Supporting Information

Extra- vs. Intracellular Delivery of CO: Does it Matter for a Stable, Diffusible Gasotransmitter?

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Materials and Methods

Reagents. All reagents were obtained from commercial sources and used as received unless otherwise noted. Bovine serum albumin (BSA, heat shock fraction) was purchased from Sigma-Aldrich. Distilled or doubly distilled water (ddH_2O) was used in all experiments. Compound **1** was prepared following the literature procedure.[1] The procedure for the synthesis of **3** was adapted from a literature source.[2]

Physical Methods. Nuclear magnetic resonance spectra were collected on a Brüker Avance III HD 500 MHz NMR. Chemical shifts are reported relative to the residual solvent peaks of DMSO-d_6 (^{1}H: δ 2.50, ^{13}C: δ 39.52). Multiplicity is reported as follows: s (singlet); d (doublet); t (triplet); m (multiplet); *J* values are given in Hz. An IR spectrum of **3** was collected using a Shimadzu FTIR-8400 spectrometer. UV-vis spectra were recorded at ambient temperature using a CARY 50Bio or a Hewlett-Packard 8453A diode array spectrophotometer. Fluorescence emission spectra were collected using a Shimadzu RF-530XPC spectrometer in the range of 400-800 nm, with the excitation wavelengths corresponding to the absorption maxima of the analyte. The spectra were collected using 1.0 cm quartz cells with excitation and emission slit widths set at 1.5 or 3.0 nm. Mass spectral data were collected at the Mass Spectrometry Facility, University of California, Riverside. ESI/APCI mass spectra were recorded on an Agilent LCTOF (2006) with a Windows XP based operating system. A Rayonet photoreactor equipped with RPR-4190A lamps (8 lamps) was used for all photochemical reactions. Quantum yields were determined using ferrioxalate as a standard to measure photon flux (~10^{16}-10^{17} photons/second).[3-4] Carbon monoxide was quantified as previously described.[5] The RP-HPLC system consisted of a LC-2030 pump with a LC-2040 PDA detector and a C18 column (50 x 4.6 mm) was used in chromatographic studies.

Steady-State and Time-Resolved Emission data were collected at room temperature using an Edinburgh FLS980 spectrometer. The deaerated sample was first bubbled with N_2 for 30 minutes before spectra were obtained. The sample was excited using light output from a housed 450 W Xe lamp passed through a single grating (1800 l/mm, 250 nm blaze) Czerny-Turner monochromator. Emission from the sample was passed through a single grating (1800 l/mm, 500 nm blaze) Czerny-Turner monochromator and finally detected by a peltier-cooled Hamamatsu R928 photomultiplier tube. The dynamics of emission decay were monitored by using the FLS980's time-correlated single-photon counting capability (1024 channels; 100 ns window) with data collection for 5,000 counts. Excitation for TCSPC was provided by an Edinburgh EPL-405 ps pulsed light emitting diode (405 ± 10 nm, pulse width 57.6 ps) operated at 10 MHz. Emission was passed through a 420 nm long-pass filter and then a single grating (1800 l/mm, 500 nm blaze) Czerny−Turner monochromator and finally detected by a Peltier-cooled Hamamatsu R928 photomultiplier tube. Time-resolved emission data were fit using Edinburgh software package with an exponential reconvolution function fit using an IRF obtained from solvent and cuvette only. Quantum Yield data was collected at room temperature using a Hamamatsu Quantaurus- QY Spectrometer. The sample was excited using a 405 nm output from a 150 W Xenon arc lamp and detected by a cooled 1024 channel CCD detector.

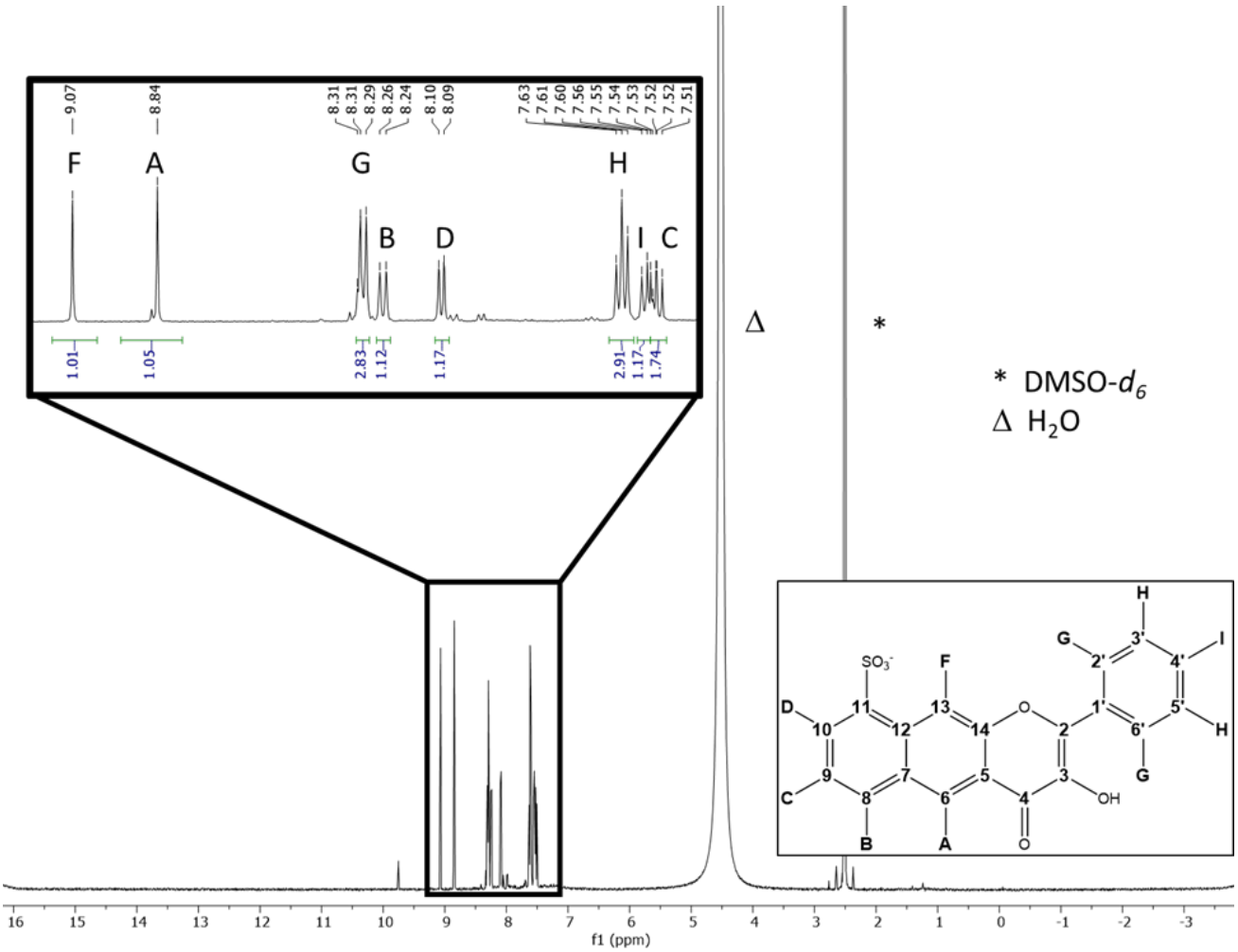


Figure S1A. ^{1}H NMR (500 MHz) spectrum of **3** in DMSO-d_6.

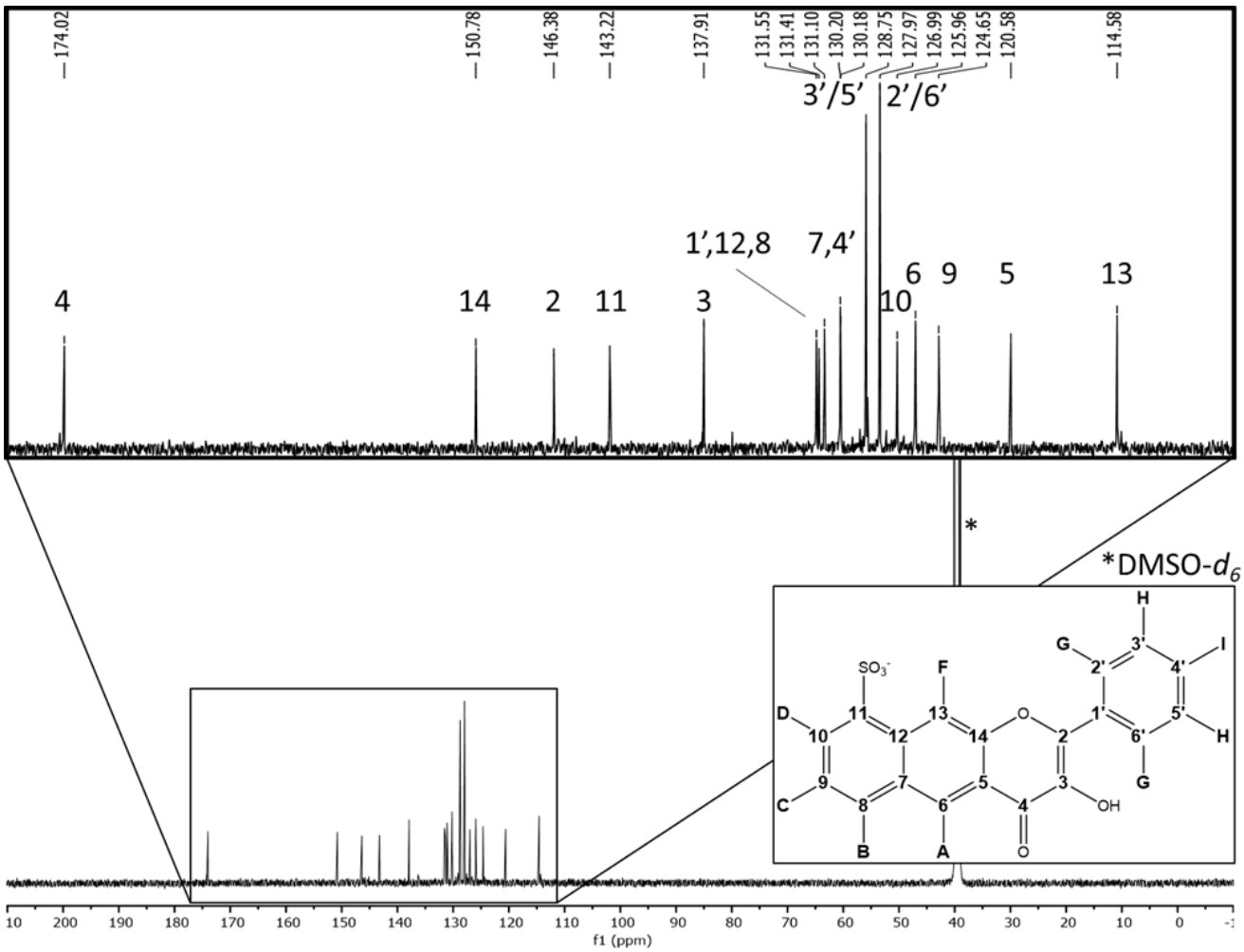


Figure S1B. $^{13}C\{^{1}H\}$ NMR (125 MHz) spectrum of **3** in DMSO-d_6.

