SUPPORTING INFORMATION

A near-infrared light-activatable Ru(II)-coumarin photosensitizer active under hypoxic conditions

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1. Synthesis and characterization of the compounds

*Synthesis of compound **6***

Coumarin 153 (1 g, 3.23 mmol) and Lawesson's reagent (791 mg, 1.94 mmol) were dissolved in toluene (50 mL) and heated at 100 °C for 15 h. After evaporation under reduced pressure, the residue was purified by column chromatography (silica gel, 0-40% DCM in hexanes) to give a maroon solid (1.14 mg, 95%). TLC Rf (DCM) 0.9. ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 7.12 (1H, s), 7.10 (1H, s), 3.33 (4H, m), 2.98 (2H, t, *J* = 6.4 Hz), 2.78 (2H, t, *J* = 6.2 Hz), 1.99 (4H, m). ^{13}C{^{1}H} NMR (101 Hz, $CDCl_3$) δ(ppm) 197.7, 155.4, 147.0, 133.2 (q, *J* = 32 Hz), 122.8 (q, *J* = 277 Hz), 122.1, 120.9, 119.6 (q, *J* = 6.0 Hz), 106.4, 105.2, 50.2, 49.8, 28.1, 21.2, 20.6, 20.3. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{16}H_{15}F_3NOS$ 326.0821, found 326.0816.

*Synthesis of compound **7***

To a solution of 4-pyridylacetonitrile hydrochloride (380.2 mg, 2.46 mmol) and NaH (60% dispersion in mineral oil, 245.9 mg, 6.15 mmol) in dry acetonitrile (100 mL) under an Ar atmosphere and protected from light, a solution of coumarin **6** (400 mg, 1.23 mmol) in dry acetonitrile (20 mL) was added. After the mixture was stirred for 3 h at room temperature, silver nitrate (459.5 mg, 2.70 mmol) was added, and the reaction mixture was stirred at room temperature for 2 h under an Ar atmosphere and protected from light. The crude product was evaporated under reduced pressure and purified by column chromatography (silica gel, 0−1.2% MeOH in DCM) to give 471.4 mg of a red golden solid (yield 94%). TLC: Rf (10% MeOH in DCM) 0.33. ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 8.62 (2H, d, *J* = 6.4 Hz), 7.70 (2H, d, *J* = 6.4 Hz), 7.09 (1H, s), 7.00 (1H, s), 3.30 (4H, m), 2.86 (2H, t, *J* = 6.4 Hz), 2.76 (2H, t, *J* = 6.3 Hz), 1.99 (4H, m). ^{13}C{^{1}H} NMR (101 Hz, DMSO-d_6) δ(ppm) 160.9, 150.6, 150.5, 150.0, 146.5, 140.0, 133.1 (q, *J* = 32 Hz), 122.7, 122.4, 122.3 (q, *J* = 277 Hz), 121.6, 119.4, 118.7, 111.0 (q, *J* = 6.0 Hz), 106.7, 103.3, 87.0, 50.2, 49.4, 27.8, 21.5, 21.3, 20.7. HRMS (ESI-TOF) *m/z* $[M + H]^+$ calcd for $C_{23}H_{19}F_3N_3O$ 410.1475, found 410.1475.

*Synthesis of compound **9***

Bromoacetyl bromide (331 μL, 3.80 mmol) was added to a cooled down solution (-10 ºC) of *N*-Boc-1,3-diaminopropane hydrochloride (400 mg, 1.90 mmol) in a 1:1 (v/v) mixture of DCM/$NaHCO_3$ sat. (60 mL). The mixture was vigorously stirred at room temperature for 3 h. The DCM was evaporated under reduced pressure and the aqueous phase was extracted with AcOEt (2 × 100 mL). The organic phase was washed consecutively with aqueous solutions of sat. $NaHCO_3$ (2 x 50 mL), HCl 5% (2 x 50 ml) and brine (2 x 50 mL). The combined organic fractions were dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was purified by column chromatography (silica gel, 0-60% AcOEt in hexanes) to give 420.6 mg of white solid (yield 71%). TLC: Rf (50% AcOEt in hexanes) 0.53. ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm) 7.08 (1H, br s), 4.85 (1H, br s), 3.86 (2H, s), 3.34 (2H, q, *J* = 6.3 Hz), 3.18 (2H, q, *J* = 6.3 Hz), 1.66 (2H, qt, *J* = 6.3 Hz), 1.44 (9H, s). ^{13}C{^{1}H} NMR (101 Hz, $CDCl_3$) δ(ppm) 166.1, 156.8, 79.6, 37.2, 36.9, 30.1, 29.3, 28.5. HRMS (ESI-TOF) *m/z* $[M + Na]^+$ calcd for $C_{10}H_{19}BrN_2NaO_3$ 317.0471, found 317.0472.

*Synthesis of compound **8***

Compound **9** (180.2 mg, 0.61 mmol) was added to a solution of coumarin **7** (100 mg, 0.24 mmol) in AcOEt/ACN 1:1 (50 mL). The mixture was stirred for 48 h at 60 °C protected from light. The crude product was evaporated under reduced pressure and purified by column chromatography (silica gel, 0−10% MeOH in DCM) to give 156 mg of a blue solid (yield, 97%). TLC: Rf (10% MeOH in DCM) 0.43. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) 8.75 (2H, d, *J* = 6.6 Hz), 8.56 (1H, br s), 8.18 (2H, d, *J* = 6.6 Hz), 7.16 (1H, s), 6.99 (1H, s), 6.82 (1H, s), 5.28 (2H, s), 3.40 (4H, m), 3.13 (2H, q, *J* = 6.6 Hz), 2.95 (4H, m), 2.81 (2H, m), 1.92 (4H, m), 1.57 (2H, qt, *J* = 6.9 Hz), 1.37 (9H, s). ^{13}C{^{1}H} NMR (101 Hz, DMSO-d_6) δ(ppm). 164.9, 164.5, 155.6, 151.0, 148.2, 147.8, 145.0, 135.0 (q, *J* = 32 Hz), 122.2, 121.9 (q, *J* = 276 Hz), 121.4, 117.6, 107.6 (q, *J* = 6.0 Hz), 106.1, 103.6, 82.9, 77.5, 60.1, 49.7, 48.8, 37.5, 36.9, 29.3, 27.1, 20.8, 20.2, 19.6. HRMS (ESI-TOF) *m/z* $[M]^+$ calcd for $C_{33}H_{37}F_3N_5O_4$ 624.2792, found 624.2793.

*Synthesis of compound **4***

A cooled down solution of hydrochloric acid in dioxane (4 M, 17 mL) was added to coumarin **8** (33.3 mg, 0.047 mmol)). The reaction mixture was stirred for 25 min at room temperature under an Ar atmosphere and protected from light. After removal of the solvent, several co-evaporations from acetonitrile were carried out. The crude product was used without further purification in the next step since HPLC-MS analysis revealed that the removal of the Boc group was quantitative. Analytical HPLC (10 to 70% B in 30 min; system A): Rt = 14.25 min. LRMS (ESI-TOF) *m/z*: $[M]^+$ Calcd for $C_{28}H_{29}F_3N_5O_2$ 524.23, found 524.18.

*Synthesis of compound **2.***

Methyl trifluoromethanesulfonate (17 μL, 0.15 mmol) was added to a solution of coumarin **7** (20 mg, 0.049 mmol) in DCM (20 mL) under an argon atmosphere. The mixture was stirred overnight at room temperature and protected from light. The reaction mixture was evaporated under reduced pressure and purified by column chromatography (silica gel, 0−9% MeOH in DCM) to give 16.7 mg of a blue solid (yield 60%). TLC: Rf (10% MeOH in DCM) 0.36. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) 8.80 (2H, d, *J* = 7.0 Hz), 8.16 (2H, d, *J* = 7.0 Hz), 7.15 (1H, s), 6.97 (1H, s), 4.23 (3H, s), 3.40 (4H, m), 2.89 (2H, t, *J* = 6.3 Hz), 2.80 (2H, t, *J* = 6.0 Hz), 1.91 (4H, m). ^{13}C{^{1}H} NMR (101 Hz, DMSO-d_6) δ(ppm) 164.5, 150.8, 147.6, 147.4, 144.5, 122.1, 121.9, 121.4, 119.1, 117.6, 107.6, 106.2, 103.4, 82.8, 49.7, 48.8, 46.6, 27.0, 20.8, 20.3, 19.6. Analytical HPLC (5 to 100% B in 2.5 min, isocratic 100% B for 1.3 min; system B): Rt = 2.5 min. HRMS (ESI-TOF) *m/z* $[M]^+$ calcd for $C_{24}H_{21}F_3N_3O$ 424.1631, found 424.1634.

*Synthesis of compound **1b***

A solution of Ru(II) complex **1a** (25.5 mg, 0.023 mmol) in MeOH (25 mL) was mixed with an aqueous solution of NaOH 1.5 M (75 mL). The mixture was stirred for 72 h at 40 ºC and protected from light. After evaporation of the MeOH under reduced pressure, the aqueous phase was neutralized with an aqueous saturated solution of NH_4Cl (pH between 7-8). Then, the aqueous phase was extracted with DCM (3 x 15 mL) and the combined organic fractions were dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was used without further purification since HPLC-MS analysis revealed that the hydrolysis of the ester group was quantitative. Analytical HPLC (5 to 100% B in 2.5 min, isocratic 100% B

for 1.3 min; system B): Rt = 2.9 min. LRMS (ESI-TOF) *m/z*: $[M]^+$ Calcd for $C_{52}H_{37}N_{10}O_2Ru$ 935.21, found 935.3.

