Supplementary Material

Reconfigurable Logic Operations for Fluorescent Sensing of Drug Resistant and/or Hypoxic Cancer Cells

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1 General Information

In all experiments, reagents and solvents were purchased and used without further purification, unless stated otherwise. For the purifications of the compounds by column chromatography, silica stationary phase (230–400 mesh, SiliCycle Inc., Canada) was used. For thin layer chromatography (TLC), 0.25 mm thick precoated silica gel plates (60F254, Merck, Germany) were used. ^{1}H Nuclear Magnetic Resonance (NMR) and ^{13}C NMR spectra were recorded on a Bruker instrument (400 MHz) at Necmettin Erbakan University, Science and Technology Research and Application Center (BITAM). Chemical shifts (δ) are reported in parts per million (ppm) and referenced to the residual solvent peak. Coupling constants (J) are reported in Hertz (Hz). Abbreviations for multiplets are given as: br = broad, d = doublet, m = multiplet, s = singlet, t = triplet. High-resolution mass spectrometry was carried out using Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight Q-TOF LC/MS of Atatürk University, the Eastern Anatolia Advanced Technology Research and Application Centre (DAYTAM, Erzurum, Turkey). HEP3B human hepatocellular carcinoma cell line was used for cell culture experiments. Cells were grown in high glucose Dulbecco s Modified Eagle s Medium (DMEM) supplied with 10% FBS and 0.1% gentamycin at 37 °C, under 5% CO_2 atmosphere in a sterile humidified incubator. Time resolved fluorescence lifetime spectroscopy analysis were performed at National Nanotechnology Research Center, Bilkent University

2 Supplementary Figures

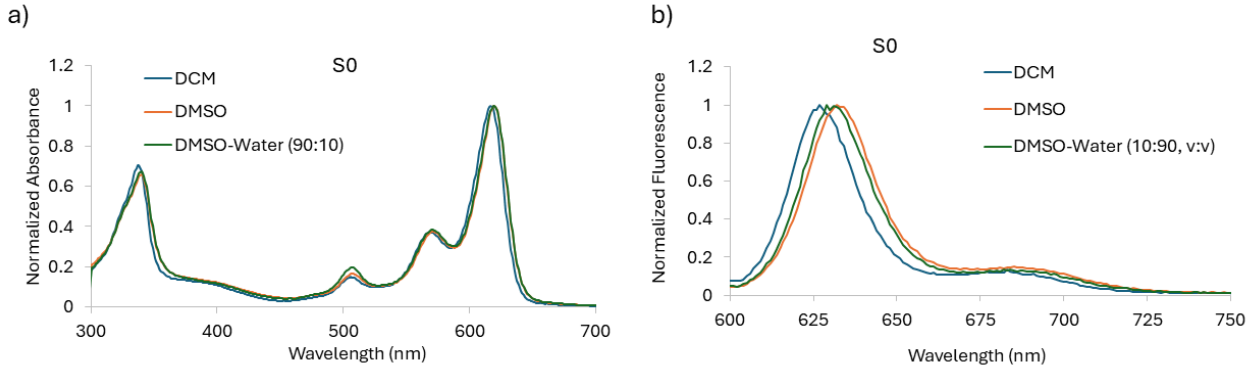


Figure S1. Normalized UV-Vis absorbance (a) and fluorescence spectra (b) of **S0** in dichloromethane (DCM), dimethyl sulfoxide (DMSO) and DMSO: Phosphate Saline Buffer (PBS) mixture (90:10 by volume). $\lambda_{excitation}$ is 600 nm. Only a 3 nm hypochromic shift in the emission of **S0** is observed in dichloromethane.

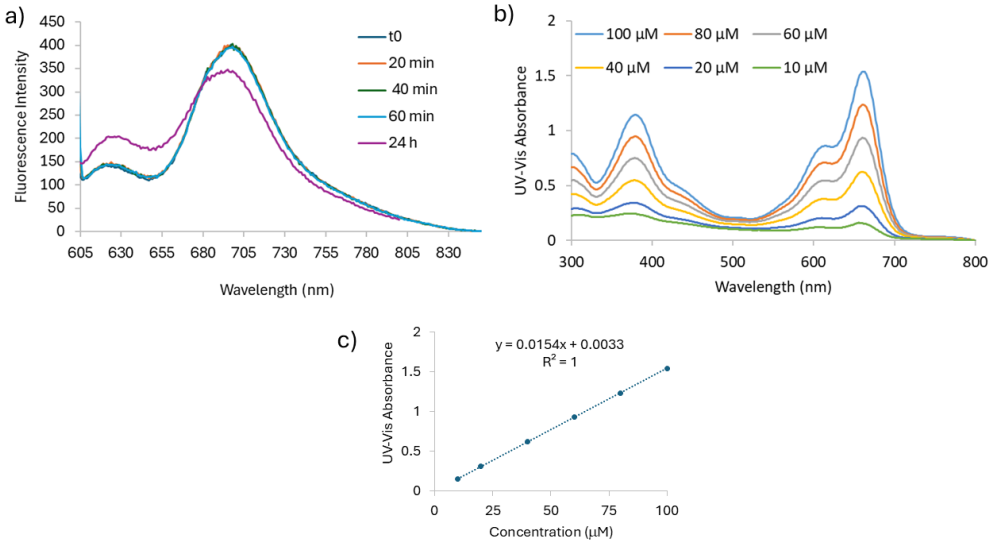


Figure S2. Change in fluorescence spectra of **M2** over 24 h in DMSO: PBS mixture (75:25 by volume) (a). Concentration dependency of UV-Vis Absorbance spectra of **M2** (b, c) in DMSO: PBS mixture. $\lambda_{excitation}$ is 600 nm.

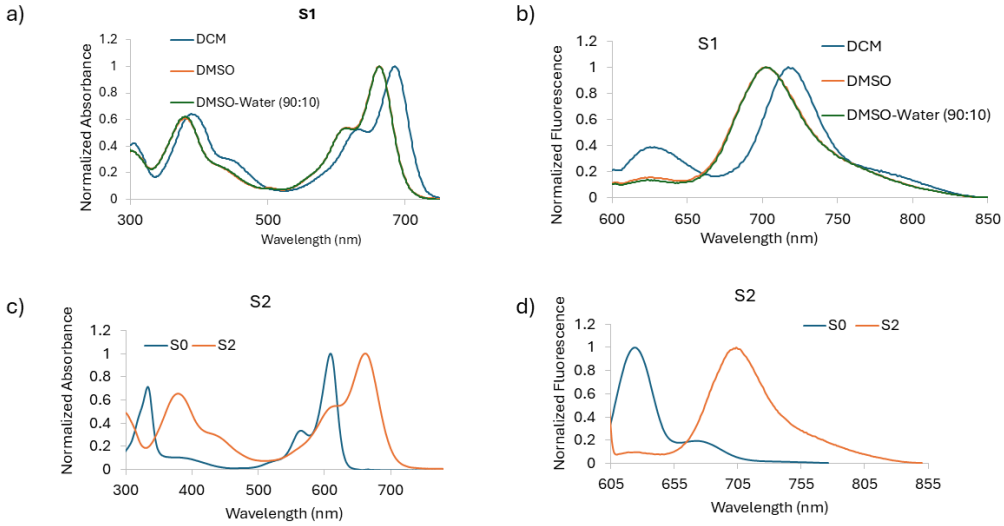


Figure S3. Normalized UV-Vis absorbance (a) and fluorescence spectra (b) of **S1** in DCM, DMSO and DMSO: PBS mixture (90:10). Normalized UV-Vis absorbance (c) and fluorescence spectra (d) of **S0** and **S2** in MeCN. λ_{exc} is 600 nm.

Table S1. Spectroscopic characterization of **S0**, **S1**, **S2**, **M1** and **M2**

Compound	Solvent	λ_{max} (abs), nm	λ_{max} (flo), nm
M2	DCM	685	713
M2	MeCN	660	703
M2	DMSO	663	702
M2	DMSO:PBS[a]	662	697
M1	DMSO:PBS[a]	643	628, 677
M1	DMSO	646	676
S0	DCM	617	627
S0	DMSO	620	632
S0	DMSO:PBS[b]	620	629
S1	DCM	685	717
S1	DMSO	663	703
S1	DMSO:PBS[b]	663	702
S2	MeCN	662	704

[a]25% PBS in DMSO, [b]10% PBS in DMSO.

Table S2. Additional spectroscopic characterization of **S0**, **S1**, **S2**, **M1** and **M2** in DMSO.

Compound	ε, $M^{-1}cm^{-1}$ [a]	Φ_F[b]	τ_F (ns)[c]
M2	71000	0.13	2.13
M1	77000	0.14	2.08
S0	15200[7]	0.67	4.87
S1	81000	0.20	2.38
S2	67000	0.27	1.08[d]

[a] For each molecule molar absorptivity (ε) are calculated for maximum absorbance wavelengths in DMSO, which are tabulated in Table 1. [b]Calculations were done by exciting samples at 589 nm and using Zinc Phthalocyanine as reference compound.[8] [c] Fluorescence lifetime (τ_F) of the compounds were obtained using time-resolved fluorescence spectrometry and by using 625 nm laser source. [d] For this compound fluorescence lifetime is calculated using 2 exponentials and the second τ is determined to be 4.34 ns.