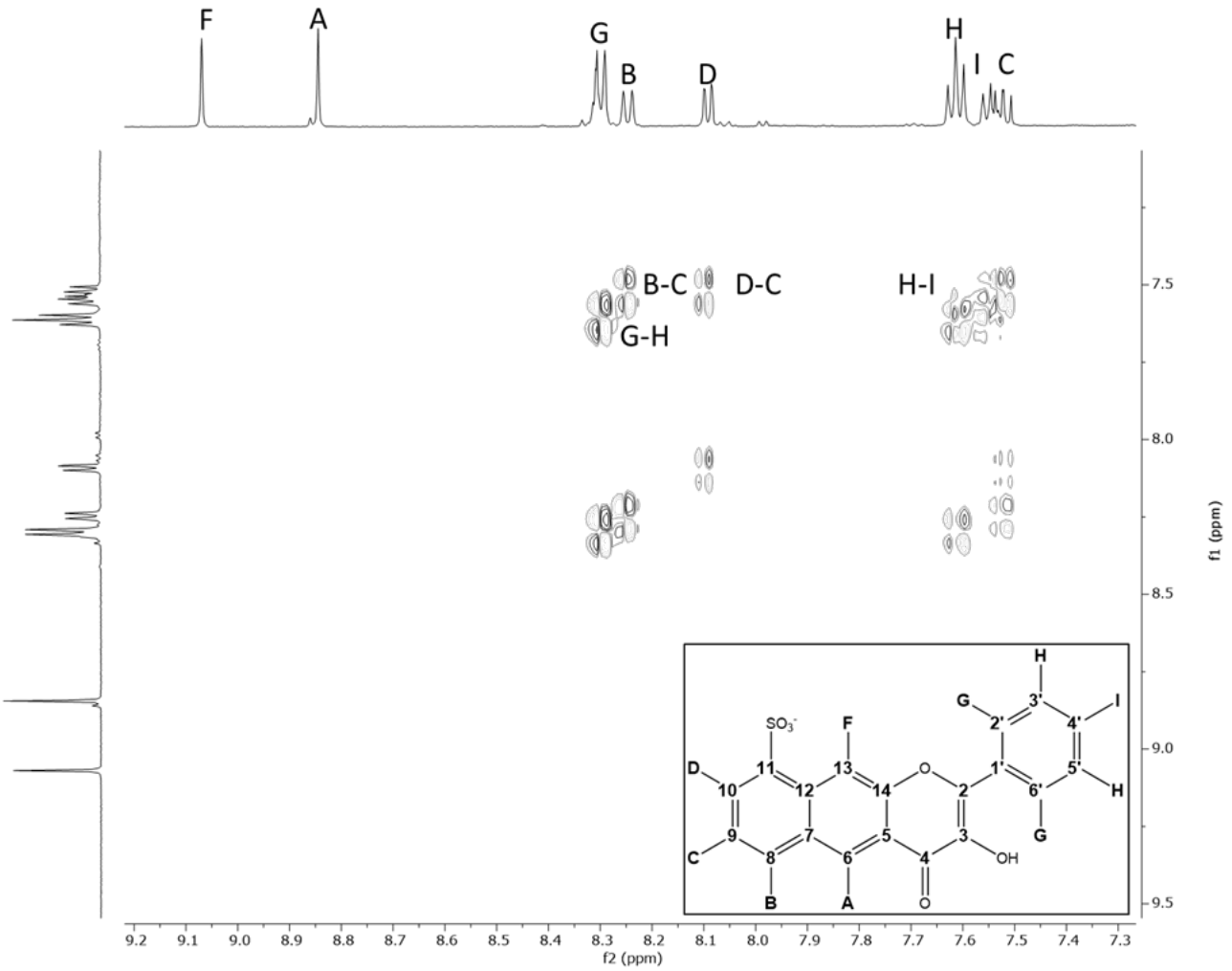


Figure S1C. 2D-COSY spectrum of **3** in DMSO-d_6.

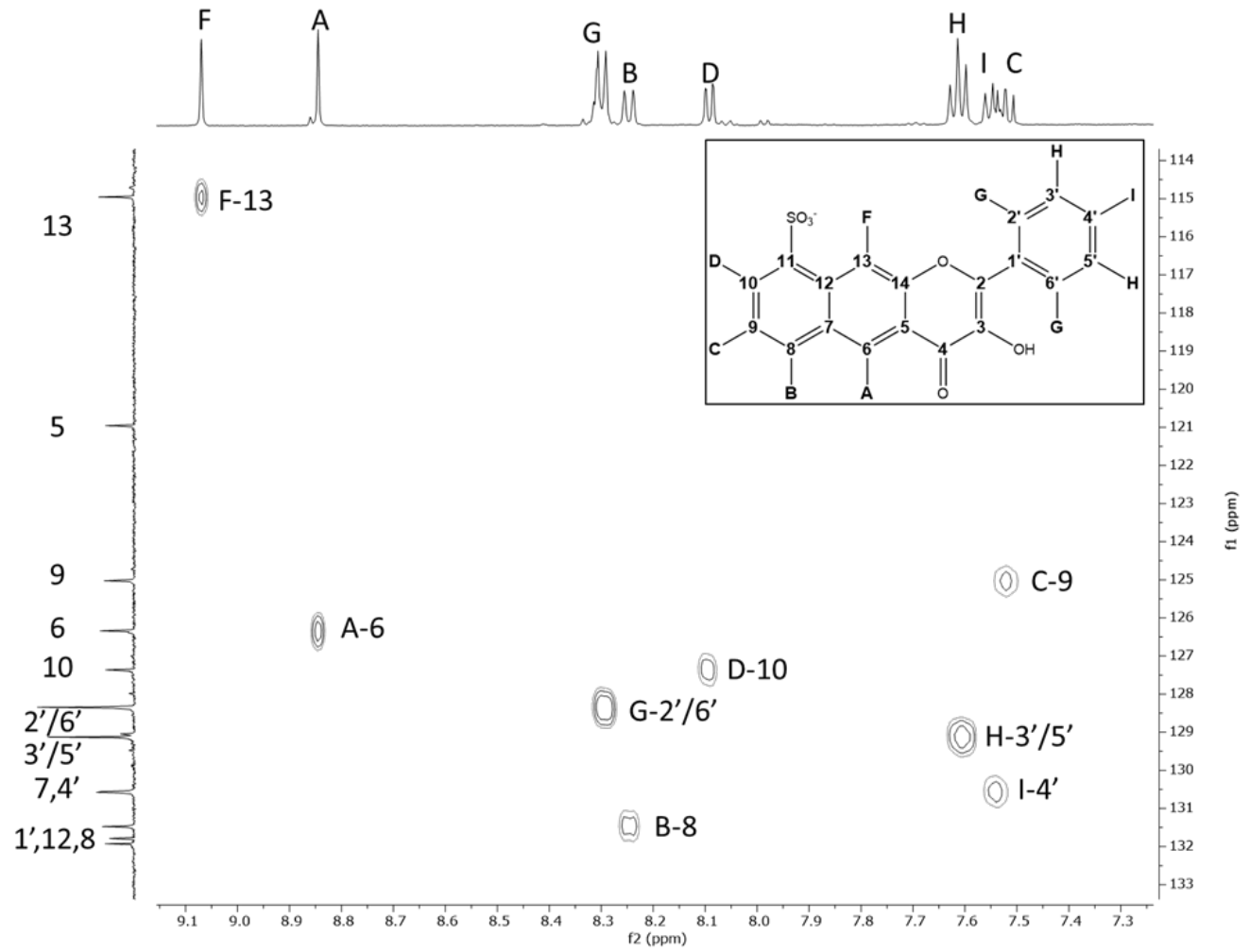


Figure S1D. HSQC spectrum of **3** in DMSO-d_6.

