SUPPORTING INFORMATION

Synthesis, Characterization and Application of a Highly Hydrophilic Triarylmethyl Radical for Biomedical EPR

Urikhan Sanzhaeva,^{†,‡} Martin Poncelet, ^{†,§} Oxana Tseytlin, ^{†,‡} Mark Tseytlin, ^{†,‡} Marieta Gencheva,^{†,#} Timothy D. Eubank, ^{†,#} Valery V. Khramtsov, ^{†,‡} Benoit Driesschaert*^{†,§}

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

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

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

Email: benoit.driesschaert@hsc.wvu.edu

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

Table of Contents

1.	Synthetic procedures	S2
2.	MS spectra from HPLC-MS analysis	S5
3.	DMPO spin trapping	S7
4.	UB3LYP/6-31G* optimized structure of dFT-(SO ₃) ₃	
5.	Stability of dFT-(SO ₃) ₃ in presence of Asc/GSH and in cell lysate	S10
6.	HRMS	S11
7.	g-factors	S13
8.	Spectral simulations	S14

1. <u>Synthetic procedures</u>

a. Synthesis of dFT-(SO₃)₃



Deuterated Finland trityl synthesized using reported protocol¹ (1 g, 1 mmol, 1 eq.) was added to 200 mL of water, then NaOH (120 mg, 1mmol, 3 eq.) was added. The green solution was deoxygenated by bubbling nitrogen for 30 minutes. Then, Na₂SO₃ (25.2 g, 0.2 mol, 200 eq.) and K₂Cr₂O₇ (2.9 g, 10 mmol, 10 eq.) were added at once. The solution was stirred for 30 minutes under nitrogen. Then, TFA (30 mL) was added and the solution was poured on the top of a C18 column (360 gram). **dFT-(SO₃)**₃ was purified using a gradient from 100% H₂O containing 0.1% TFA to 20% ACN in water containing 0.1% TFA. The acetonitrile was removed under reduced pressure and the remaining aqueous solution was freeze-dried to afford 1.09 g of **dFT-(SO₃)**₃ (95% yield).

Then, **dFT-(SO₃)**³ was dissolved in water, titrated to pH 7.4 with NaOH and freeze-dried again to afford **dFT-(SO₃)**³ sodium salt as a green powder.

HRMS (ESI) calcd for $[C_{37}H_3D_{36}O_9S_{15}]^+$: 1143.0664 m/z, found: 1143.0707 (see section 6)

b. Ipso-substitution by S-, N-, P- nucleophiles

b1. Glutathione as nucleophile



dFT-(SO₃)₃ sodic salt (50 mg, 0.041 mmol, 1 eq.) was dissolved in 100 mL of phosphate buffer (100 mM, pH=7.4) at room temperature. Then, K₂IrCl₆(20 mg, 0.041 mmol, 1 eq.) was added to the solution and stirred 10 seconds. Reduced glutathione (253 mg, 0.83 mmol, 20 eq.) was added and the solution was stirred for 1 hour at room temperature. The solution was acidified with TFA to pH<2 and the solution was poured onto a Hypersep C18 cartridge (3 cm diameter, 3 cm length). The product was purified using a gradient from 100% water containing 0.1% TFA to 60% ACN in water containing 0.1% TFA. The acetonitrile was removed under reduced pressure and the remaining aqueous solution was freeze-dried. Then the residue was dissolved in water, titrated to pH 7.4 with NaOH and freeze-dried again to afford 17 mg the mono substituted derivative **6**, 29% yield.

HRMS (ESI) calcd for $[C_{47}H_{19}D_{36}N_3O_{12}S_{15}]^+$: 1369.1850 m/z, found: 1369.1844 m/z (see section 6).

X-Band EPR spectra were recorded and the hyperfine splitting constants were determined by spectral simulation using SpinFit. The experimental acquisition parameters were: ModAmp 0.020 G, ModFreq 10.00 kHz, ConvTime 20.00 ms, Power 0.02993 mW, SweepWidth 1 G, 1024 pts, 50 μ L of deoxygenated solution (100 μ M) in phosphate buffer (100 mM, pH=7.4), \geq 95% estimated paramagnetic purity (see section 8 for spectrum and simulation).

b2. Dimethylamine as nucleophile



dFT-(SO₃)₃ sodic salt (50 mg, 0.041 mmol, 1 eq.) was dissolved in 100 mL of phosphate buffer (100 mM, pH=7.4) at room temperature. Then, K₂IrCl₆(20 mg, 0.041 mmol, 1 eq.) was added to the solution and stirred 10 seconds. Dimethylamine (55 μ L, 0.83 mmol, 20 eq.) was added and the solution was stirred for 1 hour at room temperature. The solution was acidified with TFA to pH<2 and the solution was poured onto a Hypersep C18 cartridge (3 cm diameter, 3 cm length). The product was purified using a gradient from 100% water containing 0.1% TFA to 60% ACN in water containing 0.1% TFA. The acetonitrile was removed under reduced pressure. Then the residue was dissolved in water, titrated to pH 7.4 with NaOH and freeze-dried again to afford 18 mg the mono substituted derivative **7**, 38% yield.