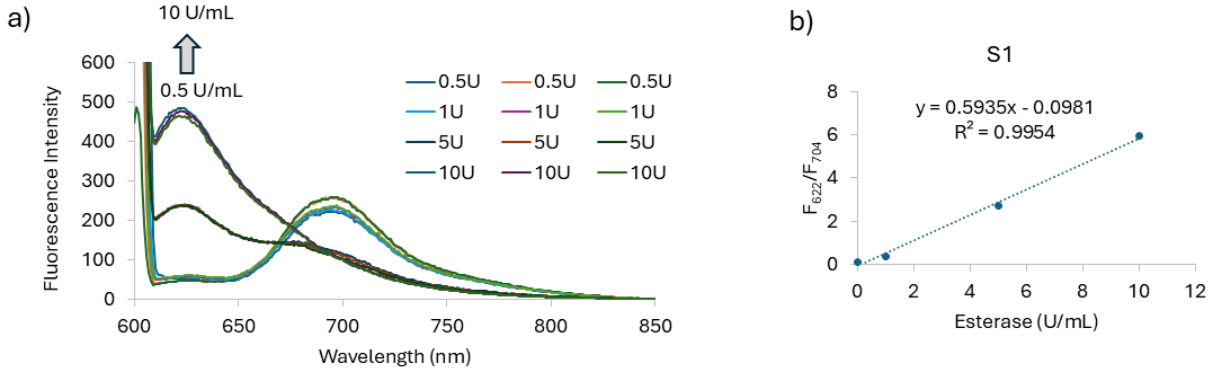


Figure S4. Fluorescence spectra (a) and ratio of fluorescence intensity at 622 nm to 704 nm (b) of **S1** (3.75 μM) in the presence of varying concentrations of carboxylesterase enzyme (E, 0.5-10 U/mL). Spectra were obtained after 30 min incubation by exciting the samples at 600 nm. (n = 3)

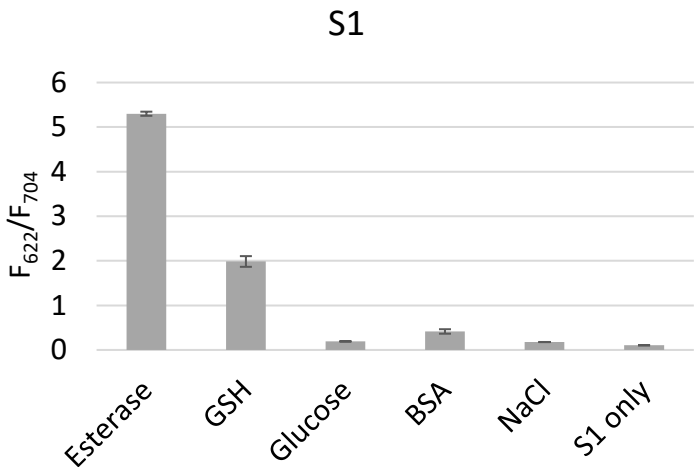


Figure S5. Ratio of fluorescence intensity at 622 nm to 704 nm when **S1** (3.75 μM) is incubated with abundant cellular components for 30 min. Spectra were obtained by exciting the samples at 600 nm. Esterase (10 U/mL), glutathione (GSH, 0.1 mM), glucose (0.5 mM), Bovine Serum Albumin (BSA, 20 mg/mL), NaCl (120 mM). Except GSH, no significant change in the fluorescence ratio was obtained. (n = 3)

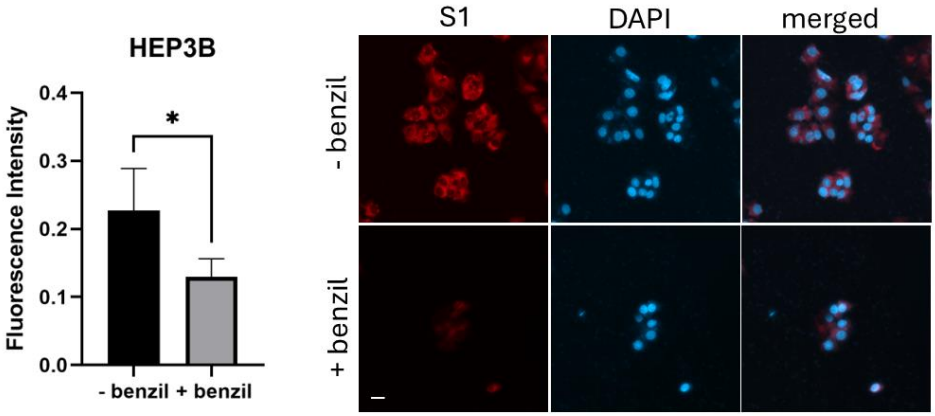


Figure S6. Fluorescence analysis of HEP3B cells after incubation with **S1** esterase sensor (4 μM) for 30 min. Benzil (200 μM) is applied to the cells 2 h prior to probe application to inhibit esterase activity. Scale bar 100 μm. n = 4, * $p \leq 0.05$.

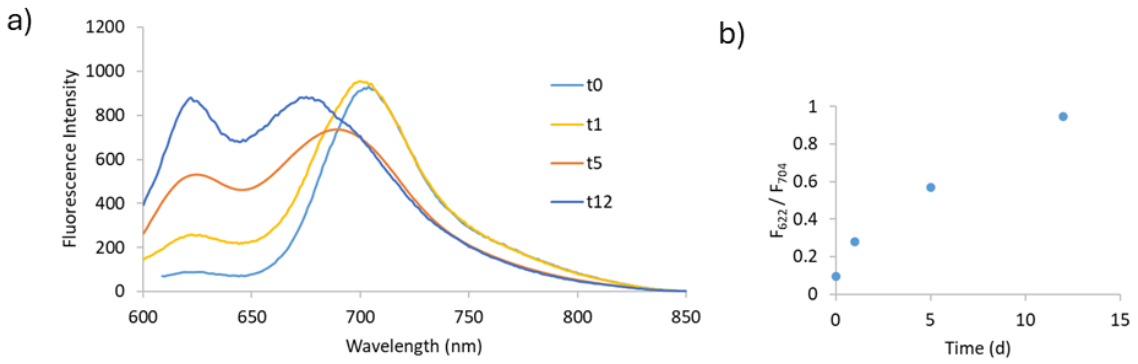


Figure S7. Response of **S2** to nitroreductase (NTR, 18 µg/mL) and reduced form of Nicotinamide adenine dinucleotide (NADH) cofactor (0.5 mM) over a period of 12 days. Peak at 622 nm (a) and the ratio of this peak to initial **S2** peak emission at 704 nm (b), is shown to rise in time however complete conversion is not achieved within the experimental time scale. The new peak at 622 nm is attributed to **S0** emission and indicates the generation of this molecule.

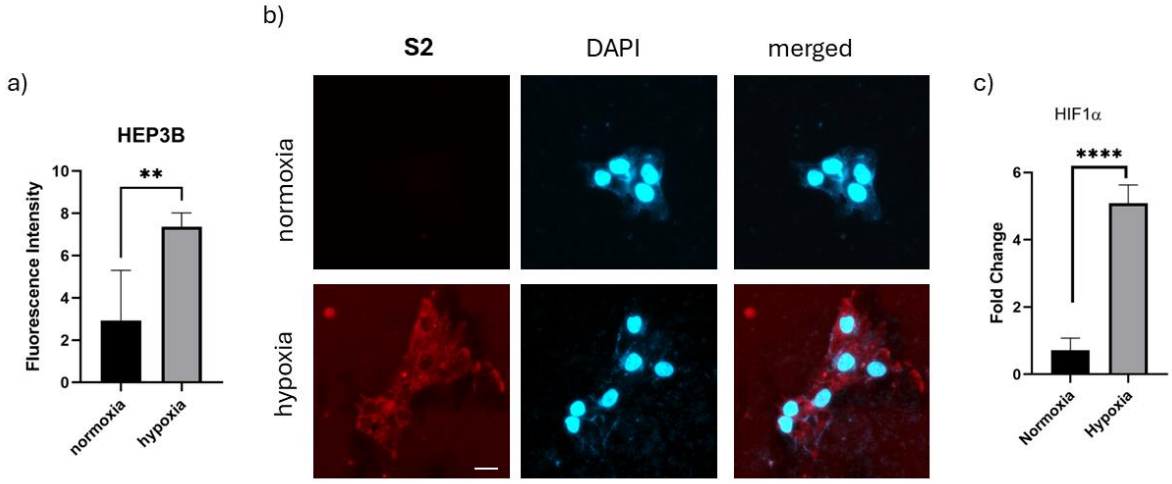


Figure S8. Fluorescence analysis of HEP3B cells after incubation with **S2** nitroreductase sensor (3.2 µM) for 75 min (a, b). $CoCl_2.6H_2O$ (100 µM) is applied 24 h before probe application to mimic hypoxic microenvironment and HIF1α mRNA level is analyzed by RT-PCR to verify hypoxia (c). Scale bar 20 µm. n = 4, ** $p \leq 0.01$.

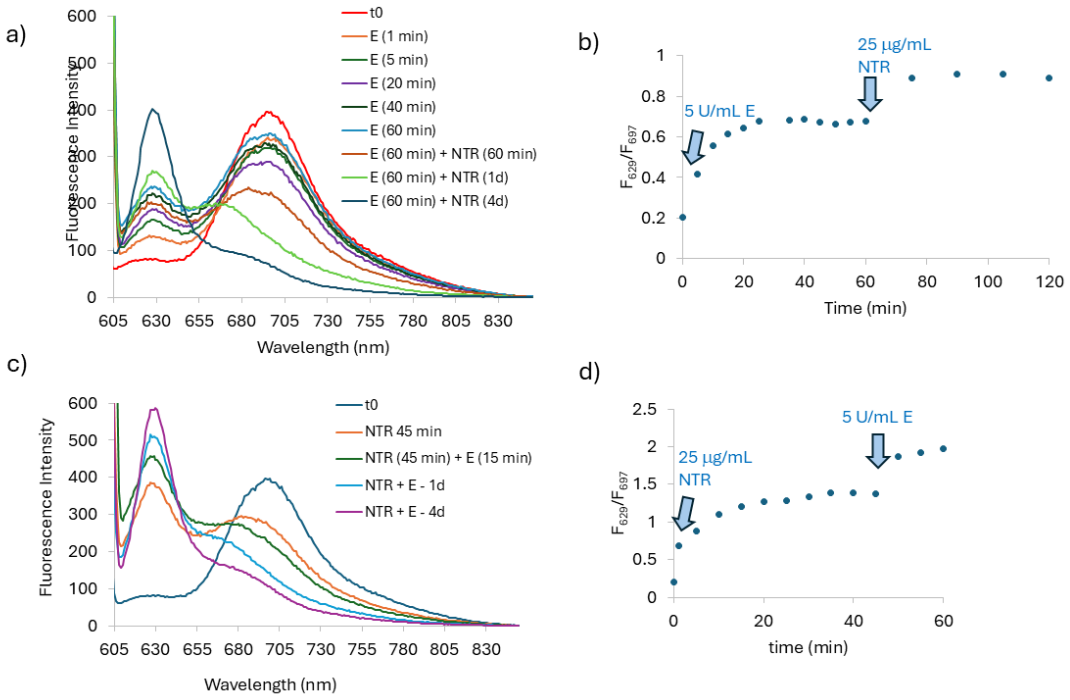


Figure S9. Fluorescence response of **M2** (5 µM) to NTR and E enzymes (25 µg/mL and 5U/mL, respectively) Enzymes were applied sequentially and the spectra is obtained by exciting at 600 nm.