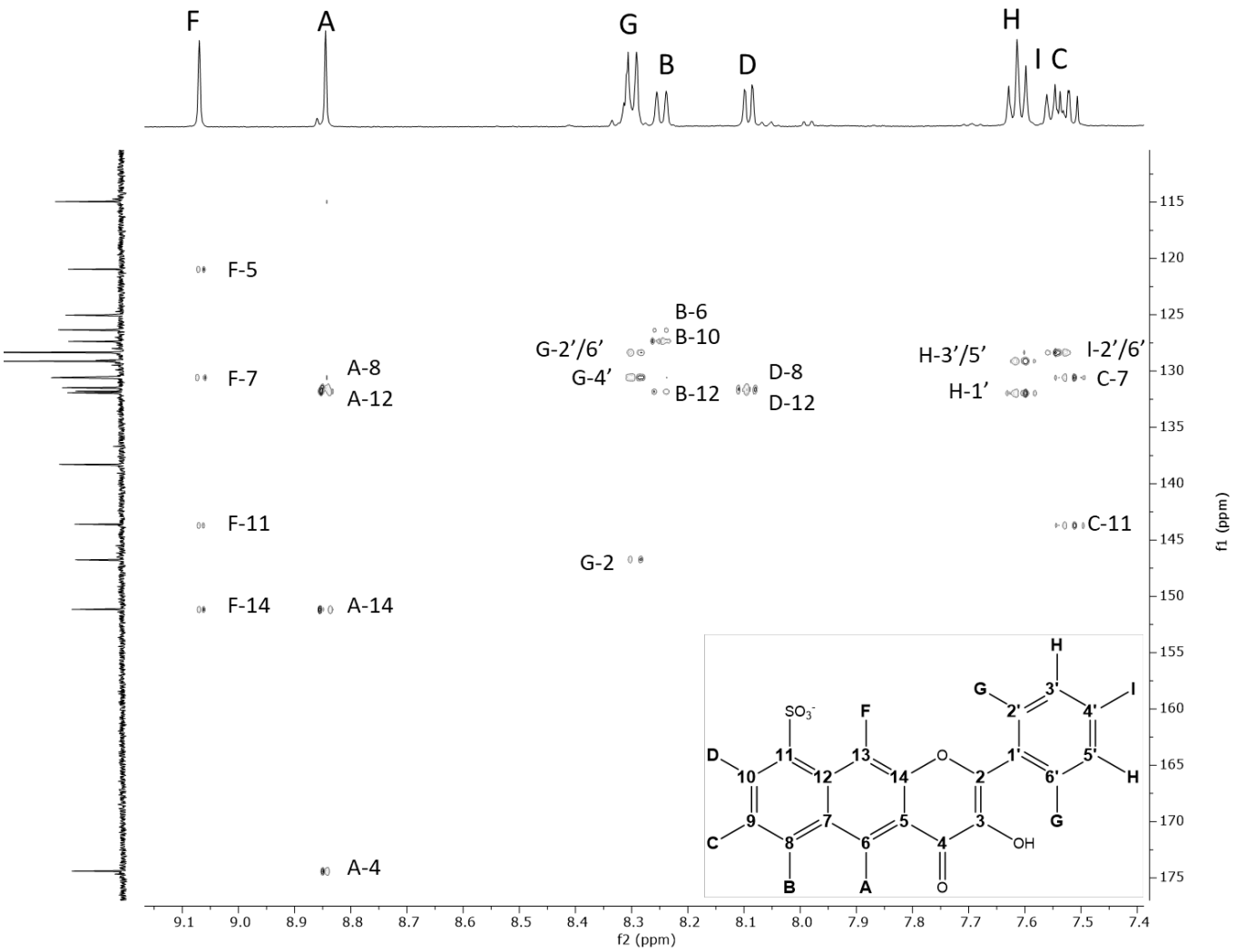


Figure S1E. HMBC spectrum of **3** in DMSO-d_6.

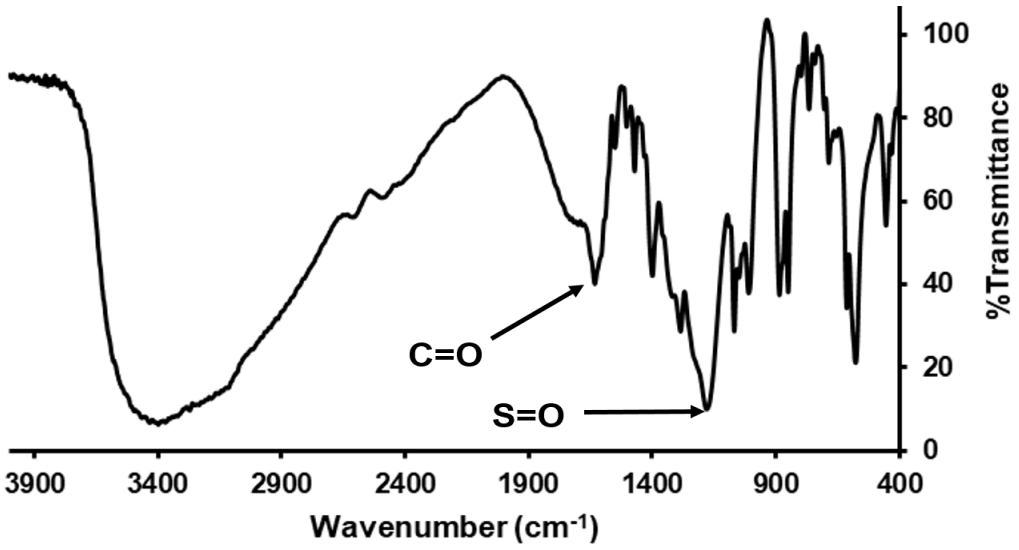
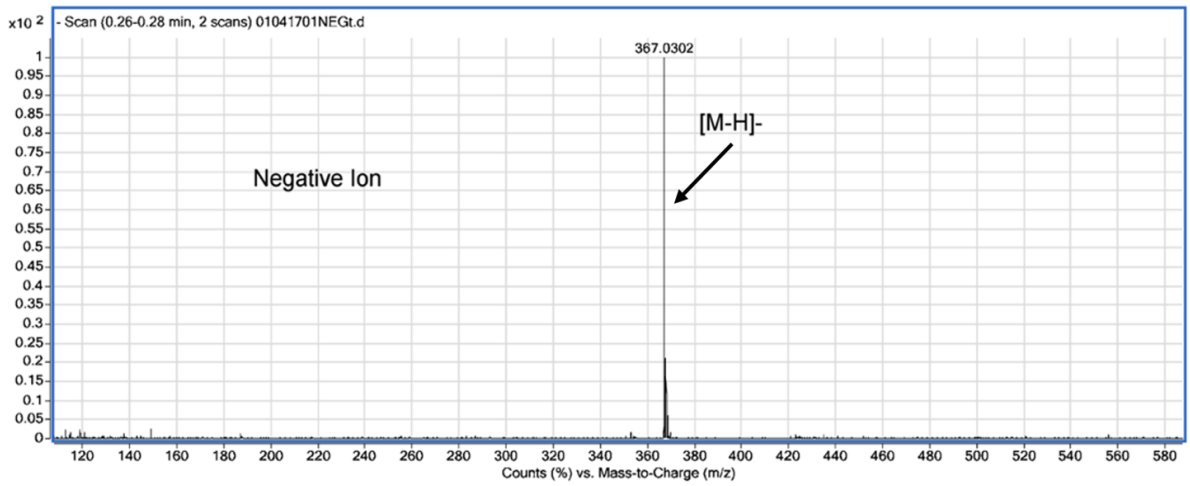


Figure S2. FTIR spectrum of **3** in KBr. Assignments of the $\nu_{C=O}$ (1637 cm^{-1}) and $\nu_{S=O}$ (1181 cm^{-1}) vibrations were made on the basis of literature precedent.[6] The IR spectrum also indicates a broad water band at ~3400 cm^{-1} which correlates with the presence of water in the sample, as is indicated by the elemental analysis (1.8 eq).



Measured Mass	368.038			
Element	Low Limit	High Limit		
C	5	30		
H	5	20		
S	0	2		
O	3	9		
Formula	Calculated Mass	mDaError	ppmError	RDB
C16 H16 O6 S2	368.0388	-0.8	-2.3	9
C19 H12 O6 S	368.0355	2.5	6.9	14

Figure S3. ESI/APCI-MS of **3.**

LC conditions: The RP-HPLC system consisted of a LC-2030 pump with a LC-2040 PDA detector and a C18 column (50 x 4.6 mm). The 50:50 (v/v) mixture of methanol-water (with 0.1% TFA in water) was used as the mobile phase in the chromatography experiment. The chromatographic test was conducted under the flow rate of 0.5 mL/min, the detection wavelength of 254 nm, the column temperature of 22.1 °C and the injection volume of 1 µL. In brief, a solution of **3** (2 mM) in ddH_2O was diluted to a final concentration of 20 µM and analyzed using HPLC.

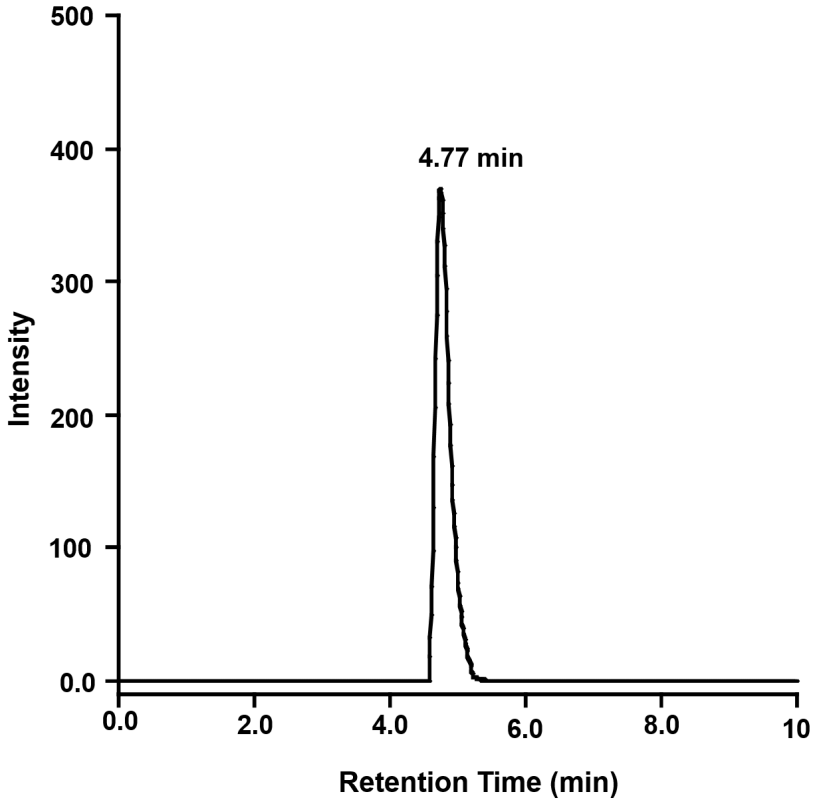


Figure S4. HPLC chromatogram of **3**.

