Electronic Supplementary Material (ESI) for ChemComm.

Supporting information for

Polysulfide-Triggered Fluorescent Indicator Suitable for Super-Resolution Microscopy and Application in Imaging

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General experimental methods:

All the commercial reagents were used as received without further purification. Solvents were dried using standard procedures. HPLC grade solvents were used for spectral measurements. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400/500 MHz FT NMR (Model: Advance-DPX 400/500) using TMS as an internal standard. High-resolution mass spectra were recorded on JEOL JM AX 505 HA mass spectrometer. UV-Vis spectra were recorded using Shimadzu UV-1800 spectrometer. Fluorescence measurements were carried out on quanta master-400 steady state fluorescence spectrometer. Na_2S_2 was purchased from Dojindo molecular technologies Inc. Japan. Quantum yield was calculated by using Rhodamine B as reference standard.[2] All the Structured Illumination Microscopy (SIM) and Wide Field Fluorescence Microscopy experiments were performed by using Delta Vision OMX-SIM (GE Healthcare). The Post processing SIM reconstructions were performed by using Soft Worx software. Images were analysed and processed using ImageJ software.

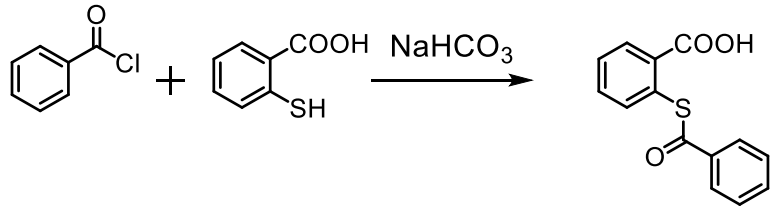
General experimental methods for UV-Vis and fluorescence studies:

A stock solution of **MB-S$_n$** (5×10^{-3}M) was prepared in HPLC grade acetonitrile and the same solution was used for all the studies after appropriate dilution with 20 mM Phosphate buffer solution (pH 7.4). For spectroscopic measurements, stock solution of the probe was diluted by using Phosphate buffer: CH_3CN (9:1 v/v) mixture and the effective final concentration of the probe was made as 10 μM. CTAB (5 mM) was prepared in ethanol and 50 μM of CTAB was used for all the studies. After a thorough screening of the different solvent/buffer combinations, we found that 10 μM of **MB-S$_n$** in Phosphate buffer: CH_3CN (9:1 v/v) and 50 μM of CTAB provided the best results. Hence, this combination was used for all the spectral measurements. Emission measurements were done using λ_{Ext}= 530 nm with the emission slit widths of 4/4 nm.

All the ROS, RNS used in the study were freshly prepared. Concetration of H_2O_2 was determined by measuring the absorption at 240 nm (ε = 43.6 $M^{-1}cm^{-1}$).[4] Perchlorate concentration was determined by measuring the absorption at 292 nm (ε = 360 $M^{-1}cm^{-1}$).[5] Hydroxyl radicals were generated *in situ* by the Fenton reaction of $FeSO_4$ with H_2O_2.[6] Final concentration of H_2O_2 and Fe^{II} was 200 μM and 50 μM respectively. $^{1}O_2$ was generated by the reaction of NaOCl with H_2O_2. Final concentration of OCl^- and H_2O_2 was 50 μM and 200 μM respectively. Superoxide was prepared by adding 1mg of KO_2 to 1ml of dry DMSO followed by vigorous stirring.[7] Na_2S_2 stock solution (5 mM) was prepared in phosphate

buffer. Na_2S_2 is highly unstable and readily decomposes in the buffer, so solutions were freshly prepared as and when required and used immediately. CTAB is necessary to ensure the stability of Na_2S_2. Hence, 50 μM of CTAB was used for the spectroscopic measurements.

Synthesis: BODIPY core and **MB-OH** were prepared by following our previous reports.[1,2]



Scheme 1: Synthetic route for 2-(benzoylthio)benzoic acid.

Synthesis 2-(benzoylthio)benzoic acid: Thiosalicylic acid (1.5 g, 9.72 mmol) was dissolved in 25 ml of sodium bicarbonate (3 g, 35.71 mmol). The mixture was cooled to 0°C and benzoyl chloride (1.15 ml, 9.72 mmol) and solid sodium carbonate (1.5 g, 14.15 mmol) was added. The reaction mass was stirred at 0° C for 30 minutes and then allowed to warm to room temperature. Reaction was stirred at room temperature for 1 hour. It was acidified with conc. HCl, which resulted in a pure white precipitate. It was filtered and washed with water to afford 2-(benzoylthio)benzoic acid as white solid 72% Yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm) 7.95 (2H, d, J = 7.6 Hz), 7.91 (1H, d, J = 7.2 Hz), 7.73-7.70 (1H, t) , 7.63-7.57 (5H, m). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm) 189.33, 167.94, 137.48, 136.54, 136.38, 134.74, 132.28, 130.88, 130.42, 129.75, 127.54, 126.86. HRMS (ESI): m/z calculated for $C_{14}H_{11}O_3S$ [M+H] 259.0423 found 259.0419. (Data matched with literature report[3])

Synthesis of MB-S_n: 2-(benzoylthio)benzoic acid (150 mg, 0.58 mmol) was dissolved in 20 ml of dry CH_2Cl_2 . It was cooled to 0° C and *N,N′*-Dicyclohexylcarbodiimide (DCC) (121 mg, 0.58 mmol) was added. It was stirred at the same temperature for 1 hour. Then MB-OH (200 mg) and a catalytic amount of 4-dimethylaminopyridine (DMAP) was added. The reaction mass was allowed to warm to room temperature and stirred overnight. The reaction mass was filtered in order to remove the urea impurity and subsequently concentrated under reduced pressure. Crude was purified by silica gel flash column chromatography using EtOAc and hexane. The compound was eluted at 13% EtOAc. Off-pink solid yield 18%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 1.41 (3H, s), 1.45 (3H, s), 2.62 (3H, s), 6.03 (1H, s), 6.63 (1H, s), 7.23 (2H, d, J = 6.7 Hz), 7.32 (3H, d, J = 7.3 Hz), 7.37-7.41 (1H, m), 7.50-7.56 (6H, m), 7.66-7.71 (5H, m), 8.22-8.24 (3H, m). ^{13}C NMR ($CDCl_3$, 100 MHz): δ (ppm) 14.42, 14.57,

29.72, 117.51, 119.38, 121.55, 122.00, 127.60, 127.90, 128.14, 128.46, 128.83, 129.01, 129.15, 129.64, 133.84, 134.46, 137.23, 142.35, 152.23, 155.87, 164.60, 189.36. HRMS (ESI): m/z calculated for $C_{40}H_{31}N_2O_3BF_2S$ [M+Na] 691.2009 found 691.2003.