HRMS (ESI) calcd for [C₃₉H₈D₃₆NO₆S₁₄]⁺: 1106.1518 m/z, found: 1106.1587 m/z (see section 6).

X-Band EPR spectra were recorded and the hyperfine splitting constants were determined by spectral simulation using WinSim. The experimental acquisition parameters were: ModAmp 0.020 G, ModFreq 30.00 kHz, ConvTime 40.00 ms, Power 0.09464 mW, SweepWidth 4 G, 4096 pts, 50 μ L of a deoxygenated solution (500 μ M) in phosphate buffer (100 mM, pH=7.4), \geq 90% estimated paramagnetic purity (see section 8 for spectrum and simulation).

b3. Tri-n-butylphosphine as nucleophile



dFT-(SO₃)₃ sodic salt (50 mg, 0.041 mmol, 1 eq.) was dissolved in 100 mL of phosphate buffer (100 mM, pH=7.4) at room temperature. Then, K₂IrCl₆ (20 mg, 0.041 mmol, 1 eq.) was added to the solution and stirred 10 seconds. Tri-nbutylphosphine (207 μ L, 0.83 mmol, 20 eq.) was added and the solution was stirred for 1 hour at room temperature. The solution was acidified with TFA to pH<2 and the solution was poured onto a Hypersep C18 cartridge (3 cm diameter, 3 cm length). The product was purified using a gradient from 100% water containing 0.1% TFA to 60% ACN in water containing 0.1% TFA. The acetonitrile was removed under reduced pressure and the remaining aqueous solution was freeze-dried. Then the residue was dissolved in water, titrated to pH 7.4 with NaOH and freeze-dried again to afford 11 mg the mono substituted derivative **8**, 20% yield.

HRMS (ESI) calcd for [C₄₉H₂₉D₃₆O₆PS₁₄]⁺: 1264.2863 m/z, found: 1264.2937 m/z (see section 6)

X-Band EPR spectra were recorded and the hyperfine splitting constants were determined by spectral simulation using WinSim. The experimental acquisition parameters were: ModAmp 0.020 G, ModFreq 30.00 kHz, ConvTime 40.00 ms, Power 0.09464 mW, SweepWidth 7 G, 4096 pts, 50 μ L of a deoxygenated solution (500 μ M) dissolved in 50% MeOH and 50% of phosphate buffer (100 mM, pH=7.4), \geq 95% estimated paramagnetic purity (see section 8 for spectrum and simulation).

b4. Trimethylphosphite as nucleophile



dFT-(SO₃)₃ sodic salt (50 mg, 0.041 mmol, 1 eq.) was dissolved in 100 mL of phosphate buffer (100 mM, pH=7.4) at room temperature. Then, K₂IrCl₆(20 mg, 0.041 mmol, 1 eq.) was added to the solution and stirred 10 seconds. Trimethylphosphite (98 μ L, 0.83 mmol, 20 eq.) was added and the solution was stirred for 1 hour at room temperature. The solution was acidified with TFA to pH<2 and the solution was poured onto a Hypersep C18 cartridge (3 cm diameter, 3 cm length). The product was purified using a gradient from 100% water containing 0.1% TFA to 60% ACN in water containing 0.1% TFA. The acetonitrile was removed under reduced pressure and the remaining aqueous solution was freeze-dried. Then the residue was dissolved in water, titrated to pH 7.4 with NaOH and freeze-dried again to afford 24 mg the mono substituted derivative **9**, 48% yield.

HRMS (ESI) calcd for [C₃₉H₇D₃₆O₉PS₁₄]: 1170.1000 m/z, found: 1170.0996 m/z (see section 6)

X-Band EPR spectra were recorded and the hyperfine splitting constant was determined by spectral simulation using WinSim. The experimental acquisition parameters were: ModAmp 0.020 G, ModFreq 30.00 kHz, ConvTime 40.00 ms, Power 0.09464 mW, SweepWidth 5.0 G, 2048 pts, 50 μ L of a deoxygenated solution (100 μ M) in phosphate buffer (100 mM, pH=7.4), \geq 95% estimated paramagnetic purity (see section 8 for spectrum and simulation).