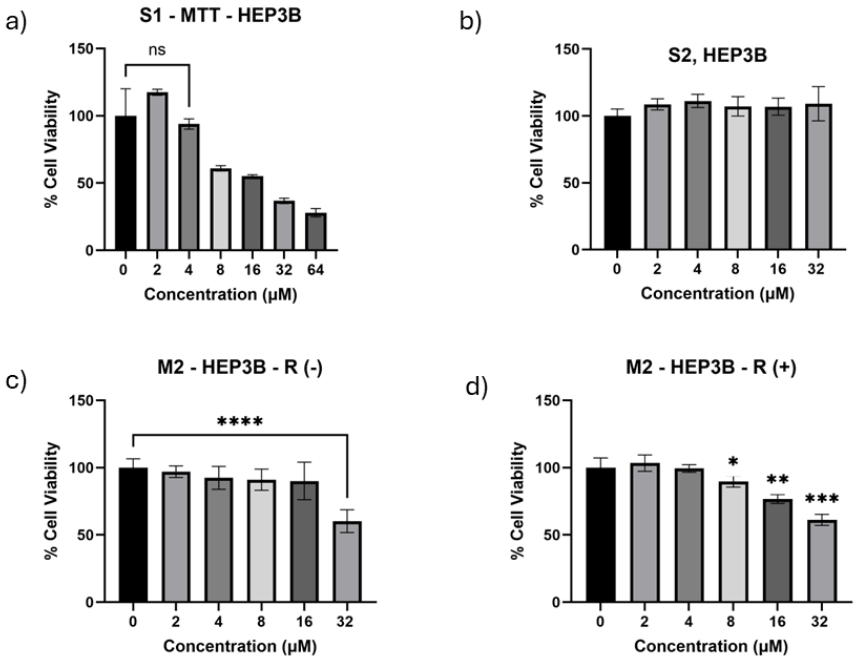


Figure S10. Cytotoxicity analysis of probes **S1**, **S2** and **M2** on HEP3B cells recorded after 24 h of incubation. **M2** toxicity is analyzed in normal HEP3B cells (R(-)) and sorafenib resistant HEP3B cells (R(-)).Compounds are non-toxic at and below the application concentration (4 µM). n = 3 for **S1**, n = 4 for **S2** and n = 6 for **M2**.

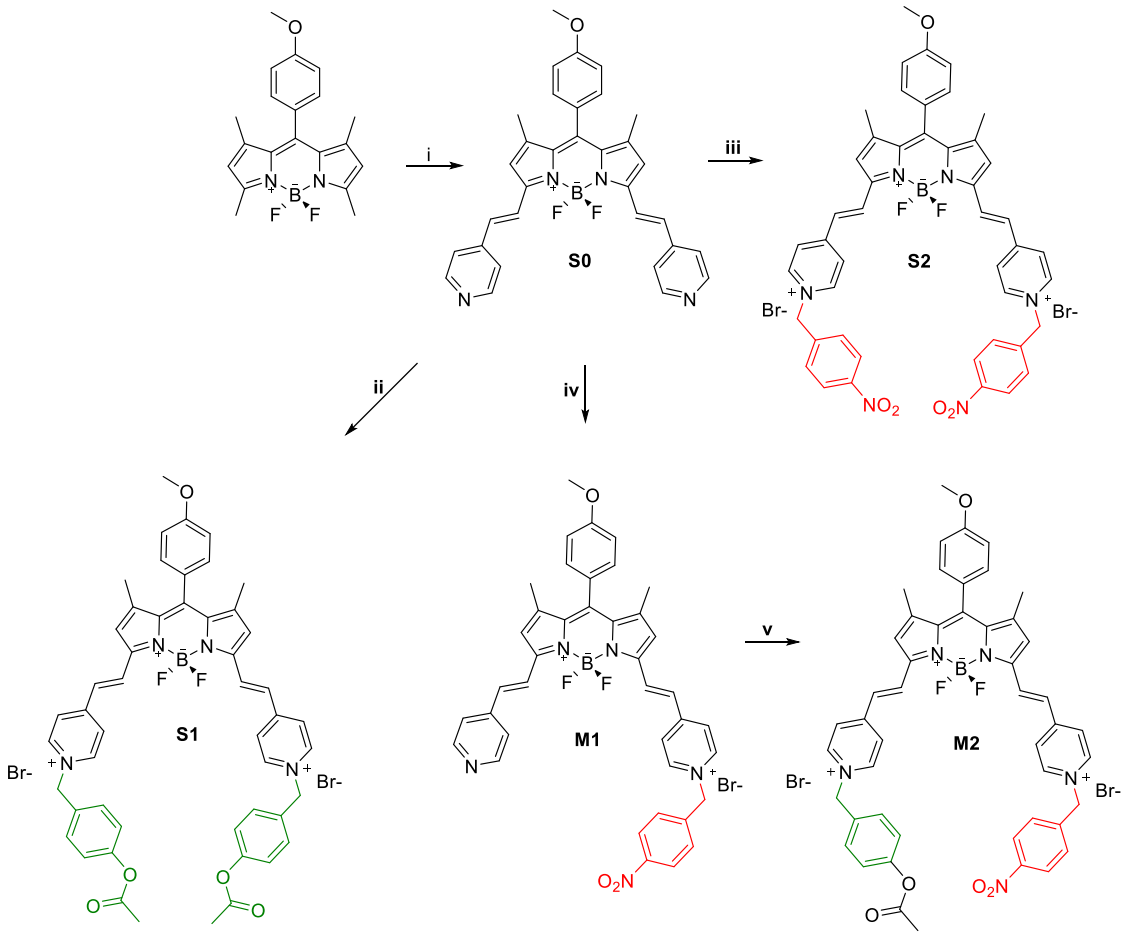
3 Experimental Methods

3.1 Synthesis

Compounds were synthesized as shown in Scheme S1. 1,3,5,7-Tetramethyl-8-(4-methoxyphenyl)-4,4′-difluoroboradiazaindacene and 4-acetoxy benzyl bromide were synthesized as described in literature.[1,2]

Synthesis of S0: 4-methoxyphenyl BODIPY derivative (1,3,5,7-Tetramethyl-8-(4-methoxyphenyl)-4,4′-difluoroboradiazaindacene) was synthesized as described in literature.[1] 1,3,5,7-Tetramethyl-8-(4-methoxyphenyl)-4,4′-difluoroboradiazaindacene (1.19 mmol, 420 mg) was dissolved in 5 mL benzene. 4-pyridine carboxaldehyde (4.26 mmol, 400 μL), acetic acid (AcOH, 0.45 mL) and piperidine (0.40 mL) were added. The solution was refluxed using a Dean Stark apparatus for about 1h until the color turned into blue green. Product formation was checked with Thin Layer Chromatography (TLC) using acetone and hexane mixture as mobile phase (1:2, V:V). When the starting BODIPY compound was completely consumed, the reaction mixture was extracted with dichloromethane and water. Organic layer was collected, dried over Na_2SO_4. Solvent was evaporated under vacuo and the crude sample was purified with silica column chromatography using acetone and hexane mixture as mobile phase (1:2, V:V). The blue-green product was obtained with 3.7% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, J = 6.2 Hz, 4H), 7.82 (d, J = 16.3 Hz, 2H), 7.40 (d, J = 6.3 Hz, 4H), 7.16 (d, J = 8.7 Hz, 2H), 7.09 (d, J = 16.4 Hz, 2H), 6.99 (d, J = 8.7 Hz, 2H), 6.62 (s, 2H), 3.83 (s, 3H), 1.46 (s, 6H).



Scheme S1. Synthesis of probes. i) 4-pyridine carboxaldehyde, piperidine, acetic acid, benzene, reflux with Dean-Stark apparatus; ii) 4-acetoxybenzyl bromide, DMF, RT; iii) 4-nitrobenzyl bromide, MeCN, 80ºC; iv) 4-nitrobenzyl bromide, DMF; v) 4-acetoxybenzyl bromide, DMF, RT.

Synthesis of S1: **S0** (56 μmol, 30 mg) was dissolved in 5 mL dimethyl formamide (DMF). 5 mole equivalent 4-acetoxy benzyl bromide was added. Reaction mixture was stirred at room temperature (RT) for 2 days. Solvent was partially removed through rotary evaporator and ethyl acetate was added to precipitate the product. Solid products were washed with ethyl acetate several times. Dark green products were obtained in quantitative yields.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.16 (d, J = 6.5 Hz, 4H), 8.25 (d, J = 6.3 Hz, 4H), 7.94 (d, J = 16.4 Hz, 2H), 7.88 (d, J = 16.3 Hz, 2H), 7.61 (d, J = 8.6 Hz, 4H), 7.40 (d, J = 8.5 Hz, 2H), 7.32 – 7.02 (m, 8H), 5.87 (s, 4H), 3.86 (s, 3H), 2.28 (s, 6H), 1.54 (s, 6H).

MS (ESI): m/z Theoretical value: 415.17255 $(M)^{2+}$; experimental value: 415.16751 (Δ: 12.1 ppm).

MS (ESI): m/z Theoretical value: 911.27020 $(M+Br^-)^+$; experimental value: 911.26048 (Δ: 10.67 ppm).

Synthesis of S2: **S0** (56 μmol 30 mg) was dissolved in 10 mL acetonitrile (MeCN). 4-nitrobenzyl bromide (0.22 mmol 48 mg) was added and the mixture was stirred at 80°C for 2 days. The solvent was concentrated under vacuo and 1 mL ethyl acetate was added to precipitate the product. Solid product was washed several times with ethyl acetate. Dark green products were obtained in quantitative yields.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.11 (d, J = 6.5 Hz, 4H), 8.29 (d, J = 8.3 Hz, 4H), 8.25 (d, J = 6.6 Hz, 4H), 7.93 (d, J = 16.8 Hz, 2H), 7.86 (d, J = 16.5 Hz, 2H), 7.75 (d, J = 8.5 Hz, 4H), 7.38 (d, J = 8.3 Hz, 2H), 7.17 (m, 4H), 6.00 (s, 4H), 3.84 (s, 3H), 1.52 (s, 6H).