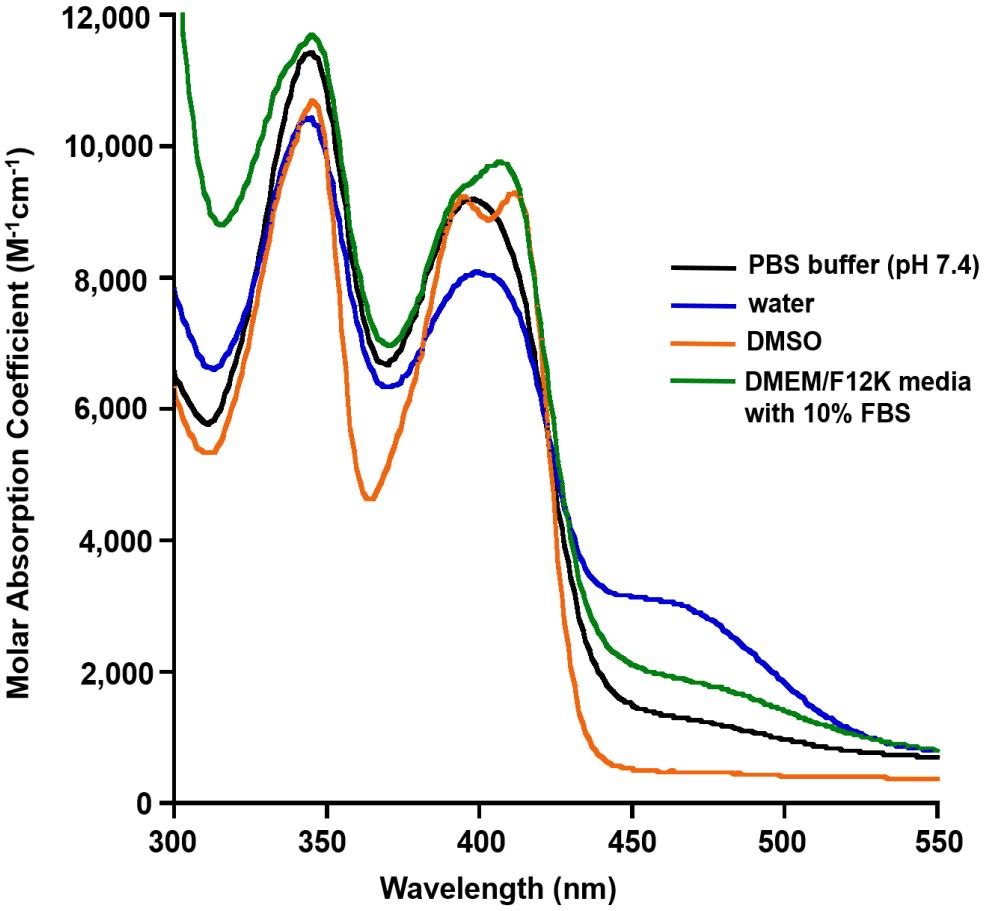


Figure S5. Absorption spectral features of **3** in various solvents. The increase in absorbance in the 450-500 nm range suggests that partial deprotonation of the 3-OH moiety of **3** may occur in aqueous environments.

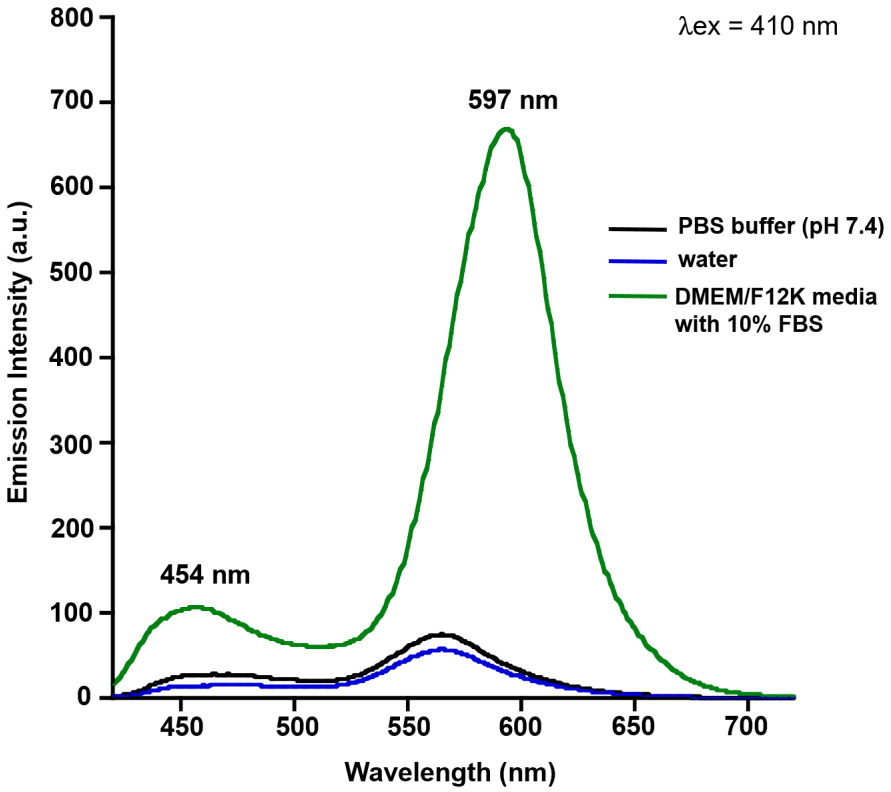


Figure S6. Emission spectra of **3** (0.1 mM) in PBS (10 mM, pH 7.4), water and DMEM/F12K media with 10% FBS (λ_{ex} = 410 nm, slit width = 3.0 nm).

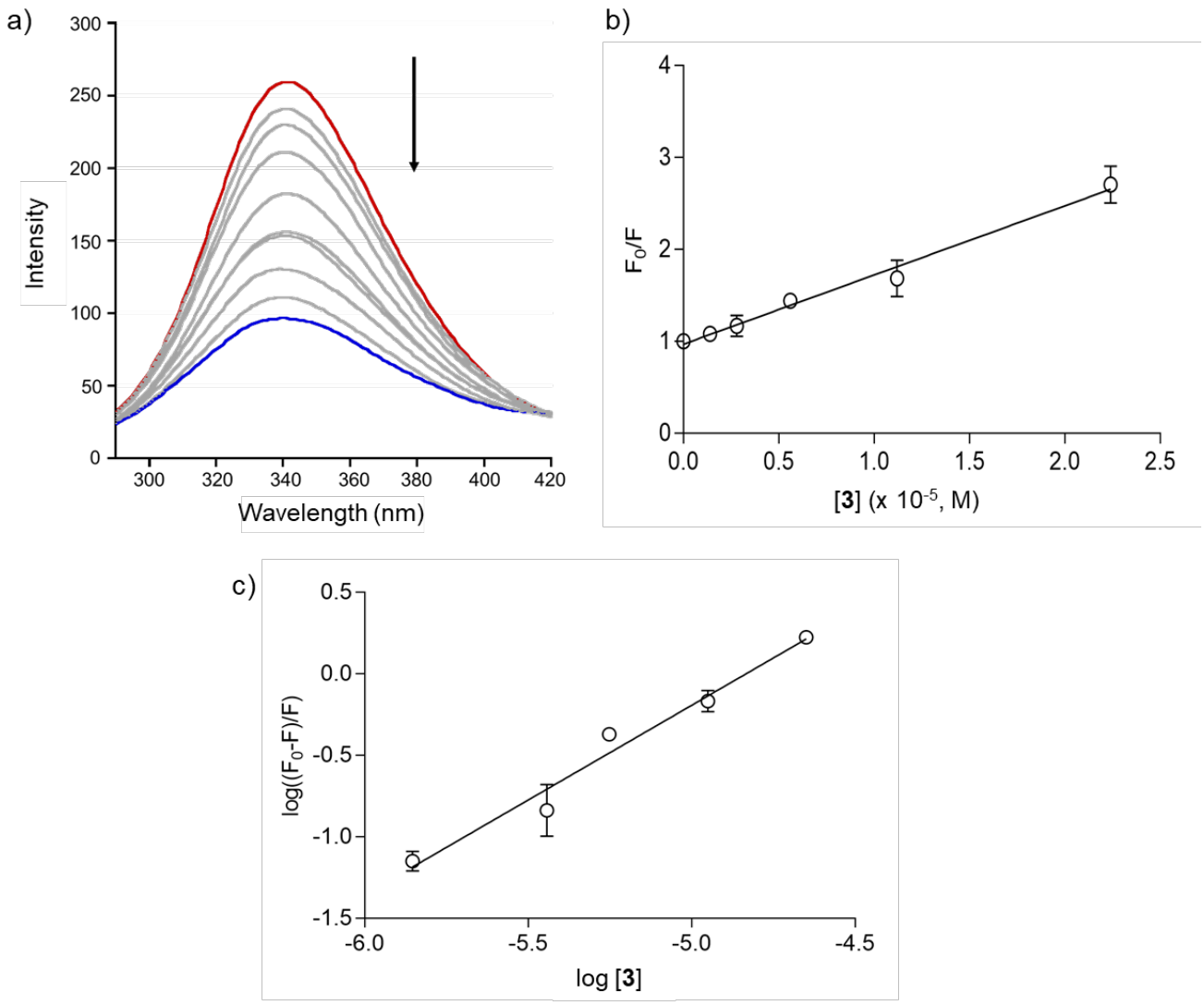


Figure S7. a) Fluorescence emission of spectrum of BSA (1.4 μM) in presence of various concentrations of **3** in TRIS buffer at pH = 7.4; T = 295 K, λ_{ex} = 282 nm. [**3**] = 0, 1.4, 2.8, 4.2, 5.6, 11.2, 13.3, 15.75, 16.8, and 22.4 μM. b) Stern-Volmer plot of data for titration of BSA with **3**. c) Modified Stern-Volmer plot of data for titration of BSA with **3**.[7]

