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Electronic Supporting Information

Detection of subcellular nitric oxide in the mitochondria by a pyrylium probe: assays in cell cultures and peripheral blood

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Fluorescence measurements. 10 μM solutions of **2** in PBS 10 mM, pH 7.4 (20 % DMF as a cosolvent) were prepared. For the kinetic analysis, different equivalents of DEA NONOate were added, and the absorption spectra/emission spectra/maximum fluorescence intensity was measured over time. DEA NONOate is a commercial NO donor with a half-life of 16 minutes at 22-25 °C in 0.1 M phosphate buffer (pH 7.4). It liberates 1.5 moles of NO per mole of the parent compound. For the concentration dependence study, different aliquots (3 ml each) of 10 μM solutions of **2** were titrated by adding increasing volumes of 12.25 mM DEA/NONOate stock solution (in NaOH 10 mM). The fluorescence spectra were measured after 20 minutes of reaction at the corresponding excitation wavelength (480 nm).

Preparation of NO (g). Gaseous nitric oxide (NO) was synthesized by a reaction between KI (1 M) and $NaNO_2$ (1 M) catalyzed by acid, according to the following reaction:

$$2\ NO_2^-\ (aq) + 2\ I^-\ (aq) + 4\ H^+(aq) \rightarrow 2\ NO\ (g) + I_2\ (s) + 2\ H_2O\ (l)$$

Selectivity test. Different analytes (final concentration: 500 μM): NO, ClO^-, H_2O_2, NO_2^- NO_3^-, O_2^-, $HO^\cdot$, $^1O_2$, ascorbic acid (AA), dehydroascorbic acid (DHA), and $ONOO^-$ were added to 10 μM solutions of **2** in PBS 10 mM, pH 7.4 (20 % DMF as a cosolvent). The resulting solutions were kept at ambient temperature for 20 min and then the emission spectra were recorded (λ_{exc} = 480 nm). Aqueous solutions of $NaNO_2$, $NaNO_3$, AA and NaClO were prepared freshly and used as sources of NO_2^-, NO_3^-, AA and ClO^-, respectively. Dimethyl sulfoxide solutions of KO_2 and DHA were used as sources of O_2^- and DHA. H_2O_2 was diluted promptly from 35 % wt solution. Hydroxyl radicals were generated by the reaction of Fe^{2+} with H_2O_2 (molar ratio of Fe^{2+} to H_2O_2 is 1:10).[1] Nitric oxide (NO) was generated from DEA/NONOate (stock solution 12.25 mM in 10 mM NaOH). Singlet oxygen ($^1O_2$) was generated from ClO^- and H_2O_2.[2] $ONOO^-$ was prepared by reaction of NO_2^- and H_2O_2.[3]

Cell cultures and treatments. HT29 human colon cancer cell line and RAW 264.7 (ATCC® TIB-71™) murine macrophage cell line were employed to test probe **2** in an *in vitro* setting. Cell cultures were maintained in DMEM high glucose supplemented with 10 % inactivated fetal bovine serum, 2 mM L-glutamine, 1% P/E (penicillin and streptomycin) at 37 ºC, in a cell culture incubator with a humidified 5 % CO_2/95 % air atmosphere. Cell cultures were used for experiments at passage number lower than 20.

Effects on Cell Growth/Viability. The cytotoxic effects of probe **2** were assessed using the MTT assay in RAW 264.7 cells. Briefly, cells were grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µL culture medium per well in a humidified atmosphere (37 °C, 5 % CO_2/95 % air), before the incubation with different concentrations of **2**, from 0 to 1000 µM (n = 3). After the incubation period (48 h), 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each well and incubated for 3 h in a humidified atmosphere (37 °C, 5% CO_2). The purple formazan crystals formed were then dissolved by the addition of 100 µL of DMSO into each well. Using a microplate reader (Multiskan FC, Termo Scientific), the absorbance of the samples at 550 nm was measured.

Visualization of Exogenous NO in HT29 living cells. 10 mM stock solution of **2** in DMSO was prepared. HT29 cells were seeded (5×10^4 cells/mL) in a sterile µ-Slide 4-well-chamber slide (Ibidi, Inycom, Madrid, Spain). After 24 h incubation (37 °C in a 5 % CO_2/95 % air), the culture medium was removed and freshly prepared serum-free medium with **2** (10 µM, 0.1% DMSO as the cosolvent) was added. Cells were then incubated at 37 °C for 30 minutes (5 minutes before this time end, 0.01 mg/ml Hoechst 33342 was added to stain nuclei). For the positive control group, **2**-loaded cells were then supplied with NO (100 µM SNAP as the donor) in PBS for another 60 min. In all experiments, the cells were washed with PBS (3 × 0.5 mL/well) before the fluorescence imaging using a

confocal microscope. In colocalization analysis, **2** was coincubated with Mitotracker Green FM (ThermoFisher M7514, 75 nM).

Flow Cytometry Analysis of NO in HT29 cells. HT29 cells were seeded into a six-well plate at the density of 1×10^5 cells/ml. After 24 h incubation (37 °C in a 5 % CO_2/95 % air), the culture medium was removed and freshly prepared serum-free medium with **2** (10 μM, 0.1% DMSO as the cosolvent) was added. Cells were then incubated at 37 °C for 30 minutes, washed with PBS (3 × 1 mL/well), and further incubated with SNAP (100 μM), for 1 hour. The well-containing only cells was used as the blank group, and the group of **2**-loaded cells was employed as control. Before flow cytometry analysis, cells were collected and washed with PBS (3 × 1 mL/tube or Eppendorf). All the measurements were performed three times.

Visualization of Endogenous NO in Raw 264.7 macrophage cells. 10 mM stock solution of **2** in DMSO was prepared. RAW 264.7 macrophage cells were seeded (5×10^4 cells/ mL) in a sterile μ-Slide 4-well-chamber slide (Ibidi, Inycom, Madrid, Spain). After 24 h incubation (37 °C in a 5% CO_2/95% air), the culture medium was removed and fresh serum-free medium containing 20 μg/mL of LPS was added. After incubation at 37 °C overnight, LPS activated RAW cells were incubated with fresh serum-free culture medium containing **2** (10 μM, 0.1% DMSO as the cosolvent) for another 30 minutes (5 minutes before this time end, 0.01 mg/ml Hoechst 33342 was added to stain nuclei). The cells were washed with PBS (3 × 0.5 mL/well) before the fluorescence imaging using a confocal microscope. In a control experiment, the cells were treated with 20 μg/mL LPS and 50 μM iNOS inhibitor L-NMMA overnight and then incubated with probe **2** (10 μM, 0.1% DMSO as the cosolvent) for 30 min (5 minutes before this time end, 0.01 mg/ml Hoechst 33342 was added to stain nuclei).

Flow Cytometry Analysis of NO in inflamed macrophage cells. RAW 264.7 cells were seeded into a six-well plate at the density of 1×10^5 cells/ml. After 24 h incubation (37 °C in a 5 % CO_2/95 % air), the culture medium was removed and freshly prepared serum-free medium with LPS (20 μg/mL) was added. Cells were then incubated at 37 °C overnight, washed with PBS (3 × 1 mL/well), and further incubated with **2** (10 μM, 0.1% DMSO as the cosolvent), for 30 minutes. **2**-stained cells were washed with PBS (3 × 1 mL/well) and removed from the plate using a cell scraper. The well-containing cells only was used as the blank group, and the group of **2**-loaded cells was employed as control. Before flow cytometry analysis, cells were washed with PBS (3 × 1 mL/tube). In a control experiment, the cells were treated with 20 μg/mL LPS and 50 μM iNOS inhibitor L-NMMA overnight and then incubated with probe **2** (10 μM, 0.1% DMSO as the cosolvent) for 30 min. All the measurements were performed three times.