MS (ESI): m/z theoretical value: 402.15215 $(M)^{2+}$; experimental value 402.14996 (Δ: 5.4 ppm).

Synthesis of M1: 60 mg (0.11 mmol) **S0** was dissolved in DMF (5 mL). 7 mg (33 μmol, 0.3 mole equivalent) 4-nitrobenzyl bromide was added. The reaction mixture was stirred at RT for 16 h and the progress of the reaction was checked with TLC using dichloromethane and methanol mixture as mobile phase (90:10; V:V). Reaction mixture was purified using silica column chromatography and dichloromethane and methanol mixture as mobile phase (90:10; V:V). Recovered **S0** was further reacted using the same procedure and this reaction step was repeated 6 times. 40 mg dark green product was obtained with 54% yield after all the repeated steps.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.08 (d, J = 6.2 Hz, 2H), 8.68 (b, 2H), 8.39 – 8.22 (m, 5H), 7.98 (d, J = 16.3 Hz, 1H), 7.86 – 7.67 (m, 4H), 7.55 (b, 2H), 7.39 (d, J = 8.2 Hz, 2H), 7.18 (s, 2H), 7.14 (d, J = 16.1 Hz, 2H), 6.00 (s, 2H), 3.86 (s, 3H), 1.54 (s, 3H), 1.51 (s, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) 160.7, 154.6, 152.4, 151.1, 148.3, 145.7, 145.5, 143.1, 142.7, 142.1, 137.2, 130.3, 129.8, 125.8, 125.3, 124.7, 121.6, 120.8, 115.3, 55.8, 55.4, 15.2, 14.9.

MS (ESI): m/z theoretical value: 668.26450 $(M)^+$; experimental value 668.26857 (Δ: 6.09 ppm).

Synthesis of M2: Compound **M1** (7 μmol 5 mg) was dissolved in 5 mL dimethyl formamide (DMF). 6 mole equivalent 10 mg 4-acetoxybenzyl bromide was added and the reaction was allowed to stir at RT for 2 days. When the compound **M1** was consumed, DMF evaporated under vacuo. The solid

product was washed with ethyl acetate several times. Dark green product was obtained in quantitative yield.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.27 (dd, J = 6.7, 4.8 Hz, 4H), 8.40 – 8.16 (m, 6H), 8.04 – 7.90 (m, 4H), 7.85 (d, J = 8.8 Hz, 2H), 7.67 (d, J = 8.7 Hz, 2H), 7.38 (d, J = 8.5 Hz, 2H), 7.30 – 7.20 (m, 4H), 7.16 (d, J = 8.8 Hz, 2H), 6.13 (s, 2H), 5.94 (s, 2H), 3.84 (s, 3H), 2.27 (s, 3H), 1.49 (s, 6H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 169.6, 163.4, 162.8, 160.8, 157.5, 152.1, 151.7, 151.6, 148.3, 145.7, 145.4, 144.6, 142.0, 135.9, 135.3, 132.4, 132.1, 130.5, 130.4, 129.8, 128.5, 125.5, 124.6, 124.3, 123.1, 121.4, 115.4, 55.8, 34.8, 21.3.

MS (ESI): m/z theoretical value: 408.66181 $(M)^{2+}$; experimental value 408.66540 (Δ: 8.78 ppm).

3.2 Spectral Characterization

Enzymes used for analysis were nitroreductase enzyme (NTR, Sigma-Aldrich, E. Coli, N9284) and carboxylesterase enzyme (E, ECN: 3.1.1.1, Porcine Liver, Sigma, 15 U/mg). Together with nitroreductase enzyme, NADH was used as electron donating cofactor. Stock solutions of Probes **S0**, **S1**, **M1** and **M2** were prepared in DMSO, stock solution of **S2** was prepared in acetonitrile. For each experimental analysis, absorbance of the stock solutions is checked to determine exact concentrations from the Beer-Lambert Law (Formula 1).

$$A = \varepsilon C l \qquad \text{(Formula 1, Beer-Lambert Law)}$$

Where, A is the absorbance value, ε is extinction coefficient, C is concentration, *l* is 1 cm light path of the quartz cuvette. For **S1**, enzyme detection limit (EDL) was determined using Formula 2. Same analysis with **S2** cannot be done since this enzyme displays very slow and incomplete conversion.

$$EDL = 3\sigma/s \qquad \text{(Formula 2)}$$

Where σ is the standard deviation of experimental values of lowest experimental value (0.5U enzyme applied probe), s is the slope of the graph plotted with respect to enzyme concentration. Fluorescence quantum yields of the compounds were measured using Zinc Phthalocyanine (ZnPc) as reference compound and calculations were done using the Formula 3:[3]

$$\Phi_F(s) = \Phi_F(r)\,(A_r\, I_s\, n_s^2)/(A_s\, I_r\, n_r^2) \qquad \text{(Formula 3)}$$

Where r refers to reference compounds, s refers to samples. Φ_F is the fluorescence quantum yield, and this value is 0.28 for ZnPc in ethanol. Samples were excited at 589 nm. I refers to the integrated

fluorescence area, which is calculated using Origin Software. n is the refractive index of the solvents. For ethanol this value is 1.361. Samples **S1** and **M2** were dissolved in DMSO, the n value for this solvent is 1.4793. **S2** is dissolved in acetonitrile, the n value for this solvent is 1.344.

Time resolved fluorescence lifetime spectroscopy analysis were performed at National Nanotechnology Research Center, Bilkent University using Horiba Jobin Yvon Nanoled source. LUDOX (AS-40 colloidal silica 40 wt. % suspension in water) was used to obtain instrument response function. For **S0**, 625 nm LED light source was used. For **M1**, 650 nm laser diode was used as light source and finally, for **S1**, **S2** and **M2** 657 nm laser diode was used as light source.

Probes are not soluble in apolar solvents such as hexane. Solubility is low in ethyl acetate. Spectroscopic analysis and selectivity of the probes for the most abundant cellular components were analyzed in 25% water in DMSO for **S1** and **M2** and for **S2** acetonitrile is used for that purpose. To visualize **S0** formation effectively a relatively high organic solvent ratio is preferred. Fluorescence spectra are recorded by exciting the samples at 600 nm unless otherwise stated. To measure the response of **S1** to abundant cellular components, 3.75 μM probe, 10 U/mL carboxylesterase enzyme, 0.5 mM of glucose, 120 mM NaCl, 20 mg/mL BSA, 0.1 mM GSH were used.

3.3 Preparing Drug Resistant HEP3B Cells

Drug resistant human liver cells (HEP3B R (+)) was prepared through long term exposure of cells with increasing doses of sorafenib drug. The same drug dose was repeatedly given to cells for weeks and the dose increased slightly. RNA isolation was done when the dose reaches 2.75 μM to check the level of drug resistance marker gene Multi-Drug Resistance 1 (MDR1) which encodes membrane pump responsible for drug efflux.[4] Statistically significant overexpression of this gene compared to untreated HEP3B cells is considered as gain of resistance. Details of gene expression analysis are given below.

3.4 Cell Viability Analysis

HEP3B human hepatocellular carcinoma cells were counted using Thoma counting chamber and $5x10^4$ cells/well were seeded on 96-well plate in HG-DMEM supplemented with 10% FBS and 0.1% gentamycin and incubated at 37^{o}C in humidified incubator for at least 24 h. Then different doses of the probes were given to the cells from the stock samples. Probe stocks were prepared in DMSO and diluted in 100 μL/well cell medium. The final DMSO concentrations in the cell do not exceed 1%. Cells were further incubated for 24 h. 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT, 5 mg/mL) was added to each well and the cells were further incubated in the dark for 4h. 100 mL DMSO was added to each well to dissolve resulting formazan crystals. After 15 min incubation absorbance at 570 nm was recorded using ELISA plate reader (Bio-tek Instruments, ABD). Experiments were done with at least three replicates. Average values of each dose were normalized to untreated cell control group to determine relative viability. The Formula 4 is used to determine percent cell viability:

$$\% \text{ Cell Viability} = A_s/A_r*100 \qquad \text{(Formula 4)}$$

Where, A_s is the absorbance of the dose applied cells at 570 nm and A_r is the absorbance of untreated cells at 570 nm.

Same analyses were performed with sorafenib resistant HEP3B cells.

3.5 Gene Expression Analysis

Sorafanib drug resistance of HEP3B is confirmed by MDR1 gene expression analysis and the level of Human Carboxylesterase enzyme (HCE2) in resistant cells are also verified by Real Time Polymerase Chain Reaction (RT-PCR) method. Gene expression was analyzed when the sorafenib dose reaches 2.75 μM concentration. To isolate RNA from cells, cells were seeded on 6-well plate in HG-DMEM supplemented with 10% FBS and 0.1% gentamycin and incubated at 37^{o}C for at least 24 h. Medium of the wells were discarded and 500 μL Trizol was added to each well followed by 10 min incubation at RT. Cells were scraped with a scraper and transferred to an Eppendorf. 100 μL chloroform was added, and the sample was incubated at RT for 15 min. Tubes were centrifuged at 14.000 rpm for 20 min at 4^{o}C. Supernatant was transferred to a new Eppendorf and 200 μL isopropanol and 200 μL 10 M NaCl solution were added. Following 10 min incubation at RT, tubes were centrifuged at 14.000 rpm for 15 min at 4^{o}C. Supernatant was discarded. The Pellet was washed with 500 μL cold ethanol, centrifuged at 10.000 rpm for 10 min. and the procedure was repeated for 3 times. The pellet was dried and dissolved in 40 μL RNase free water. To remove DNA contamination sample was treated with DNase and RNA was quantified using a nanodrop. cDNA was synthesized using commercial c-DNA synthesis kit (Applied Biosystems™) and RT-PCR was performed using CFX96 Touch Real-Time PCR Detection System (BioRAD) and SYBR Green Supermix kit (Bio-Rad). The primer set used for PCR analysis is given in Table S2. β-Actin was used as reference housekeeping gene.

