM-(OMe)$_2$**

Then, **pTAM-(OMe)$_2$** (150 mg, 0.135 mmol) was dissolved in 50 mL of dichloromethane and cooled down to 0°C. Bromotrimethylsilane (270 uL, 2 mmol, 15 eq.) was added at 0°C, then stirred overnight at room temperature. The solvent was evaporated under reduced pressure, then 50 mL methanol were added, stirred 15 min at room temperature, and then evaporated. The final compound was purified by chromatography, using a prepacked a C18 column. The column was eluted with 30% acetonitrile in water containing 0.1% TFA to afford 137 mg of pure **pTAM** (95% yield).

3. MS spectra from HPLC-MS

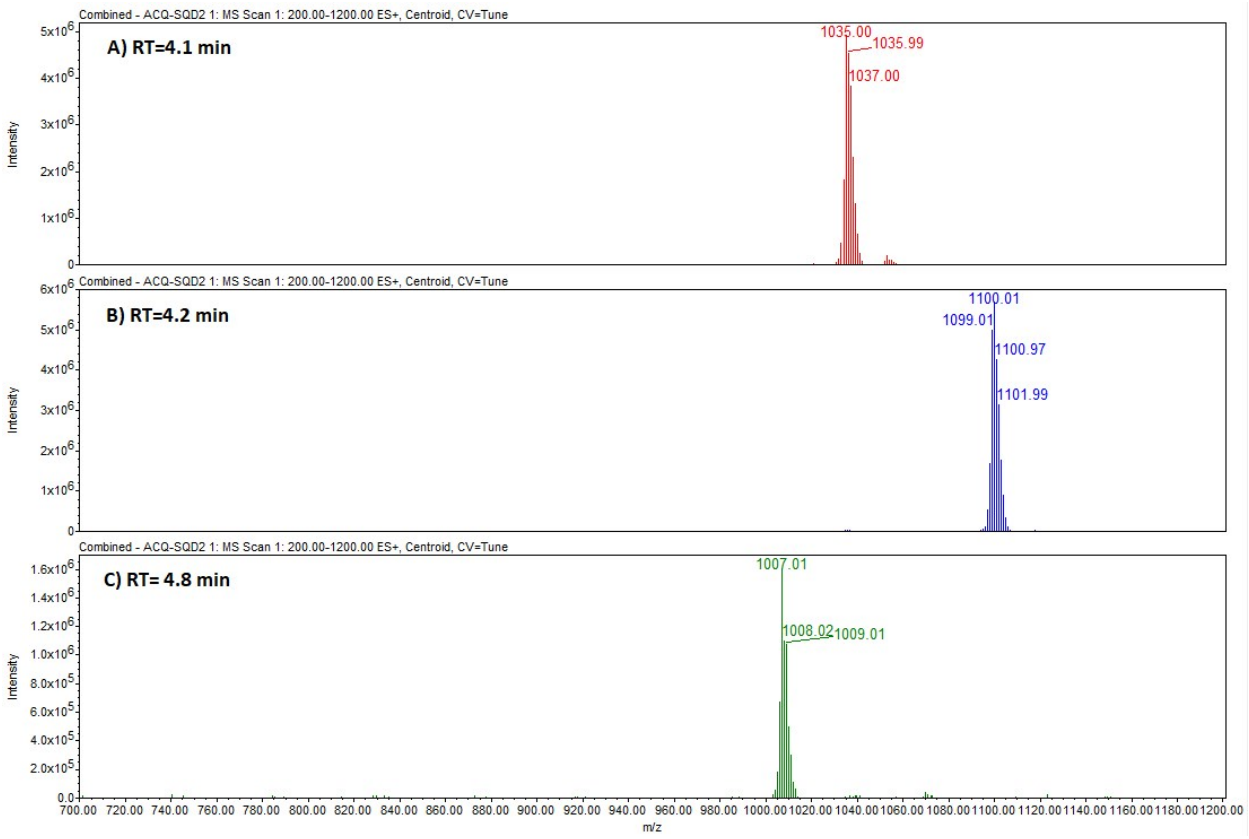


Figure S6. ESI MS spectra extracted from the three peaks of the HPLC chromatogram in Figure 2B.

