Supporting Information for

Leucomethylene blue probe detects a broad spectrum of reactive oxygen and nitrogen species

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1. General Information

All the reactions, work-up and chromatography were performed under open flask conditions unless mentioned otherwise. The work-up and the isolation of products were carried out in a fume hood using standard techniques. Oven dried glassware was used for all the reactions. Flash chromatography was performed on silica gel (0.04 – 0.063 mm) by standard techniques. Organic solutions were concentrated under reduced pressure on a Büchi rotary evaporator (water bath temp $\leq$ 37°C) under protection from light, by covering the round bottomed flask with a black cloth. Thin-layer chromatography was carried out using Merck

silica gel 60 F254 aluminium plates with F-254 indicator and separated bands were visualized under UV light (254 nm, 320 nm). Technical grade solvents, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), hexane, and methanol were distilled before their use for in column chromatography. Reagent grade chemicals were obtained from various vendors, Alfa Aesar, BLDPharm, fluorochem, TCI, Acros organics and ABCR and used, unless otherwise stated, without further purification used as received. 3,7-Dinitro-10H-phenothiazine 5-oxide[1] and 4-iodo-2,6-tertbutylphenol[2] were prepared according to literature procedures.

Spectroscopy: (^{1}H-, and ^{13}C-) NMR spectra were recorded on a Varian AV300 (300 MHz), AV400 (400 MHz) or AV600 (600 MHz) spectrometers (in ^{13}C: 75, 100 or 151 MHz) in DMSO-d_6, and CD_3OD and were reported relative to the solvent residual signal (^{1}H: C*H*D_2SOCD_3, δ(H) 2.50 ppm, ^{1}H: C*H*D_2OD, δ(H) 3.31 ppm; δ(^{13}CD_3SOCD_3) = 39.5 ppm, δ(^{13}CD_3OD)=49.0 ppm). Data were reported in the following order: chemical shift (δ) in ppm; multiplicities are indicated s (singlet), bs (broad singlet), d (doublet), t (triplet), m (multiplet); coupling constants (*J*) are in Hertz (Hz), rounded to the nearest 0.1 Hz. UV/Vis spectroscopy: *Shimadzu UV-2600 spectrometer*, in 10mm quartz cells, and *TECAN* (infinite 200) in transparent 96-well plates. Fluorescence spectroscopy: *TECAN* (infinite 200) (λ_{exc}) and emission wavelengths (λ_{Em} (intensity)) in nm reported. Mass Spectrometry: ESI Thermo Fisher LTQ-Orbitrap XL, positive ion mode, *m/z* (rel. intensity %) or a Finnigan SSQ 7000 mass spectrometer (EI or CI).

HPLC: Performed on Agilent 1260 Infinity equipped with a Diode Array Detector with Multiple Wavelengths, using a reversed phase column (C18, 3.5 µm, 4.6*150 mm). A gradient elution was used with eluent A (0.1 % trifluoro acetic acid in ACN) increasing from 5 % to 50 % in 8 minutes (eluent B was H_2O with 0.1 % trifluoroacetic acid). The flow rate was 0.8 mL/min, and the detection wavelengths were 254, 370, 500, 670 nm.

2. Cell culture / in vitro ROS induction procedure

Macrophage cell lines (J774.A1) were obtained from ATCC. Cells were grown in 1 g/mL glucose containing DMEM medium (low Glucose medium, from Gibco Life Tech) supplemented with 10% FCS and 1% pencillin/streptomycin (P/S)). For microscopy imaging, 40,000 cells were seeded on No. 1.5 coverslips placed 24 well plates, placed them in incubator (37 °C, with 5% CO_2) for 24h to attach the cells to coverslips. After PBS washing

and exchanging medium, respective amount of dye solutions, in either DMSO or H_2O were added into medium. The cells further incubated for 5h, and washed thrice with PBS, fixed with 4% formalin for 25 min. The fixed cells labelled with DAPI (1 µg mL^{-1}) or WGA-488 (1.25 µg mL^{-1}), followed by washed thrice PBS. The fixed cells coverslips mounted on microscope slides with mowiol for imaging.

For ROS induction, 100 ng/mL of LPS (bacterial lipopolysaccharide), 20 ng/mL of interferon-γ (IFN-γ) were added to the cell culture medium and incubated for 24h. After washing thrice with PBS and exchanging the medium, the dye solutions were added to the ROS-producing cells, and used same procedure as above for preparing slides for imaging.

3. Microscopic imaging procedure

a) Fluorescence microscopy

Microscopy was performed with an Axio Imager M2 microscope, equipped with an Axio Cam MR3- and Axio Cam ICc-1-camera (Carl Zeiss, Germany). J774A.1 cells (both ROS-induced, normal) were fed with BHP-LMB (10 nmol mL^{-1}) and incubated. Nuclei were stained with DAPI (1 µg mL^{-1}) after fixation of cells with 4% formalin. The fluorescent signals were detected blue channel (for DAPI; λ_{ex} = 365 nm, λ_{em} = 445/50 nm), and red channel (Cy5; λ_{ex} = 665/45 nm, λ_{em} = 725/50) nm for the formed methylene blue.

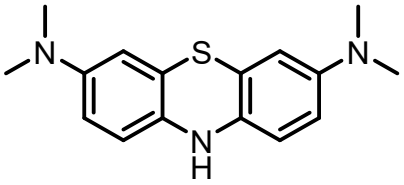
b) Confocal laser scanning microscopy (CFLSM).

CFLSM was performed on a Leica TCS SP8 microscope equipped with a HC PL APO CS2 93x/1.30 GLYC (Leica Microsystems, Germany). Preparation of samples, including washing is described above. Membrane labelled WGA-488 (WGA-Alexafluor 488, 1.25 µg mL^{-1}) dye was excited at λ_{ex} = 488 nm using a variable excitation wavelengths laser. The fluorescent signal was detected at λ_{em} = 504 – 586 nm. The ROS detection (the formed Methylene blue) was detected with excited λ_{ex} = 660 nm; the fluorescent signal was detected at λ_{em} = 674 – 789 nm.

4. Experimental procedures

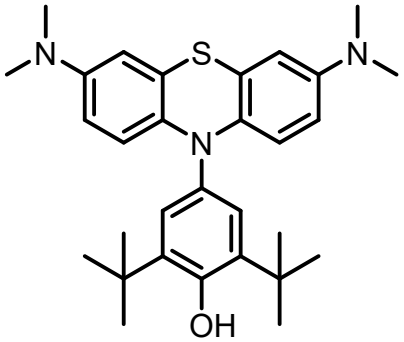
4.1. Synthesis of BHP-LMB (1)

Synthesis of leuco-methylene blue (LMB)



Methylene blue (0.56 g, 1.88 mmol) and K_2CO_3 (1.04 g, 7.5 mmol 4 eq.) were dissolved in DCM (10 mL) and H2O (5 mL) under N_2 atmosphere. A solution of $Na_2S_2O_4$ (1.31 g, 7.5 mmol, 4 eq.) in H_2O (5 mL) was added to the reaction under N_2 atmosphere. The reaction was stirred at 40 °C for 1h. The organic layer was dried over Na_2SO_4 and the solvent was removed in vacuo. The product was isolated as a yellow solid (323 mg, 1.25 mmol, 60%). The compound was used without further analysis or purification.

Synthesis of 4-(3,7-bis(dimethylamino)-10H-phenothiazin-10-yl)-2,6-di-tert-butylphenol (BHP-LMB; 1):



The crude product from above reaction was dissolved in degassed toluene (4 mL) and added to 2,6-tertbutyl-4-iodophenol (439 mg, 1.32 mmol, 1.17 eq.), NaOtBu (152 mg, 1.58 mmol, 1.4 eq.), DPPF (61 mg, 0.11 mmol, 10 mol%) and Pd_2dba_3 (51 mg, 0.056 mmol, 5 mol%) under nitrogen atmosphere. The reaction was stirred at 110°C for 18h. The reaction mixture was diluted with sat. NH_4Cl solution and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and the solvent was removed on silica. The crude product was purified by flash column chromatography yielding the title compound as a pale-turquoise solid (64 mg, 0.13 mmol, 10%).