The Hypoxic microenvironment was verified by elevated expression of HIF1α. After 24 h $CoCl_2.6H_2O$ application to mimic hypoxic microenvironment,[5] RNA was isolated from HEP3B cells using GeneJEt RNA purification kit (Thermo Scientific). cDNA was synthesized (Takara: 6110A-50) and relative gene expression was analyzed with RT-PCR (SYBR Green Supermix, Bio-Rad). β-Actin was used as reference housekeeping gene.

Table S2. Primer sequences

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
MDR1	CCCATCATTGCAATAGCAGG	TGTTCAAACTTCTGCTCCTGA
HCE2	AACCTGTCTGCCTGTGACCAAGT	ACATCAGCAGCGTTAACATTTTCTG
HIF1α	TAT GAG CCA GAA GAA CTT TTA GGC	CAC CTC TTT TGG CAA GCA TCC TG
ACTB	CAC CAT TGG CAA TGA GCG GTTC	AGG TCT TTG CGG ATG TCC ACG T

3.6 Fluorescence Microscopy Imaging

For fluorescence cell imaging with **S1** sensor, human hepatocyte (HEP3B) cells are seeded on 6-well plate with a cell density of $20x10^5$ cells/well using the High-glucose DMEM medium supplemented with 10% FBS and 0.1% gentamycin antibiotic. Cells were incubated in humidified incubator at 37°C for at least 24 h. At this point, one group of cells were treated with benzil (200 μM, esterase inhibitor) for 2h under the same incubation conditions and others are untreated.[6] Then, 4 μM **S1** is applied to all wells. After 30 min incubation, cells are washed with Phosphate Saline Buffer (PBS) three times and cells are fixed with 4% formaldehyde for 20 min. Cells are washed again with PBS three times and the cells are incubated with nucleus stain DAPI (7.5 μM) for 10 min. After washing three times with PBS, 1 mL PBS was added and cell images are obtained using Zeiss microscopy, Axiocam 305 camera. Spectra are recorded selecting DAPI (excitation wavelength 353 nm, emission wavelength 465 nm) and AF610 channels (excitation wavelength 612, emission wavelength 630 nm).

For **S2** probe analysis, HEP3B cells are seeded on 6-well plate with a cell density of cells/well using the High-glucose Dulbelco Modified Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 0.1% gentamycin antibiotic. One group of cells are applied to hypoxic microenvironment for 24 h prior to probe application. For this purpose, 100 μM $CoCl_2.6H_2O$ was applied to cell medium.[5] After at least 16 h, **S2** (3.2 μM) was given to both normoxic and hypoxic cells for 75 min under before mentioned incubation conditions. Then after washing three times with PBS, cells were fixed with 4%

formaldehyde for 20 min. Cells are washed again with PBS three times and the cells are incubated with nucleus stain DAPI (7.5 μM) for 10 min. After washing three times with PBS, 1 mL PBS was added and cell images are obtained using Zeiss microscopy, Axiocam 305 camera. Spectra are recorded selecting DAPI (excitation wavelength 353 nm, emission wavelength 465 nm) and AF610 channels (excitation wavelength 612, emission wavelength 630 nm).

Finally for the cellular fluorescence analysis of **M2** sensor (4 μM), either sorafenib resistant HEP3B cells (R (+)) or normal HEP3B cells (R (-)) are used. Cells are either exposed to hypoxic environment at least 16 h prior to probe addition using 100 μM $CoCl_2.6H_2O$ or incubated under normoxic conditions. For appropriate groups, esterase inhibitor benzil (200 μM) is added to the cell medium 2 h prior to probe addition. Following 30 min probe incubation cells are fixed with 4% formaldehyde for 20 min., washing three times with PBS followed by DAPI staining (7.5 μM) was performed as described for other probes and cell images are obtained using Zeiss microscopy, Axiocam 305 camera. Spectra are recorded selecting DAPI (excitation wavelength 353 nm, emission wavelength 465 nm) and AF610 channels (excitation wavelength 612, emission wavelength 630 nm).

For each sample, at least 4 independent cell photos are analyzed using ImageJ software to elucidate the relative fluorescence intensity. Stock solutions of all probes are prepared in DMSO and the probe is applied to cell culture medium in such a way that the final DMSO level is less than 1%.

3.7 Statistical Analysis

Experiments are performed with at least 3 replicates and the data is presented as mean values and standard deviations are displayed. The statistical significance of the compared data is analyzed using GraphPad Prism 10 software using unpaired student t test. P values smaller or equal to 0.05 are considered statistically significant (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

4. NMR and QTOF-LC/MS Characterizations of the Compounds

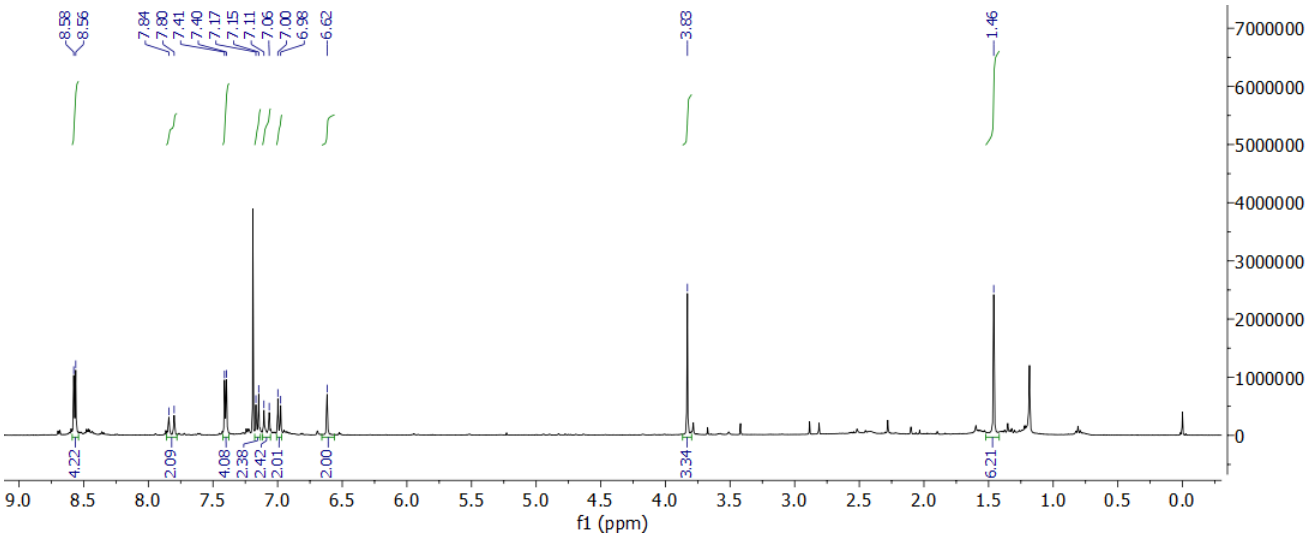


Figure S11. ^{1}H NMR spectrum of compound **S0** (400 MHz, $CDCl_3$).

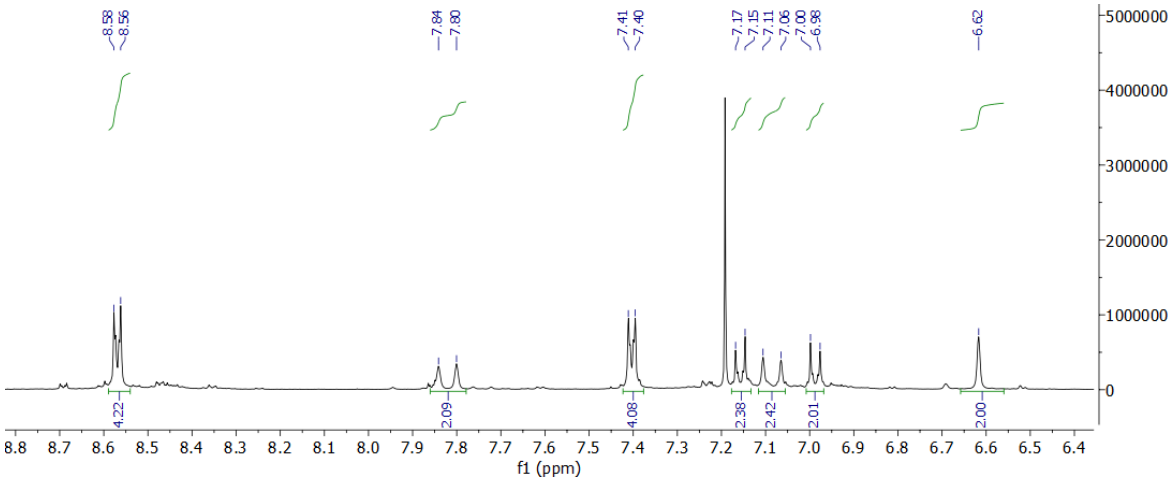


Figure S12. ^{1}H NMR spectrum of compound **S0** (aromatic region, 400 MHz, $CDCl_3$).

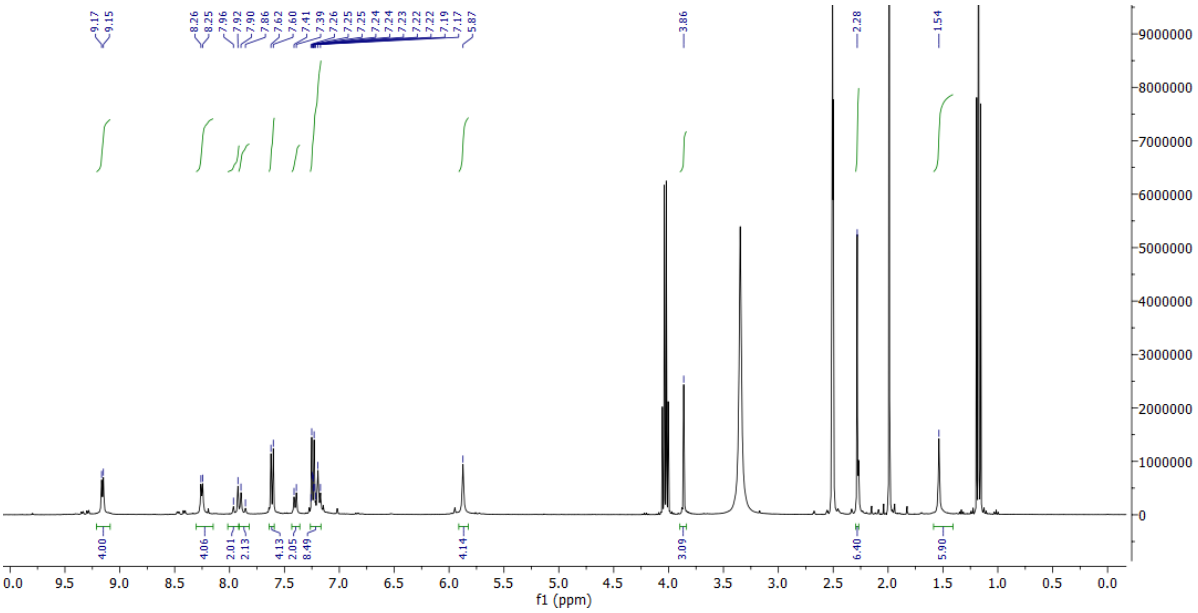


Figure S13. ^{1}H NMR spectrum of **S1** (400 MHz, DMSO-d_6).