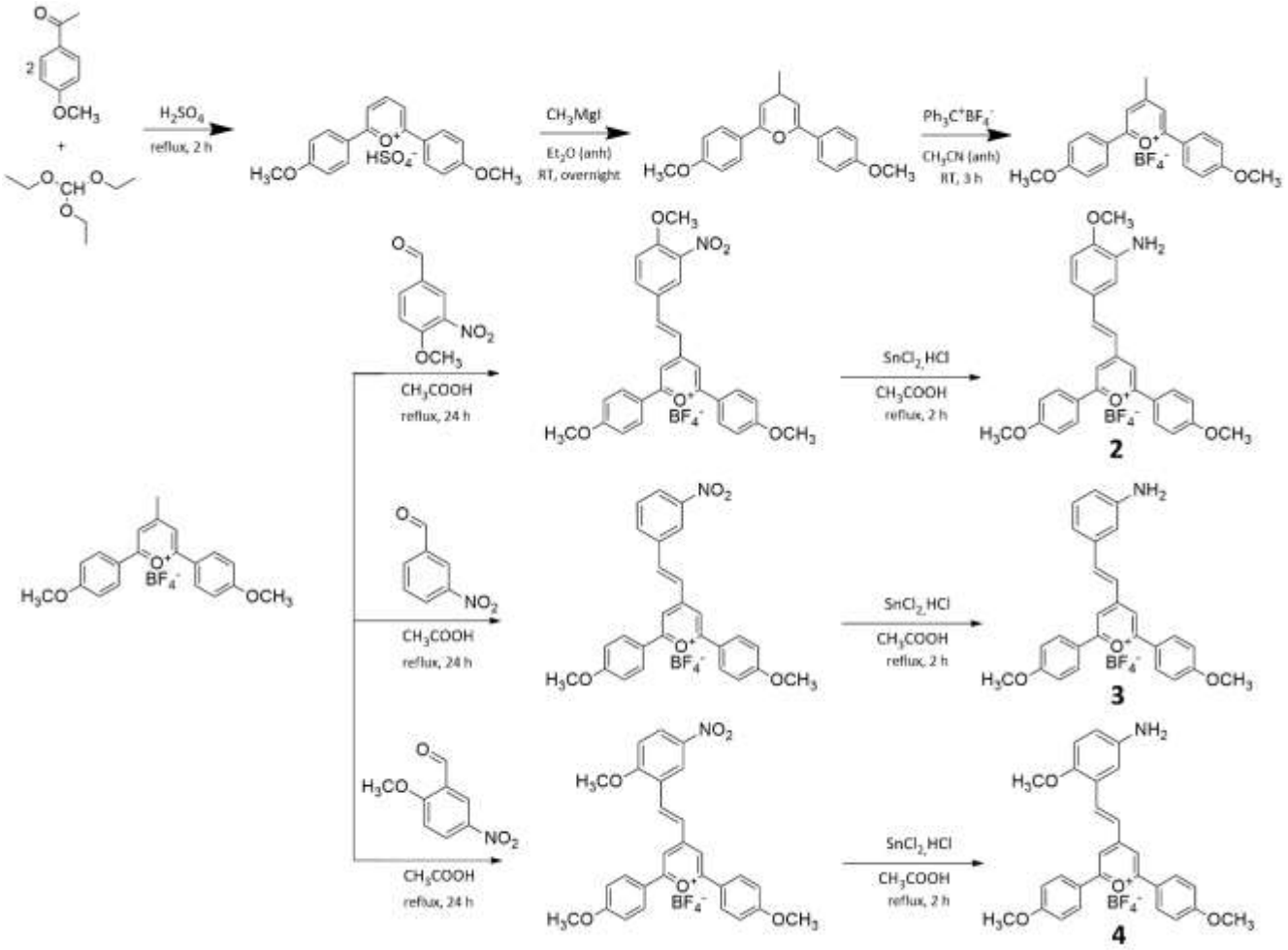
Intracellular nitric oxide (NO) analysis by flow cytometry in peripheral blood leukocytes. Whole heparin anti-coagulated peripheral blood diluted 1:1 in 100 µl of RPMI-1640 medium (Gibco, Thermo Fisher Scientific) or RPMI + Lipopolysaccharide 1 µg/ml (LPS, Sigma-Aldrich) were incubated in a U-button 96-well plate for 0 (ex-vivo), 24 or 48 hours in a cell incubator at 37 °C and 5% CO_2. Blood cells were labelled with CD8-BV605 (SK1, Becton-Dickinson, BD), CD16-V450 (3G8, BD), CD45-APC-H7 (2D1, BD), CD56-BV711 (NCAM16.2, BD), CD64-APC (10.1, BD), and HLA-DR-BV786 (L243, Biolegend) monoclonal antibodies during 5 min at 37 °C and then transferred to a flow cytometry tube containing 1.5 ml pre-warmed RPIM with 10 μM **2** and immediately acquired during 30 minutes at a low flow rate (time was recorded). During the acquisition, the tube was incubated in a 37 °C water bath protected from light. The level of NO was evaluated as mean fluorescence intensity (MFI) of **2** using FACSLyric and DIVA 9.0 software (BD). Photomultiplier voltages were previously

adjusted using CS&T beads (BD). Double thresholds in FSC (Forward side scatter) and CD45 APC-H7 were set to differentiate leukocytes (CD45+) from red blood cells and platelets (CD45-). The MFI of **2** was evaluated in the PE-channel (586/42 nm) excited by the blue laser (488 nm) and in the V500-channel (528/45) excited by the violet laser (405 nm). Staining with other 488 nm excited fluorochromes was ruled out to avoid compensation with the PE-channel.