4. Mechanism for the formation of QM

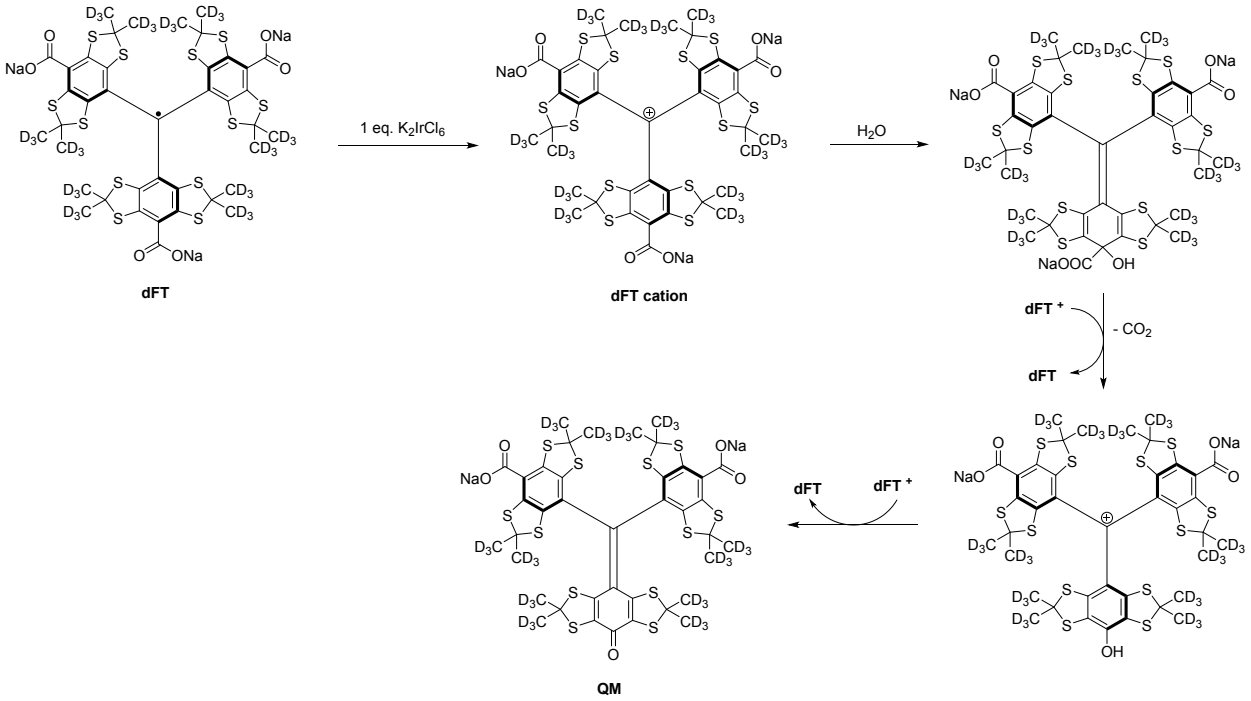


Figure S7. Proposed mechanism for the formation of QM

5. Confirmation of the extracellular localization of pTAM

1.7 x 10^7 exponentially growing MDA-MB-231 cells were divided between two tubes. The cells were collected by centrifugation at 1000 g/5 min/4˚C and washed twice in 1 mM phosphate buffer, pH 7.4 /136 mM NaCl. Each pellet was resuspended in buffer and adjusted to yield 200 µM pTAM and 100 uL total volume cell suspension. Gd-DTPA was added to one of the tubes to a final concentration of 10 mM. The cells were incubated for 30 min on ice, after which the cell suspension was transferred into glass EPR capillaries, and the spectra were recorded. The viability of the cells during the measurements was confirmed with Trypan Blue after collecting the spectra. EPR acquisition parameters were as follow: Power 0.4743 mW, ModFreq 30.00 kHz, ModAmp 0.030 G, SweepTime 20.48400 s, SweepWidth 8.0 G, ConvTime 20.00 ms.

6. MTT assays

Exponentially growing MDA-MB-231 cells at 60-70% confluency were treated with increasing concentrations of pTAM in a 96-well plate. After 24h of incubation, the medium was removed and replaced with medium containing MTT. The MTT assay was performed as described in the manufacturer's protocol (Vybrant MTT Cell Proliferation Assay Kit, ThermoFisher). Medium containing equivalent concentrations of pTAM without the presence of cells was used as a background control. Absorbance was measured at 570 nm and also at 630 nm, to correct for the presence of cell debris. The assay was performed in triplicate.

7. pTAM Calibration (L-Band), Fitting Software & in vivo measurement

The software is available at the following link: https://github.com/tdg0013/pTAMFittingApp/releases

The fitting application requires "MATLAB Runtime version 9.8 (R2020a)", which is free and included in the installation files on GitHub. It does not use the full MATLAB software.

For calibration samples, 800 µL pTAM solutions were placed into a 1.5 mL conical tube. The sample tubes were placed in the middle of the surface resonator coil of the spectrometer. The temperature was controlled using a circulation thermostat. All spectra were recorded at 37°C. Nitrogen/oxygen gas mixtures were controlled using a temperature and gas controller (Noxygen, Germany). To control the oxygen concentration, solutions were bubbled with defined N_2/O_2 mixtures for 30 min. The bubbling was stopped just before recording the spectra. A focus on the high-field part of the spectrum was recorded for each condition. Parameter settings for the spectrometer were as follows: mod. freq.; 100 kHz, mod. Ampl.; 0.004 mT, scan time; 30s, number of points; 4096, sweep width; 0.0960 mT and non-saturating power.