^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 7.16 (s, 1H), 6.97 (s, 2H), 6.48 (t, *J* = 2.6 Hz, 2H), 6.40 (dd, *J* = 9.1, 2.8 Hz, 2H), 6.18 (d, *J* = 8.9 Hz, 2H), 2.76 (s, 12H), 1.39 (s, 18H).

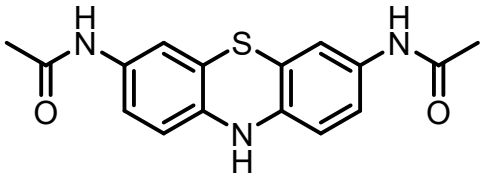
^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 152.5 (s, C_{quat}), 146.3 (s, C_{quat}), 141.4 (s, C_{quat}), 135.5 (s, C_{quat}), 133.9 (s, C_{quat}), 124.9 (s, CH), 121.4 (s, C_{quat}), 117.2 (s, CH), 111.8 (s, CH), 110.9 (s, CH), 40.6 (s, CH_3), 34.7 (s, C_{quat}), 30.3 (s, CH_3).

IR (neat, cm^{-1}) 3624, 2955, 2873, 2793, 2323, 2134, 2005, 1915, 1711, 1597, 1481, 1434, 1357, 1305, 1231, 1138, 1056, 991, 961, 930, 887, 834, 797, 698, 656.

HRMS (EI) calculated for $C_{30}H_{39}N_3OS$ 489.28084 (M^+), found 489.27926.

4.2. Synthesis of hydrophilic BHP-LMB (2)

Synthesis of N,N'-(10H-phenothiazine-3,7-diyl)diacetamide (3):

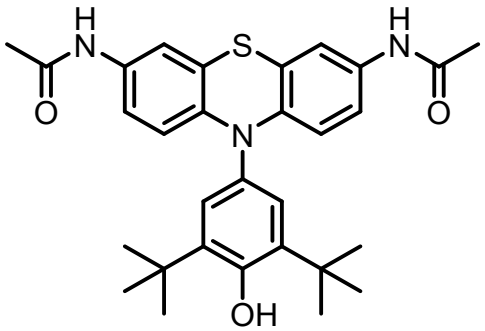


3,7-Dinitro-10H-phenothiazine 5-oxide (3.1 g, 10 mmol) and Fe powder (5.1 g, 90 mmol, 9 eq.) were dissolved in AcOH (50 mL) and refluxed overnight. The reaction mixture was filtered through a plug of silica (eluent: ethyl acetate). The solvent was removed on silica and the crude product was purified by flash column chromatography ethyl acetate/ethanol (10:1) yielding the desired product as a grey solid (2.90 g, 9.2 mmol, 92%).

^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 9.72 (s, 2H), 8.39 (s, 1H), 7.23 (d, *J* = 2.3 Hz, 2H), 7.10 (dd, *J* = 8.5, 2.3 Hz, 2H), 6.59 (d, *J* = 8.5 Hz, 2H), 1.97 (s, 6H).

NMR data corresponds to literature[3].

Synthesis of N,N'-(10-(3,5-di-tert-butyl-4-hydroxyphenyl)-10H-phenothiazine-3,7-diyl)diacetamide (4):



3 (313 mg, 1.0 mmol), 2,6-tertbutylphenol (619 mg, 3.0 mmol, 3 eq.) and $NaIO_4$ (107 mg, 0.5 mol, 50 mol%) were dissolved in DCM (2.5 mL) and AcOH (0.5 mL). The reaction vessel was sealed and heated to 40 °C. The reaction mixture was stirred for 24 h at 40 °C. Solvent was removed on silica and the crude product was purified by flash column chromatography pure ethyl acetate yielding the desired product as a light green solid (174 mg, 0.34 mmol, 34%).

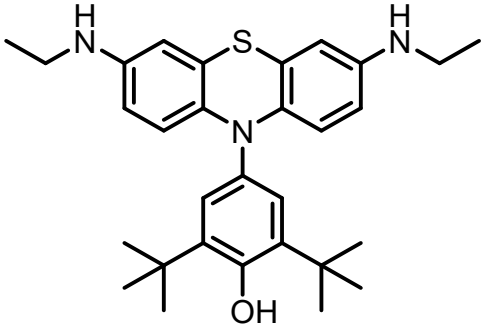
^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.79 (s, 2H), 7.38 (d, *J* = 2.3 Hz, 2H), 7.34 (s, 1H), 7.03–6.98 (m, 4H), 6.08 (d, *J* = 8.9 Hz, 2H), 1.97 (s, 6H), 1.40 (s, 18H).

^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 167.8 (s, C_{quat}), 153.5 (s, C_{quat}), 141.7 (s, C_{quat}), 139.8 (s, C_{quat}), 134.1 (s, C_{quat}), 132.0 (s, C_{quat}), 126.2 (s, CH), 118.7 (s, C_{quat}), 118.1 (s, CH), 117.1 (s, CH), 115.5 (s, CH), 34.7 (s, C_{quat}), 30.3 (s, CH_3), 23.8 (s, CH_3).

IR (neat, cm^{-1}) 3632, 3266, 3101, 3054, 2951, 2871, 2625, 1645, 1598, 1536, 1475, 1434, 1403, 1368, 1302, 1254, 1151, 1118, 1082, 1013, 972, 935, 897, 864, 805, 770, 744, 717, 662.

HRMS (ESI) calculated for $C_{30}H_{35}N_3O_3S$ 517.23936 $(M)^+$, found 517.23938.

Synthesis of 4-(3,7-bis(ethylamino)-10H-phenothiazin-10-yl)-2,6-di-tert-butylphenol (5):



BH_3SMe_2 (0.34 mL, 0.68 mmol, 2.5 eq.) was added dropwise to a solution of **4** (140 mg, 0.27 mmol) in toluene (4 mL) at 0°C under N_2 atmosphere. The mixture was stirred for 15 min at 0°C and then stirred for 2 h at 110°C. After cooling down to room temperature EtOH was added slowly to quench the excess of BH_3SMe_2 and the reaction mixture was stirred for 30 minutes at room temperature. The solvents were removed in vacuo. The crude product was purified by flash column chromatography hexane/ethyl acetate (7:3) to yield the desired product as a dark blue solid (115 mg, 0.23 mmol, 87%).

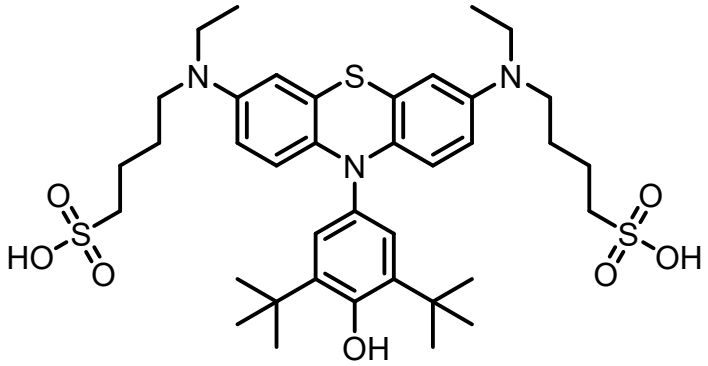
^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 6.96 (s, 1H), 6.88 (s, 2H), 6.37 (d, *J* = 2.5 Hz, 2H), 6.32 (d, *J* = 8.7 Hz, 2H), 6.27 (dd, *J* = 8.7, 2.5 Hz, 2H), 5.24 (t, *J* = 5.6 Hz, 2H), 2.97-2.91 (m, 4H), 1.34 (s, 18H), 1.10 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 151.2 (s, C_{quat}), 144.9 (s, C_{quat}), 140.9 (s, C_{quat}), 136.1 (s, C_{quat}), 134.3 (s, C_{quat}), 124.4 (s, C_{quat}), 122.1 (s, CH), 119.9 (s, CH), 110.8 (s, CH), 110.0 (s, CH), 37.7 (s, CH_2), 34.7 (s, C_{quat}), 30.4 (s, CH_3), 14.4 (s, CH_3).