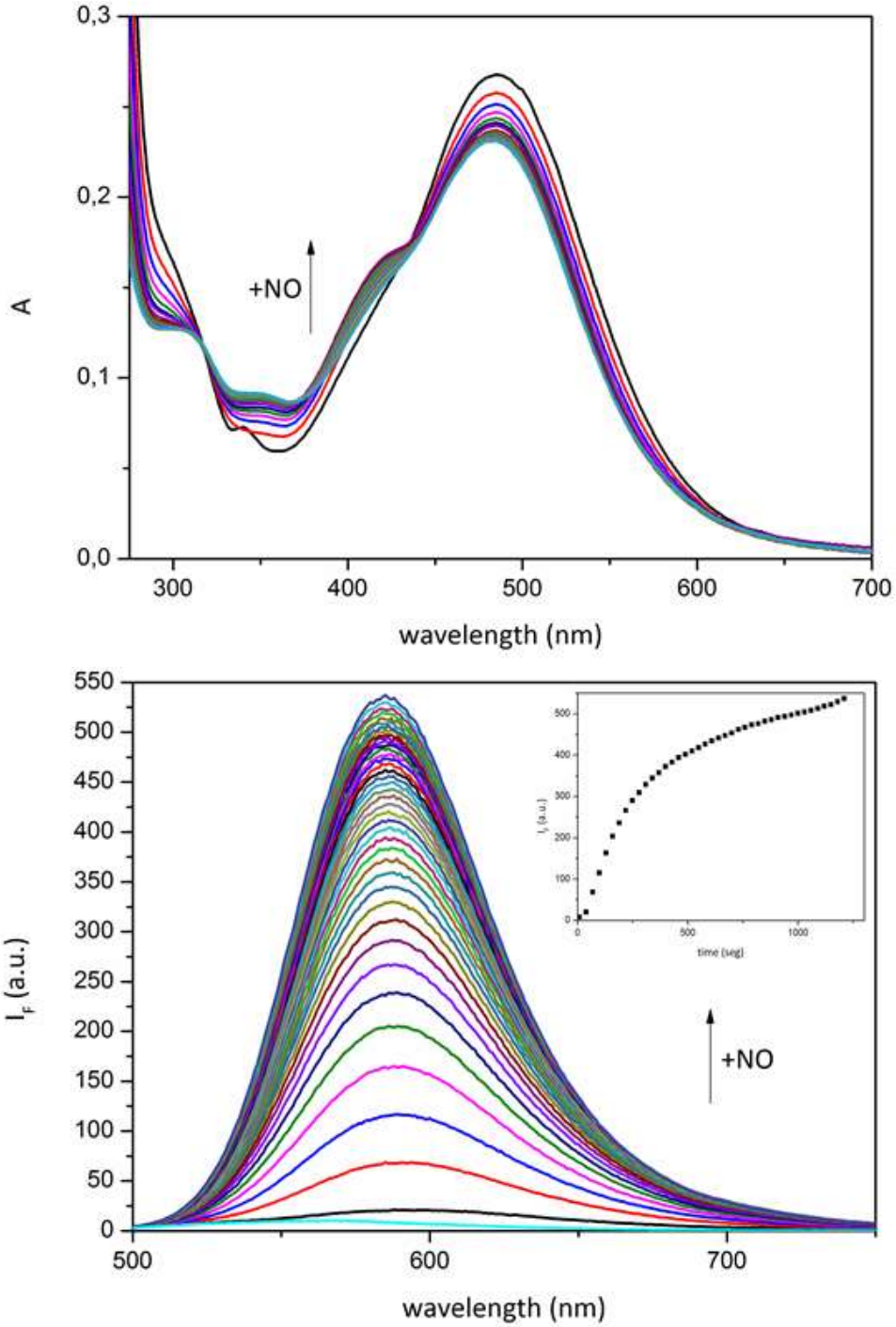
Table S1. Spectroscopic data of **2** and its reaction product with NO.

Compd.	**Solvent**	**λ_{abs} (log ε) (nm)**	**λ_{em} (nm)**	**ϕ_F**
2	PBS-DMF 8:2 pH = 7.4	485 (5.43)	-	-
2 + NO	PBS-DMF 8:2 pH = 7.4	420 -s- 485 (5.36)	585	0.01

s = shoulder



Scheme S1. Synthetic route for compounds **2-4**.



F**igure S1**. Evolution over time of the absorption (up) and emission (down) spectra of 10 µM solutions of **2** in PBS 10 mM (pH 7.4, 20 % DMF as a cosolvent) with 50 equivalents of DEA NONOate. λ_{exc} was set at 480 nm.

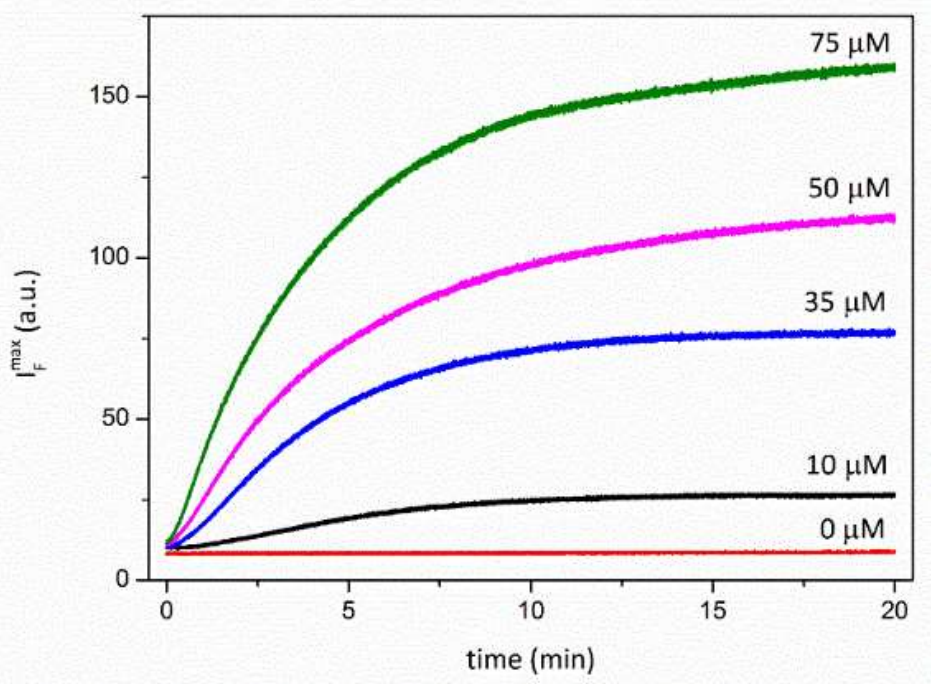


Figure S2. Evolution over time of the fluorescence intensity at 585 nm of 10 µM solutions of **2** in PBS 10 mM (pH 7.4, 20 % DMF as a cosolvent) after the addition of different equivalents of NO. λ_{exc} was set at 480 nm.

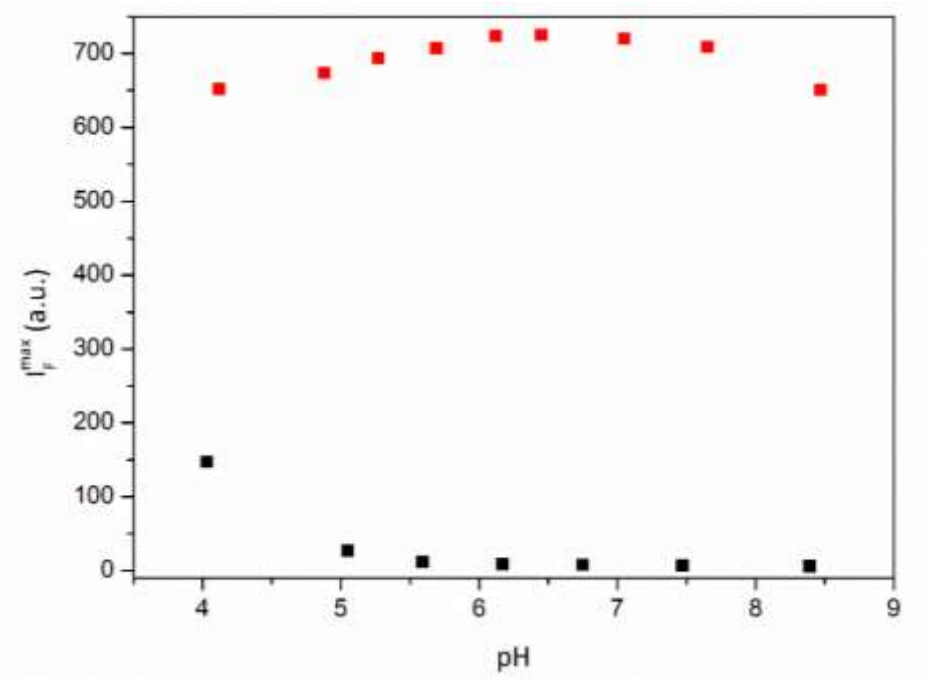


Figure S3. pH response of **2** + NO (50 equivalents, red dots) and **2** (black dots).