Reaction of MB-S_n with Na_2S_2:

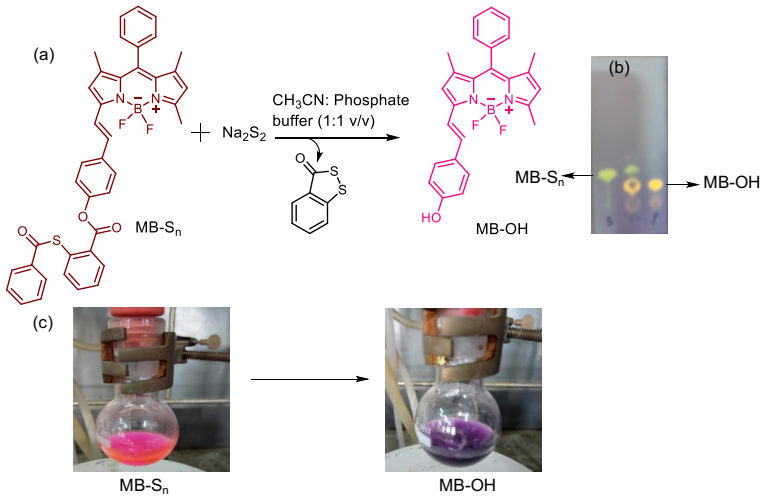


Fig. S1: (a) Reaction of **MB-S_n** with Na_2S_2. (b) Thin layer chromatography analysis under hand held 365 nm UV-lamp showing the formation of **MB-OH**. (c) Corresponding colour change in the reaction mass observed after the addition of Na_2S_2.

MB-S_n (0.015 mmol) was dissolved in 2 ml CH_3CN and 2 ml of Phosphate buffer (pH 7.4) containing 200 µM of CTAB. To this Na_2S_2 (0.15 mmol) was added. Immediate change in the colour from pale pink to purple was observed. The reaction mass was further stirred for 30 minutes and it was monitored by TLC. The reaction mass was diluted with EtOAc organic layer was separated and dried over sodium sulphate and concentrated. It was purified by preparatory chromatography method. ^{1}H NMR ($CDCl_3$, 400 MHz): δ (ppm) 1.39 (3H, s), 1.42 (3H, s), 2.60 (3H, s), 6.00 (1H, s), 6.58 (1H, s), 6.81 (2H, d, J = 8.5 Hz), 7.22 (1H, d, J = 16.5 Hz), 7.31 (2H, m), 7.33-7.52 (6H, m). HRMS (ESI): m/z calculated for $C_{26}H_{24}N_2OBF_2$ [M+H] 429.1944 found 429.1945.

Absorption spectrum of MB-S_n and emission spectrum of MB-OH

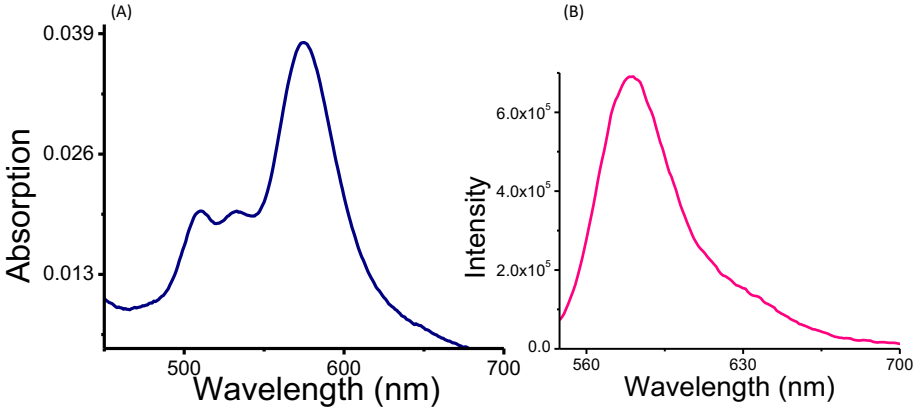


Fig. S 2: (A) Absorption spectra of **MB-S_n** (10 µM). (B) Emission spectra of MB-OH (5 µM) in phosphte buffer (20 mM) : CH_3CN (9:1 v/v), at pH 7.4 containing 50 µM of CTAB.

Absorption spectrum of MB-S_n and MB-OH and absorption changes of MB-S_n in the presence of Na_2S_2

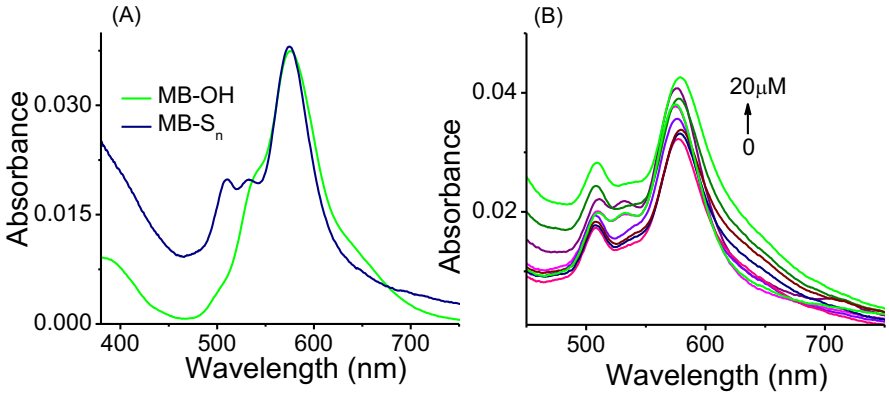


Fig. S 3: (A) Absorption spectra of **MB-S_n** and **MB-OH** (10 µM each) in phosphate buffer: Acetonitrile (9:1 v/v) containing 50 µM CTAB. (B) Absorption changes of **MB-S_n** in the presence of varying concentrations Na_2S_2 (0-20 µM).