*Synthesis of Ru(II)-COUPY conjugate **3***

To a solution of Ru(II) complex **1b** (17 mg, 15.7 μmol) and HATU (6.0 mg, 15.7 μmol) in anhydrous DMF (4 mL) under an Ar atmosphere, DIPEA (9 μL, 47.2 μmol) was added and the mixture stirred for 10 min under Ar at room temperature and protected from light. After addition of a solution of coumarin **4** (26.0 mg, 43.6 μmol) and DIPEA (14 μL, 78.7 μmol) in anhydrous DMF (3 mL), the reaction mixture was stirred for 2.5 h at room temperature under Ar and protected from light. After evaporation under reduced pressure, the crude was purified by column chromatography (neutral aluminium oxide, 0-2.5% MeOH in DCM) to give 8.2 mg of dark blue solid (yield: 34%). TLC: Rf (10% MeOH in DCM) 0.50. ^{1}H NMR (400 MHz, CD_3OD) δ(ppm) 9.60 (1H, dd, *J* = 8.2, 1.2 Hz), 9.38 (2H, m), 9.34 (1H, dd, *J* = 8.2, 1.2 Hz), 9.16 (2H, m), 9.14 (2H, m), 8.70 (1H, dd, *J* = 5.6, 1.2 Hz), 8.63 (1H, m), 8.55 (2H, m), 8.42 (1H, dd, *J* = 5.6, 1.2 Hz), 8.19 (1H, dd, *J* = 5.6, 1.2 Hz), 8.12 (2H, m), 7.94 (1H, m), 7.77 (3H, m), 7.66 (2H, m), 7.55 (1H, m), 7.26 (2H, m), 7.16 (5H, m), 7.11 (1H, s), 6.69 (1H, d, *J* = 2.0 Hz), 6.44 (1H, d, *J* = 2.0 Hz), 5.20 (2H, m), 3.75 (4H, m), 3.66 (4H, m), 3.57 (2H, m), 2.85 (2H, m), 1.99 (4H, m), 1.73 (2H, m), 1.61 (2H, m), 1.48 (2H, m), 1.36 (2H, m), 1.00 (3H, t, *J* = 7.2 Hz). Analytical HPLC (5 to 100% B in 2.5 min, isocratic 100% B for 1.3 min; system B): Rt = 2.77 min. HRMS (ESI-TOF) *m/z* $[M]^{2+}$ Calcd for $C_{80}H_{64}F_3N_{15}O_3Ru$ 720.7151; Found 720.7170.

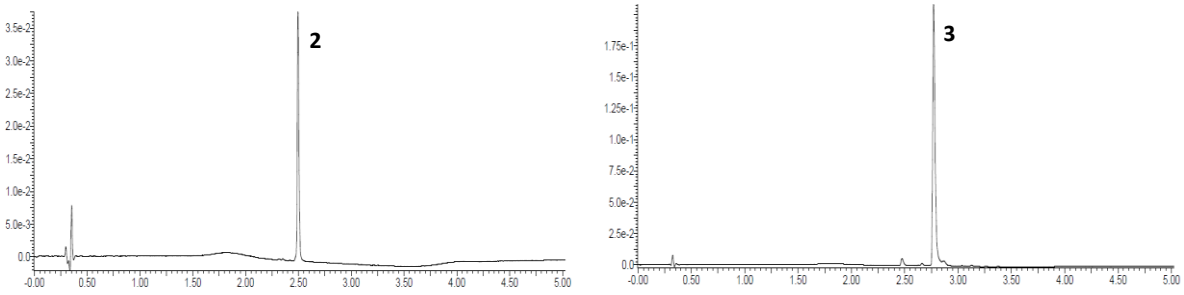


Figure S1. Reversed-phase HPLC traces of purified COUPY **2** (left) and Ru(II)-COUPY conjugate **3** (right).

2. Photophysical characterization

For photophysical measurements, all solvents used were spectroscopic grade. Absorption spectra were recorded on a Jasco V-730 UV-Vis spectrophotometer at room temperature. Molar absorption coefficients (ε) were determined by direct application of the Beer-Lambert law, using solutions of the compounds in each solvent with concentrations ranging from 10^{-6} to 10^{-5} M. Emission spectra were measured on a Photon Technology International (PTI) Quantamaster fluorimeter. Fluorescence quantum yields (Φ_F) were measured by a comparative method using Cresyl violet in ethanol (Φ_F = 0.54)[i] as a standard for compounds **2** and **3**. Then, optically matched solutions of the probes and the appropriate standard were prepared and fluorescence spectra were recorded. The absorbance of the sample and the standard solutions was set below 0.1 at the excitation wavelength (540 nm for cresyl violet and compounds **2** and **3**) and Φ_F was calculated using the following Equation (1):

$$\Phi_{F,x} = \Phi_{F,Std.} \times \left(\frac{Area_x}{Area_{Std.}}\right) \times \left(\frac{{\eta_x}^2}{{\eta_{Std.}}^2}\right) \qquad (1)$$

where $Area_x$ and $Area_{Std.}$ are the integrated fluorescence for the sample and the standard and η_x and $\eta_{Std.}$ are the refractive index of the sample and the standard solution, respectively. The uncertainty in the experimental value of Φ_F has been estimated to be approximately 10%.

Singlet oxygen quantum yield determination

Singlet oxygen generation was studied by time-resolved near-infrared phosphorescence by means of a customised setup. Briefly, a pulsed Nd:YAG laser (FTSS355-Q, Crystal Laser, Berlin, Germany) working at 1 or 10 kHz repetition rate at 355 nm (0.5 μJ per pulse) or 532 nm (1.2 μJ per pulse) was used to excite the sample. A 1064-nm rugate notch filter (Edmund Optics) and an uncoated SKG-5 filter (CVI Laser Corporation) were placed in the laser path to remove any NIR emission. The light emitted by the sample was filtered with a 1000-nm long-pass filter (Edmund Optics) and later by a narrow bandpass filter at 1275 nm (BK-1270-70-B, bk Interferenzoptik). A thermoelectric-cooled NIR-sensitive photomultiplier tube assembly (H9170-45, Hamamatsu Photonics, Hamamatsu, Japan) was used as detector. Photon counting was achieved with a multichannel scaler (NanoHarp 250, PicoQuant). The time dependence of the $^1O_2$ phosphorescence with the signal intensity $S(t)$ is described by Equation 2, in which τ_T and τ_Δ are the lifetimes of the photosensitizer triplet state and of $^1O_2$ respectively, and S_0 a preexponential parameter proportional to Φ_Δ.

$$S_{1275}(t) = S_{1275}(0) \times \frac{\tau_\Delta}{\tau_\Delta - \tau_T} \times \left(e^{-t/\tau_\Delta} - e^{-t/\tau_T}\right) \qquad (2)$$

The Φ_Δ values of the different samples were obtained by comparing S_0 values of optically matched samples and using an appropriate reference, by means of equation 3.

$$\Phi_{\Delta,\text{sample}} = \Phi_{\Delta,\text{ref}} \times \frac{S_{0Sample}}{S_{0Ref}} \qquad (3)$$

The same setup was used to monitor the phosphorescence of the complex and the conjugate, except that the red-sensitive Hamamatsu H5783 photosensor module was used for detection.

Transient absorption spectroscopy

The excited-state behaviour of the of the Ru(II) complex 1a, COUPY coumarin 2, and the Ru(II)-COUPY conjugate 3 were studied using a home-built nanosecond laser flash photolysis system with transient absorption detection. To this end, the 2nd harmonic of a Q-switched pulsed Nd:YAG laser (Surelite I-10, Continuum, Santa Clara, CA), operating at 0.5 Hz repetition rate and emitting 5-ns, 10 mJ light pulses at 532 nm, was used to excite the samples in argon- and air-saturated aqueous solutions. Transient absorption was monitored at 625 nm using a white-light beam probe produced by a CW 75 W Xe lamp (Photon Technology International (PTI), Notthingham, NJ) in a right-angles geometry, which was then passed through a dual-grating monochromator (mod. 101, PTI) and detected with a Hamamatsu R928 photomultiplier appropriately wired. The signal was fed to a WaveSurfer 454 oscilloscope (Teledyne Lecroy, Chestnut Ridge, NY) for digitizing and averaging and finally transferred to a PC for data storage and analysis. The transients' decay kinetics were analyzed using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, CA) using the Levenberg-Marquardt non-linear regression algorithm.

Superoxide anion radical generation using DHR123

All compounds (10 µM) were prepared in PBS (0.2 % DMSO). To this solution, DHR123 was added so that its final concentration was 10 µM. Then the samples were irradiated in 1.0 x 0.5 cm cuvette by green light (505 nm centered LED) for indicated time intervals. Immediately, the fluorescence spectra were collected by using a Photon Technology International (PTI) fluorimeter. The excitation wavelength was set to 500 nm, the excitation and emission slit widths were 2 nm, and the integration time was set to 1 s.

Photostability

Photostability studies were performed by monitoring absorbance of aqueous solutions (0.2 % DMSO) of the compounds irradiated at 37 ºC in a custom-built irradiation setup from Microbeam, which includes a cuvette, thermostated cuvette holder, and mounted high-power LED of red light (620±15 nm; 130 mW cm^{-2}).