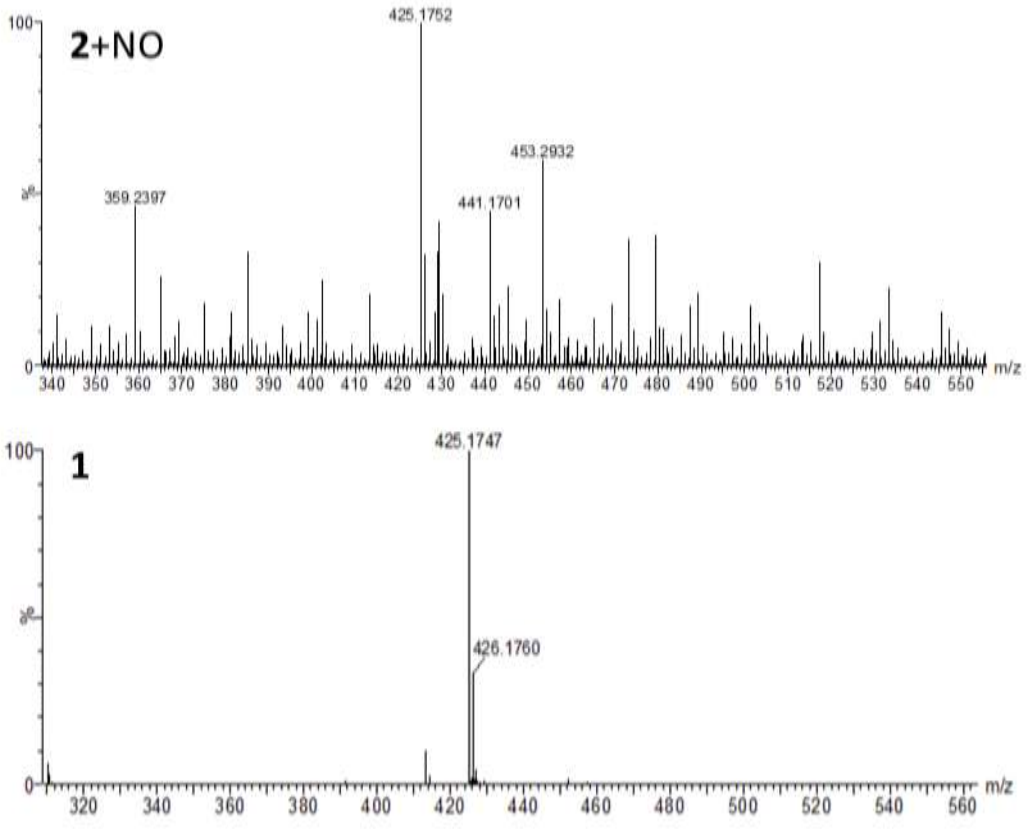


Figure S4. High resolution mass spectra of **2** after the reaction with an excess of gaseous NO (top) and its deamination product, model compound **1** (bottom).

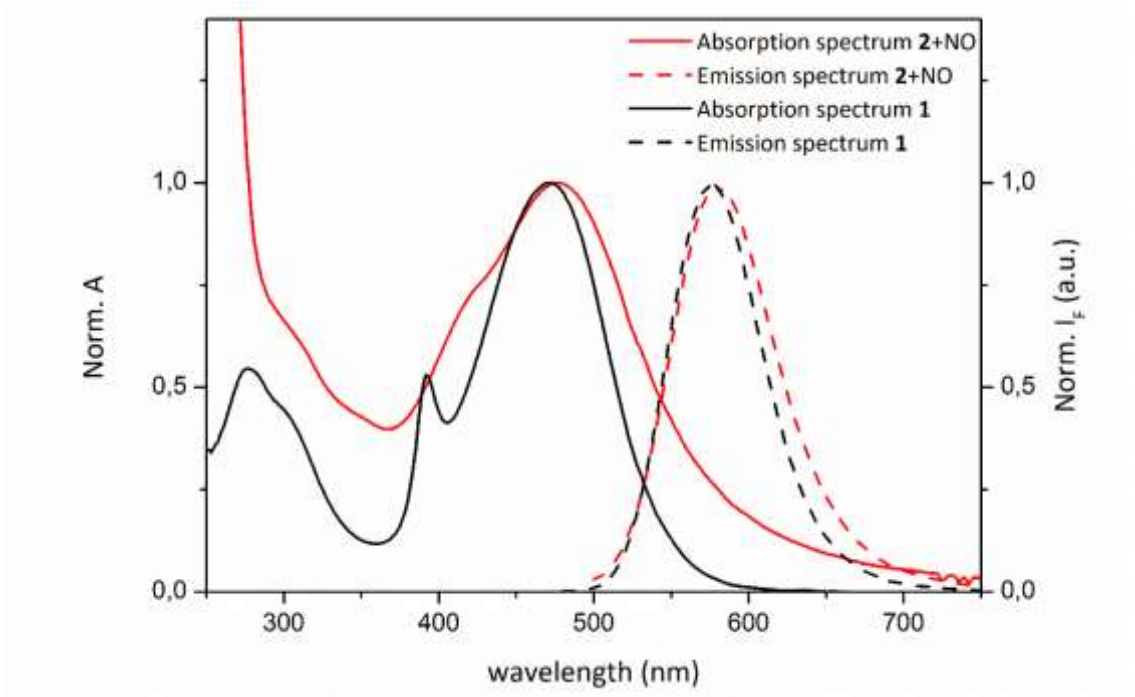


Figure S5. Normalized absorption and emission spectra of compound **2**+NO and model compound **1** in PBS (10 mM, pH 7.4, 20 % DMF as a cosolvent). λ_{exc} was set at 480 nm.

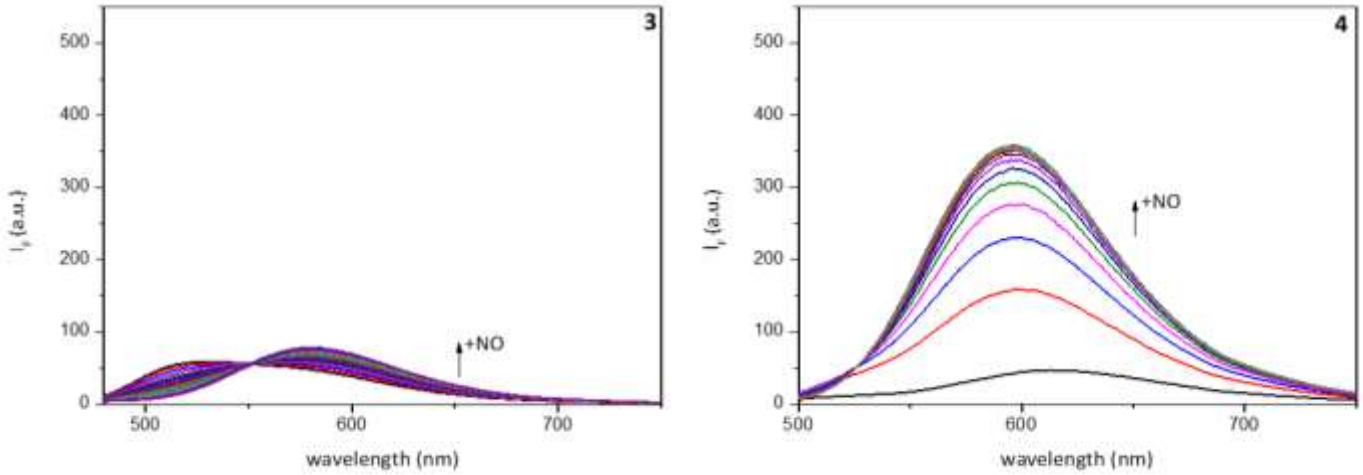


Figure S6. Evolution over time of the emission spectrum of 10 µM solutions of compounds **3** and **4** in PBS 10 mM (pH 7.4, 20 % DMF as a cosolvent) with 50 equivalents of DEA NONOate. λ_{exc} was set at the absorption maximum for each compound.