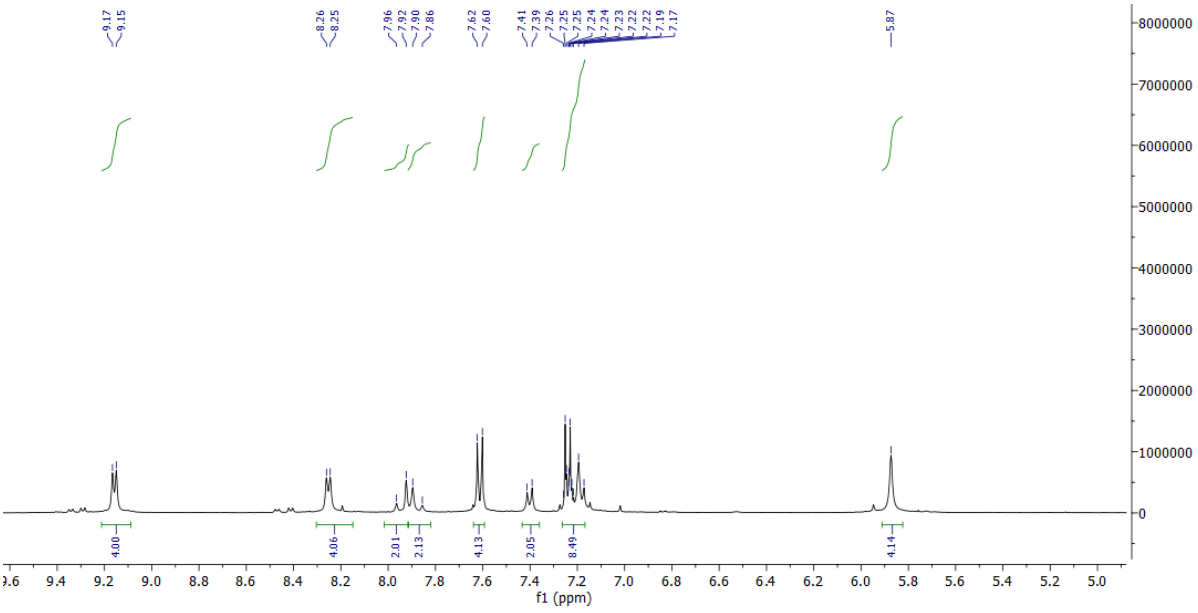


Figure S14. ^{1}H NMR spectrum of **S1** (aromatic region, 400 MHz, DMSO-d_6).

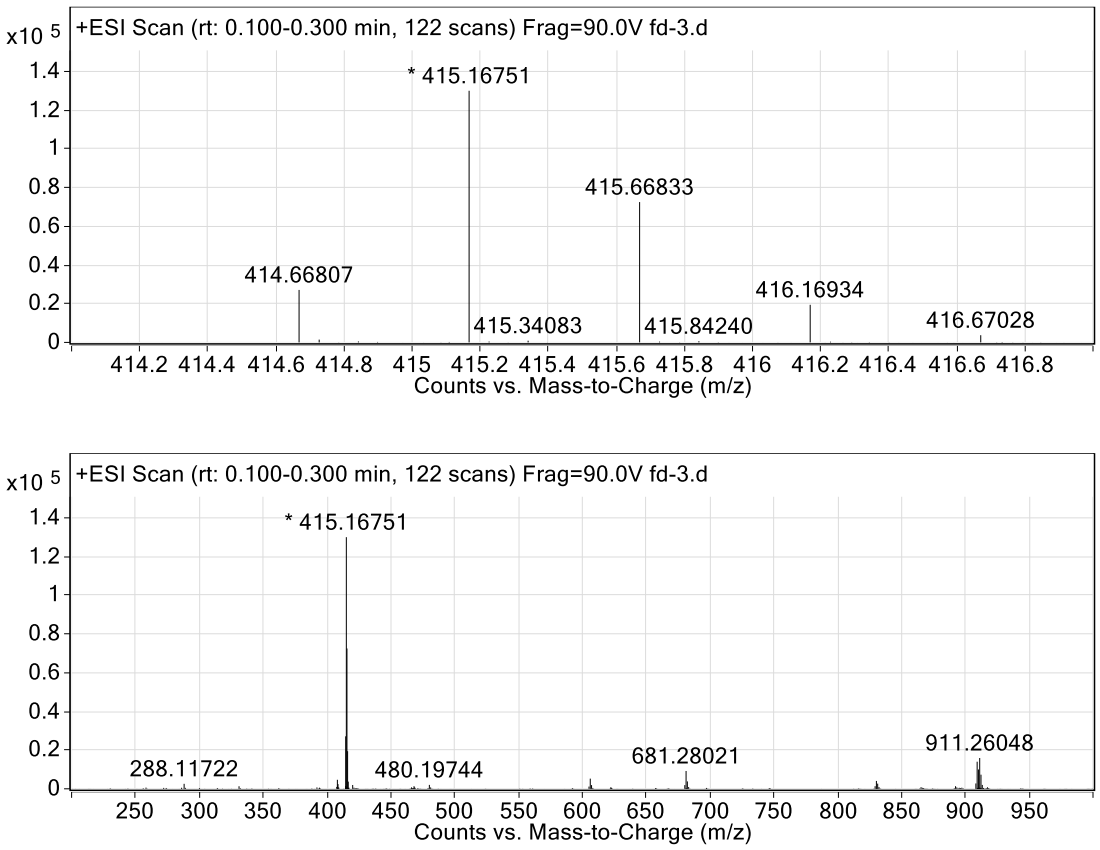


Figure S15. QTOF-LC/MS Spectra of **S1**

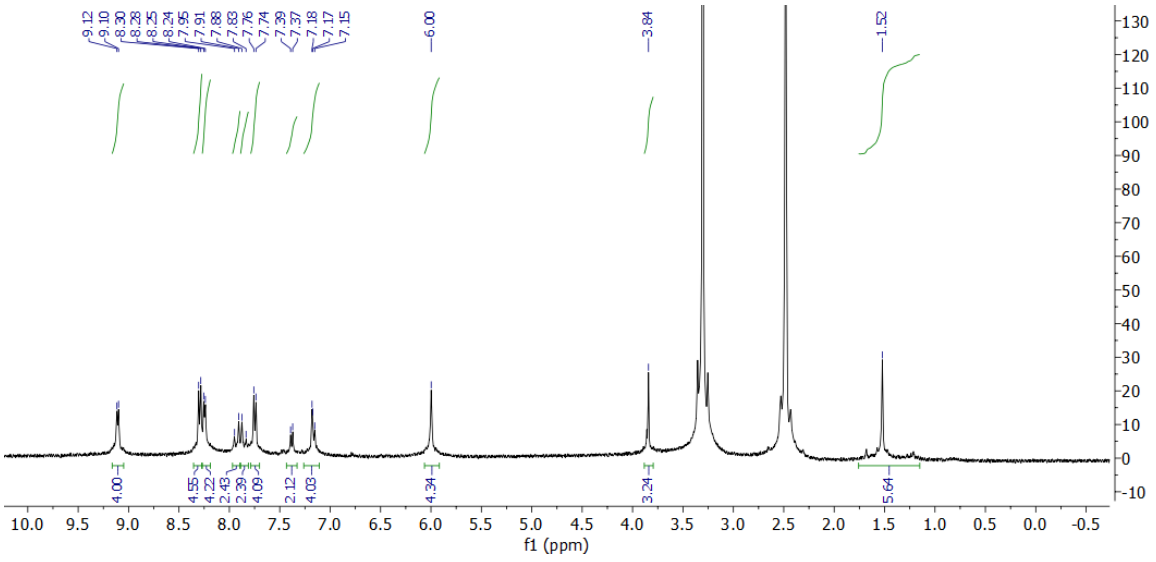


Figure S16. ^{1}H NMR spectrum of **S2** (400 MHz, DMSO-d_6).

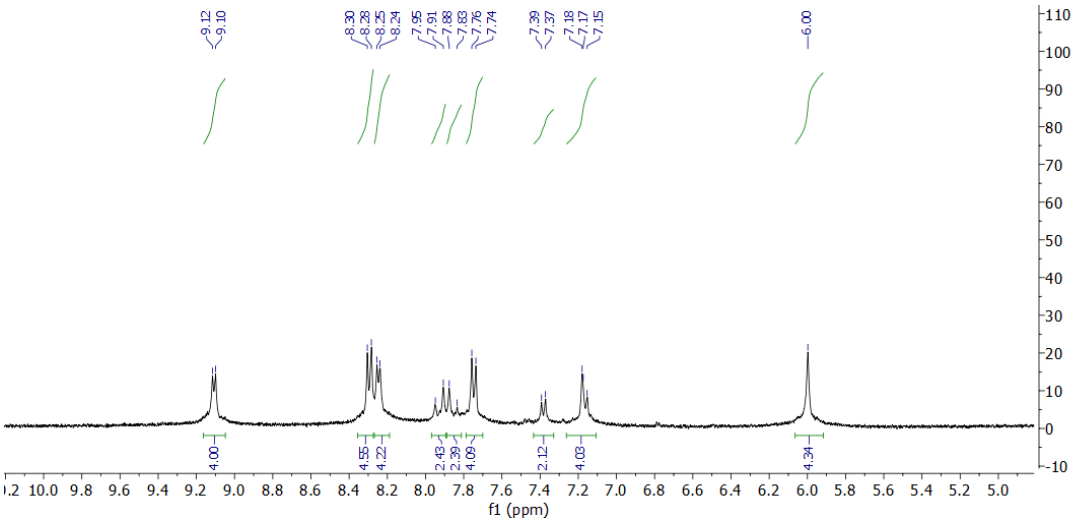


Figure S17. ^{1}H NMR spectrum of **S2** (aromatic region, 400 MHz, DMSO-d_6).