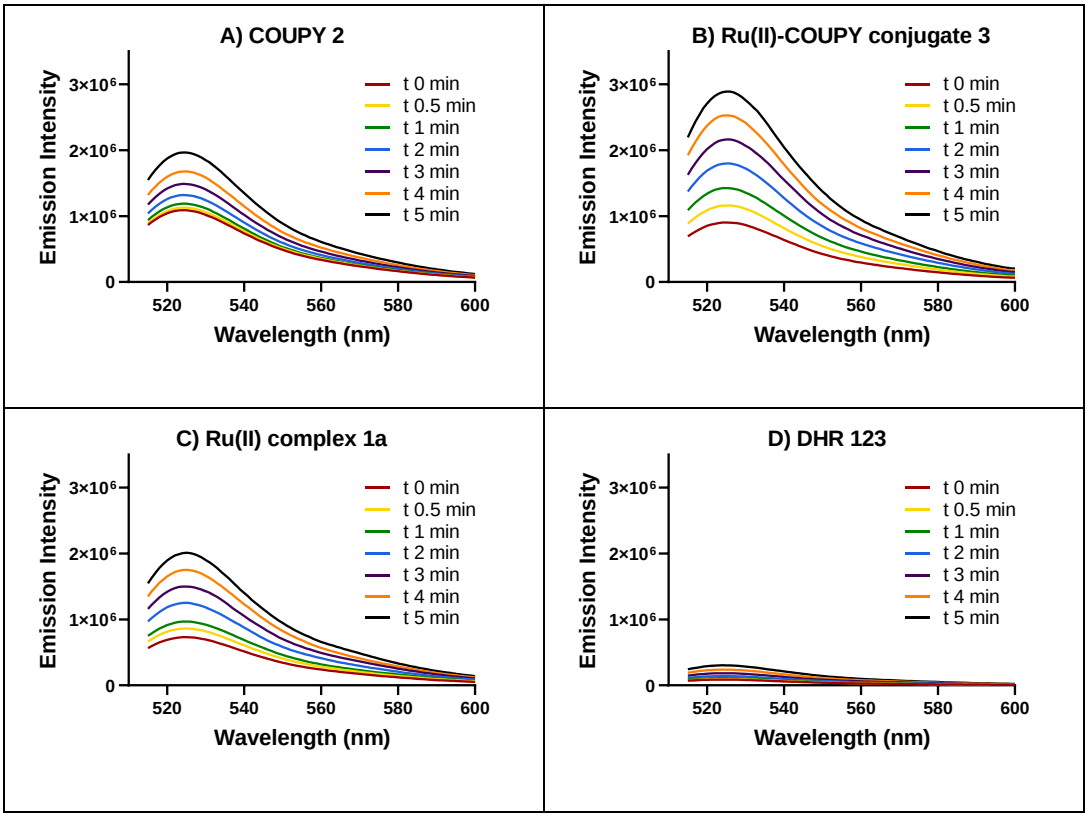


Figure S2. Fluorescence spectra of DHR123 induced by irradiation with visible light (505 nm) for 5 min, in the presence of COUPY coumarin **2** (A), Ru(II)-COUPY conjugate **3** (B), Ru(II) complex **1a** (C), or without any compound (DHR 123 alone, D) in PBS.

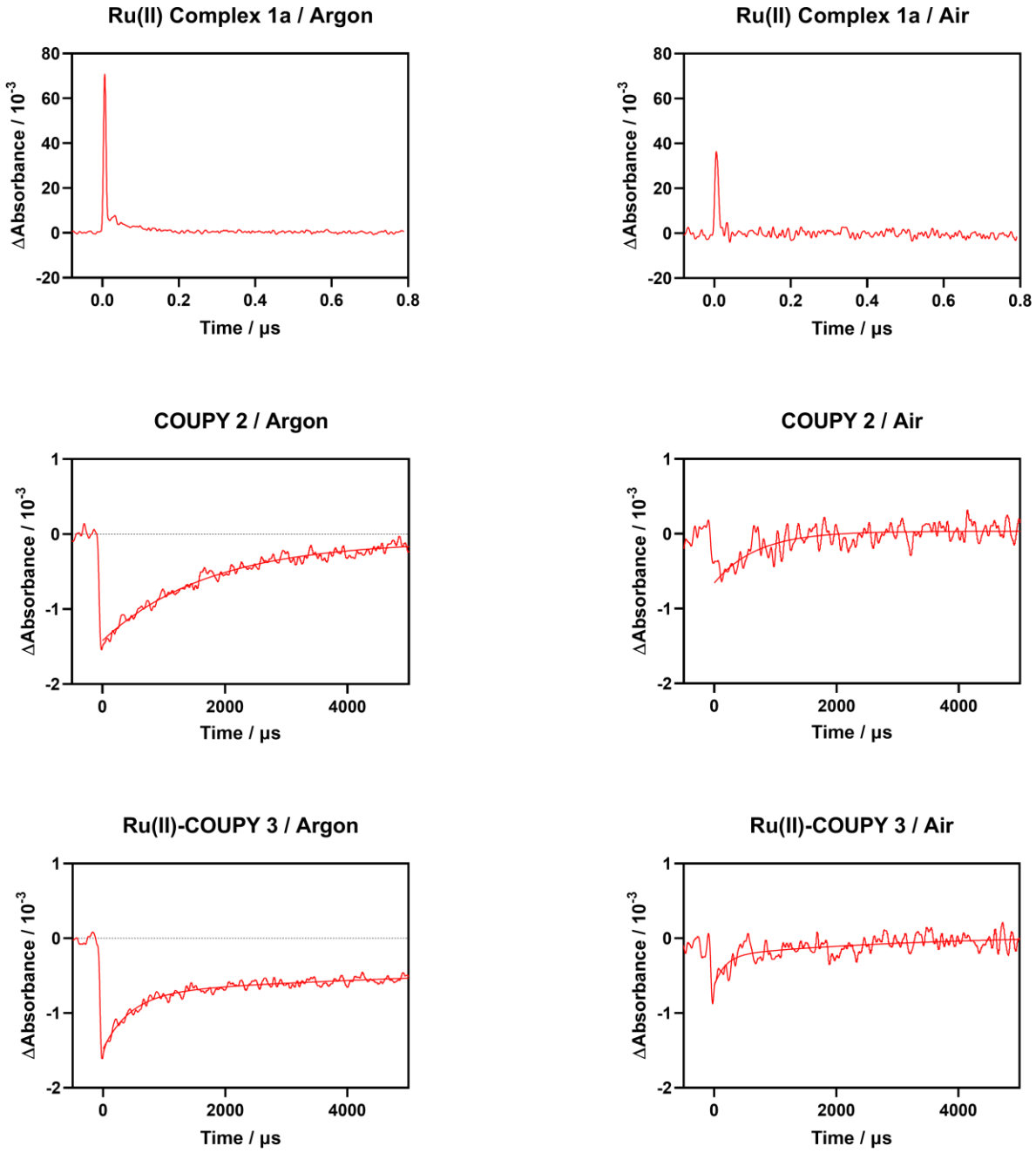


Figure S3. Transient absorption signals obtained upon laser flash photolysis at 532 nm of the Ru(II) complex **1a**, COUPY coumarin **2**, and the Ru(II)-COUPY complex **3** in argon-saturated (left) and air-saturated (right) aqueous solutions. The transients were observed at 625 nm, where the signal is maximum, corresponding to the depletion of the coumarin band.

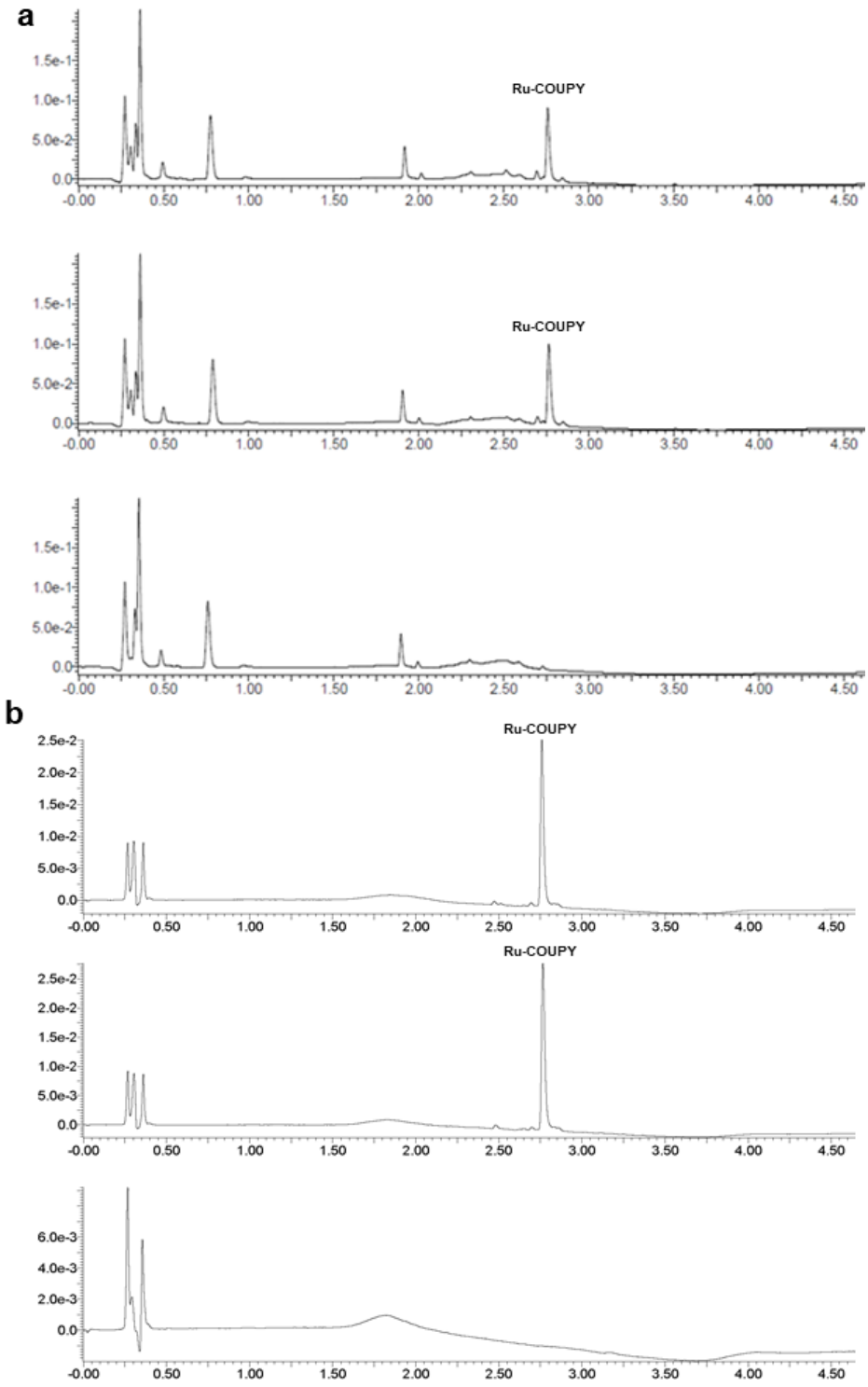


Figure S4. Stability of Ru(II)-COUPY conjugate **3** in cell culture medium (DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 $U \cdot mL^{-1}$ of penicillin-streptomycin mixture). From bottom to top: reversed-phase HPLC analysis of cell culture medium, conjugate **3** dissolved in the medium at t = 0 and after incubation for 2 h at 37°C. Detection wavelengths: 260 nm (**a**) and 600 nm (**b**).