2. <u>MS spectra from HPLC-MS analysis</u>



Figure S1. ESI⁺ MS spectrum extracted from the peak at 3.4 minutes (A) and 4.1 minutes (B) from HPLC chromatogram in Figure 2.



Figure S2. ESI⁺ MS spectrum extracted from the peak at 1.8 minutes (A) and 2.6 minutes (B) from the HPLC chromatogram in Figure 3B.

3. <u>DMPO spin trapping</u>



Figure S3. EPR spectrum of the mixture containing **DMPO** (20mM), **dFT** (200 μ M), Na₂SO₃ (200 mM) and K₂Cr₂O₇ (3 mM) under nitrogen. The arrows indicate the lines corresponding to the **DMPO-SO₃** adduct.

4. <u>UB3LYP/6-31G* optimized structure of dFT-(SO₃)</u>



Coordinates for optimized geometry of dFT-(SO₃)₃ at UB3LYP/6-31G* level of theory:

C -3.1670480000 -0.1603930000 1.4883190000 C -1.7755090000 -0.3616420000 1.3316850000 C -1.0414060000 0.2502760000 0.2774750000 C -1.7541970000 1.1050060000 -0.6105030000 C -3.1293180000 1.3199600000 -0.4515920000 C -3.8402330000 0.6589100000 0.5664880000 S -3.9323060000 -0.9988990000 2.8564720000 C -2.3568310000 -1.2631440000 3.7975440000 S -1.0164070000 -1.4206700000 2.5318050000 S -1.0085460000 1.9252840000 -1.9998220000 C -2.6008260000 2.1884460000 -2.9212810000 S -3.8691360000 2.4693370000 -1.5989890000 C -2.4631540000 -2.5768750000 4.5810630000 C -2.0827000000 -0.0747370000 4.7280690000 C -2.9489630000 0.9634610000 -3.7734370000 C -2.4835230000 3.4580150000 -3.7705850000 C 1.1826570000 2.3877170000 0.5429350000 C 2.0265380000 3.4889940000 0.2661310000 C 2.9953710000 3.3634300000 -0.7434260000 C 3.1667070000 2.1412050000 -1.4184860000 C 2.3187300000 1.0590300000 -1.1506270000 C 1.2961770000 1.1577580000 -0.1644310000 S -0.0179800000 2.6471470000 1.8192850000 C 0.8363620000 4.0919590000 2.5972340000 S 1.7500320000 4.9541240000 1.2343790000 S 4.4366930000 1.8450770000 -2.6379370000 C 3.4897470000 0.5092530000 -3.5065240000 S 2.5877220000 -0.3853850000 -2.1514200000 C 1.8109390000 3.6208010000 3.6844000000 C -0.2187920000 5.0487070000 3.1658030000 C 4.4828100000 -0.4537210000 -4.1647200000 C 2.5050020000 1.0966310000 -4.5222210000 C 0.3030850000 -2.4383990000 -0.4051480000 C 0.8338600000 -3.7316540000 -0.3105840000 C 2.0432120000 -3.9564440000 0.3719430000