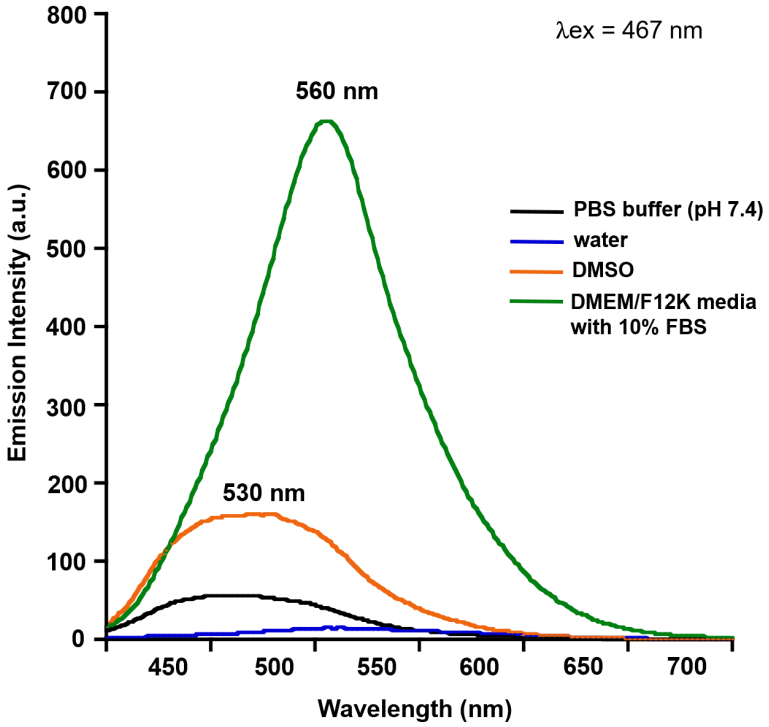


Figure S8. Emission spectra of **3** (0.1 mM) in in PBS (10 mM, pH 7.4), water and DMEM/F12K media with 10% FBS (λ_{ex} = 467 nm, slit width = 3.0 nm).

Photoreactivity of 3. A solution of **3** (~0.05 mmol) in 5.0 mL DMSO was placed in a 50 mL round bottom flask under air. The flask was placed in a Rayonet photoreactor equipped with 419 nm lamps (light intensity 2,450 lx) and illuminated until the reaction was determined to be complete (~24 h) as evidenced by the loss of the lowest energy absorption band for the compound. The solvent was then removed via lyophilization to give **5**. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.67 (s, 1H), 8.62 (s, 1H), 8.18 (d, J = 8.4 Hz, 2H), 8.15 (d, J = 8.3 Hz, 1H), 8.08 (d, J = 8.5 Hz, 1H), 7.77 (t, J = 7.5 Hz, 1H), 7.65-7.60 (m, 3H), 7.58-7.55 (m, 1H). ESI/APCI-MS (relative intensity) calcd. for $C_{18}H_{12}O_7S$ [M-H]: 372.0304; found: 372.0305 (100%).

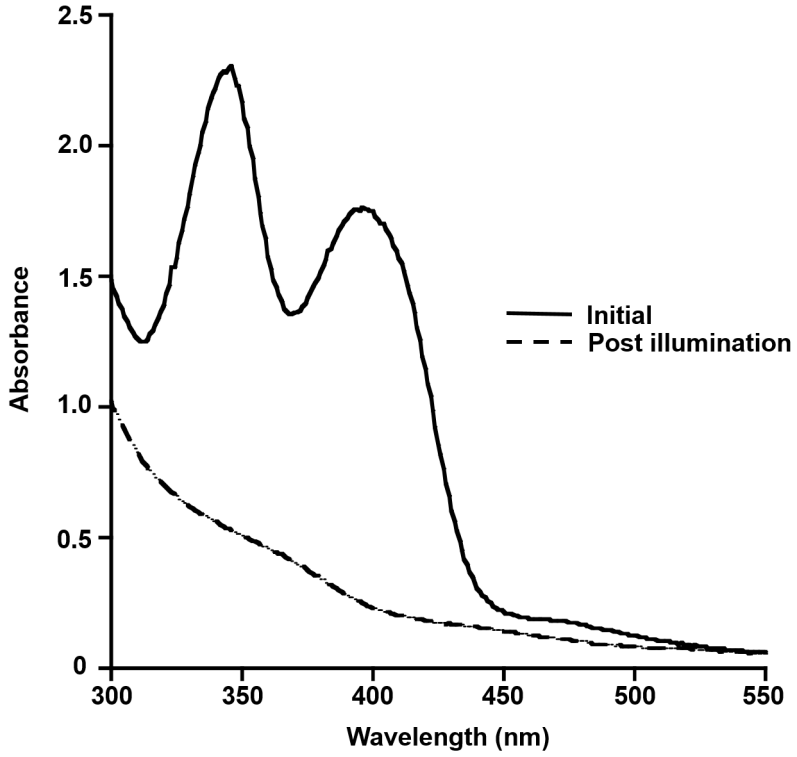


Figure S9. Absorbance of **3** prior and post 14 hours of illumination in PBS buffer (10 mM, pH = 7.4) with 3.3% final DMSO concentration.

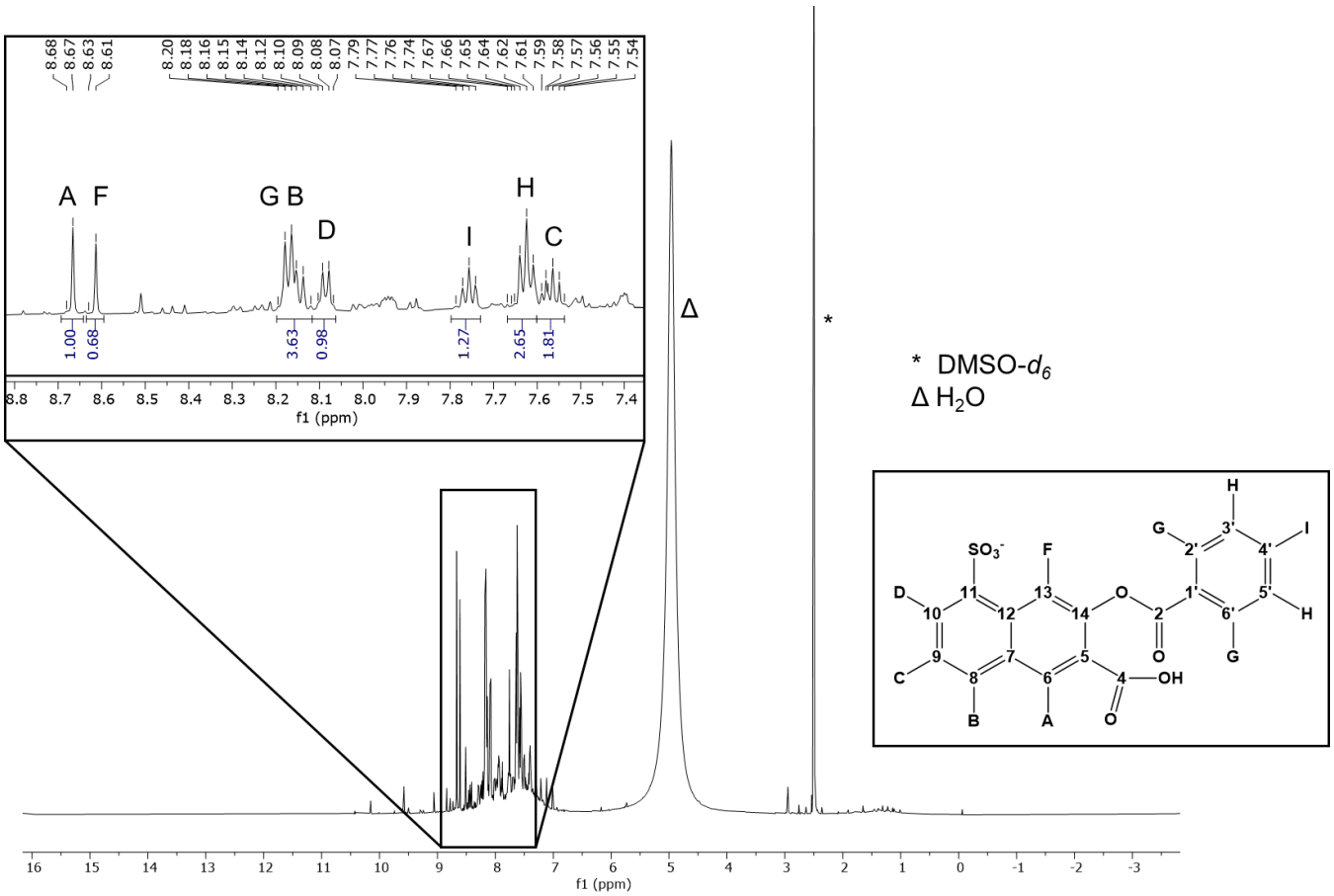


Figure S10A. 1H NMR (500 MHz) spectrum of **5** in DMSO-d_6.