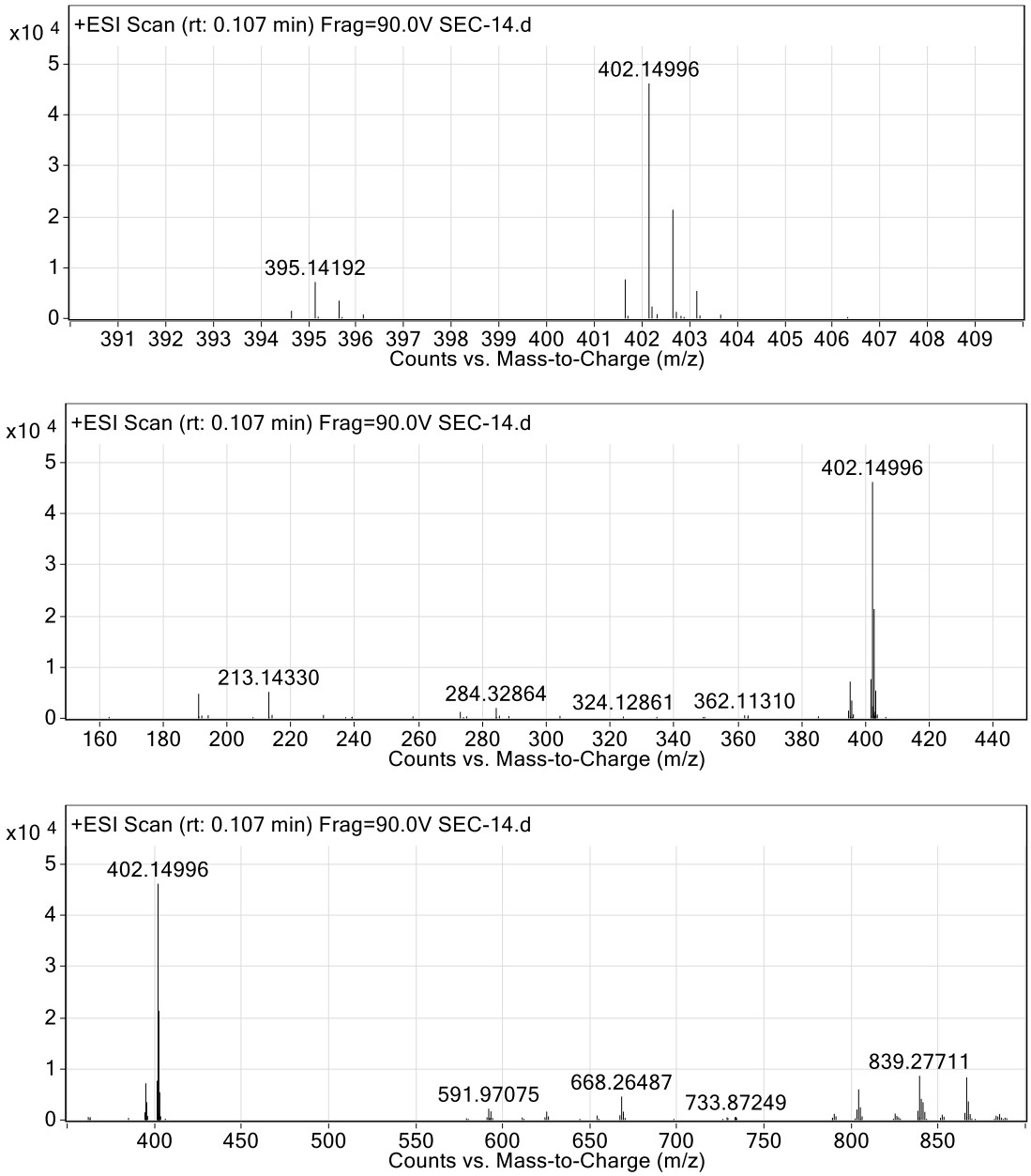


Figure S18. QTOF-LC/MS Spectra of **S2**

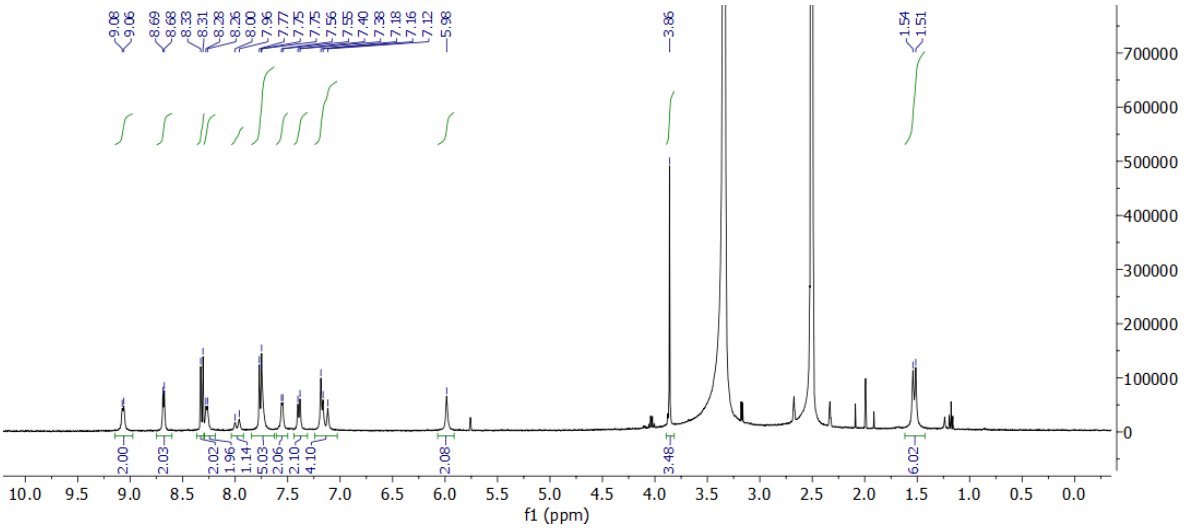


Figure S19. ^{1}H NMR spectrum of **M1** (400 MHz, DMSO-d_6).

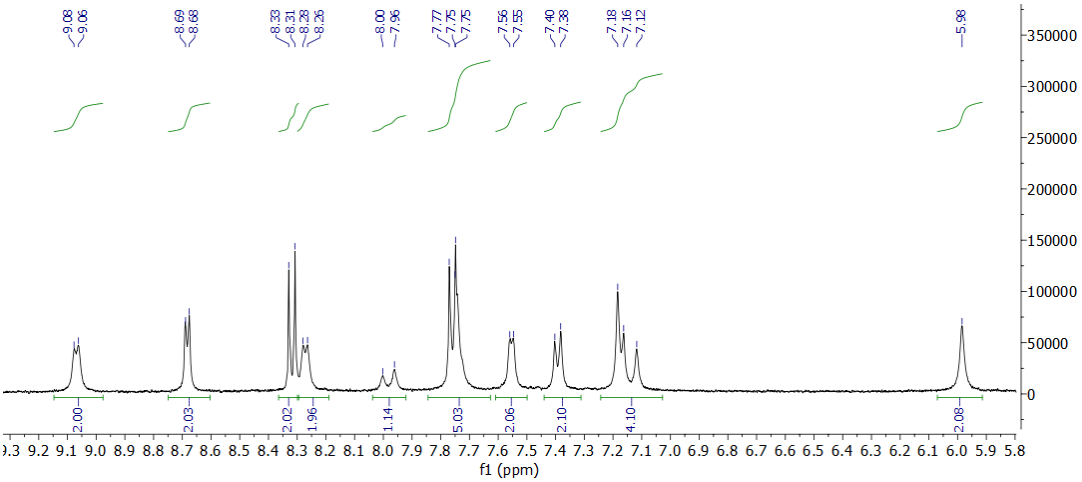


Figure S20. ^{1}H NMR spectrum of **M1** (aromatic region, 400 MHz, DMSO-d_6).

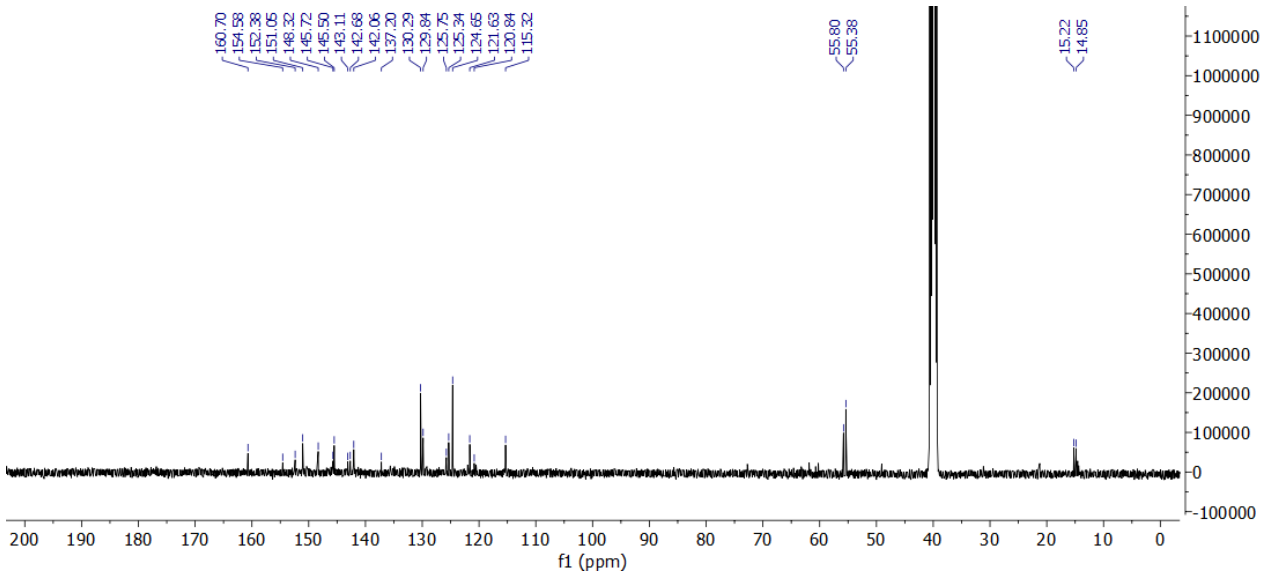


Figure S21. ^{13}C NMR spectrum of **M1** (aromatic region, 101 MHz, DMSO-d_6).