Time dependent emission study

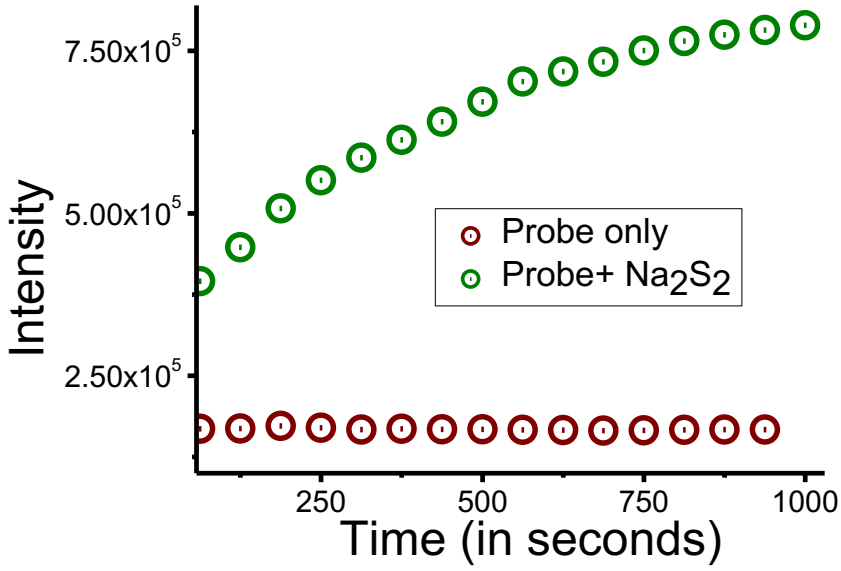


Fig. S 4: Time dependent emission response of **MB-S$_n$** (10 μM) in the absence or presence of Na_2S_2 (10 μM) in 20 mM phosphte buffer: CH_3CN (9:1 v/v), at pH7.4 containing 50 μM of CTAB. λ_{Ext} = 530 nm. Emission was monitored at 584 nm.

pH dependent study

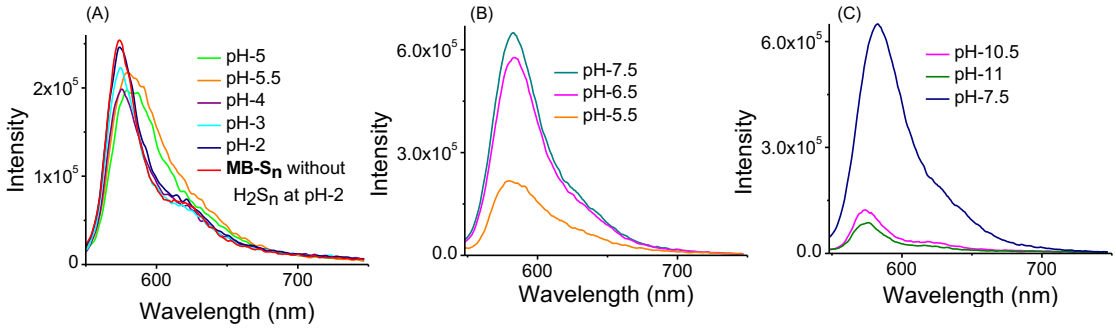


Fig. S 5: Emission response of **MB-S$_n$** (10 μM) in the presence or absence of Na_2S_2 (10 μM) at different pH range. (A) pH 2-5.5 (B) pH 5.5-7.5 (C) pH 7.5-11. Excitation-530 nm.

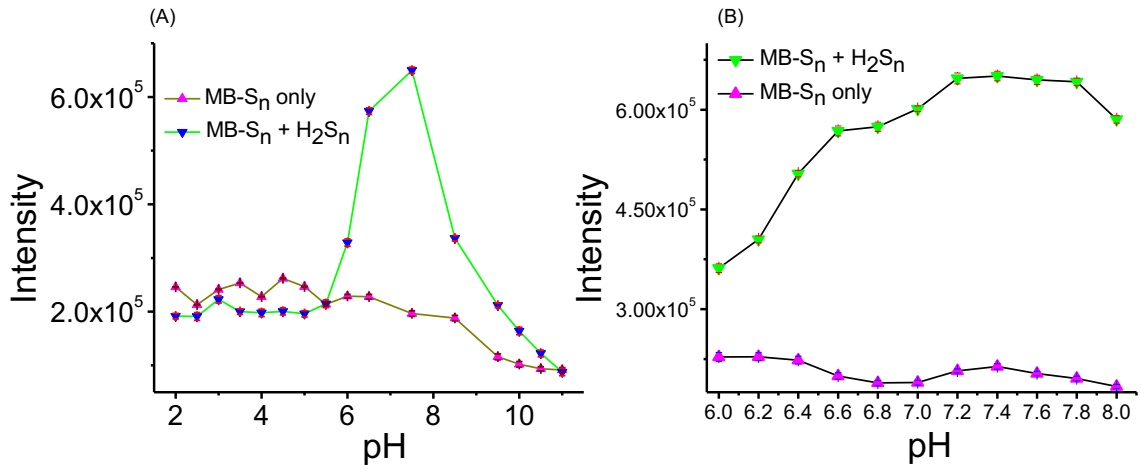


Fig. S 6: (A) Emission response of **MB-S$_n$** (10 μM) at different pH (2-11) in the absence or presence of Na_2S_2 (10 μM). (B) Emission changes of **MB-S$_n$** (10 μM) in the absence or presence of Na_2S_2 (10 μM) at physiological pH (6-8) λ_{Ext} = 530 nm.

Detection limit calculation

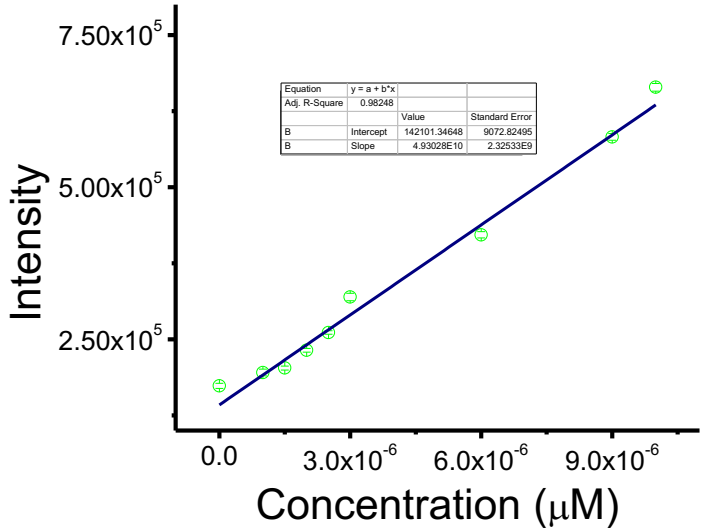


Fig. S 7 : Intensity v/s concentration plot

The detection limit was calculated based on the fluorescence titration. The standard deviation of the blank was determined by measuring the emission spectra of **MB-S$_n$** 7 times without the addition of Na_2S_2.

The detection limit (DL) of **MB-S$_n$** was determined from the following equation:

DL = K * Sb1/S

Where K = 2 or 3 (we took 3 in this case);

Sb1 is the standard deviation of the blank;

S is the slope of the calibration curve.

From the graph, we get slope = 4.93028 x 10^{10}, and the Sb1 value was found to be 427.5843.

Thus, using the above formula we get the Detection Limit = 23.65x 10^{-9} M.