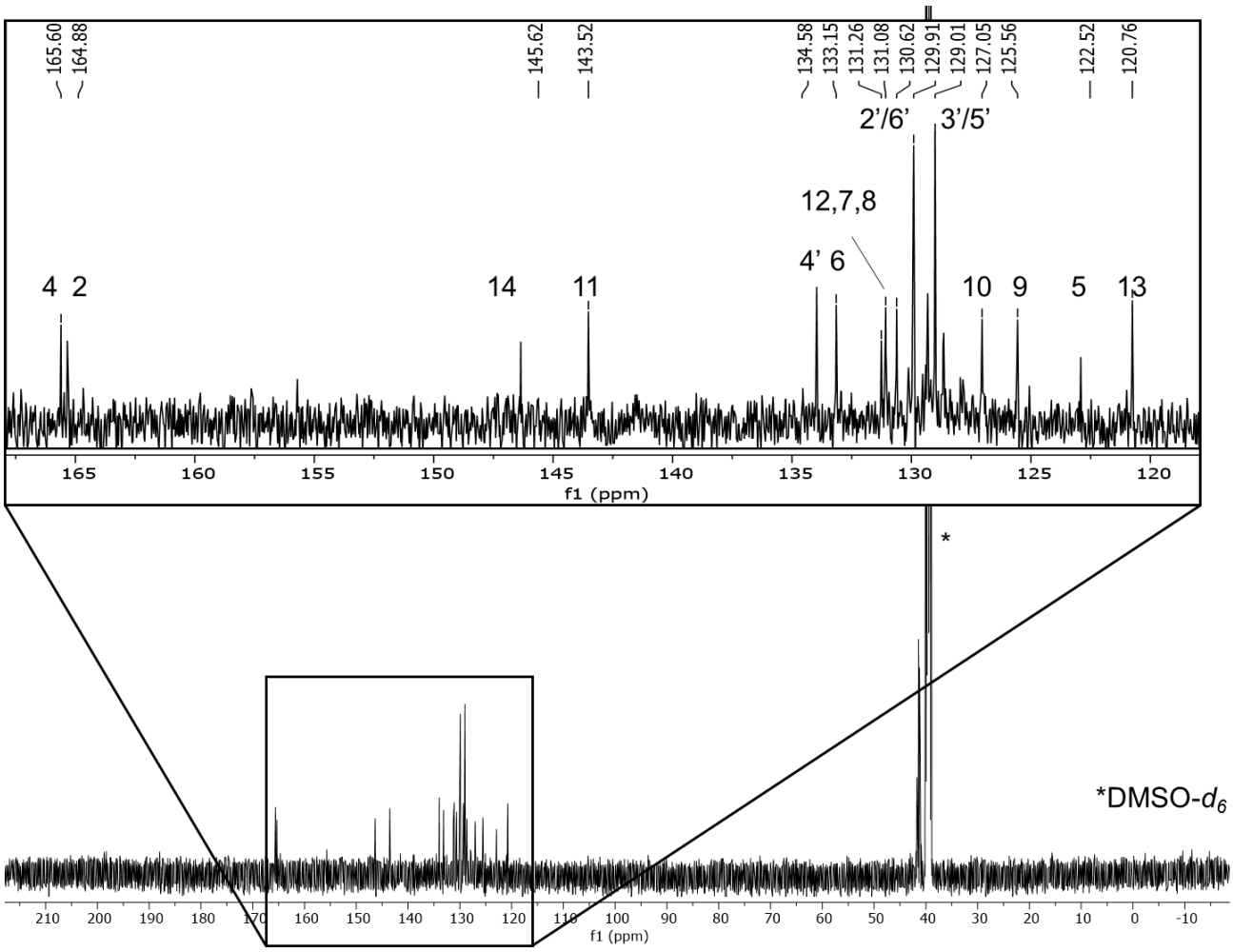


Figure S10B. $^{13}C\{^{1}H\}$ NMR (125 MHz) spectrum of **5** in DMSO-d_6.

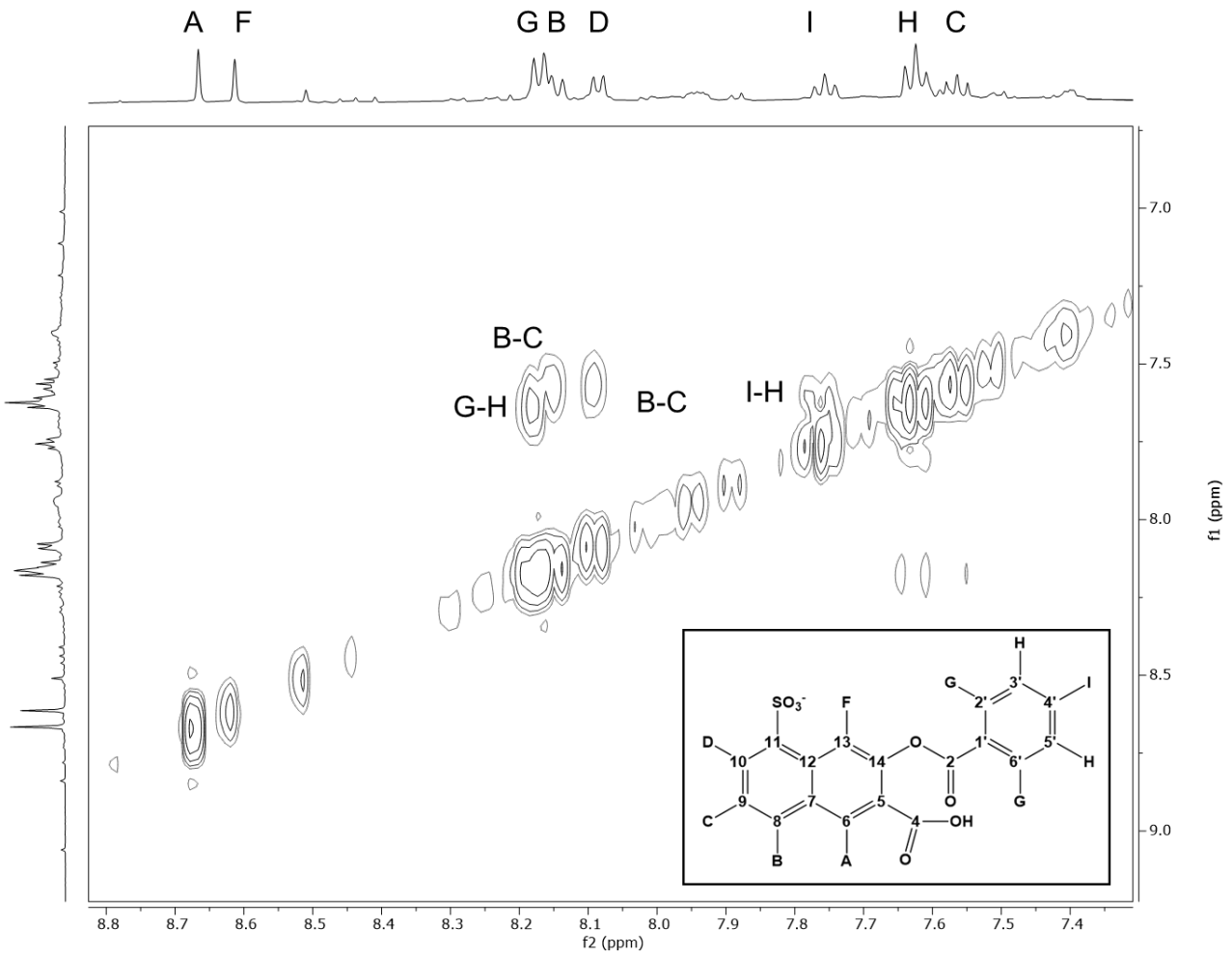


Figure S10C. 2D-COSY spectrum of **5** in DMSO-d_6.

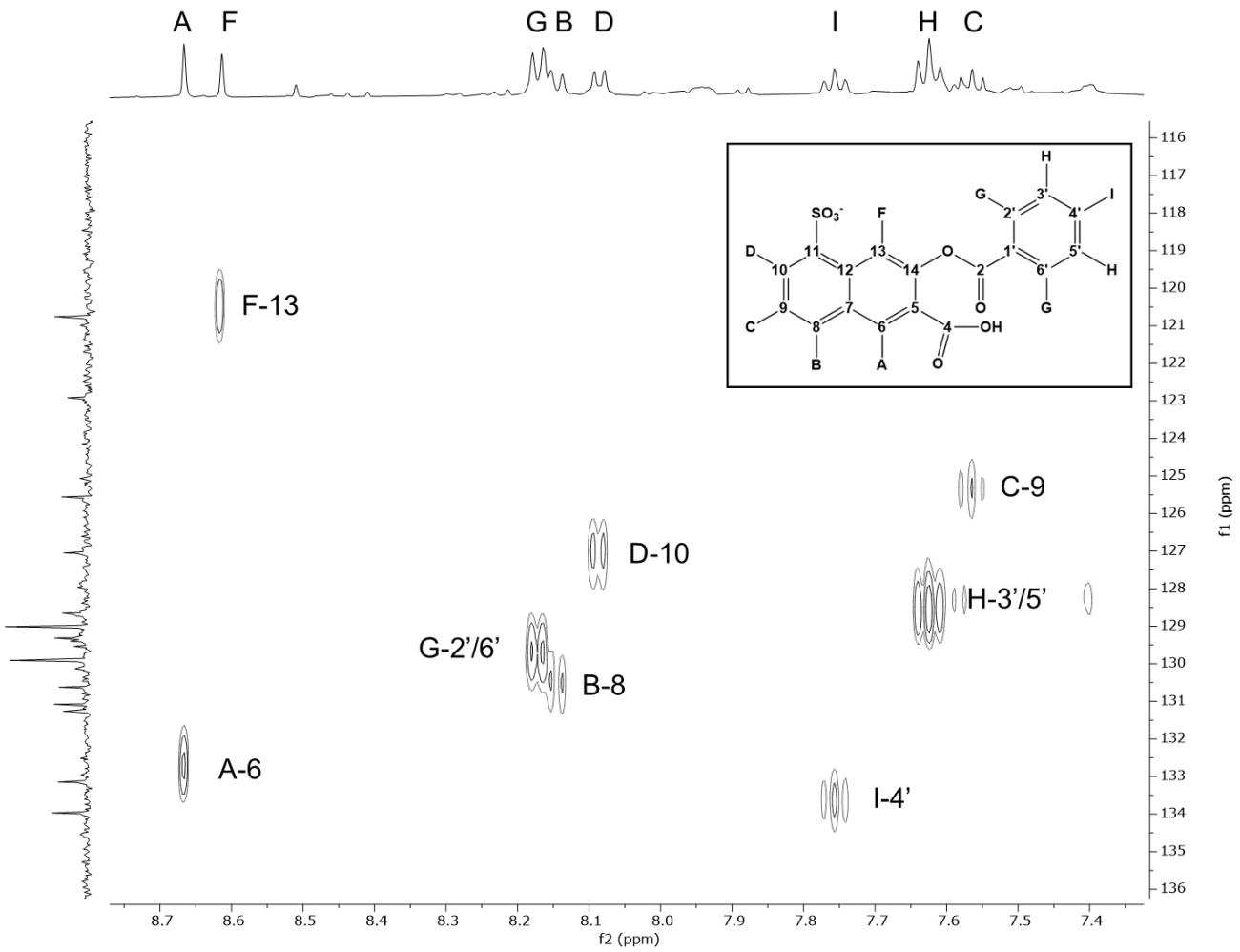


Figure S10D. HSQC spectrum of **5** in DMSO-d_6.