IR (neat, cm^{-1}) 3626, 3290, 3075, 2960, 2871, 2329, 2214, 2090, 2012, 1979, 1926, 1600, 1472, 1431, 1393, 1298, 1223, 1137, 1057, 1026, 993, 928, 889, 841, 799, 693.

HRMS (ESI) calculated for $C_{30}H_{39}N_3OS$ 489.28084 $(M)^+$, found 489.28079.

Synthesis of 4,4'-((10-(3,5-di-tert-butyl-4-hydroxyphenyl)-10H-phenothiazine-3,7-diyl)bis(ethylazanediyl))bis(butane-1-sulfonic acid) (2):



1,4 butane sultone (29 mg, 22 µL, 210 µmol, 2.3 eq.) and DIPEA (27 mg, 36 µL, 210 µmol, 2.3 eq.) were added to a solution of **5** (45 mg, 90 µmol) in acetonitrile (3 mL) under N_2 atmosphere. The mixture was stirred for 72 h at 80 °C. The crude product was purified by preparative HPLC. The title compound was isolated as a white solid (10 mg, 14 µmol, 15%).

^{1}H NMR (600 MHz, methanol-d_4) δ (ppm) 7.27 (d, *J* = 2.6 Hz, 2H), 7.16 (s, 2H), 7.08 (dd, *J* = 9.0, 2.7 Hz, 2H), 6.34 (d, *J* = 8.9 Hz, 2H), 3.62 – 3.53 (m, 8H), 2.81 (t, *J* = 7.0 Hz, 4H), 1.84 – 1.77 (m, 4H), 1.68 (s, 4H), 1.49 (s, 18H), 1.17 (t, *J* = 7.2 Hz, 6H).

HRMS (ESI) calculated for $C_{38}H_{54}N_3O_7S_3$ 760.31239 $(M-H)^+$, found 760.31018.

5. Supporting figures

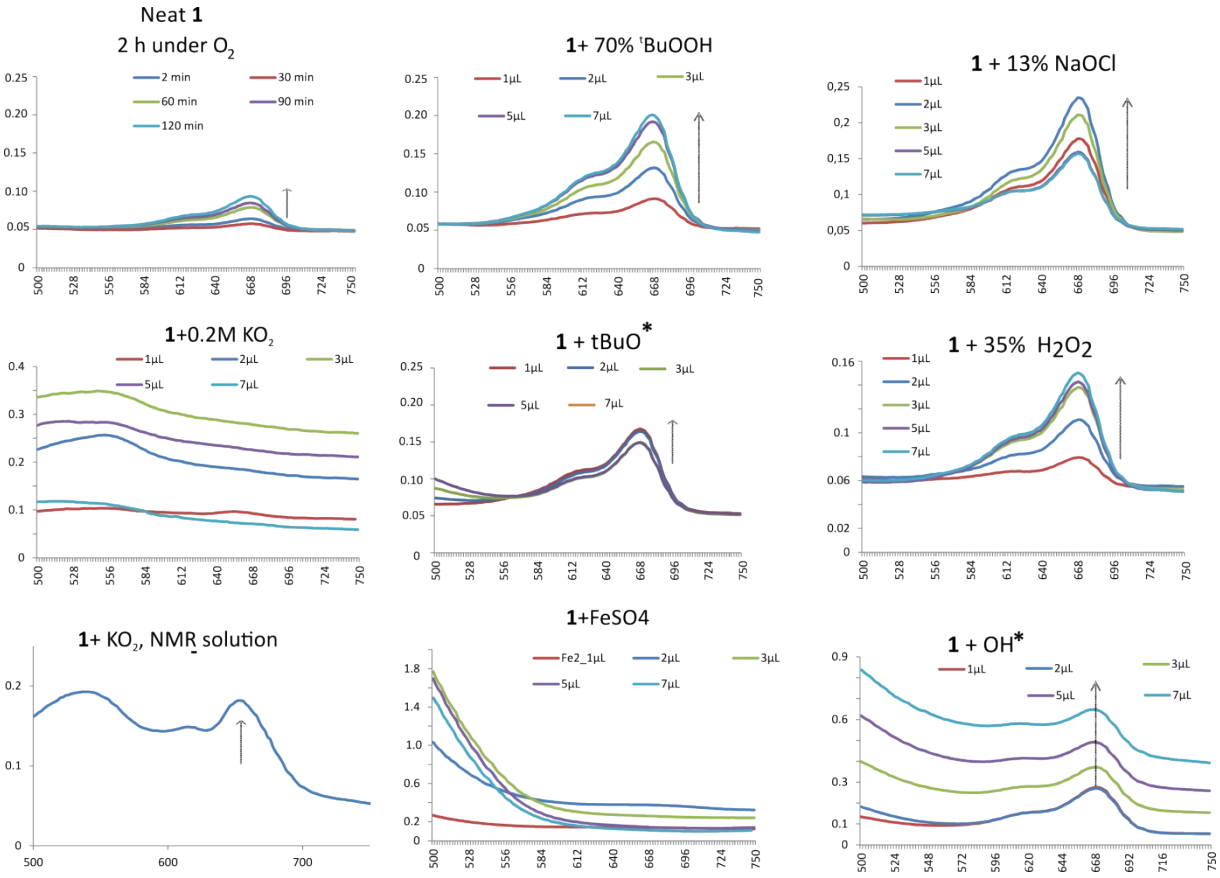


Figure S1: The reactivity of BHP-LMB **1** (25 µM, DMSO) towards a variety of ROS species and oxygen over 2 h time; Slow reaction of **1** with molecular oxygen (air O_2) with BHP-LMB used as control. The UV-VIS was recorded in about 5 min after each ROS equivalent addition; the UV-vis titration with excess of KO_2 immediately degraded the formed product. ^{1}H NMR titration of **1** with KO_2 solution is included at bottom left to confirm the formation of MB during KO_2 titration; excess KO_2 addition into UV-vis solution (25 µM) decomposed the formed MB.