Interference studies with thiols

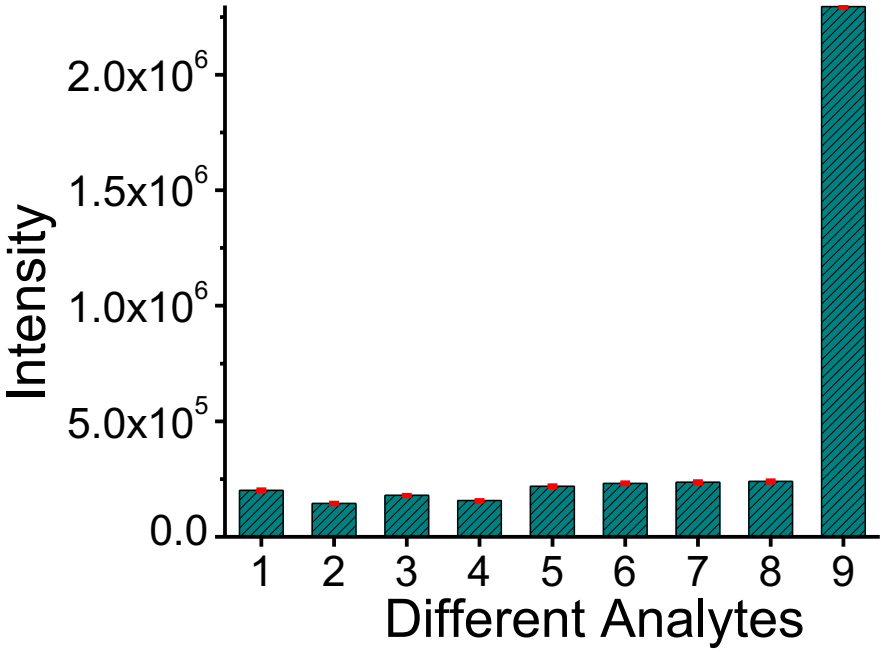


Fig. S 8: Emission response of **MB-S_n** (10 μM) in the presence of biothiols and S_8. (From 1-9 **MB-S_n** only; Cys; Homocysteine, GSH; H_2S; S_8; Cys+ S_8; GSH+ S_8; **MB-S_n**+ Na_2S_2.

Structured Illumination Microscopy Experiments with MB-S_n probe

(A) General Description:

Structured Illumination diffracts the beam into three parallel beams and they are combined by the objective to produce 3D interference fringe patterns in the sample. Multiple images are obtained by adjusting the fringe pattern and by slicing through the sample with respect to different focal planes generating a series of images of the sample and this image volume is known as Z-stack. Each frame of the Z-stack is reconstructed so that it could provide definitive information of the details of the sample which we are imaging thereby improving

the resolution close to two fold, which is not achieved by using conventional light microscopy.

(B) Sample preparation (SIM and Wide-Field Microscopy):

RAW 264.7 cells were seeded on coverslips (22 mm X 22 mm, 170 ± 5 μm square Cover glasses) placed in six well plates in DMEM culture medium containing (10% FBS and 1% Penicillin-Streptomycin) for 24 hours at 37°C, 4% CO_2. After 24 hours when 70% confluency was achieved the cells were washed with DMEM culture medium then cells were treated with **MB-S_n** (10 μM) for 25 minutes. Cells were then washed thrice with culture medium and further treated with different Na_2S_2 for 20 minutes. After that cells were washed again with Phosphate Buffer Saline (2X PBS). After carrying out the Live cell uptake of the **MB-S_n** probe and Na_2S_2, the cells were fixed with 4% PFA for 15 minutes and then washed thrice with PBS and then the coverslips were mounted using a mounting medium (Vectashield h-1000).The coverslips were then sealed using nail varnish and the samples were then imaged by Structured Illumination Microscopy and also Wide-field Fluorescence Microscopy. For the endogenous detection, cells were pre-treated with various concentration of (0-2500 nano gramas/ml) Lipopolysaccharides (16 hours). (LPS is known to stimulate H_2S_n production in cells through CSE pathway). Cells were washed and further treated with **MB-S_n** (10 μM) for 25 minutes. And images were captured by following the same procedure mentioned above.

Structured Illumination Microscopy and High Resolution Microscopy:

Instrument Specification:

The Delta Vision OMX system is a Microscope which surpasses normal microscopy resolution limits. It allows imaging beyond the surface of the coverslips by using multiple probes to retrieve exhaustive biological information from all directions. This Instrument's Structured Illumination Microscopy technology enables to image deeply the biology and resolves features which are literally close to invisible through traditional light microscopy. Delta Vision OMX is a flexible microscope and it works well with all kinds of probes including conventional fluorophores to artificially engineered fluorochromes.

Data Processing:

(A) Structured Illumination Microscopy (SIM):

Structured Illumination Microscopy (SIM) carried out by us using the Delta Vision OMX is basically a Wide Field Microscopy technique based on Moire's effect. The Resolution improvement is achieved based on the Reconstruction of the acquired image by using the inbuilt software namely Soft Worx. The Z stacks acquired during the Imaging are post processed by using the reconstruction option of Soft Worx. SIM acquisition is dependent mainly on the imaging parameters and acquisition parameters and this varies depending on the sample and in particular on the nature of the probe. The **MB-S_n** probe was Excited at 568 nm and the emission was collected at 586 nm (Alexa Flour 568 Channel of the Delta Vision OMX). In the case of **MB-S_n** probe, the Structured Illumination experimental conditions for single colour experiments were mainly dependent on the thickness of the Z stack (sections 80 to 100), section spacing (0.125 to 0.150), thickness of the sample (8 to 10). As **MB-S_n** probe is a very bright probe, for the SIM acquisition, we needed to vary the %T and exposure time. Therefore in all our Single colour experiments the exposure time was between 3 to 10 and the %T was in the range of 10 to 50. The Colocalization experiments were performed with ER Tracker Green. The Dual colour experiments were performed with Hoechst. In both of these experiments, the SIM conditions of these Co-staining agents were maintained in accordance with the **MB-S_n** probe which would be discussed in the later sections.

(B) 3D SIM Projection:

The offline processing of Structured Illumination Microscopy (SIM) images was carried out by using ImageJ software. The option Stacks (3D project) was employed in obtaining a 3D projection of the Structured Illumination Microscopy images obtained by using the Delta Vision OMX-SIM Microscope.

Colocalization SIM and Wide Field Microscopy Experiments:

(A) Colocalization Experiments with ER Tracker Green:

The Co-staining experiments with ER Tracker Green was carried out by Incubating the ER Tracker Green (1 μM) further for 30 minutes after incubating the RAW cells with **MB-S_n** (10 μM) for 25 minutes probe (10 μM) initially for 25 minutes and Na_2S_2 (12.5 μM) for a further

20 minutes. The ER-Tracker Green was excited at 488 nm and the Emission was collected in the **FITC** Channel (500 nm to 550 nm).