EPR Measurements for Oxygen Calibration

Samples were prepared with 200 µM pTAM, 137 mM sodium chloride, and pH=7. Gas composed of 21% oxygen and 79% nitrogen was bubbled through the sample for 25-30 minutes. Quadruplicate spectra were recorded for the high-field components. The process of bubbling and quadruplicate spectra recording was repeated for 6 different gas compositions between 21 and 0% oxygen.

EPR Measurements for Phosphate Calibration

Five samples were prepared with 200 µM pTAM, 137 mM sodium chloride, and varying amounts of phosphate buffer (0, 1, 4, 7, 10 mM) at approximately pH=7. Quadruplicate spectra of the high-field components were recorded for each sample at 0% oxygen.

pH

While the calibration of pO_2 and $[P_i]$ should be carried out for each spectrometer to consider for small instrument-dependent deviation, the pKa of pTAM has been measured previously to be 6.9[3-4] (37°C) and does not need to be recalibrated.

Calibrations with software

Parameter Calibration

Using the in-house developed MATLAB app, “Spectral Fitting for Monophosphonated Trityl Radicals”, the parameters/calibration selection dropdown was set to “Custom/Calibrate”. Then “pH 7 LW” button was clicked for determining the fitting parameters based on spectra recorded on anaerobic solutions with no inorganic phosphate and at approximately pH 7. This function sets the oxygen-induced linewidth (*Oxygen LW*) at 0 and the proton exchange rate (*H+ Exchange*) at 0.01. Gaussian linewidth (*Gaussian LW*) was set to 50 and allowed to vary 50. The acidic (*Lorentzian A LW*) and basic (*Lorentzian B LW*) component Lorentzian linewidths were set to 10 and allowed to vary 10. The acidic/basic component distance (*A/B Distance*) was set to 150 and allowed to vary 50. The *Acidic Fraction* was set to 0.5 and

allowed to vary 0.5. The files for the spectra recorded with 0% oxygen and no phosphate at pH 7 were selected and fitted. The resulting best fit values for *Gaussian LW*, *Lorentzian A LW,* and *Lorentzian B LW,* and *A/B Distance* for each spectrum were documented. The average values for *Gaussian LW* (41 mG), *Lorentzian A LW* (8 mG), and *Lorentzian B LW* (0.02 mG), and *A/B Distance* (122 mG) was entered for the start values and held constant for the rest of the calibration. [Note – When spectral fitting failed due to offsetting, increments of 25 (negative to move the fitting left, positive to move right) were entered into *Center offset,* and the spectrum fitting was repeated until successful.]

Oxygen Slope Calibration

The *Gaussian LW, Lorentzian A LW*, *Lorentzian B LW*, and *A/B Distance* values determined above were set constant as 41, 8, 0.02, and 122 mG, respectively. The *Oxygen LW* was set to 50 and allowed to vary 50. The *Acidic Fraction* was set to 0.5 and allowed to vary 0.5. The proton exchange rate (*H+ Exchange*) was held constant at 0.01. Each spectrum for the sample with no organic phosphate, at pH 7, and with and varying percentages of oxygen was fitted using the app. The resulting best fit values for *Oxygen LW* (mG) were plotted against oxygen partial pressure (mmHg), and the Linear Fit function in Origin Lab was used to determine the slope with the unit mG/mmHg of oxygen. The determined slope of 0.53 mG/mmHg (Figure S8) was entered in the software for Oxygen (pO_2) Slope.

Phosphate Slope Calibration

The *Gaussian LW, Lorentzian A LW*, *Lorentzian B LW*, and *A/B Distance* values determined above were set constant. The *Oxygen LW* was set constant at 0. *Acidic Fraction* was set to 0.5 and allowed to vary 0.5. *H+ Exchange* was set to and allowed to vary 6e+05. Each spectrum for the anaerobic samples with varying concentrations of phosphate was fitted. The resulting best fit for *H+ Exchange*, *Acidic Fraction*, and *Pi Term* were recorded. The resulted values for *Pi Term* were plotted against phosphate concentration), and the Linear Fit function in Origin Lab was used to determine the slope of 2.44×10^4 s^{-1}/mM of P_i (Figure S8). See ref[5] for full detail of the equations. (Note – the Pi Term is dependent on pH, and therefore requires a known value for pKa.)