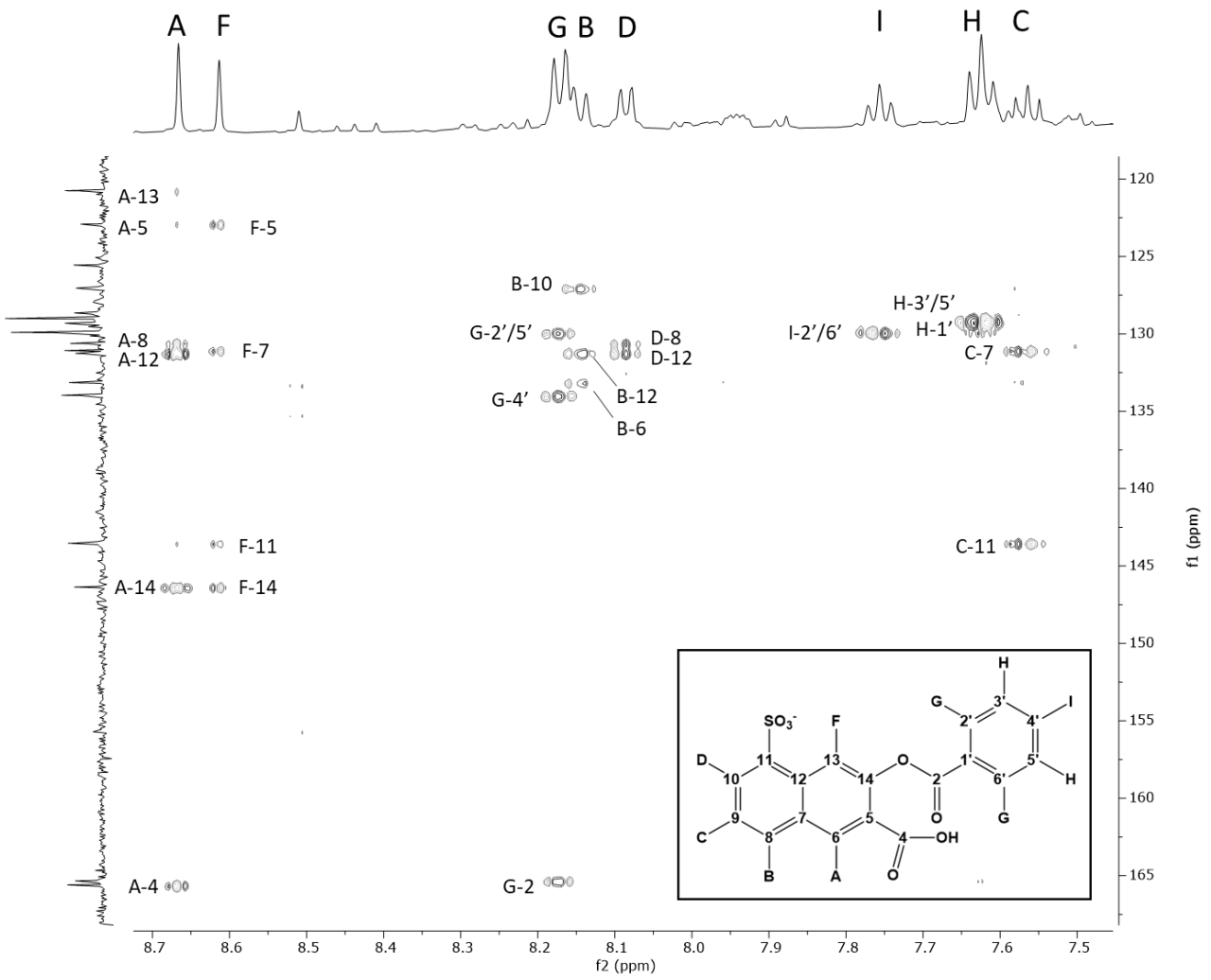


Figure S10E. HMBC spectrum of **5** in DMSO-d_6.

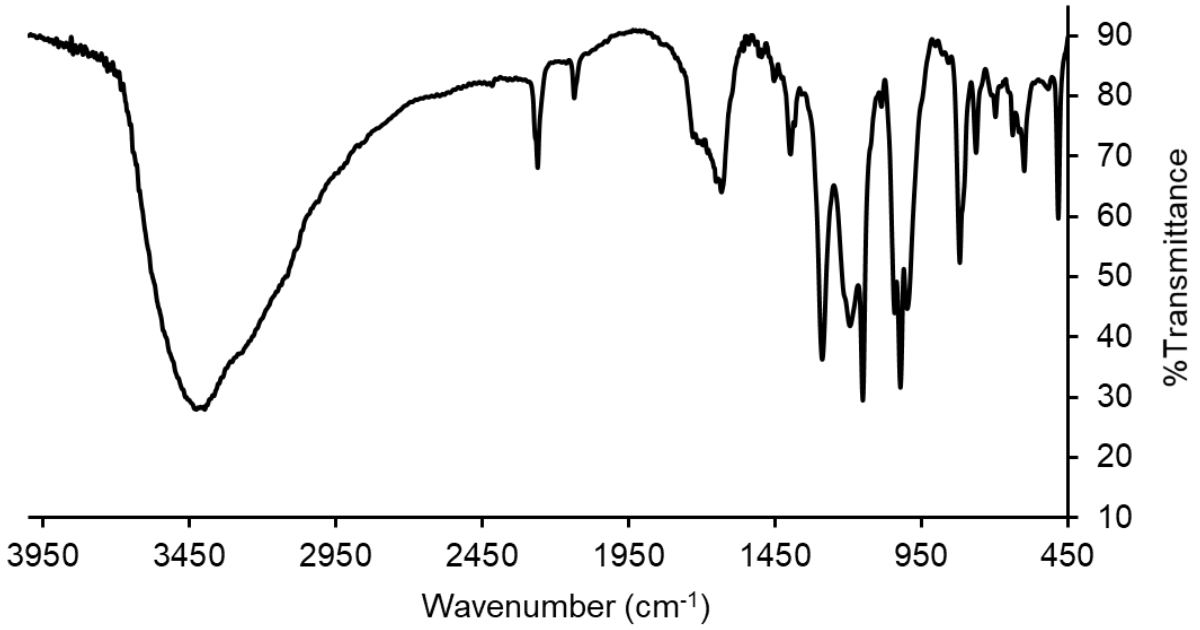
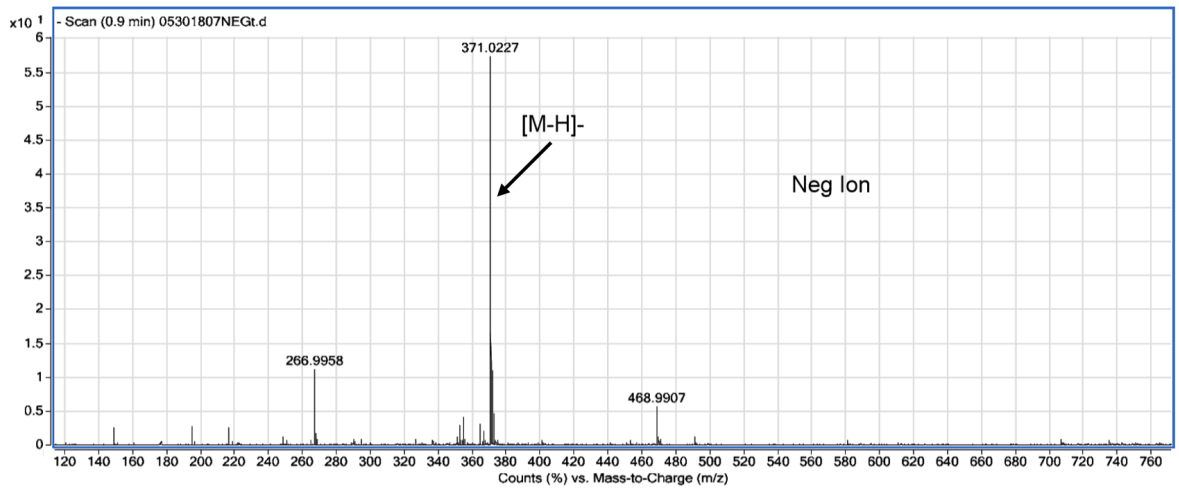


Figure S11. FTIR spectrum of **5** in KBr. Assignments of the $\nu_{C=O}$ (1721 cm^{-1}, 1626 cm^{-1}) and $\nu_{S=O}$ (1153 cm^{-1}) vibrations were made on the basis of literature precedent.[6]



Measured Mass	372.0305			
Element	Low Limit	High Limit		
C	5	50		
H	5	50		
S	1	3		
O	4	10		
Formula	Calculated Mass	mDaError	ppmError	RDB
C18 H12 O7 S	372.0304	0.1	0.3	13

Figure S12. ESI/APCI-MS of **5.**

Cell Studies

Cell culture growth parameters. Mouse macrophage cells (RAW 264.7) were obtained from ATCC (Manassas, VA) and cultured in phenol-red free DMEM-F12K media (Caisson Laboratories) supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich) at 37 °C with 5% CO_2. The media was changed every other day. All experiments were performed within 5 passages of the RAW 264.7 cells. Cells were detached by addition of 3 mL of Trypsin-EDTA 1x solution (Caisson Laboratories) for 5 min, then neutralized with 8 mL of fresh media. RAW 264.7 cells were counted using a Cell Countess instrument (ThermoFisher) according to the manufacturer's protocol.