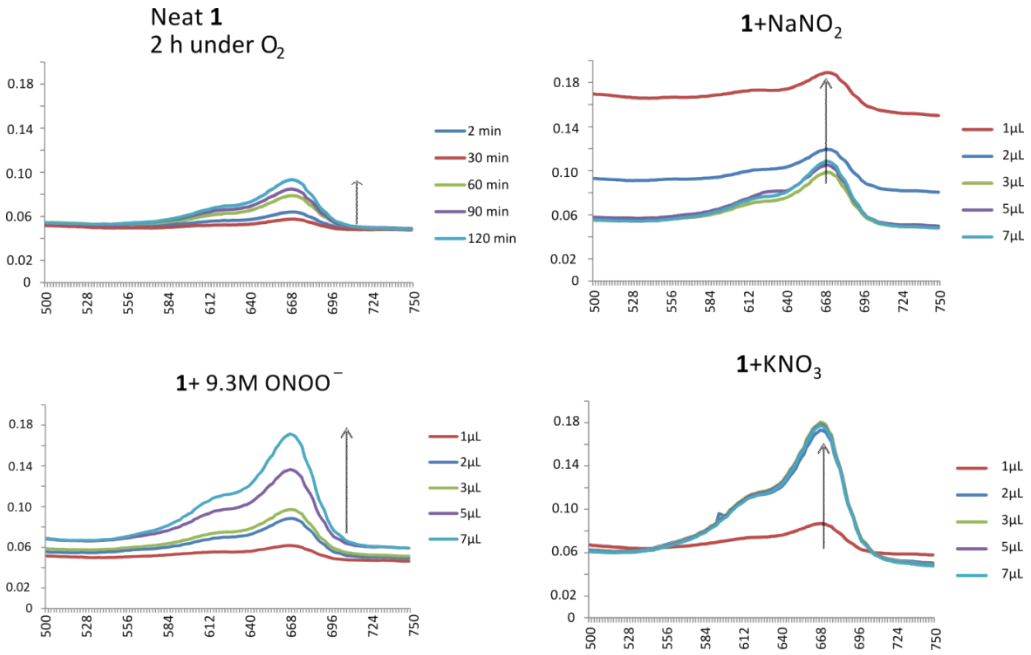


Figure S2: The reactivity of BHP-LMB **1** (25 µM, DMSO) towards a few RNS species.

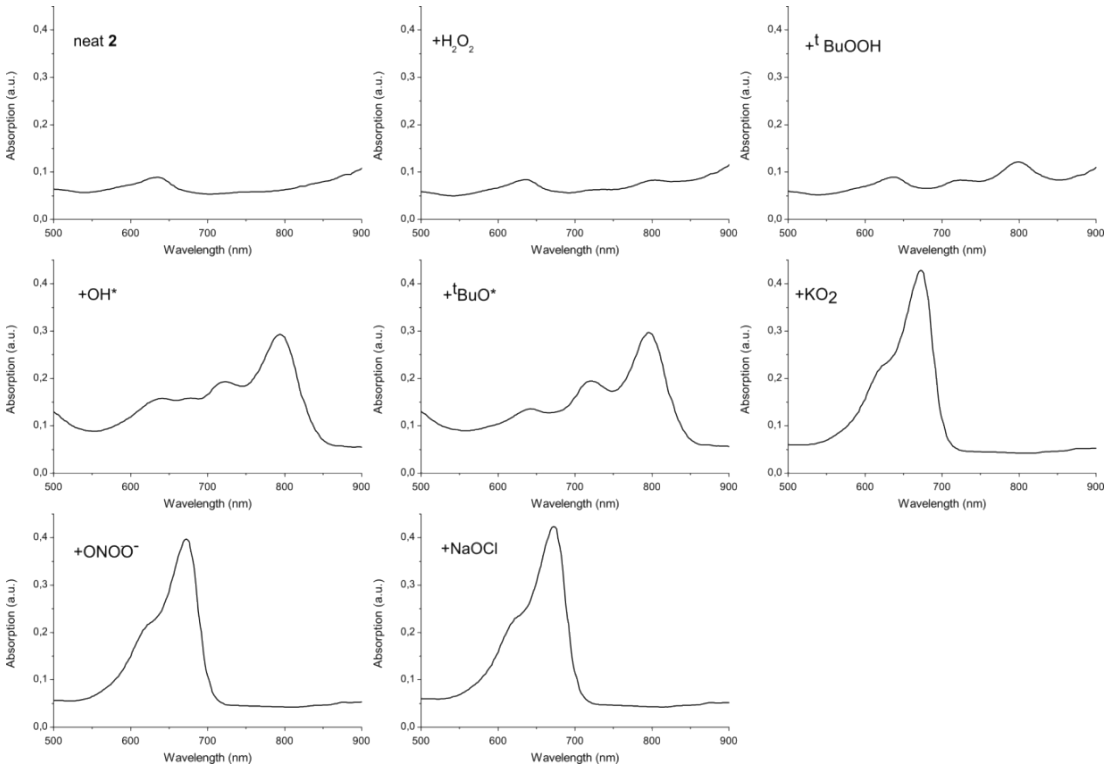


Figure S3: Detection of a variety of ROS species with water-soluble BHP-LMB **2** (20 µM, in H_2O) by UV-vis spectroscopy (excess of trigger solutions were added and within 5 minutes the spectra were recorded). It is clearly visible in water, the radical ROS triggers produced a persisting far-red absorbing species that is not formed with anionic ROS species.

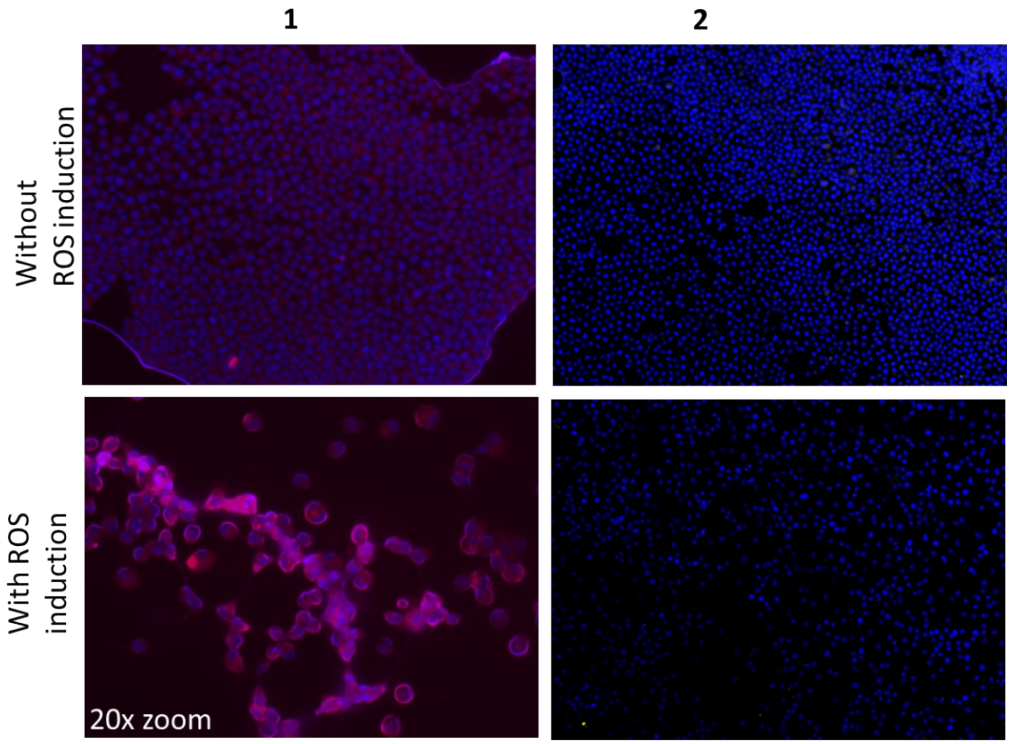


Figure S4: Microscopy images for the detection of intracellular produced ROS (induced by LPS / IFN-gamma) with BHP-LMB (**1**, **2**, 10 nmol / mL were added to the activated cells, and incubated for 6h, followed by fixing them in 4 % formalin for imaging).