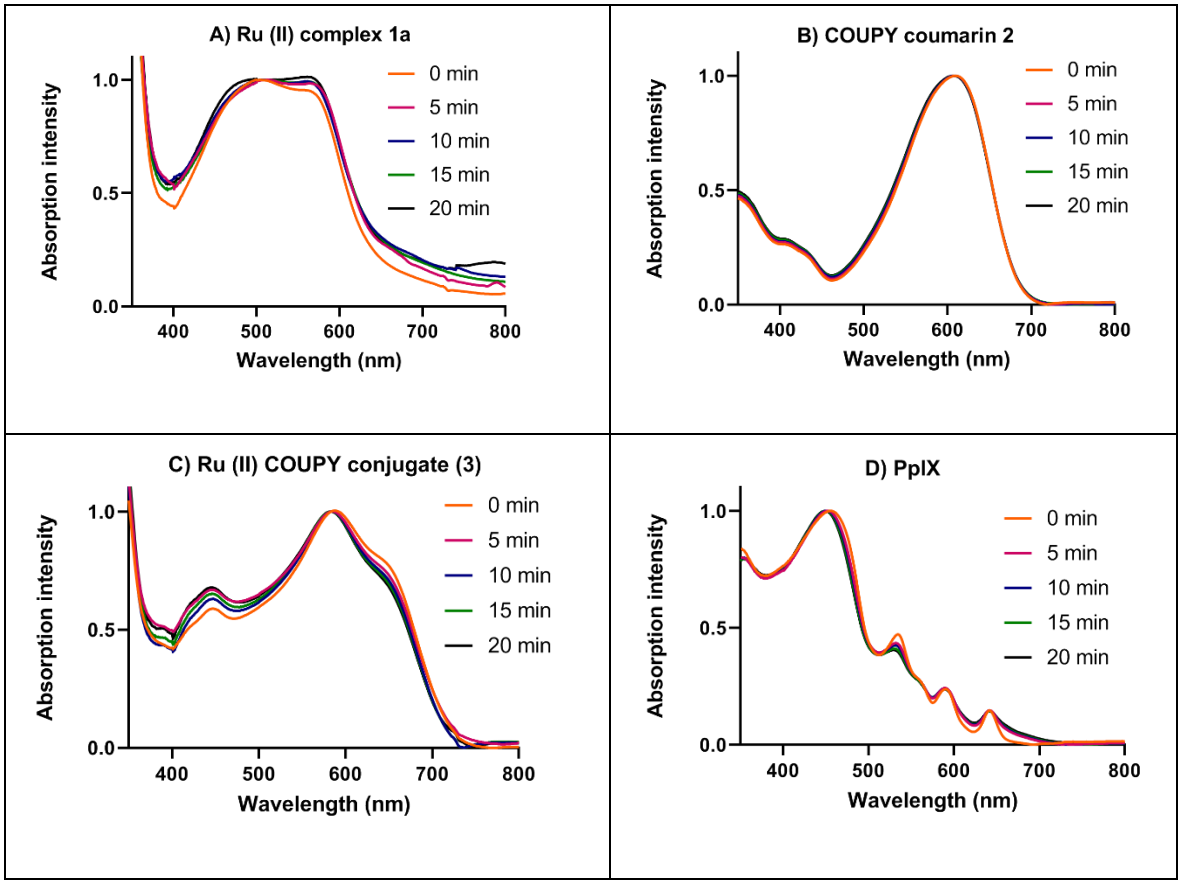


Figure S5. Photostability of the compounds **1a** (A), **2** (B) and **3** (C) upon irradiation with red LED light (620 nm, 130 mW cm^{-2}) at 37 ^{o}C in water. Protoporphyrin IX (D) was included as a reference.

3. Lipophilicity and subcellular distribution studies

Distribution coefficients

Distribution coefficients between *n*-octanol and water ($K_{O/W}$) and log *P* values of the investigated compounds (**Ru**, **COUPY** and **Ru-COUPY**) were calculated by the "shake-flask" method (adapted from [ii]). To this end, solutions of the compounds in Milli-Q H_2O-saturated *n*-octanol (4 mL, final concentration 30 μM) were prepared in centrifuge tubes from a 10 mM stock solution in DMSO. The solutions were sonicated for 5 min in an ultrasonic bath and a 2-mL aliquot of each solution was reserved in another centrifuge tube. To the remaining 2-mL of the solutions, an equal volume of *n*-octanol-saturated Milli-Q H_2O was added, and the resulting mixtures were vigorously shaken in a vortex for 15 min. Then, the octanol/water mixtures were centrifuged at 7800 rpm for 5 min to separate the phases. The UV-Vis absorption spectra of the organic phases, as well as those of the reserved aliquots were registered using a Jasco V-550 UV-Vis spectrophotometer. Log *P* values were calculated according to Equation 4:

$$\log P = \log(K_{O/W}) = log\left(\frac{A}{A_0 - A}\right) \qquad (4)$$

where A_0 refers to the absorbance of the reserved aliquots of the compounds at their maximum absorption wavelengths (Table S1, λ_{Abs}(**Ru**) = 561 nm, λ_{Abs}(**COUPY**) = 615 nm, λ_{Abs}(**Ru-COUPY**) = 623 nm) and *A* is the absorbance of the *n*-octanol phase of the corresponding octanol/water mixtures at the same wavelengths. Data are expressed as mean ± SD from two independent experiments.

Table S1. Log *P* values of **Ru**, **COUPY** and **Ru-COUPY** in *n*-octanol/water.

Compound	log *P*
Ru	+2.43 ± 0.27
COUPY	+0.36 ± 0.03
Ru-COUPY	+0.68 ± 0.08

Subcellular distribution of metal complexes by ICP-MS

Ru content distribution inside HT-29 cells was determined by inductively coupled plasma-mass spectrometry (ICP-MS) analysis. Briefly, cell pellets were obtained as described above and were fractionated using the Cell Fractionation kit (PromoCell) according to the manufacturer's

instructions, which allowed to extract membrane, cytosolic, nuclear and cytoskeleton fractions by differential centrifugation. Each sample was digested with 15% HNO_3 suprapur acid and subjected to analysis in Agilent 7900 ICP-MS. ^{99}Ru and ^{101}Ru isotopes were measured. Experiments were carried out in triplicate with n = 2 replicates.

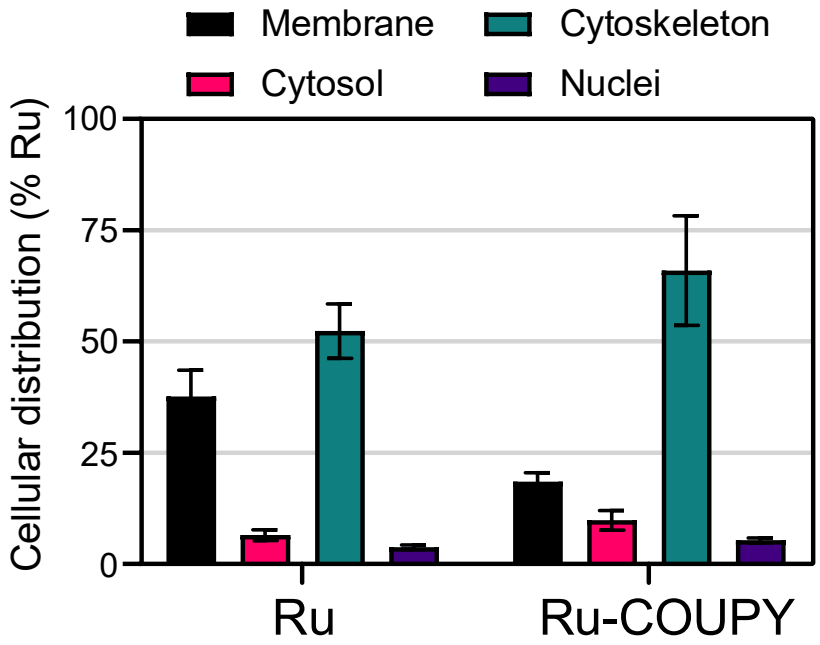


Figure S6. Percentage of Ru in different fractions (membrane, cytosol, cytoskeleton and nuclei) over total uptake in HT-29 cells after 1 h incubation at 10 µM with **Ru** or **Ru-COUPY**. Data expressed as mean ± SD from three independent measurements.