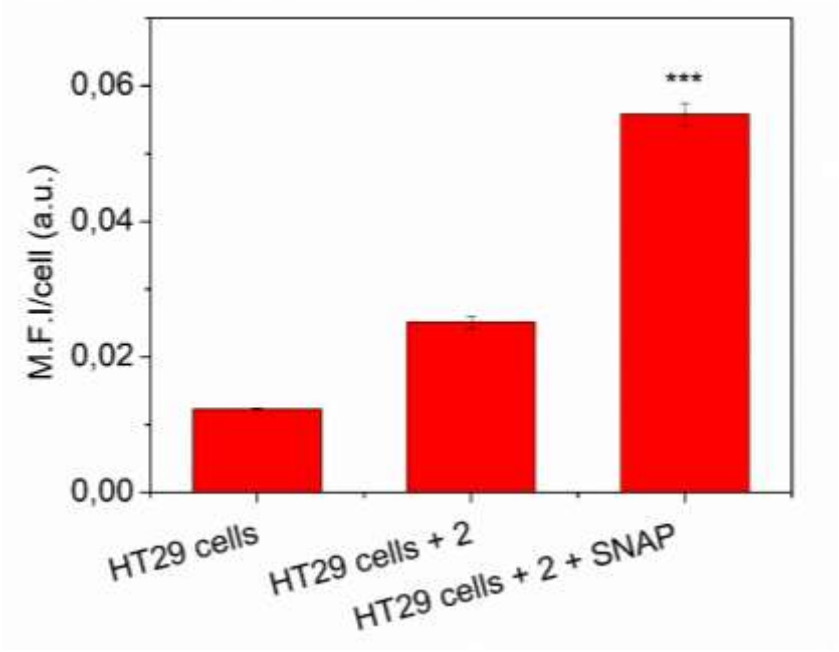


Figure S7. Mean fluorescence intensity obtained by flow cytometry of **2**-stained (10 µM, 30 min) HT29 cells exposed to SNAP (100 µM, 60 min). Excitation and emission were set at 488 nm and 585 nm, respectively. Data are shown as mean±SD, n=3. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test (*** indicates a p-value ≤ 0.001).

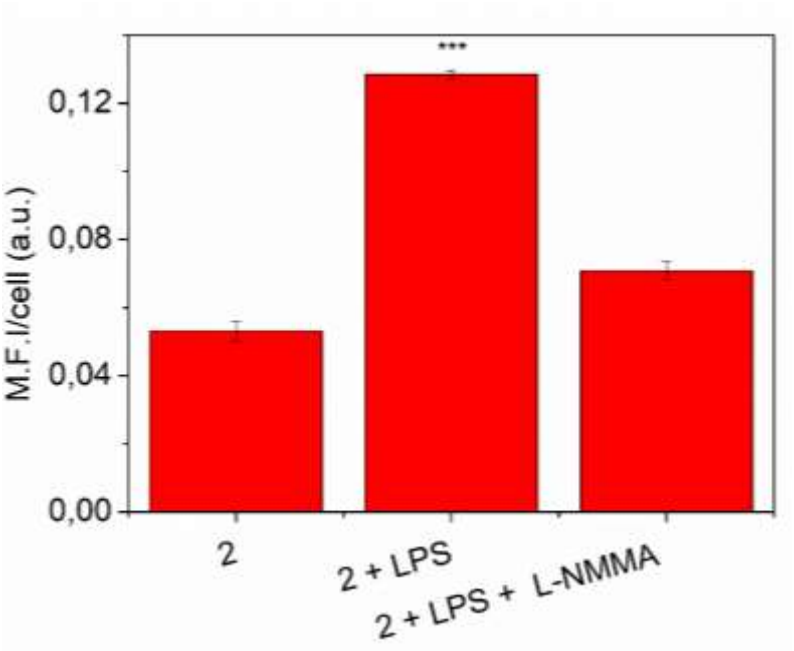


Figure S8. Mean fluorescence intensity obtained by flow cytometry of **2**-stained (10 µM, 30 min) RAW 264.7 cells exposed to LPS (20 µg/ml, overnight) and LPS+L-NMMA (20 µg/ml and 50 µM, overnight). Excitation and emission were set at 488 nm and 585 nm, respectively. Data are shown as mean±SD, n=3. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test (*** indicates a p-value ≤ 0.001).

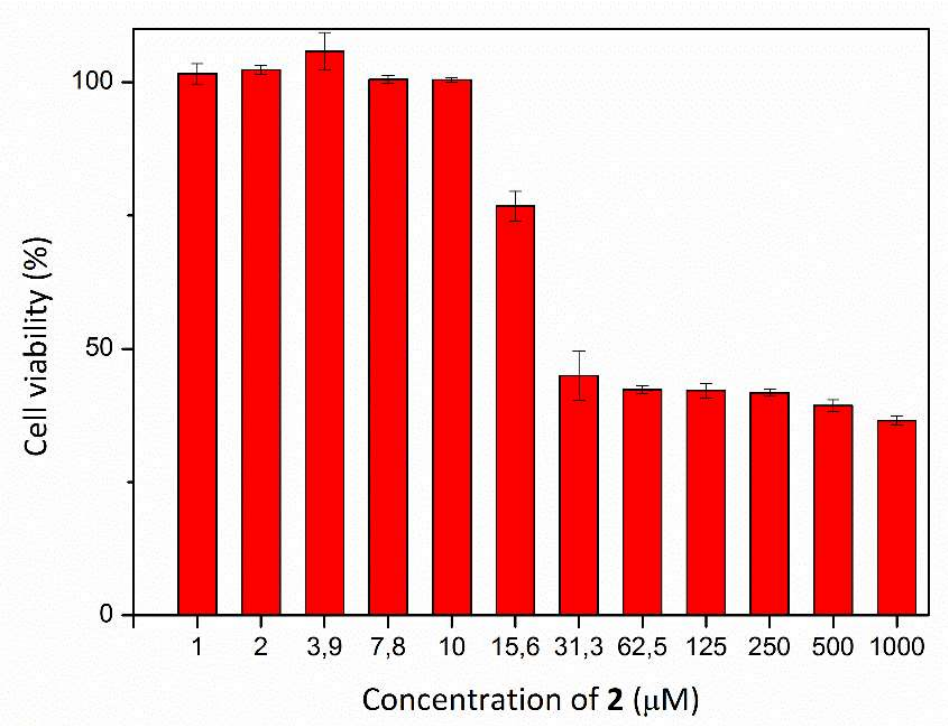


Figure S9. Viability of RAW 264.7 cells after incubation with different concentrations of probe **2** for 48 hours. Data are shown as mean±SD, n=3.