(B) Dual Colour SIM and Wide-Field Microscopy Experiments:

The Dual colour experiments with Hoechst as the nuclear stain was carried out by incubating the Hoechst-33342 (500 nM) for 30 minutes after incubating the LPS treated RAW cells with **MB-S_n** (10 µM) for 25 minutes. Hoechst was excited at 405 nm and the emission was collected in the **DAPI** channel (420 nm to 500 nm).

Cytotoxicity Assay

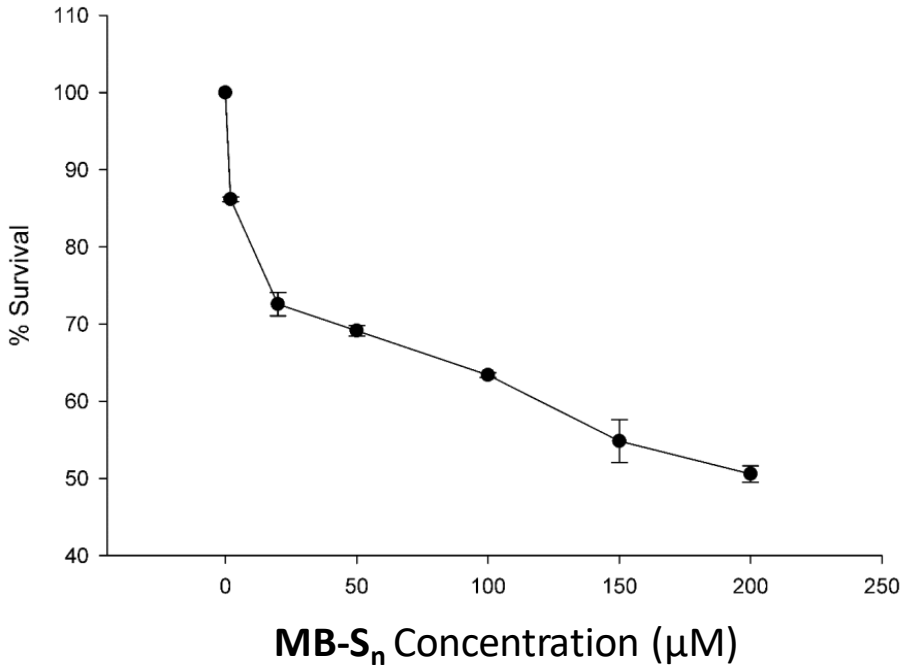


Fig. S 9: MTT assay to determine cytotoxicity of **MB-S_n**

The *in vitro* cytotoxicity of **MB-S_n** on RAW 264.7 macrophages were determined by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) assay. RAW 264.7 cells (7 x 10^3) were seeded in each well of a 96 well plate and cultured in an incubator supplied with 5% CO_2 maintained at 37°C. Cells were maintained in DMEM medium, supplemented with 10% Foetal Bovine Serum and 100 Units of Penicillin-Streptomycin antibiotics. After 24 hours, the cells were treated with different concentrations of **MB-S_n** in triplicates for 24 hours. Cells were then treated with 0.5 µg/mL of MTT reagent. The plate was then incubated for 4 hours at 37°C. 100 µl of isopropyl alcohol was added to

each well. Optical density was measured at 570 nm using Multiskan Go (Thermo Scientific) to find the concentration of the cell inhibition. IC_{50} value was found to be > 200 µM.

The formula used for the calculation of the MTT assay for evaluation of the cell viability was as follows:

Cell viability (%) = (means of Absorbance values of treated group/ means of Absorbance values of untreated control) ×100.

Wide-field fluorescence imaging

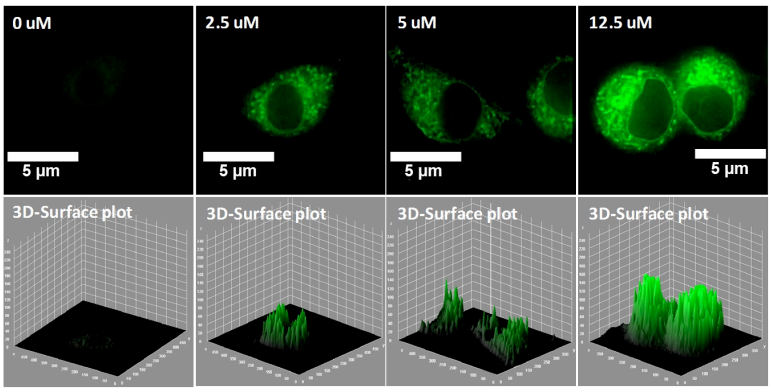


Fig. S 10: Wide-field fluorescence microscopy images of RAW 264.7 macrophages incubated with **MB-S_n** (10 µM) and various concentration of Na_2S_2. Bottom row indicates the corresponding 3D intensity profile plots.

Co-localization experiment with ER-Tracker

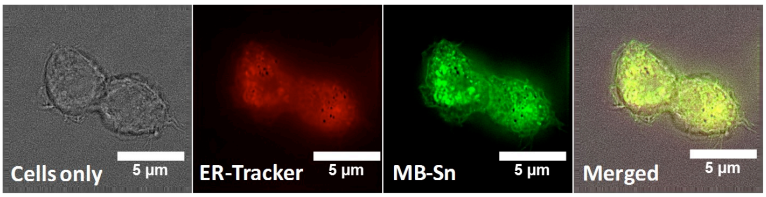


Fig. S 11: Widefield images of RAW 264.7 macrophages incubated with **MB-S_n** (10 µM) and co-stained with ER-tracker

Co-staining experiment with Hoechst-33342

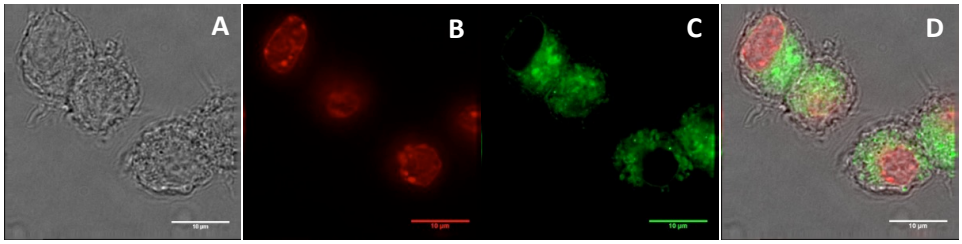


Fig. S 12: Widefield images of RAW 264.7 macrophages incubated with **MB-S$_n$** (10 μM) and co-stained with nucleus staining dye Hoeschst.