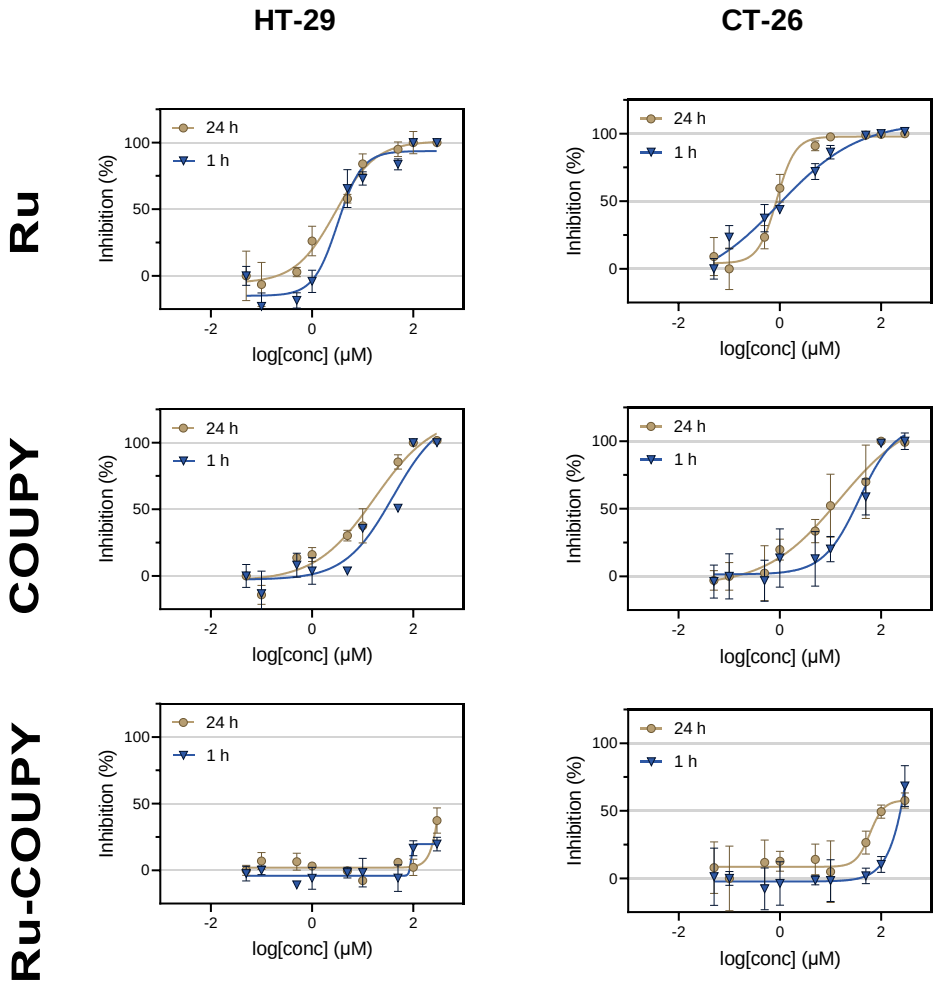
4. Photobiological studies

Table S2. IC_{50} values [µM] of selected compounds under dark or upon irradiation.[a]

	HT-29			CT-26			HeLa			A2780		
	Dark	620 nm	PI[b]	Dark	620 nm	PI	Dark	620 nm	PI	Dark	620 nm	PI
COUPY	44 ± 7	0.98 ± 0.03	45	35 ± 10	1.0 ± 0.2	35	61 ± 4	0.38 ± 0.04	161	37 ± 7	0.09 ± 0.01	411
Ru	2.4 ± 0.4	0.18 ± 0.08	39	2.1 ± 0.3	0.17 ± 0.01	12	7 ± 1	0.11 ± 0.03	63	0.8 ± 0.1	0.09 ± n.d	9
Ru-COUPY	>300	3.1 ± 0.1	>97	>300	1.0 ± 0.1	>300	>300	2.9 ± 0.3	>103	97 ± 10	0.81 ± 0.04	120

[a]Cells were treated for 1 h and exposed to red light irradiation followed by 24 h recovery period. Light irradiation: 620 nm LED source, 90 mW·cm^{-2} for 1 h. Dark analogues were kept in the dark. n.d.= not determined due to steep Hill slope. [b]PI = phototherapeutic index defined as $[IC_{50}]_{dark}/[IC_{50}]_{light}$

Table S3. IC_{50} values [µM] of selected compounds after 24 h incubation in the dark.[a]				
	HT-29	**CT-26**	**HeLa**	**A2780**
COUPY	37 ± 8	17 ± 7	49 ± 3	42 ± 8
Ru	2.0 ± 0.6	0.9 ± 0.07	0.9 ± 0.2	0.7 ± 0.08
Ru-COUPY	>300 [39 ± 4 %]	>300 [56 ± 7 %]	>300	101 ± n.d.
[a]The term >300 means that no IC_{50} was found up to that concentration. If significant cell inhibition was found at 300 µM, the corresponding cell inhibition percentage is provided in brackets. n.d.= not determined due to steep Hill slope.				



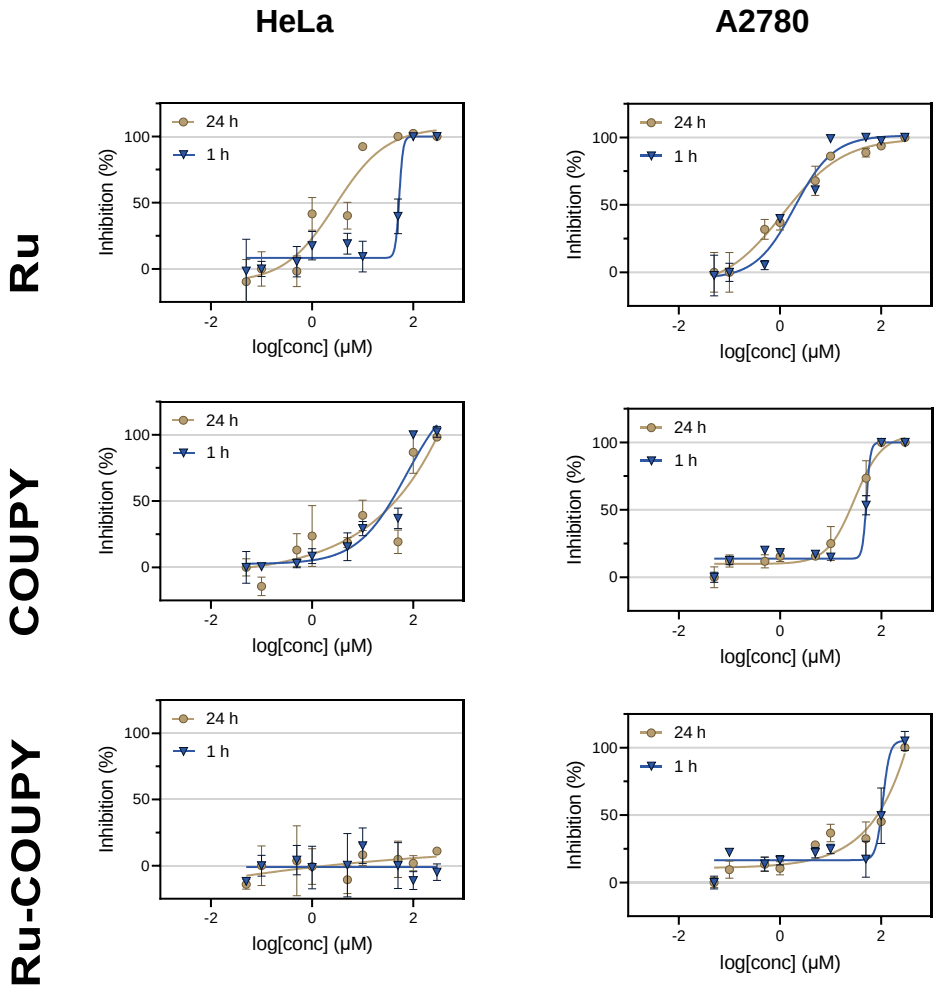


Figure S7. Comparison between dose-response curves for **Ru**, **COUPY** and **Ru-COUPY** after 1 h incubation in the dark followed by a 24 h drug-free recovery period and after 24 h incubation in the dark without recovery period against tested cancer cell lines. Data expressed as mean ± SD from three replicates and represented as inhibition of cell viability vs. logarithm of the compound concentration.

Cell death studies by flow cytometry

HT-29 cells were seeded onto 12-well plate (2×10^5 cells/well). In these experiments, 620 nm light treatments with tested compounds were applied and then analyzed by flow cytometry (FACS Calibur Beckton Dickinson). For total ROS level determination, cells were treated with the compounds at indicated concentrations for 1 h, then the 2′-7′dichlorofluorescein diacetate (DCFH-DA) probe was used for staining (10 μM for 0.5 h) prior to light irradiation. After this, cells were washed, harvested and subjected to flow cytometry λ_{exc} = 488 nm and λ_{em} = 530 ± 30 nm collected in FL1-H channel). FSC and SSC dot plots were also obtained to evaluate cell morphology upon irradiation. For apoptosis evaluation, cells were treated with the compounds at indicated concentrations for 1 h and

light irradiation was then applied. After 24 h recovery period, Annexin V-FITC staining (eBioscience) was applied according to manufacturer's instruction. FL1-H channel was used to collect emission from Annexin V-FITC-stained cells upon excitation at λ_{exc} = 488 nm. Two independent experiments were performed, and data analyzed with either FlowJo 10.8 software or Flowing Software 2.5.1 (10^4 events per sample).