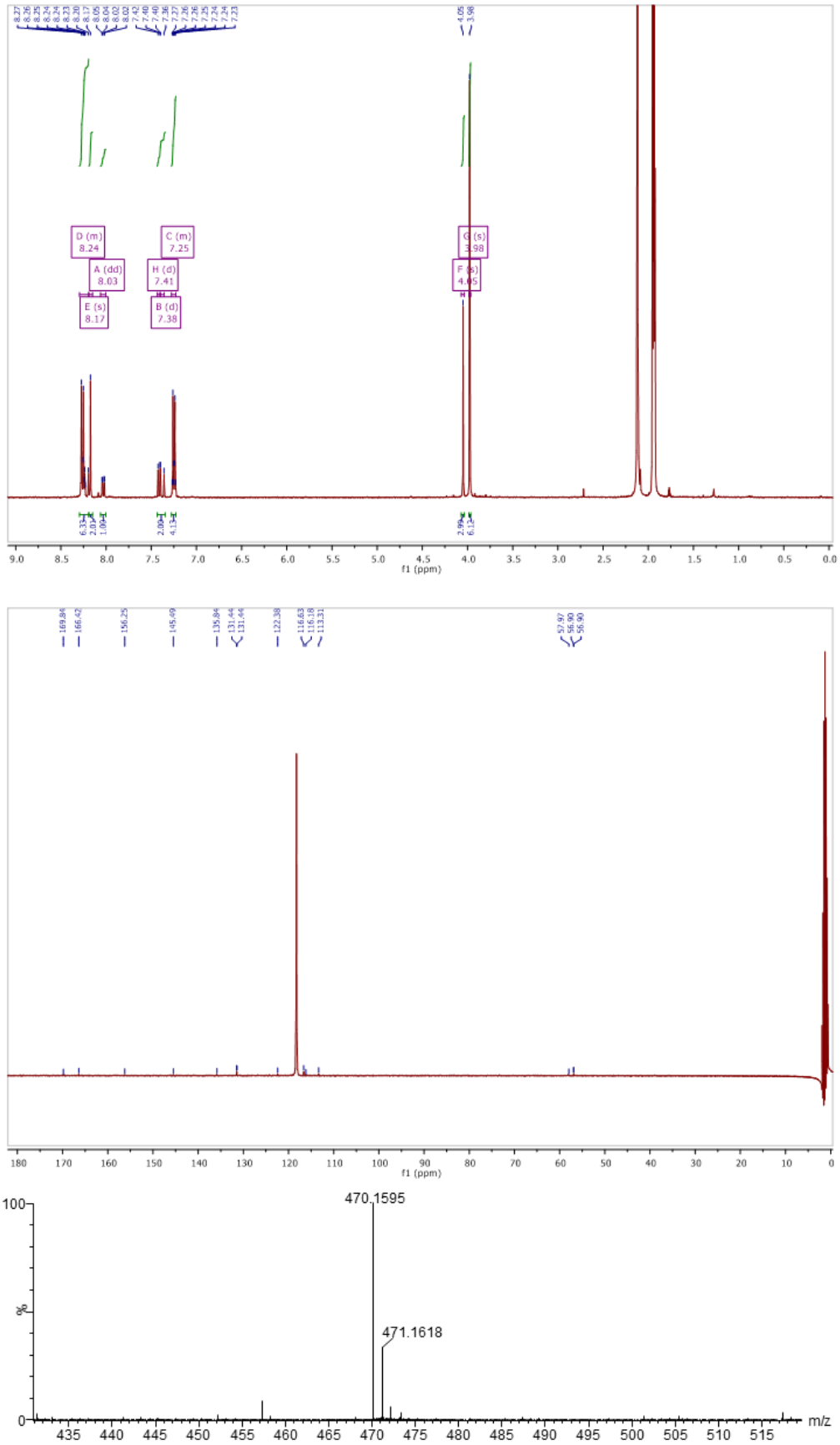


Figure S10. ^{1}H NMR, ^{13}C NMR (CD_3CN) and HRMS spectra of the precursor of compound **2**.

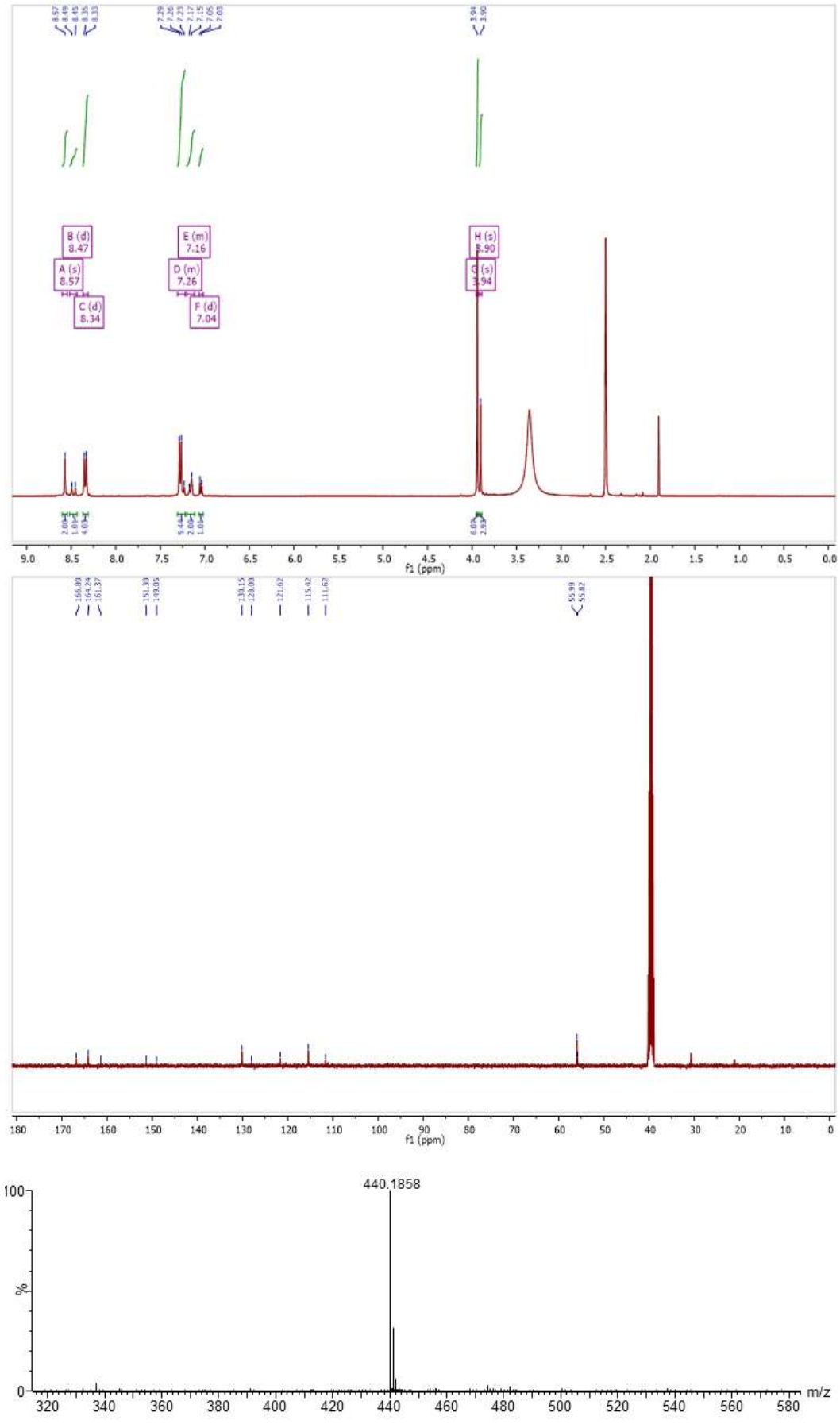


Figure S11. ^{1}H NMR, ^{13}C NMR (DMSO-d_6) and HRMS spectra of compound **2**.