Time dependent emission in cells

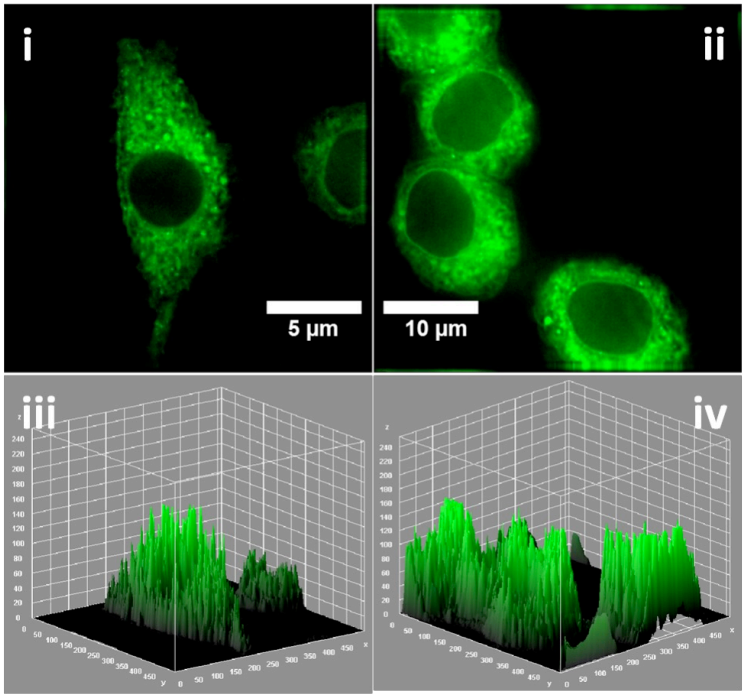


Fig. S 13: Wide-field images of RAW 264.7 macrophages with **MB-S$_n$** (10 μM) recorded after the addition of 20 μM Na_2S_2 (i) 2 minutes and (ii) 20 minutes. (iii & iv) corresponding 3D profile plots.

^{1}H NMR spectrum 2-(benzoylthio)benzoic acid:

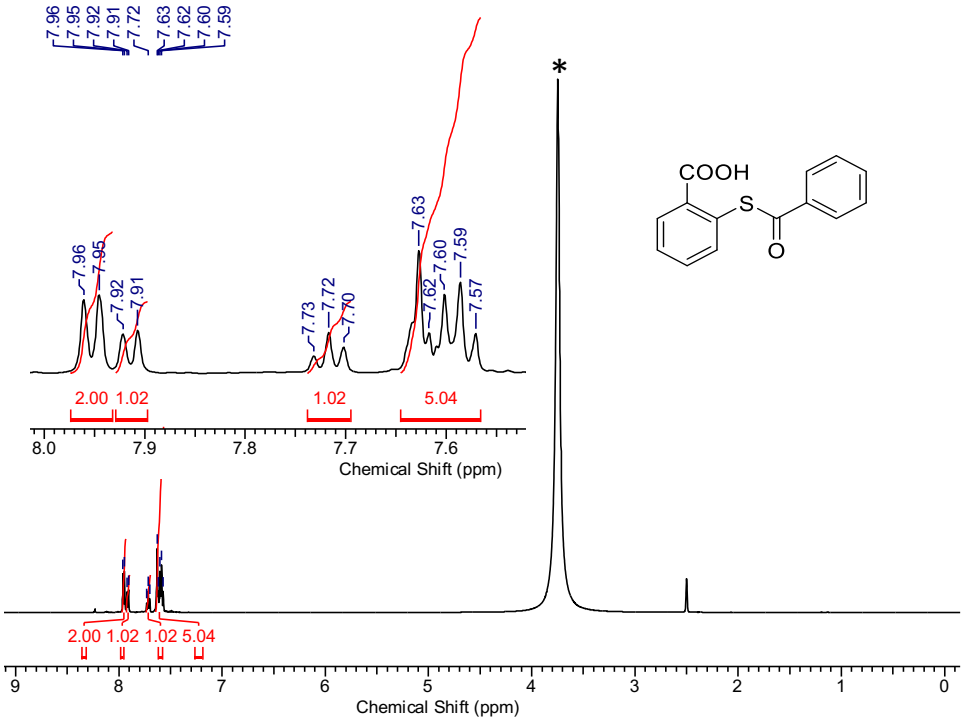


Fig. S 14: ^{1}H NMR spectrum of **2-(benzoylthio)benzoic acid** in DMSO-d_6. (* H_2O).

^{13}C NMR spectrum of 2-(benzoylthio)benzoic acid:

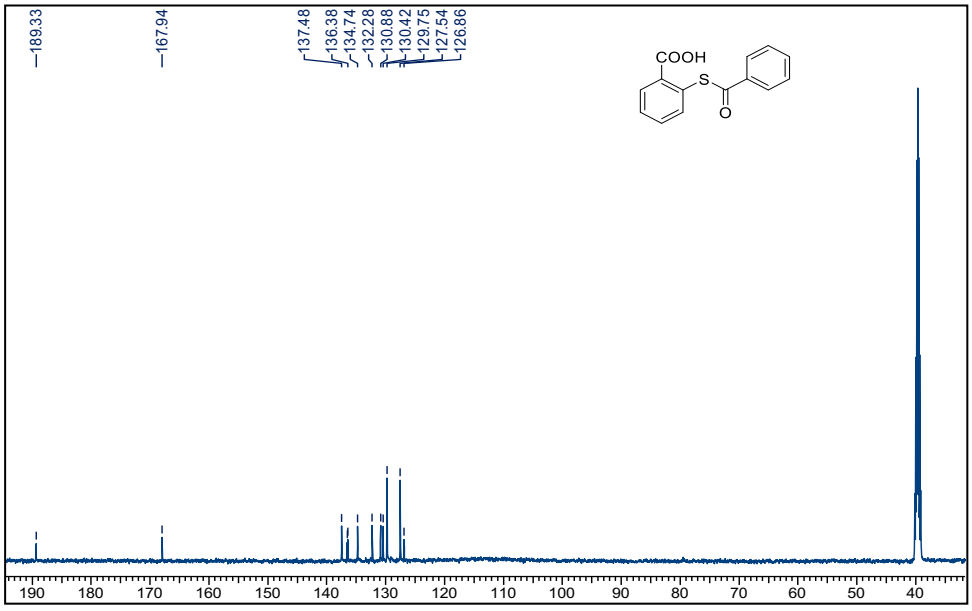


Fig. S 15: ^{13}C NMR spectrum of **2-(benzoylthio)benzoic acid** in DMSO-d_6

HRMS spectrum of 2-(benzoylthio)benzoic acid:

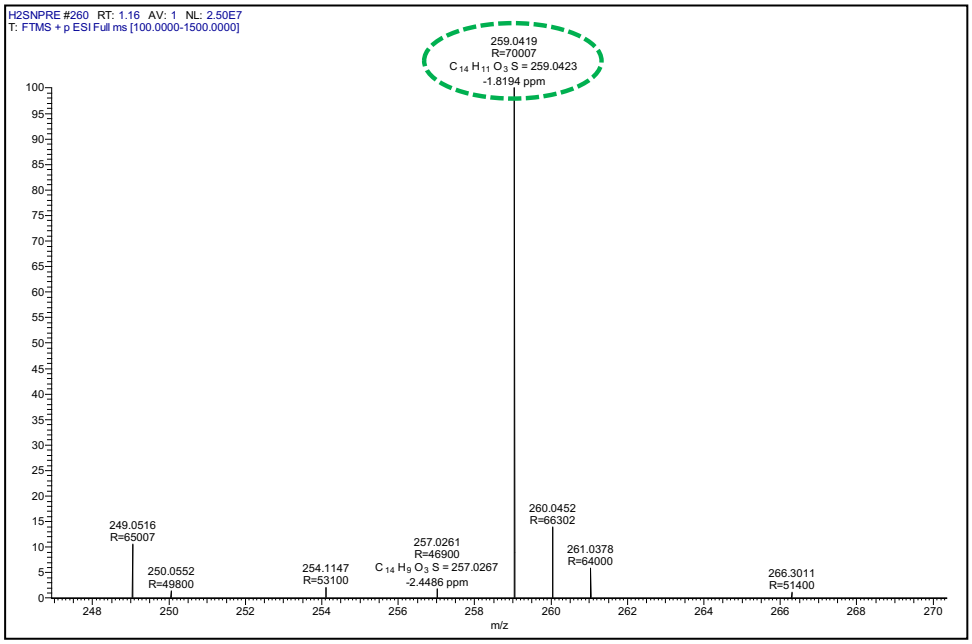


Fig. S 16: HRMS spectrum of **2-(benzoylthio)benzoic acid.**

^{1}H NMR spectrum of MB-S_n

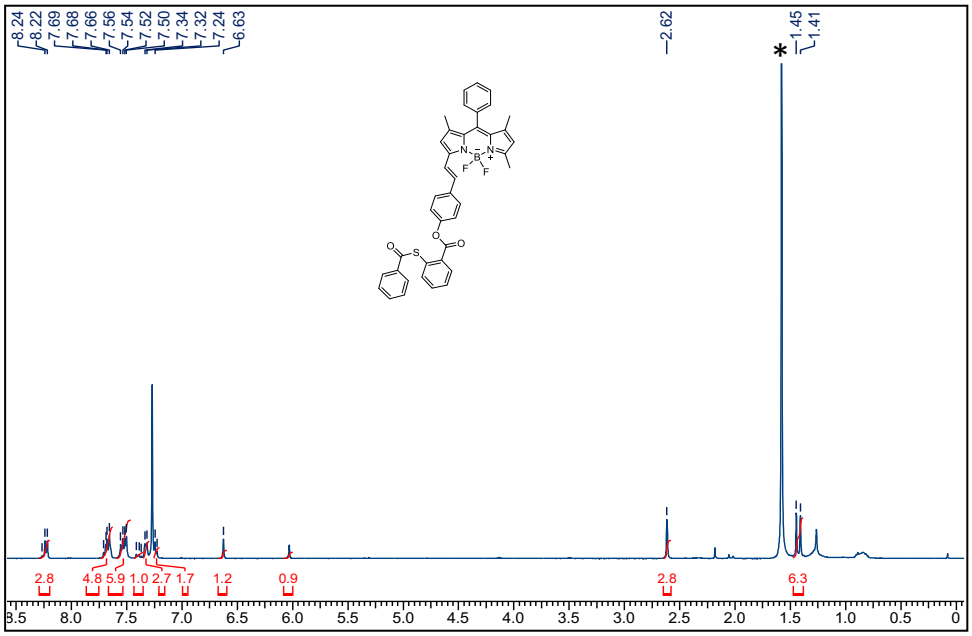


Fig. S 17: ^{1}H NMR spectrum of **MB-S_n** in $CDCl_3$ (* H_2O)