Fluorescence microscopy of 1 and 3. RAW 264.7 cells were seeded into Millicell E-Z-Slide culture chambers (EMD Millipore, Billerica, MA) at an initial density of 1.0×10^4 cells/cm^3 and allowed to adhere to the chamber slides for 24 hours. The cells were then treated with compound **1** or **3** (50 μM) for 4 h. Both compounds were prepared as a stock solution of 25 mM in DMSO and then diluted to a final concentration of 50 μM in the culture media, reaching 0.4% of final DMSO concentration. The cells were next incubated for 15 min with nuclear stain (Hoechst 33342 dye) followed by three washes with PBS buffer to remove all the residual dye. All the steps described above were performed with minimal light exposure. Cell imaging was performed using a Zeiss Axio Observer inverted microscope (Carl Zeiss Microscopy, Thornwood, NY) equipped with fluorescence detection. Images were acquired at 20x air magnification with the following excitation and emission parameters: blue channel, λ_{ex} = 310-390 nm and a detection wavelength range λ_{em} = 420-470 nm; green channel, λ_{ex} = 450-490 nm and a detection wavelength range λ_{em} = 500-550 nm. Acquired images were processed by universal adjustment to enhance contrast levels (same settings were applied for all acquired images in each detection channel) using ZEISS ZEN 2.3 Lite software.

Emission studies of the amount of 3 left in washes after cell incubation. RAW 264.7 cells were incubated with **3** (50 μM) as described above. After 4 hours of incubation in the dark, the cells were washed with media containing 10% FBS and the washes were collected and combined. Fluorescence emission studies (λ_{ex} = 410 nm, slit width = 3.0 nm) were performed and the intensity profile was compared to that of a standard solution containing **3** (50 μM).

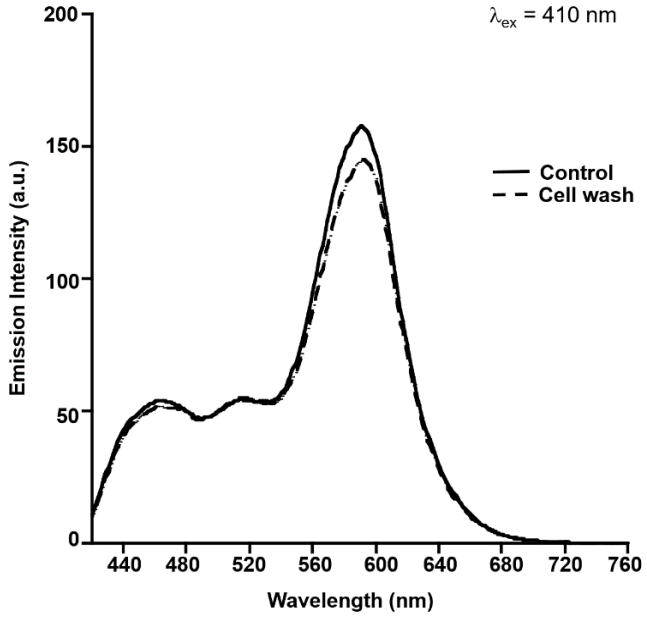


Figure S13. Emission spectra of **3** in DMEM/F12K medium enriched with 10% FBS collected from cell washes (dashed line) vs. standard solution of **3** (0.05 mM, solid line) (λ_{ex} = 410 nm, slit width = 3.0 nm). The results indicate almost quantitative recovery of **3**.

Fluorescence microscopy of intracellular CO-detection from 1 and 3. RAW 264.7 cells were seeded as described above. The cells were then treated with compound **1** or **3** (50 μM) for 4 h. The culture chambers were gently washed 3x with plain culture media to remove residual compound or were not washed. Next, a previously reported Nile red-based intracellular CO sensor (1-Ac)[8] was added to the chambers for 30 min at a final concentration of 5 μM with a final DMSO concentration of 0.2% (v/v). The stock solution of 1-Ac was made in DMSO at a concentration of 2.5 μM. The slides were subsequently subjected to illumination using a blue LED array (light density of 66,351 lx) in a temperature-controlled shaker (37 °C) for 1 h; placed at a distance of 2 cm from the light source. The slides were then incubated for 1 h in the dark at 37°C for the development of CO-sensor signal. Finally, the cells were incubated for 15 min with nuclear stain (Hoechst 33342 dye) followed by three washes with PBS buffer to remove all the residual dye. Cell imaging was performed as described above with the addition of red channel having the following parameters, λ_{ex} = 550-600 nm and a detection wavelength range λ_{em} = 598-660 nm. Acquired images were processed as described above.

MTT assays on RAW 264.7 cells. Three independent biological experiments, each with three technical replicates, were performed for **3** and **5** in the dark and under visible light-induced CO release conditions. For all experiments, MTT (Sigma-Aldrich) was prepared fresh at 5 mg/mL in sterile PBS. This solution was filtered through a 0.22 μm PES filter. RAW 264.7 cells were seeded in 96-well plates (Corning, NY) at 1.0 x 10^4 cells/well for 24 h. The cells were then treated with **3** or **5** at 0.08-100 μM with a final DMSO concentration that does not exceed 0.4% (v/v). For visible light-triggered *in situ* CO release, the plates were exposed to blue LED array (light density of 66,351 lx) for 1 h and incubated for an additional 23 h. Then, MTT solution (20 μL) was added and the cells were incubated for an additional 4 h. The metabolized formazan pellets were solubilized by adding 200 μL of DMSO and absorption values were measured using a Modulus™ Microplate reader (Turner Biosystems) at 560 and 750 nm (background). The final results were background subtracted and normalized to vehicle control (0.4% DMSO). Data analysis was performed using GraphPad Prism 7 (La Jolla, California), with values reported as mean ± SEM. IC_{50} values were calculated as nonlinear regression with a bottom constraint of zero.

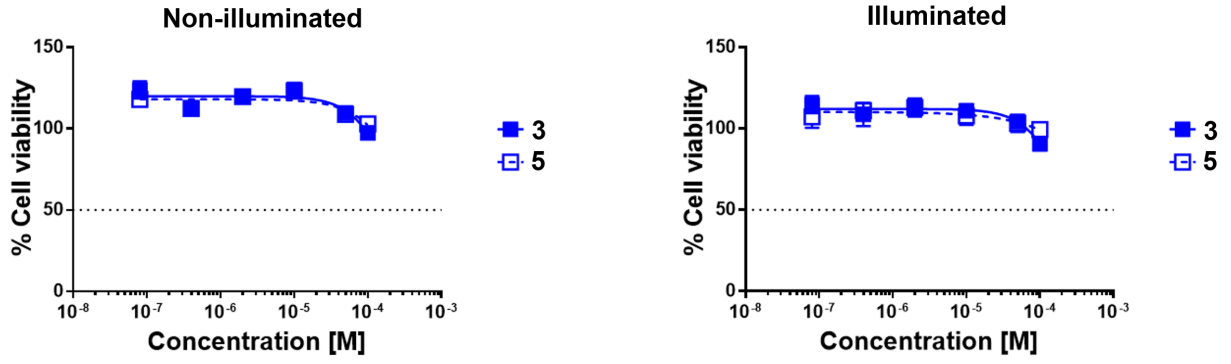


Figure S14. Plot of RAW 264.7 cell viability expressed in % cell viability vs concentration of **3** and **5**. The IC_{50} values were determined using a four-parameter nonlinear regression for assays wherein at least 50% reduction in cell viability was observed. No IC_{50} value was determined for the tested compounds. Values displayed represent the average ± SEM (standard error of the mean) of three independently replicated experiments. Some data points shown in the plot have error bars that are smaller than the size of the symbol, thus, are not seen.

TNF-α quantification. RAW 264.7 cells were seeded in 96-well plates at an initial density of 1.0 x10^4 cells/well 24 h before the experiment. On the day of the experiment, the cells were incubated with vehicle (0.4% DMSO) or **1** and **3**-**5** at a concentration range of 0.04-50 μM for 4 h protected from light. One of the two prepared plates was then subjected to illumination using a blue LED array (66,351 lx, light intensity) for 1 h at 37 °C. Both plates were then incubated for 6 additional hours in the dark at 37 °C. Thereafter, lipopolysaccharides (LPS) from E. coli O127:B8 (Sigma Aldrich) were added to the cell culture media to a final concentration of 1 μg/mL per well and the plates were incubated for one more hour. Vehicle control wells without LPS treatment were used as the negative control whereas the ones treated with LPS were used as positive control. Cell culture supernatant (50 μL) was then collected and used for determination of TNF-α concentration in each well/treatment by using a commercial ELISA kit (TNF alpha Mouse Instant ELISA™ kit (Invitrogen™)) following the supplier protocol. The results are presented as means ± SEM (standard error of the mean) from three independent experiments (2 technical replicates in each experiment for each compound). Data were analyzed by two-way ANOVA followed by Sidak's multiple comparison posthoc tests to compare effects of all treatments to the LPS positive control or to compare effects of treatment with compounds **1** and **3** under illuminated and non-illuminated conditions. A p value <0.05 was considered to be statistically significant.

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