C 2.6880830000 -2.9035530000 1.0423020000 C 2.1385590000 -1.6027080000 0.9572800000 C 0.9506680000 -1.3351330000 0.2206840000 S -1.1737210000 -2.2897250000 -1.3817950000 C -0.9317450000 -3.8797790000 -2.3102710000 S -0.1251660000 -5.0195630000 -1.0914750000 S 4.1797270000 -3.0640440000 1.9959430000 C 3.9415630000 -1.4919510000 2.9482330000 S 3.0271560000 -0.3396360000 1.8260360000 C -2.3042110000 -4.4491150000 -2.6870120000 C -0.0462790000 -3.6665370000 -3.5423030000 C 5.3176130000 -0.8933160000 3.2668120000 C 3.1331820000 -1.7547620000 4.2253770000 C 0.4025460000 0.0247050000 0.1156040000 H -1.5367850000 -2.7617290000 5.1377020000 H -2.6513970000 -3.4255330000 3.9188200000 H -3.2787730000 -2.5119760000 5.3111220000 H -2.0027890000 0.8594490000 4.1646060000 H -1.1433320000 -0.2304300000 5.2722590000 H -2.8970660000 0.0290380000 5.4553840000 H -3.0328360000 0.0628710000 -3.1581330000 H -2.1675760000 0.7946180000 -4.5238790000 H -3.9017870000 1.1234360000 -4.2930130000 H -3.4269960000 3.6490760000 -4.2954110000 H -2.2373490000 4.3298150000 -3.1590310000 H-1.7039850000 3.3296370000 -4.5305750000 S 4.0404830000 4.7492660000 -1.2283380000 H 1.2639940000 3.1101860000 4.4862920000 H 2.5535470000 2.9270400000 3.2796400000 H 2.3382530000 4.4814280000 4.1126490000 H -0.7821570000 4.5562060000 3.9670290000 H -0.9187810000 5.3778770000 2.3938660000 H 0.2679690000 5.9302340000 3.5990360000 H 5.0469380000 0.0648200000 -4.9487990000 H 3.9440360000 -1.2817260000 -4.6395400000 H 5.1868070000 -0.8660450000 -3.4372210000 H 1.9307450000 0.2906850000 -4.9944130000 H 3.0468830000 1.6418380000 -5.3048520000 H 1.8012160000 1.7816670000 -4.0405530000 S 2.7309870000 -5.6219850000 0.3472320000 H -2.1859550000 -5.4054540000 -3.2099470000 H -2.8195960000 -3.7620420000 -3.3684290000 H -2.9302070000 -4.6039050000 -1.8046710000 H -0.5275030000 -2.9619120000 -4.2311000000 H 0.9304700000 -3.2610260000 -3.2646340000 H 0.1057300000 -4.6176760000 -4.0670020000 H 5.8861650000 -1.5810190000 3.9040380000 H 5.8915070000 -0.7012210000 2.3569270000 H 5.2026400000 0.0480000000 3.8172840000 H 3.6691630000 -2.4623800000 4.8692710000 H 2.1507900000 -2.1752960000 3.9929730000 H 2.985000000 -0.8190600000 4.7777230000 S -5.6256830000 0.8933750000 0.6388080000 O -6.1060190000 0.2929280000 -0.8048750000 H -5.8902360000 0.9632890000 -1.4898540000 O -5.8944060000 2.3297100000 0.6517640000 O -6.1968900000 -0.0087280000 1.6238710000 O 2.9779790000 -5.8623310000 -1.2521630000 O 1.6932940000 -6.5481960000 0.7958470000 O 4.0610580000 -5.6098270000 0.9310480000 H 2.1036530000 -6.0513080000 -1.6582190000 O 3.6215190000 4.9518990000 -2.7962760000

O 5.4326750000 4.3086870000 -1.1763180000 O 3.5885560000 5.9595760000 -0.5641570000 H 4.0484900000 4.2315400000 -3.3105320000

E(UB3LYP/6-31G*)=-8080.81062760 Eh

<S**2>= 0.774188

Figure S4. Optimized geometry and Cartesian coordinates of **dFT-(SO₃)**₃ at the UB3LYP/6-31G* level of theory.

5. Stability of dFT-(SO₃)₃ in presence of Asc/GSH and in cell lysate



Figure S5. A) EPR time-course of **dFT-(SO₃)**₃ (50 μ M) in the presence of a mixture of ascorbate (500 μ M) and glutathione (GSH) (650 μ M) under nitrogen. B) EPR time-course of **OX063**, **dFT**, and **dFT-(SO₃)**₃ (200 μ M) incubated with lysate of MDA-MB-231 triple-negative breast cancer cell line under nitrogen at 37°C.

6. <u>HRMS</u>



Figure S6. HRMS of dFT-(SO₃)₃





Figure S7. HRMS of 6







Figure S9. HRMS of 8





7. g-factors

Table S1. g-factor determined using dFT as an internal reference

Radical	g-factor
dFT	2.00307 ^a
dFT-(SO ₃) ₁	2.00301 ^a
dFT-(SO ₃) ₂	2.00295 ^a
dFT-(SO ₃) ₃	2.00288 ^a
6	Diastereoisomer 1: 2.00292 ^b
	Diastereoisomer 2: 2.00289 ^b
7	2.00286 ^b
8	2.00291°
9	2.00291 ^b

^a – in Na₂SO₃ solution from Fig. 3A, ^b- in phosphate buffer (100 mM, pH=7.4), ^c – in 50:50 MeOH: phosphate buffer (100 mM, pH=7.4)

8. Spectral simulations





Figure S11. EPR spectra and corresponding simulations for 6, 7, 8, 9

References

1. 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.