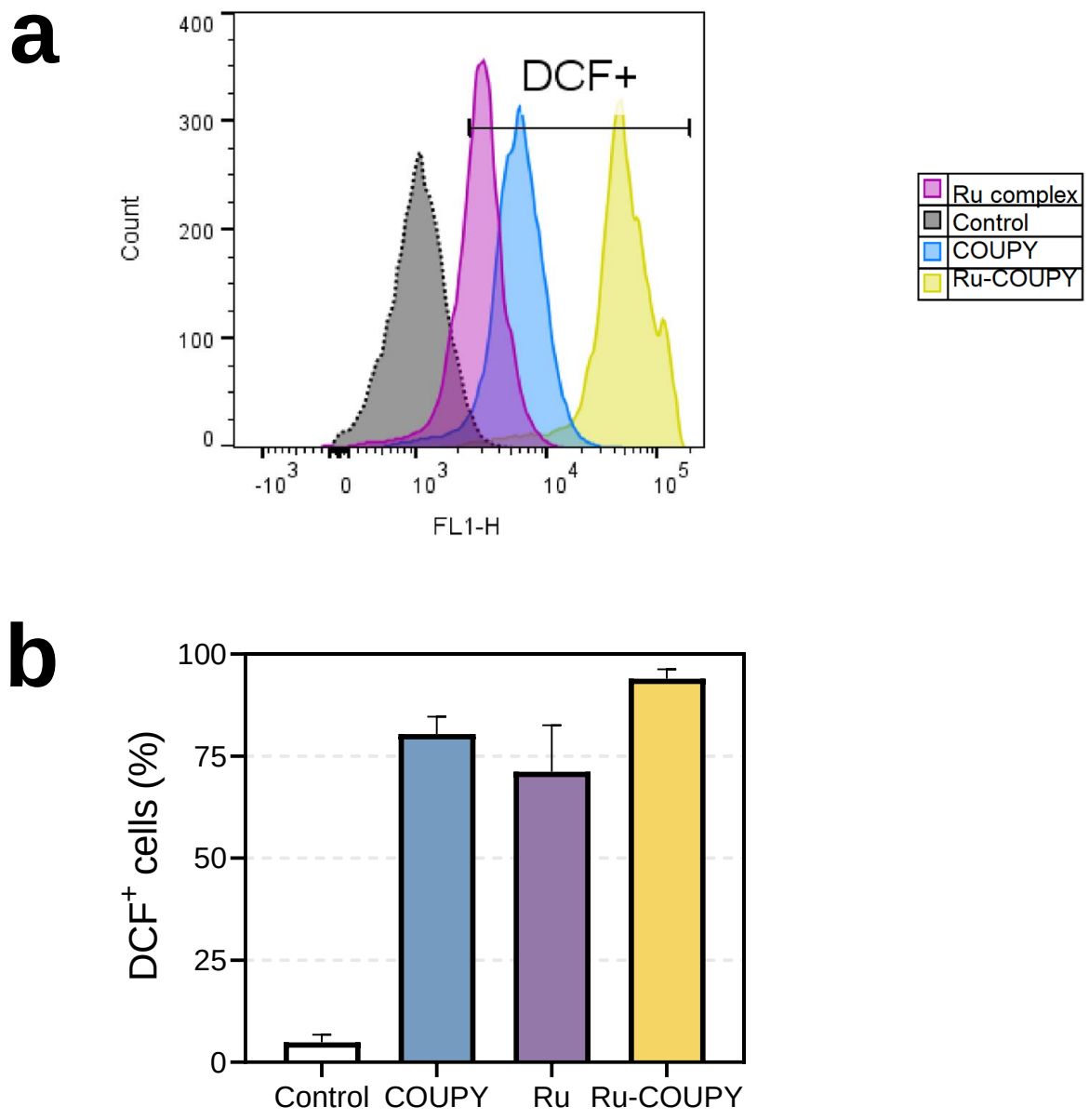


Figure S8. Determination of ROS levels by flow cytometry after red light irradiation. **a**) Representative histograms of dicholorofluorescein (DCF)-stained HT-29 cells after treatment with indicated compounds at 10 µM upon irradiation. **b**) Quantitation of ROS levels based on DCF fluorescence measured in the FL1-H channel by flow cytometry. Light irradiation conditions: 620 nm light; 90 mW·cm^{-2}, 1 h.

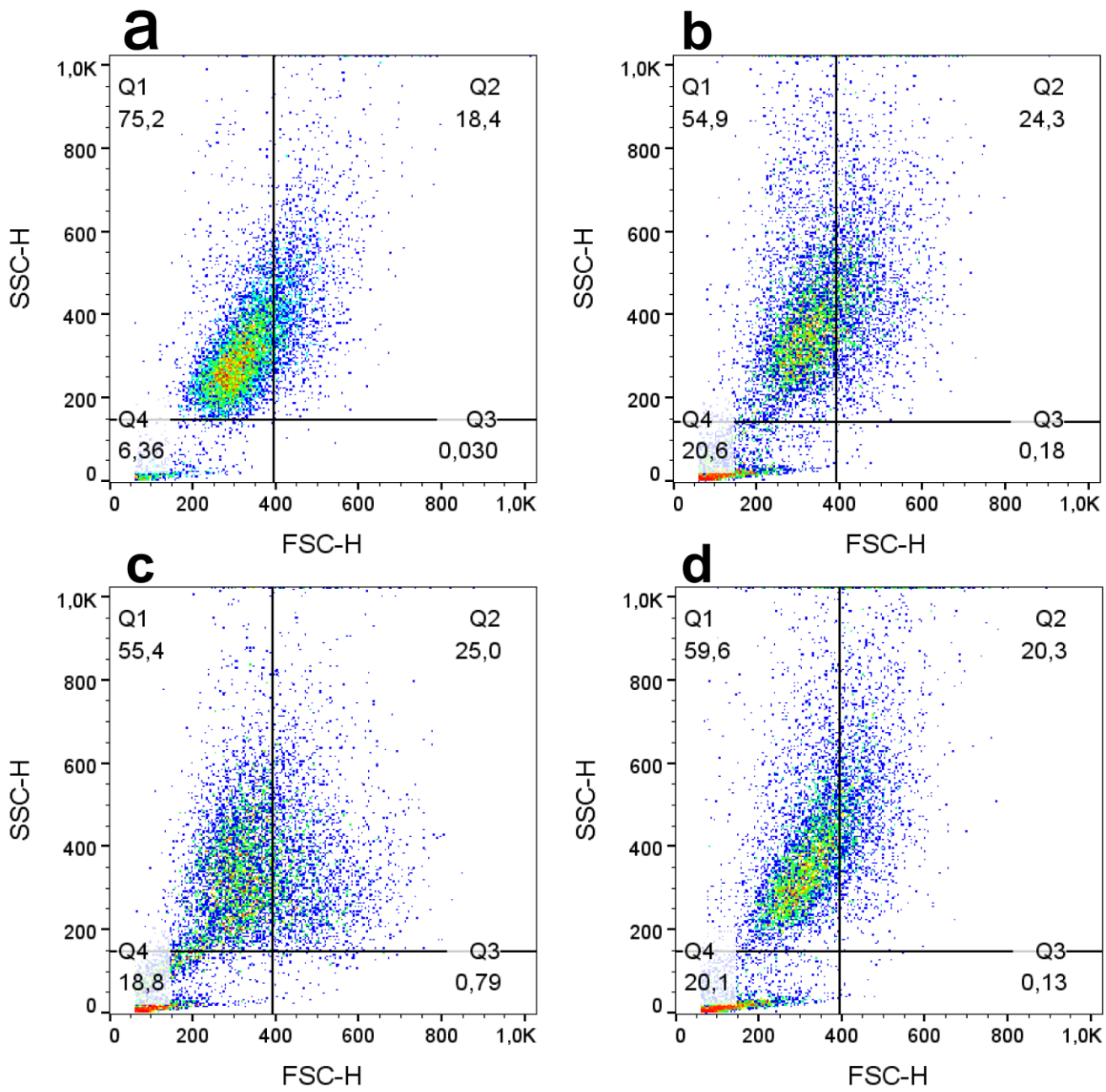


Figure S9. Representative cell size (FSC) vs. cell complexity (SSC) flow cytometry plots from irradiated, PS-free HT-29 cells (**a**) or treated with **Ru** (**b**), **COUPY** (**c**) and **Ru-COUPY** (**d**). Light irradiation conditions: 620 nm light; 90 mW·cm^{-2}, 1 h.