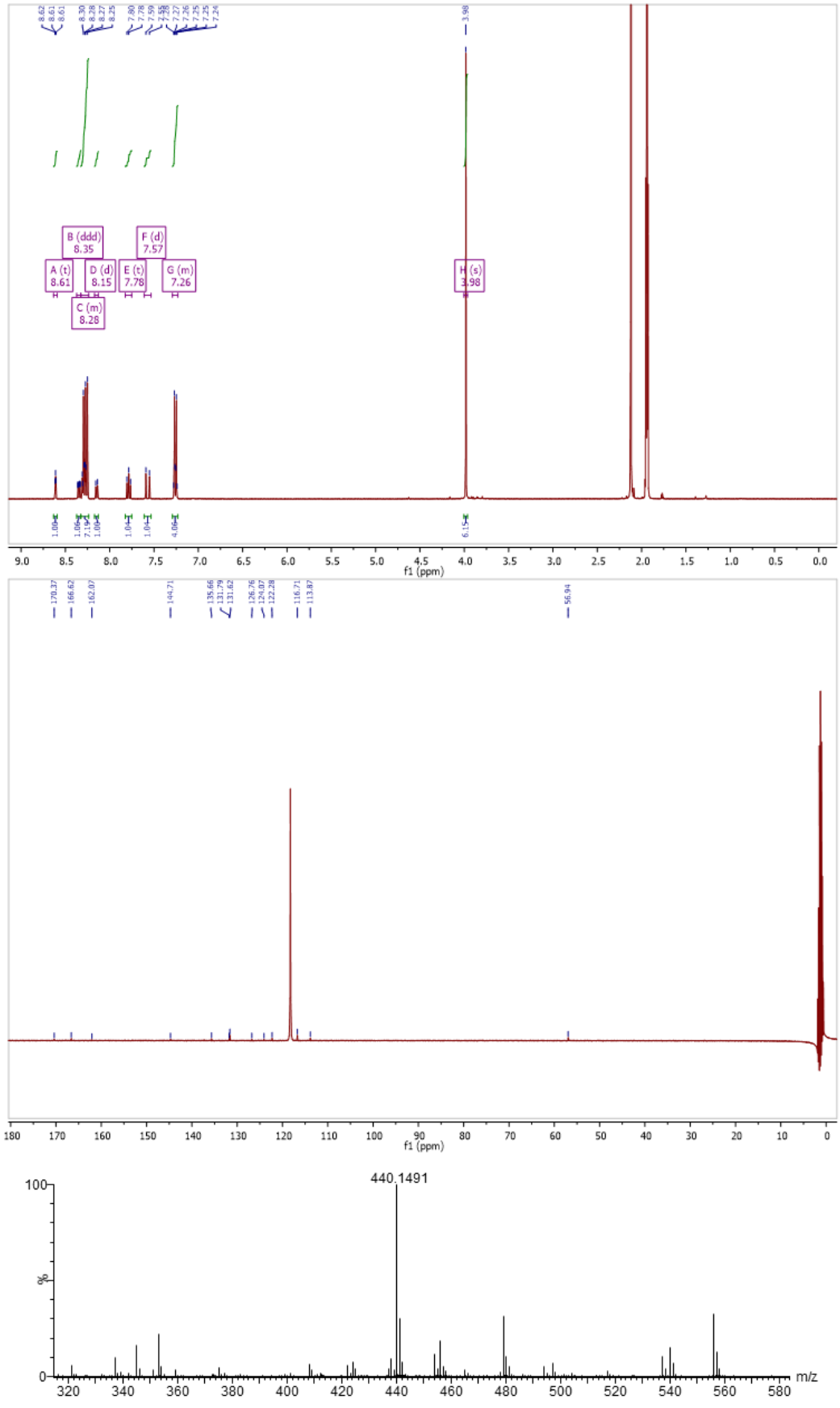


Figure S12. ^{1}H NMR, ^{13}C NMR (CD_3CN) and HRMS spectra of the precursor of compound **3**.

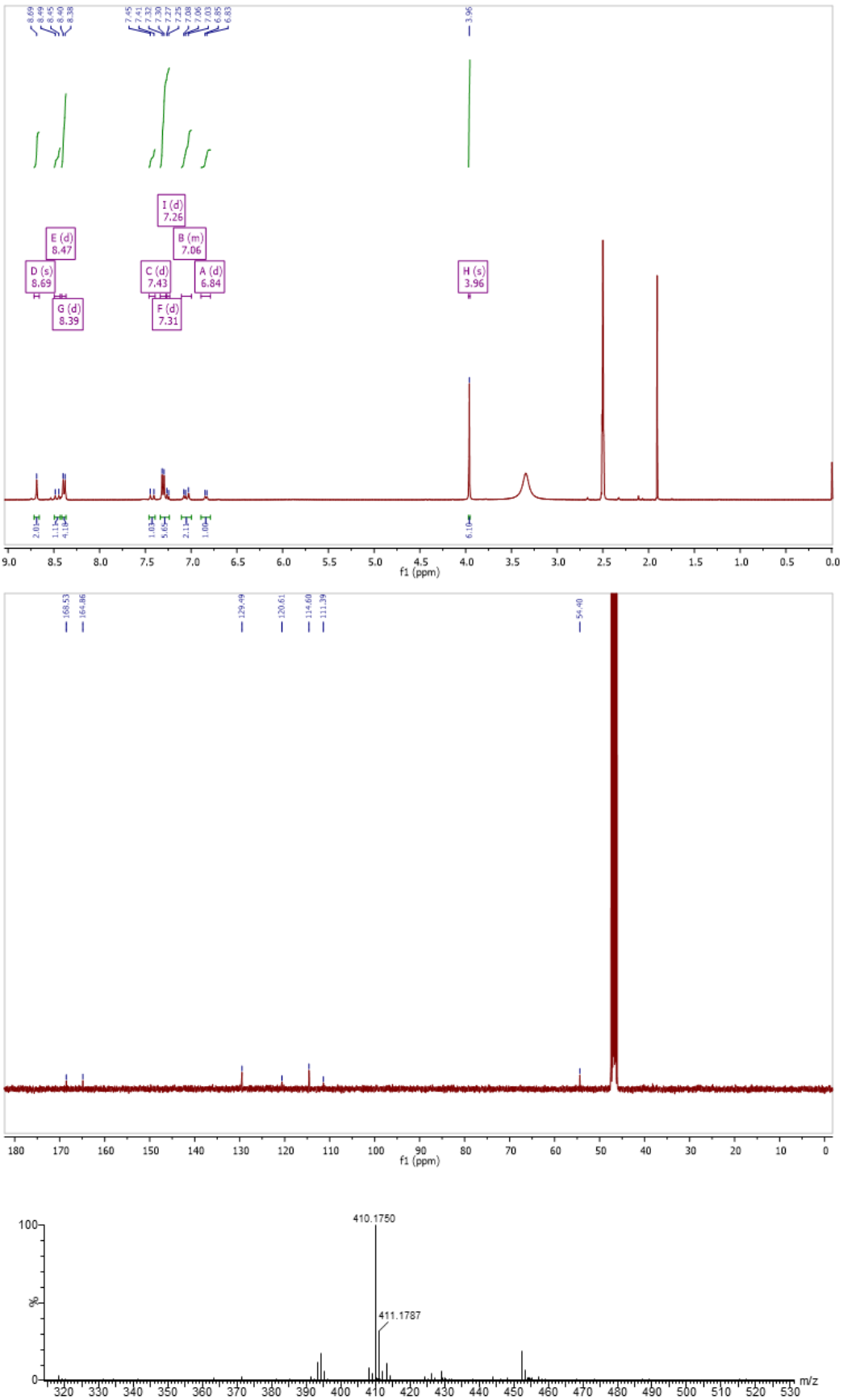


Figure S13. ^{1}H NMR, ^{13}C NMR (CD_3CN) and HRMS spectra of compound **3**.