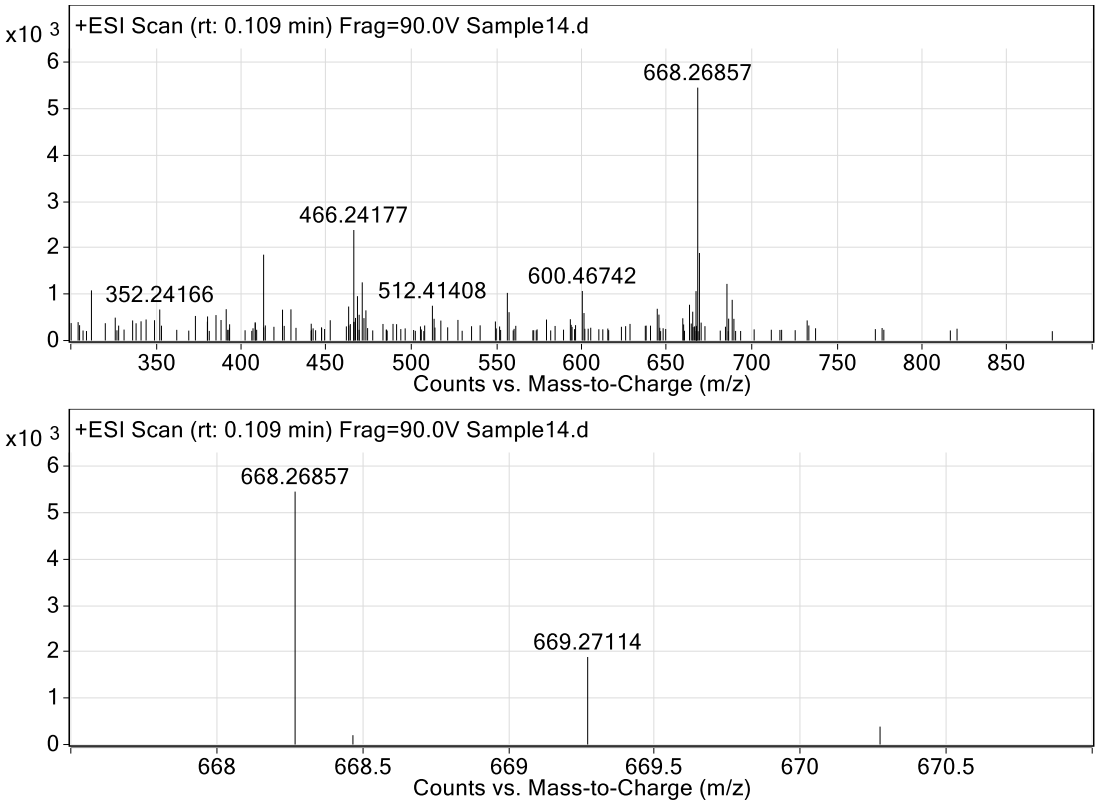


Figure S22. QTOF-LC/MS Spectra of **M1**

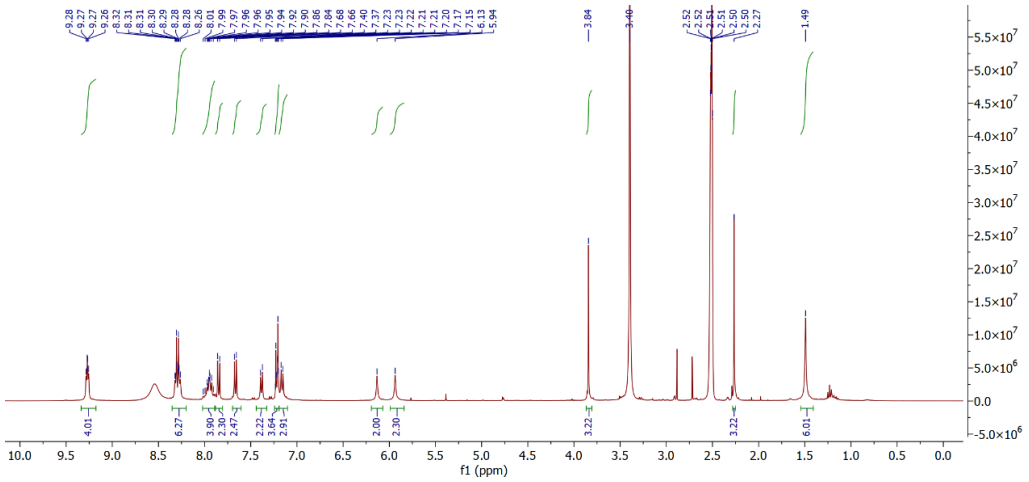


Figure S23. ^{1}H NMR spectrum of **M2** (400 MHz, DMSO-d_6).

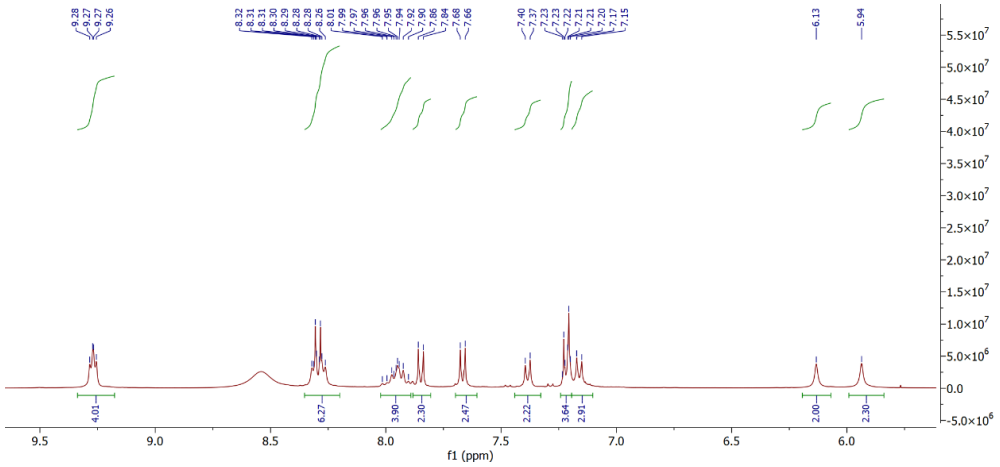


Figure S24. ^{1}H NMR spectrum of **M2** (aromatic region, 400 MHz, DMSO-d_6).

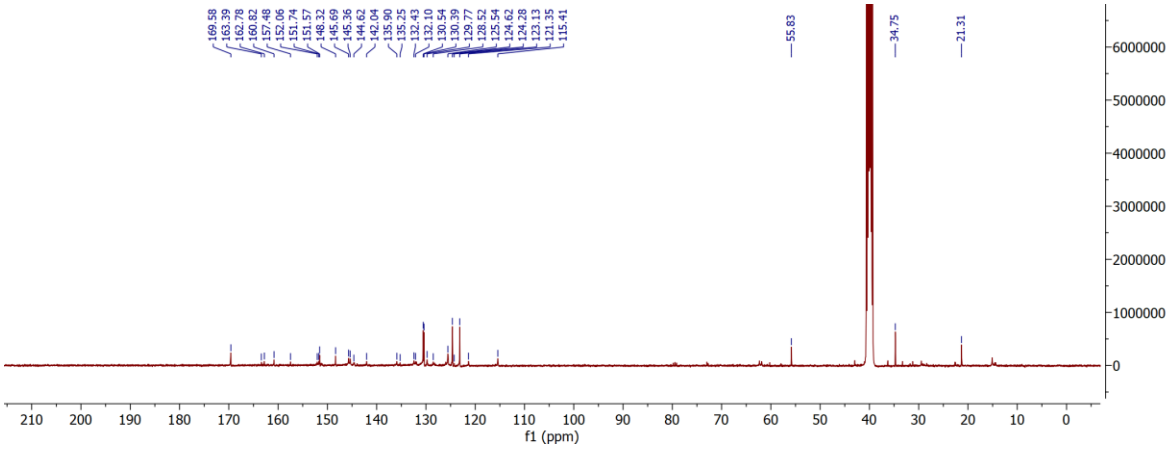


Figure S25. ^{13}C NMR spectrum of **M2** (aromatic region, 101 MHz, DMSO-d_6).

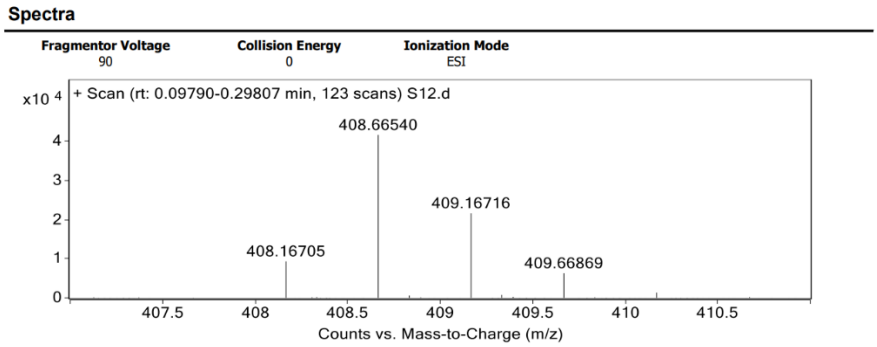


Figure S26. QTOF-LC/MS Spectra of **M2**

5. References

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