$$\text{Pi Term} = \frac{k_a\left(H^+ + K_a^B\right)}{2\pi K_a^B}$$

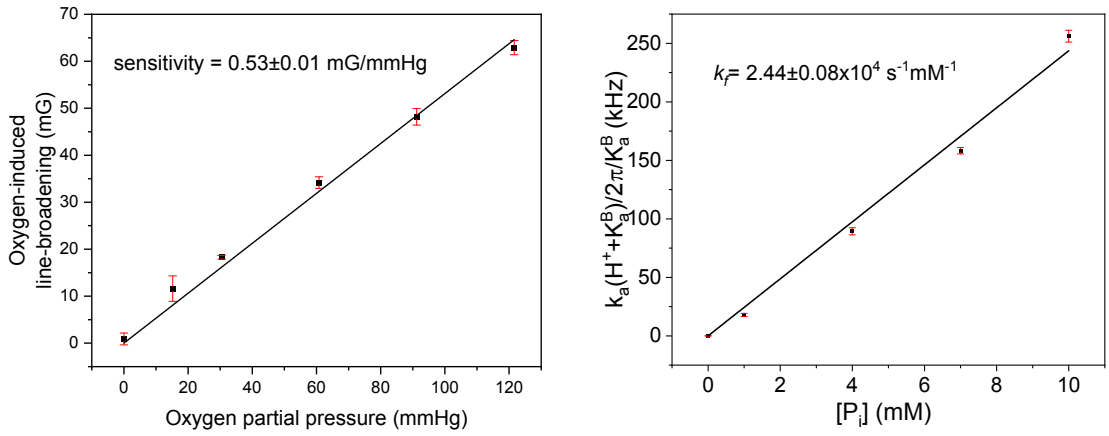


Figure S8. Oxygen and inorganic phosphate calibrations

Measuring Oxygen, pH, and [Pi] in vivo

*50 µM of a 3 mM solution of pTAM in saline was injected into the mammary gland of an MMTV-PYMT mouse under anesthesia. The mouse was placed inside the L-Band EPR spectrometer and the s*urface resonator coil placed on top of the mammary gland #4, and spectra were recorded for the high-field components. All animal work was performed in accordance with the West Virginia University Animal Care and Use Committee (WVU IACUC) approved protocol.

The *Gaussian LW, Lorentzian A LW*, *Lorentzian B LW*, and *A/B Distance* values determined above were set constant. *Oxygen LW* was set to 50 and allowed to vary 50. *Acidic Fraction* was set to 0.5 and allowed to vary 0.5. *H+ Exchange* was set to and allowed to vary 6e+05. The oxygen and phosphate slopes were entered as determined by the calibrations (Figure S8). The pKa=6.9 entered was based on the literature value.[3-4] The EPR spectra of the mouse mammary gland or mouse breast tumor with the same EPR parameters as the calibrations were fitted using the software. The resulting values for oxygen partial pressure, pH, and phosphate concentration were retrieved from the results section at the bottom of the app (Figure 3).

8. References

1. Dhimitruka, I.; Velayutham, M.; Bobko, A. A.; Khramtsov, V. V.; Villamena, F. A.; Hadad, C. M.; Zweier, J. L., Large-scale synthesis of a persistent trityl radical for use in biomedical EPR applications and imaging. *Bioorg. Med. Chem. Lett.* **2007,** *17* (24), 6801-6805.
2. Dhimitruka, I.; Grigorieva, O.; Zweier, J. L.; Khramtsov, V. V., Synthesis, structure, and EPR characterization of deuterated derivatives of Finland trityl radical. *Bioorg. Med. Chem. Lett.* **2010,** *20* (13), 3946-3949.
3. Dhimitruka, I.; Bobko, A. A.; Eubank, T. D.; Komarov, D. A.; Khramtsov, V. V., Phosphonated Trityl Probes for Concurrent in Vivo Tissue Oxygen and pH Monitoring Using Electron Paramagnetic Resonance-Based Techniques. *J. Am. Chem. Soc.* **2013,** *135* (15), 5904-5910.
4. Bobko, A. A.; Eubank, T. D.; Driesschaert, B.; Dhimitruka, I.; Evans, J.; Mohammad, R.; Tchekneva, E. E.; Dikov, M. M.; Khramtsov, V. V., Interstitial Inorganic Phosphate as a Tumor Microenvironment Marker for Tumor Progression. *Sci. Report.* **2017,** *7* (1), 41233.
5. Bobko, A. A.; Dhimitruka, I.; Zweier, J. L.; Khramtsov, V. V., Fourier Transform EPR Spectroscopy of Trityl Radicals for Multifunctional Assessment of Chemical Microenvironment. *Angew. Chem. Int. Ed.* **2014,** *53* (10), 2735-2738.