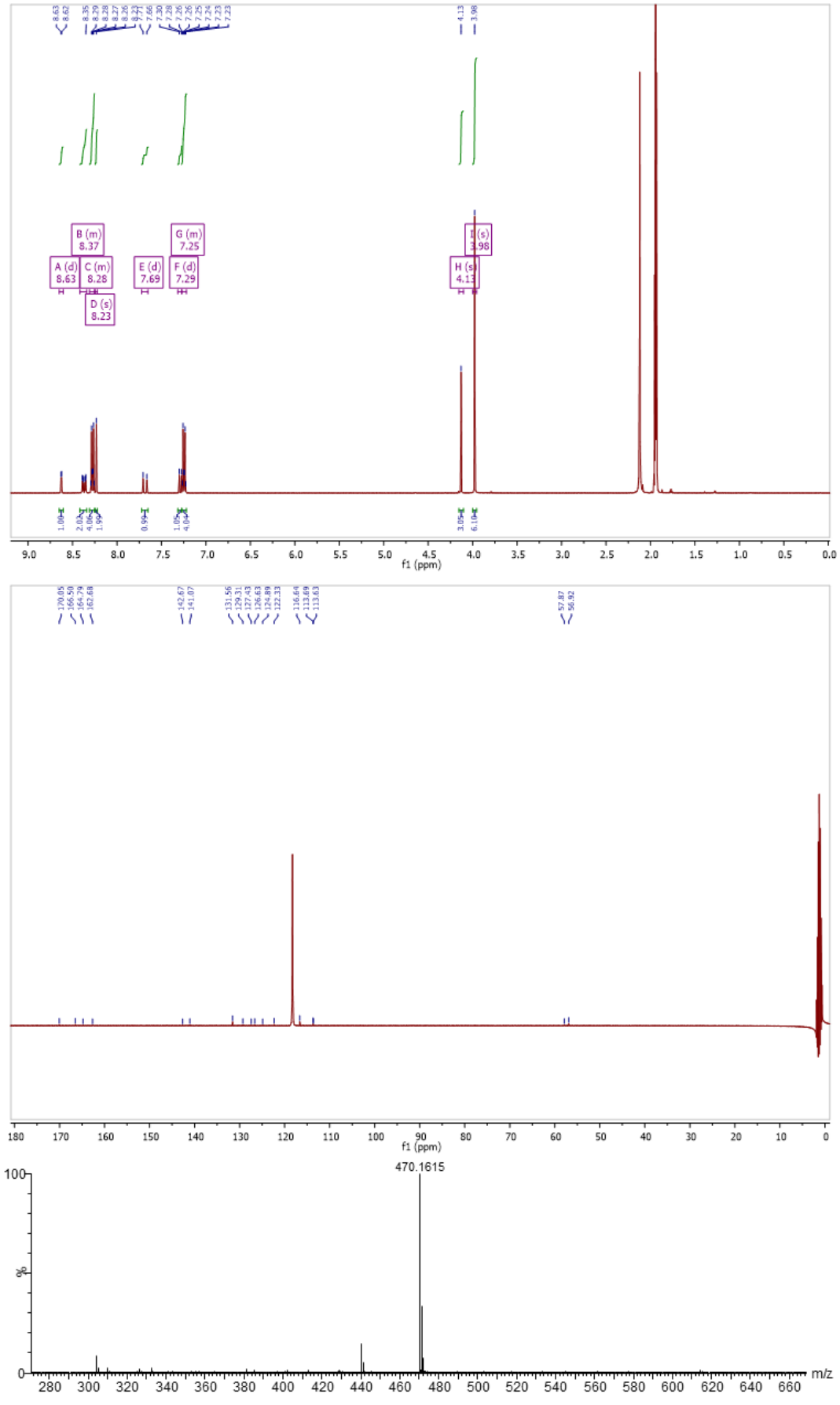


Figure S14. ^{1}H NMR, ^{13}C NMR (CD_3CN) and HRMS spectra of the precursor of compound **4**.

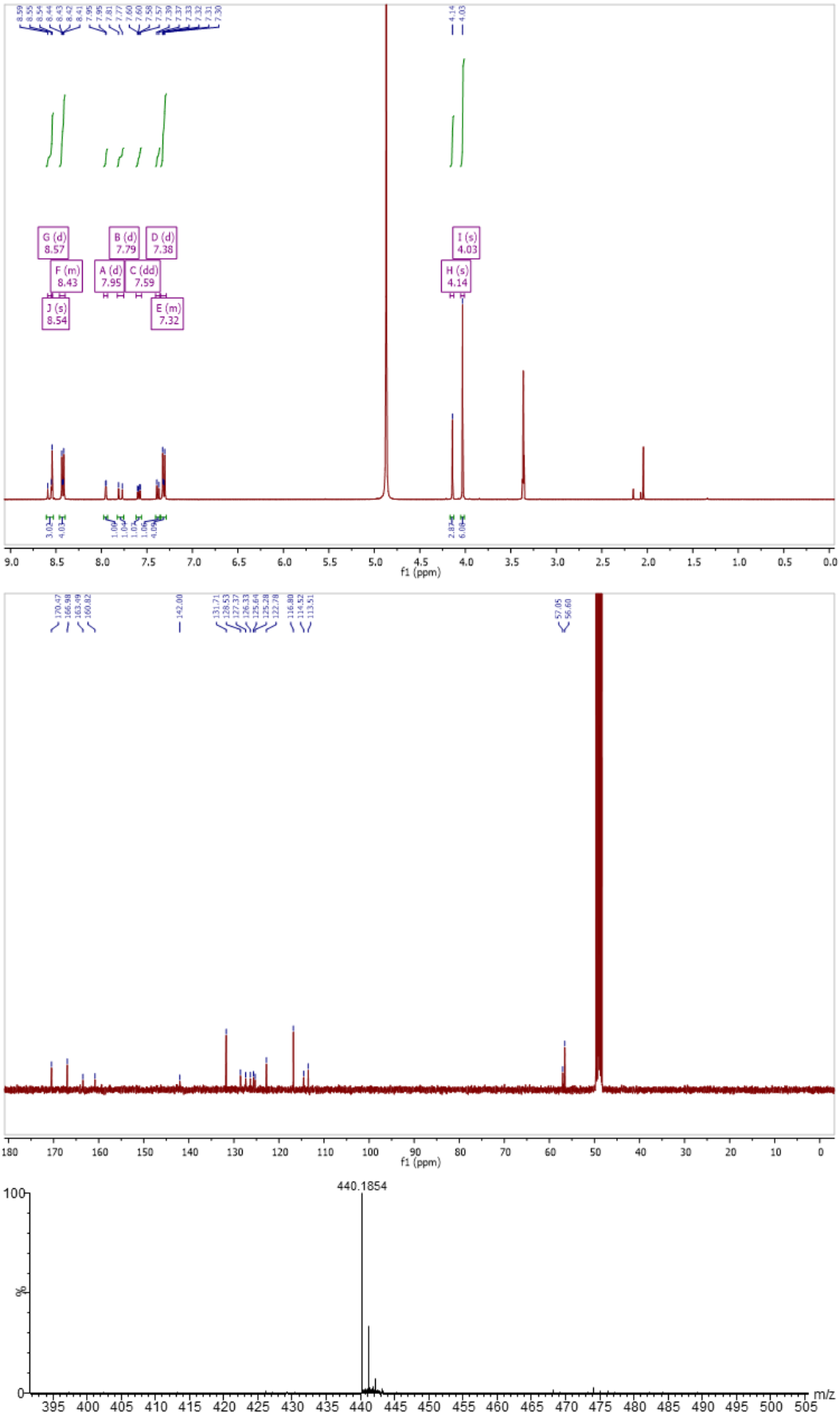


Figure S15. ^{1}H NMR, ^{13}C NMR (CD_3OD) and HRMS spectra of compound **4**.

References

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