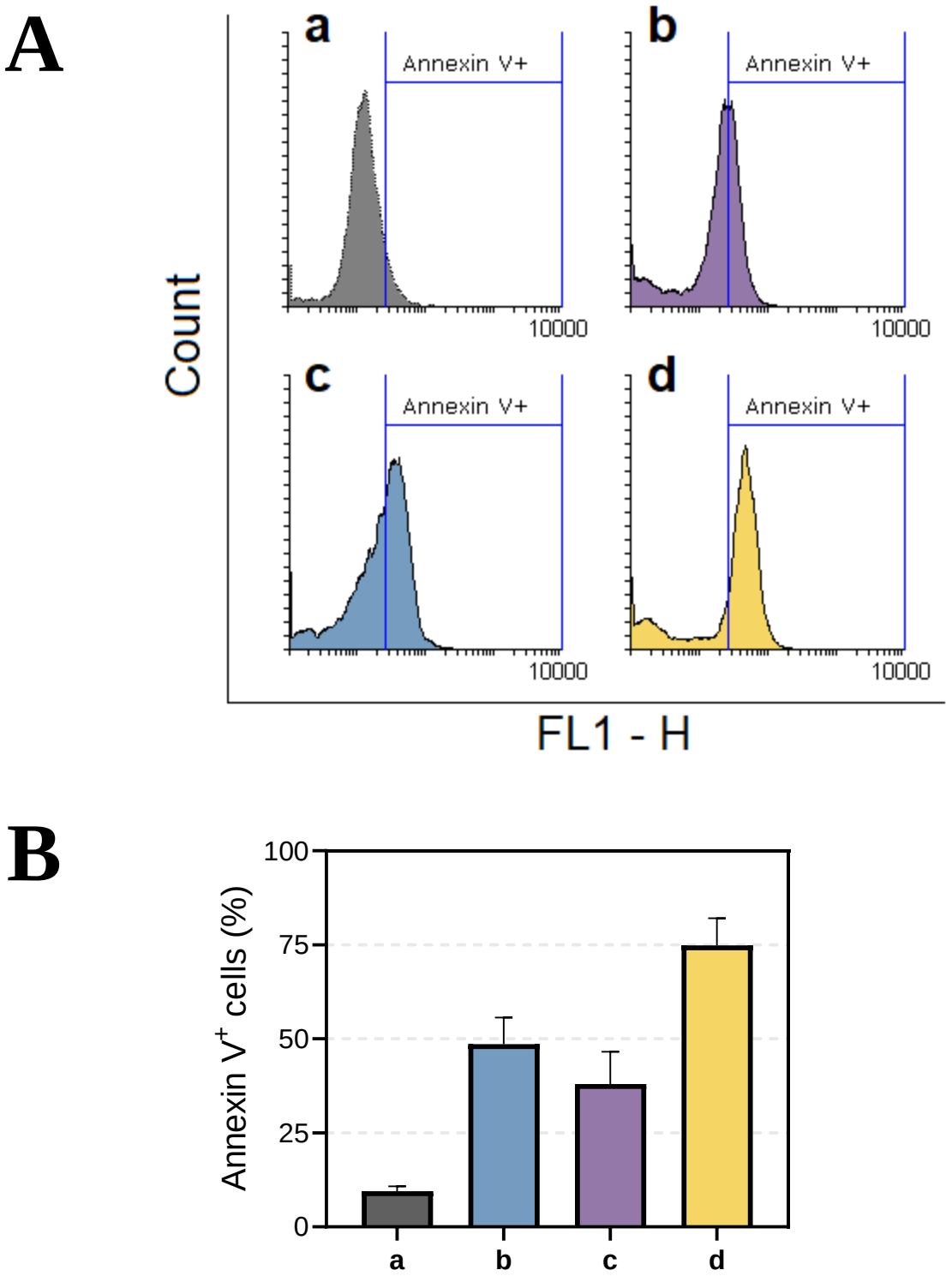


Figure S10. Evaluation of photoinduced apoptosis by flow cytometry. **A**) Representative histograms of HT-29 cells and **B**) quantitation of Annexin V-FITC stained PS-free cells (**a**) or after 10 µM treatment with **Ru** (**b**), **COUPY** (**c**) and **Ru-COUPY** (**d**). Light irradiation conditions: 620 nm light; 90 $mW \cdot cm^{-2}$, 1 h.

Table S4. IC_{50} values [µM] of compounds from chromatic screening towards HT29 cells .[a]											
	Dark	**620 nm**	**PI**	**645 nm**	**PI**	**670 nm**	**PI**	**740 nm**	**PI**	**770 nm**	**PI**
COUPY	44 ± 7	0.98 ± 0.03	45	0.7 ± 0.03	63	0.6 ± 0.1	73	6.8 ± 0.9	6	51 ± 10	0.9
Ru	2.4 ± 0.4	0.18 ± 0.08	39	0.06 ± 0.01	39	0.4 ± 0.1	6	0.5 ± 0.03	5	2.1 ± 0.3	1
Ru-COUPY	>300	3.1 ± 0.1	>97	1.2 ± 0.1	>250	4.0 ± 0.5	>75	7.1 ± 0.3	>42	254 ± 19	>1.2
PpIX	>300	0.1 ± n.d.	>3000	0.04 ± 0.01	>7500	0.6 ± 0.04	>500	>300	n.d.	n.d	n.d.
[a]Cells were treated for 1 h and exposed to light irradiation followed by 24 h recovery period. Light irradiation: 1 h, intensities described in Experimental Section. Dark analogues were kept in the dark. [b]PI = phototherapeutic index defined as $[IC_{50}]_{dark}/[IC_{50}]_{light}$											

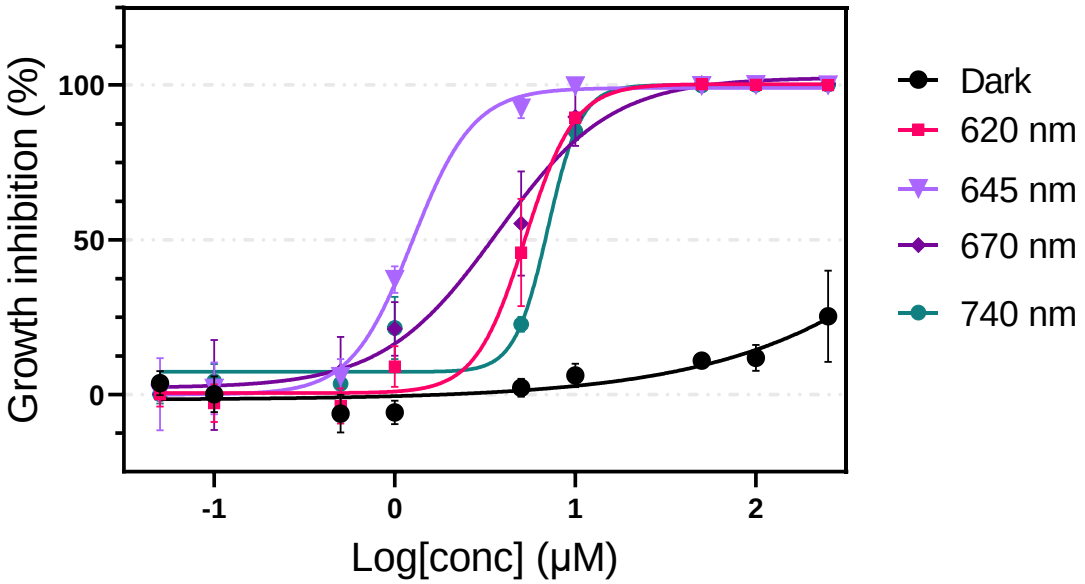


Figure S11. Dose-response curves for chromatic screening of **Ru-COUPY** in HT-29 cells.

Table S5. IC_{50} values [µM] of selected compounds towards HT-29 cells.[a]

	Normoxia (21 % O_2)			Hypoxia (2 % O_2)			
	Dark	**740 nm**	**PI[b]**	**Dark**	**740 nm**	**PI**	**HI[c]**
COUPY	44 ± 7	6.8 ± 0.9	6	47 ± 6	31 ± 8	1.5	5
Ru	2.4 ± 0.4	0.5 ± 0.03	5	11 ± 3	1.5 ± 0.4	7	3
Ru-COUPY	>300	7.1 ± 0.3	>42	>300	13 ± 2	>23	1.8
PpIX	>300	>300	n.d.	>300	>300	n.d.	n.d.

[a]Cells were treated for 1 h and exposed to red light irradiation followed by 24 h recovery period. Light irradiation: 740 nm, 100 $mW \cdot cm^{-2}$ for 1 h. Dark analogues were kept in the dark.
[b]PI = phototherapeutic index defined as [IC50]dark/[IC50]light
[c]HI = hypoxia index defined as $[IC50]_{hypoxia}/[IC50]_{normoxia}$