6. NMR Spectra

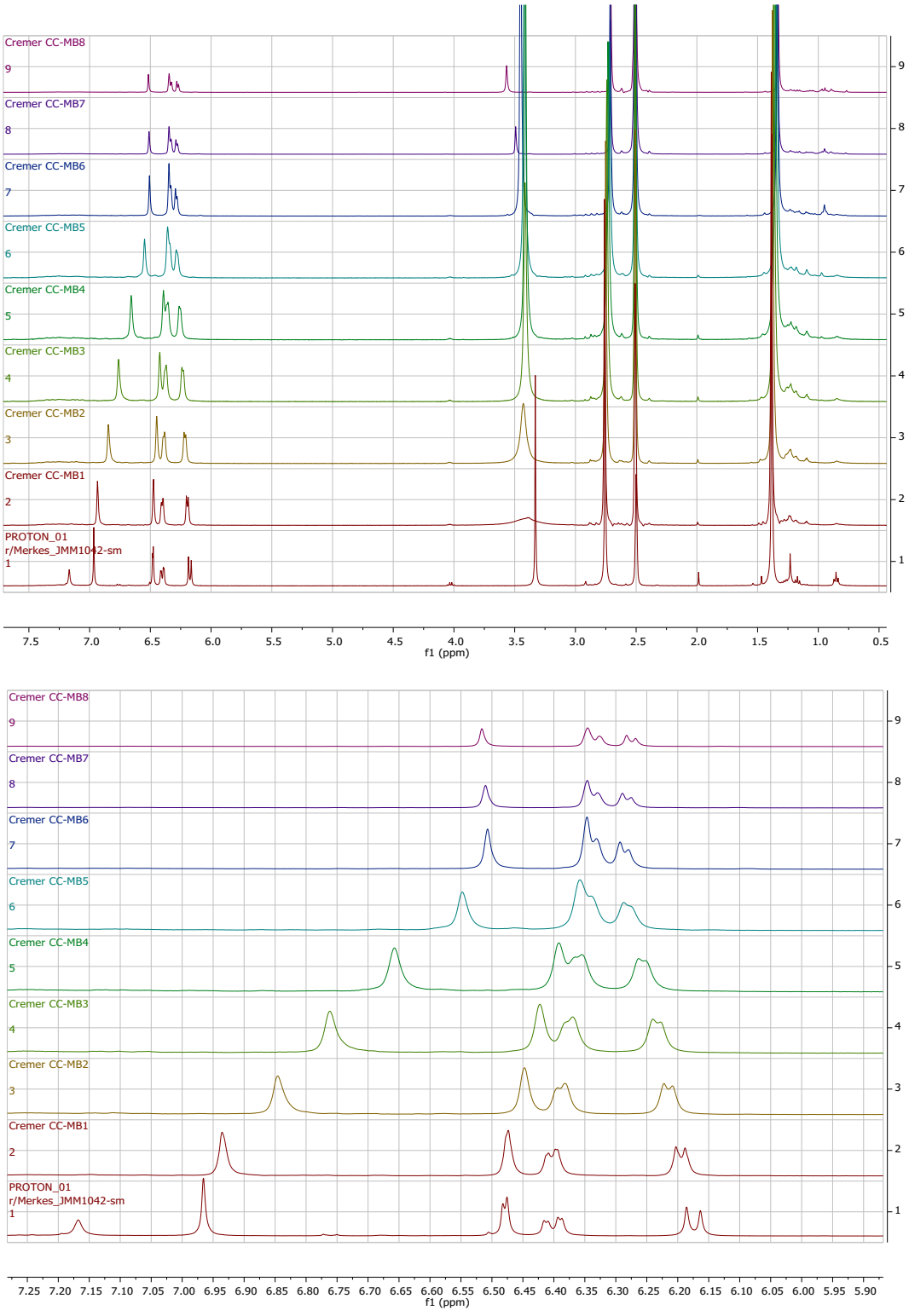
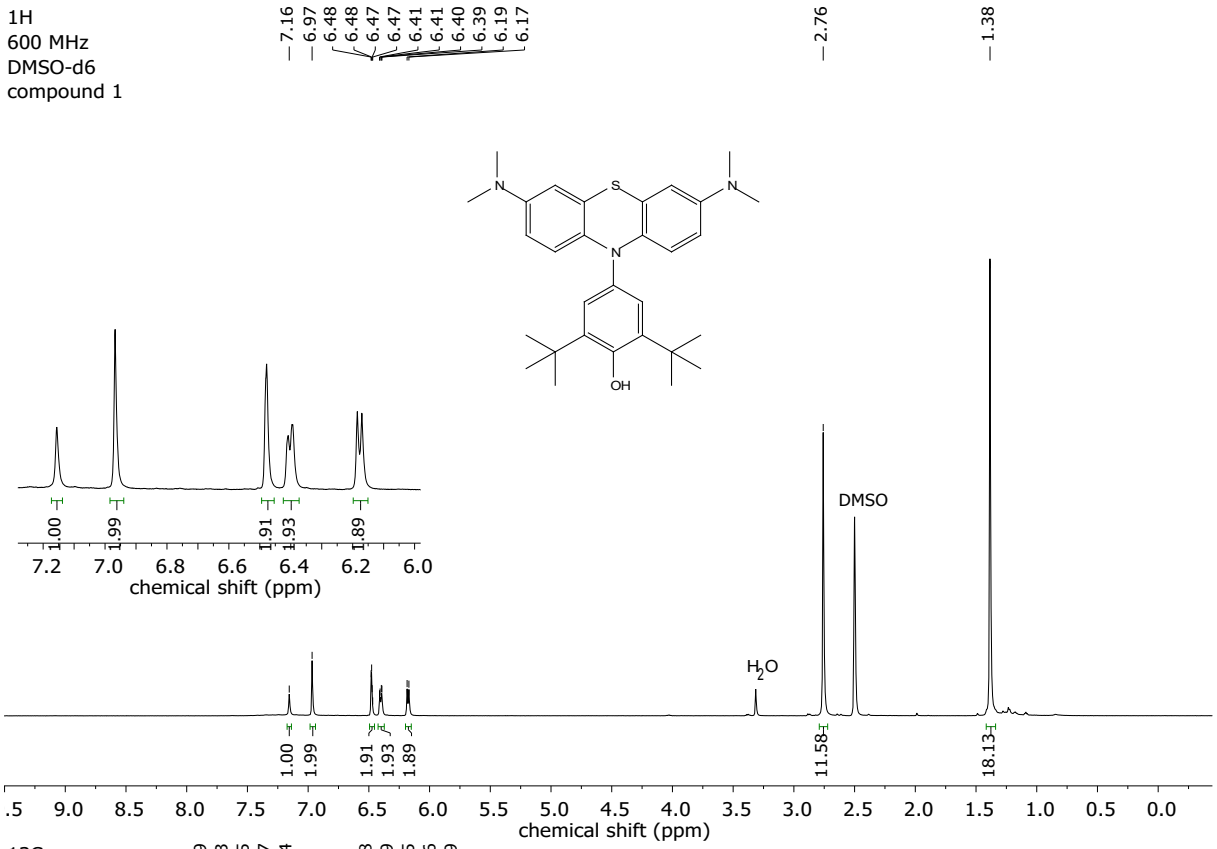
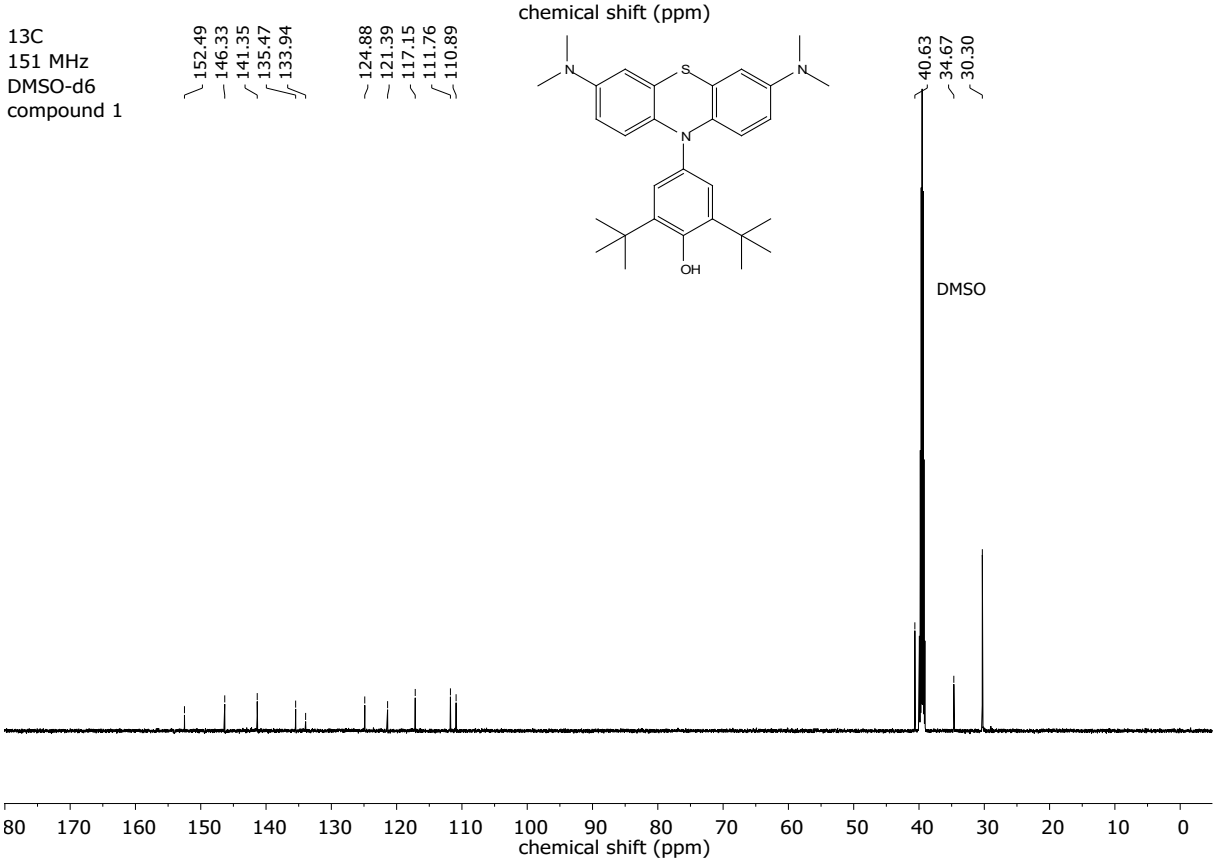