^{13}C NMR spectrum of MB-S_n

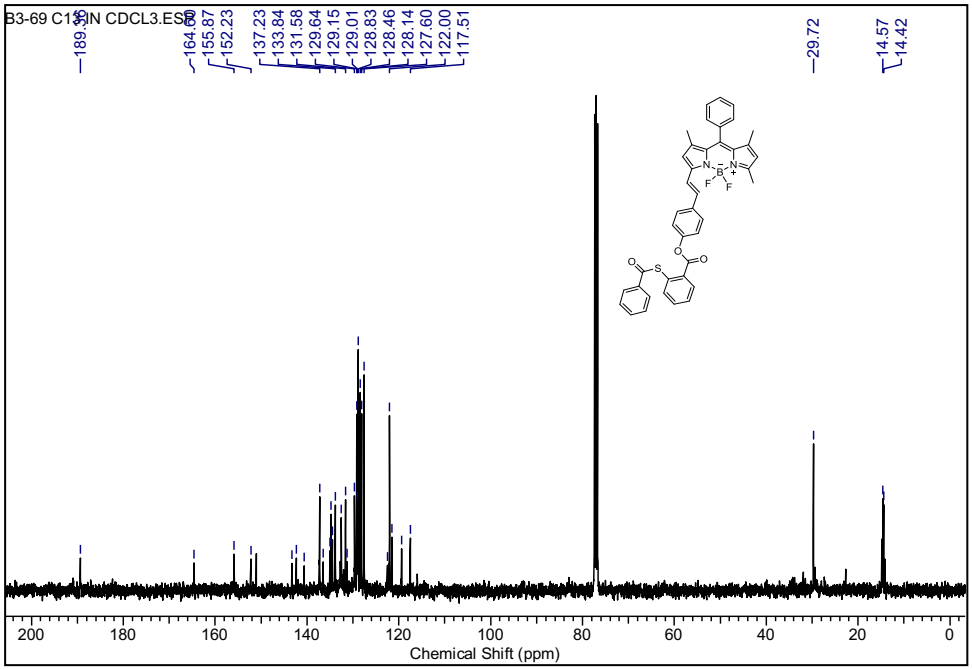


Fig. S 18: ^{13}C NMR spectrum of **MB-S_n** in $CDCl_3$

HRMS spectrum of MB-S_n

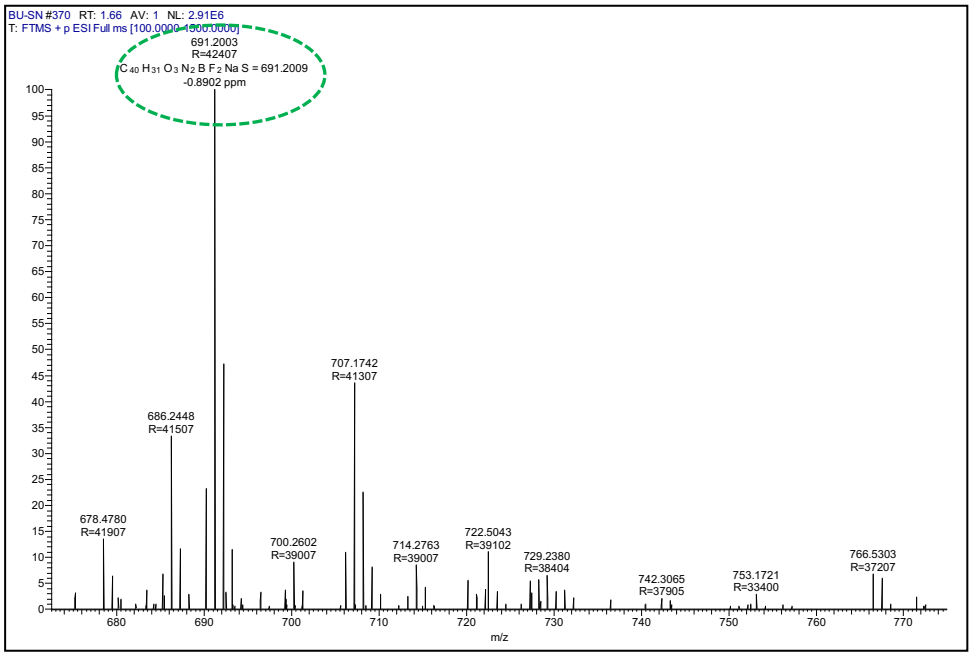


Fig. S 19: HRMS spectrum of **MB-S_n**

^{1}H NMR spectrum of the reaction product **MB-OH**

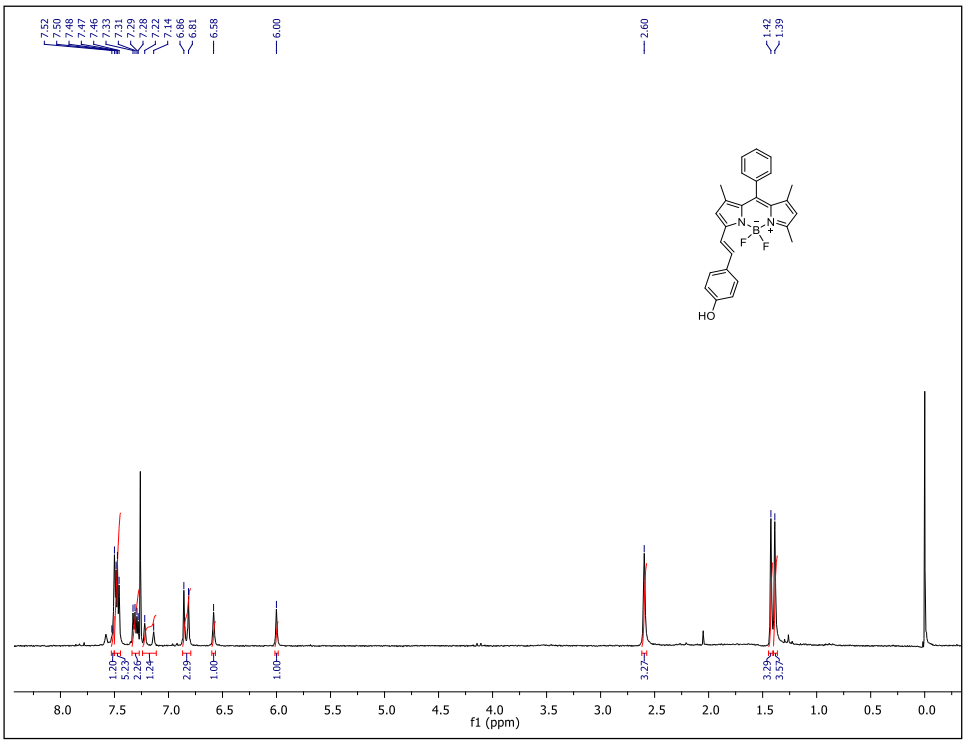


Fig. S 20: ^{1}H NMR spectrum of the reaction product **MB-OH** in $CDCl_3$

HRMS spectrum of the reaction product **MB-OH**

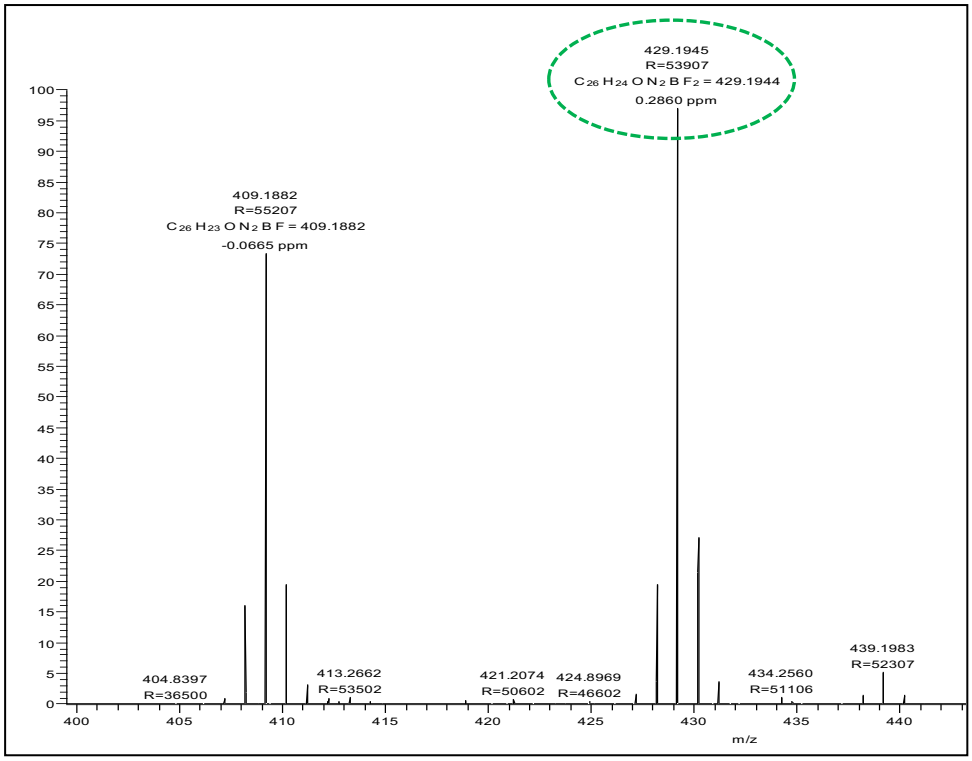


Fig. S 21: HRMS spectrum of the reaction product **MB-OH**

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