Figure S5: NMR titration of BHP-LMB (**1**; in DMSO-d_6/D_2O; conc: 34 mmol) with KO_2 (conc: 2.03 M in D_2O solution). Bottom spectrum for the reference **1**, from bottom 2 to top 9: with increasing amounts of KO_2 solution additions (0.2 equiv. to 3 equiv.); 2 to 6: with 0.2 equiv. (2 µL, 2 µL, 2 µL, 2 µL; 2 µL of KO_2) addition per measurement; 7, 8: 0.5 equiv. (5 µL, 5 µL of KO_2) and 9: 1 equiv. (10 µL of KO_2 solution) additions.


1H
600 MHz
DMSO-d6
compound 1
7.16
6.97
6.48
6.48
6.47
6.47
6.41
6.41
6.40
6.39
6.19
6.17
2.76
1.38
DMSO
H2O
1.00
1.99
1.91
1.93
1.89
11.58
18.13
chemical shift (ppm)


13C
151 MHz
DMSO-d6
compound 1
152.49
146.33
141.35
135.47
133.94
124.88
121.39
117.15
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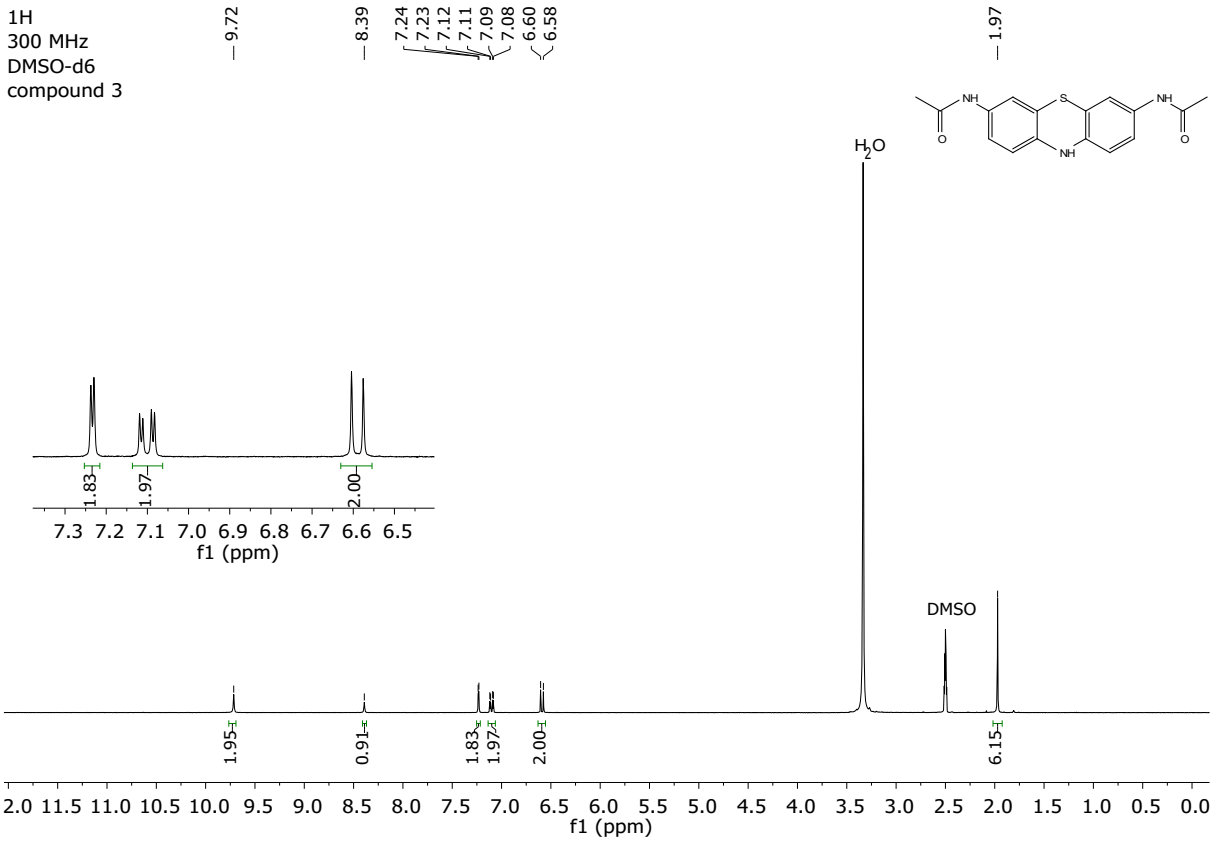
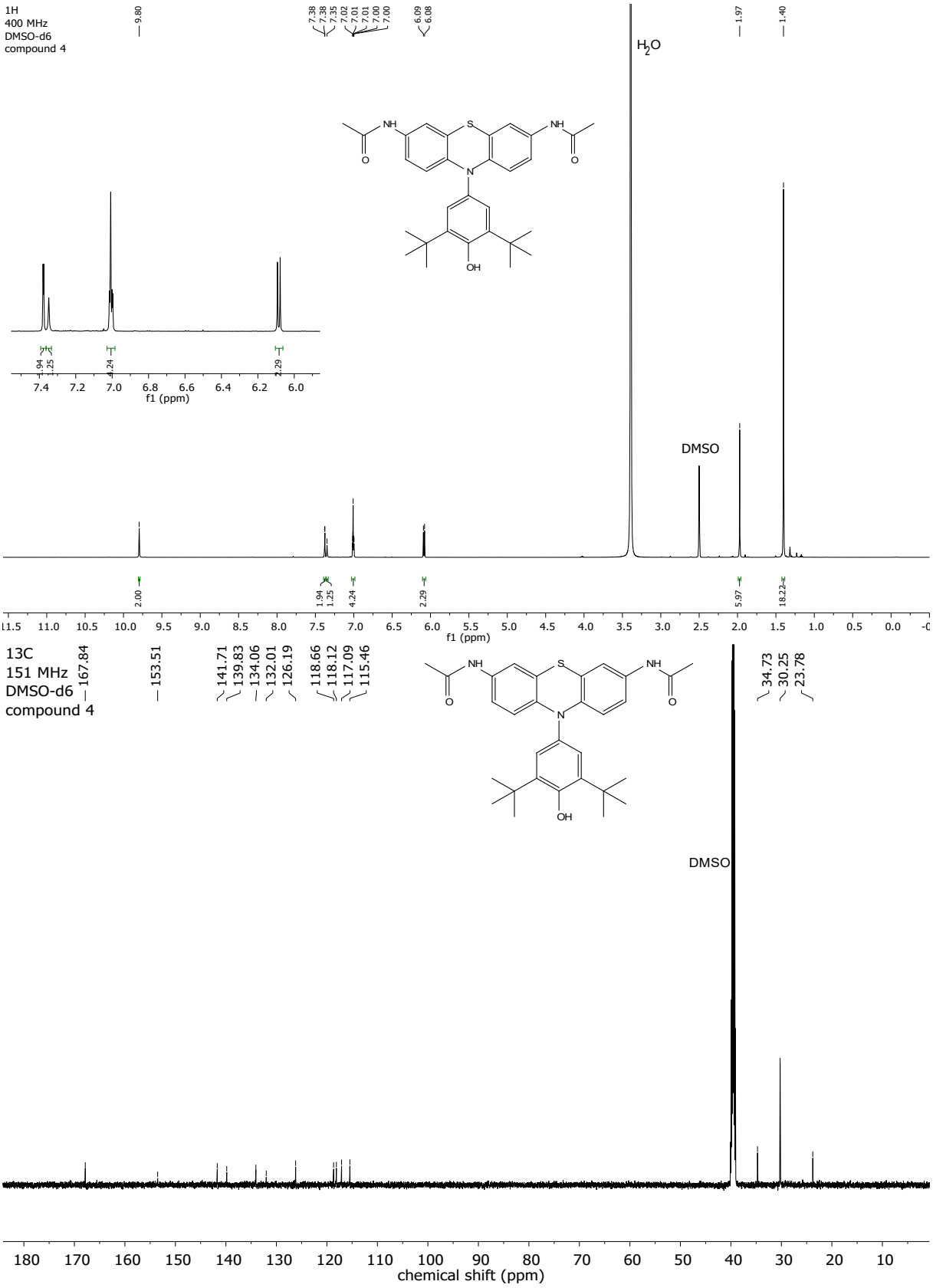
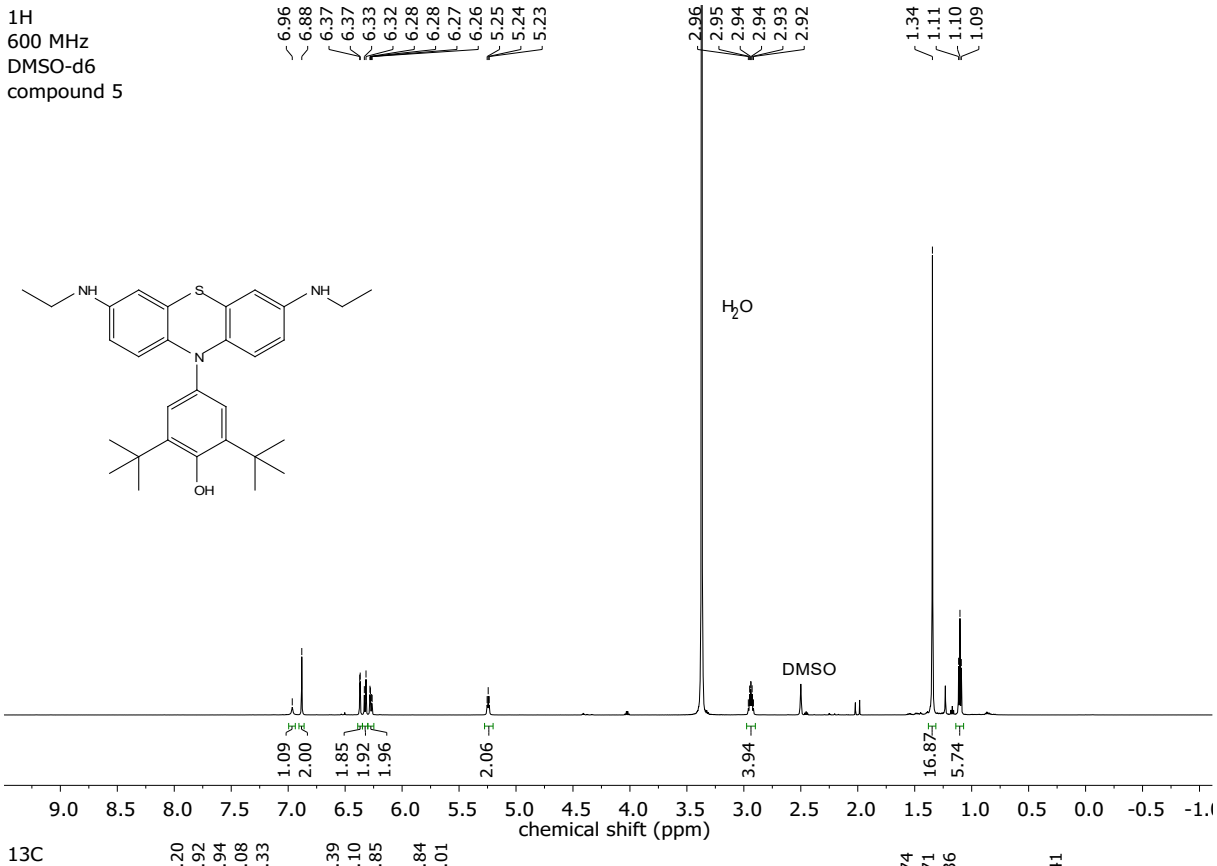
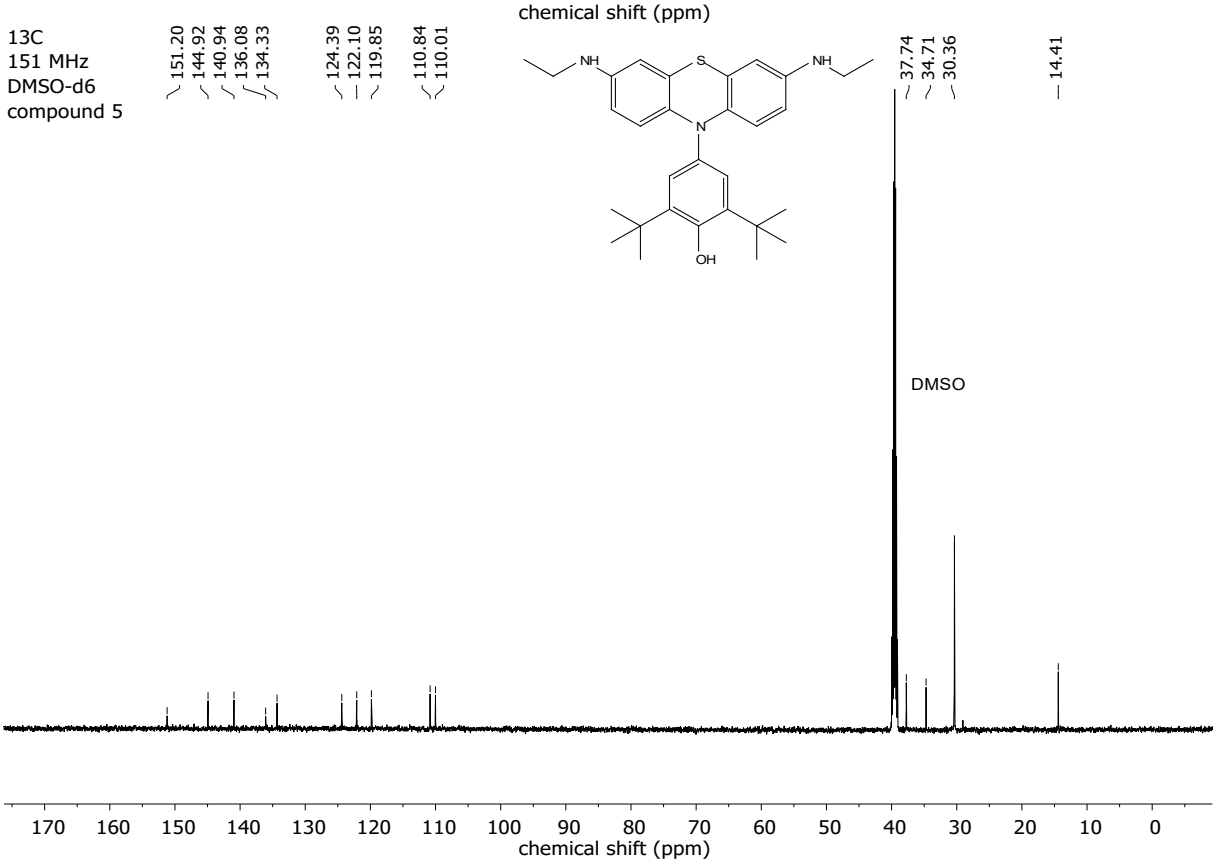
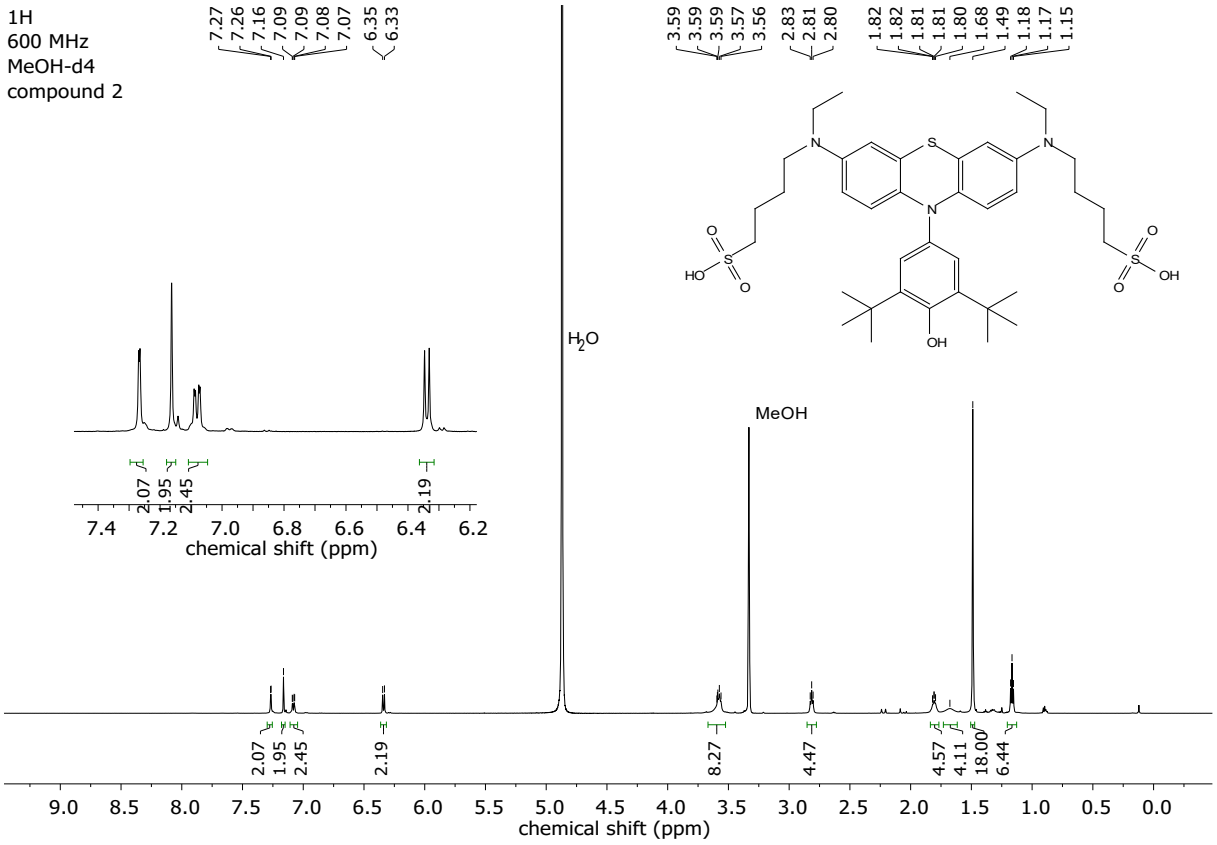
110.89
40.63
34.67
30.30
DMSO
chemical shift (ppm)


1H
300 MHz
DMSO-d6
compound 3
9.72
8.39
7.24
7.23
7.12
7.11
7.09
7.08
6.60
6.58
1.97
NH
S
NH
NH
O
O
H2O
DMSO
1.83
1.97
2.00
7.3 7.2 7.1 7.0 6.9 6.8 6.7 6.6 6.5
f1 (ppm)
1.95
0.91
1.83
1.97
2.00
6.15
2.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


1H
400 MHz
DMSO-d6
compound 4
9.80
7.38
7.38
7.35
7.02
7.01
7.01
7.00
7.00
6.09
6.08
1.97
1.40
H2O
DMSO
NH
S
NH
O
O
N
OH
2.00
1.94
1.25
4.24
2.29
5.97
18.22
f1 (ppm)
13C
151 MHz
DMSO-d6
compound 4
167.84
153.51
141.71
139.83
134.06
132.01
126.19
118.66
118.12
117.09
115.46
34.73
30.25
23.78
DMSO
chemical shift (ppm)


1H
600 MHz
DMSO-d6
compound 5
6.96
6.88
6.37
6.37
6.33
6.32
6.28
6.28
6.27
6.26
5.25
5.24
5.23
2.96
2.95
2.94
2.94
2.93
2.92
1.34
1.11
1.10
1.09
NH
S
NH
N
OH
H2O
DMSO
1.09
2.00
1.85
1.92
1.96
2.06
3.94
16.87
5.74
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
chemical shift (ppm)


13C
151 MHz
DMSO-d6
compound 5
151.20
144.92
140.94
136.08
134.33
124.39
122.10
119.85
110.84
110.01
37.74
34.71
30.36
14.41
NH
S
NH
N
OH
DMSO
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
chemical shift (ppm)



7. References:

1. Bernthsen, A., Studien in der Methylenblaugruppe. *Justus Liebigs Annalen der Chemie* **1885,** *230*, 137-211.

2. Uno, S.; Kimura, H.; Murai, M.; Miyoshi, H., Exploring the quinone/inhibitor-binding pocket in mitochondrial respiratory complex I by chemical biology approaches. *J. Biol. Chem.* **2019,** *294*, 679-696.

3. Jin, R.; Bub, C. L.; Patureau, F. W., Phenothiazinimides: Atom-Efficient Electrophilic Amination Reagents. *Org. Lett.* **2018,** *20*, 2884-2887.