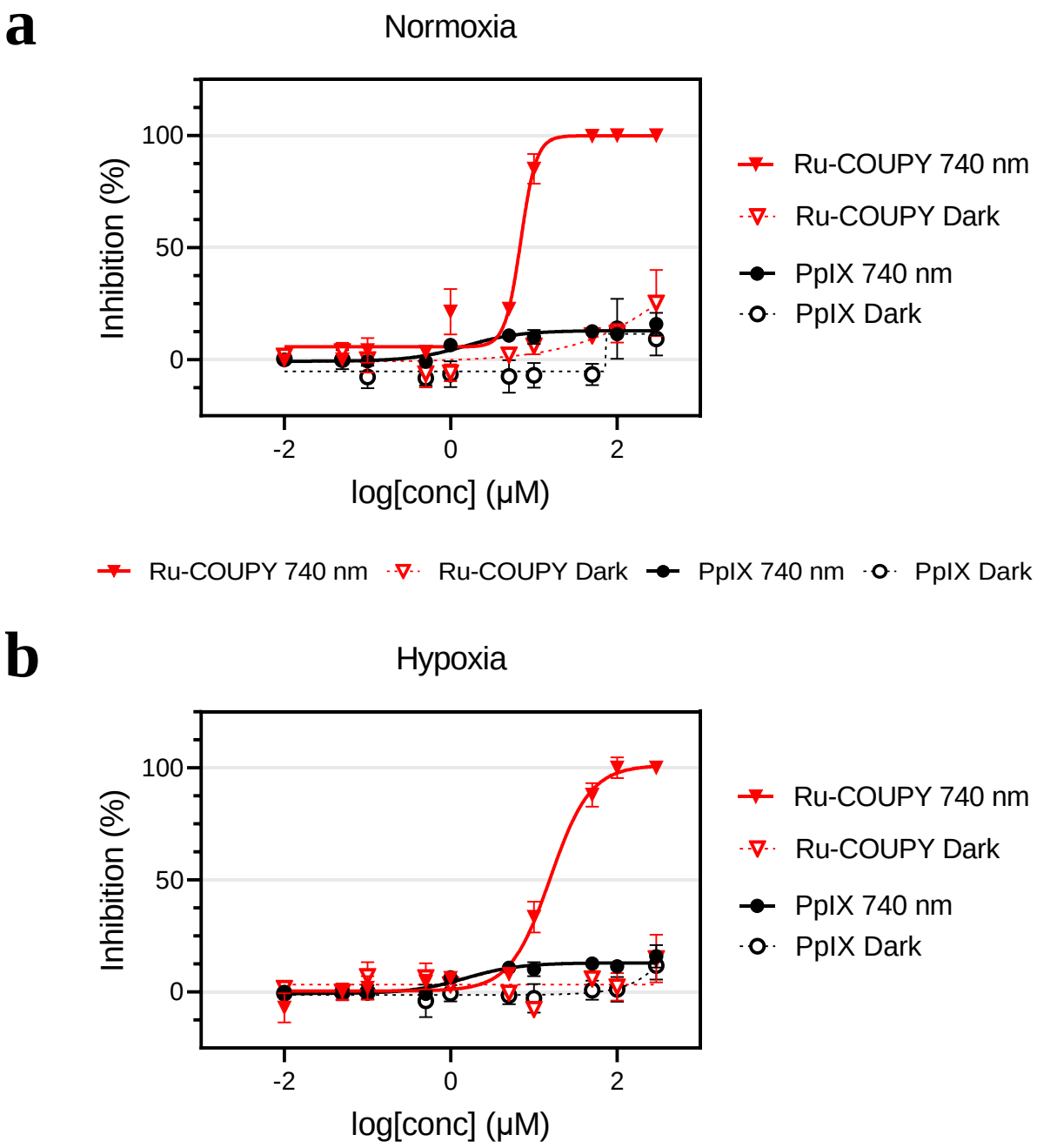


Figure S12. Dose-response curves for **Ru-COUPY** (red) and **PpIX** (black) in HT-29 upon NIR irradiation (filled symbols) or in the dark (unfilled symbols) under normoxia (**a**) and hypoxia (**b**), Light irradiation conditions: 740 nm, 100 mW cm^{-2} for 1 h.

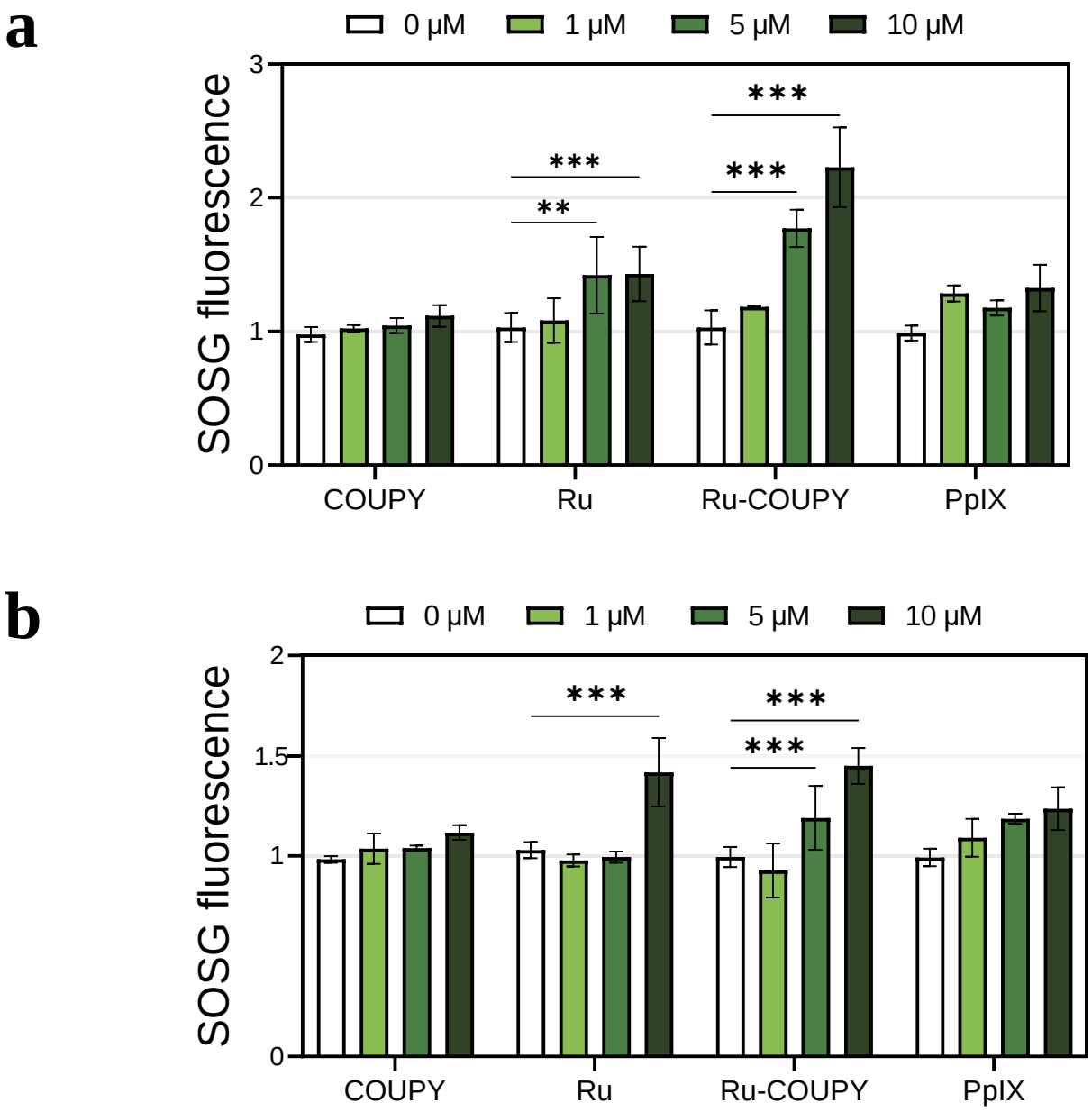


Figure S13. Singlet oxygen photogeneration (740 nm, 100 mW cm^{-2} for 1 h) in HT-29 cells in the presence of indicated photosensitizers under normoxia (**a**) and hypoxia (**b**) measured using Singlet Oxygen Sensor Green staining (SOSG, 5µM for 0.5 h). Data expressed as mean ± SD from three independent experiments and represented as normalized SOSG fluorescence. Statistical significance with respect to control (0µM) based on *p<0.05 and **p<0.01 and ***p<0.001 using 2way ANOVA test.

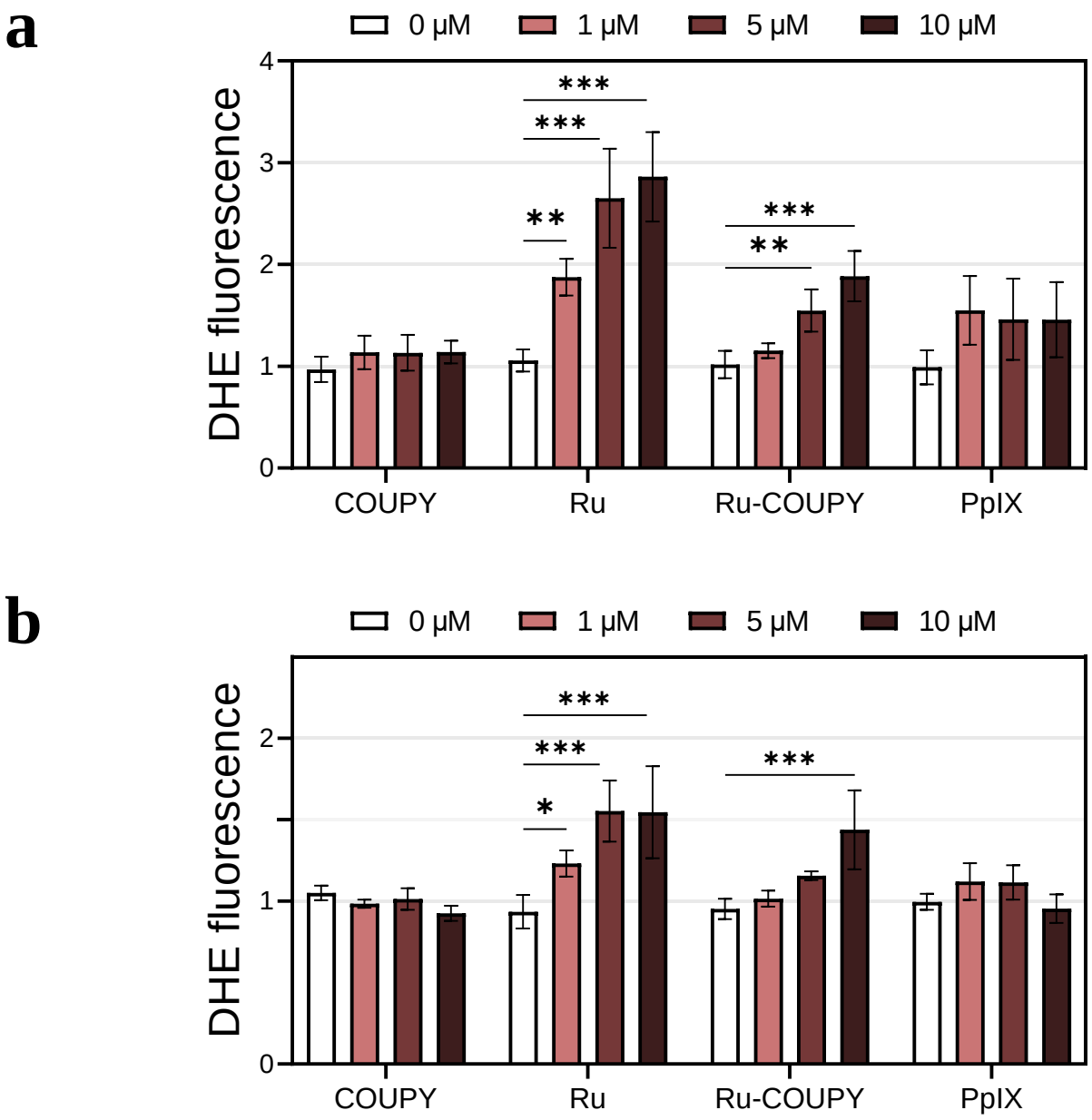


Figure S14. Superoxide anion photogeneration (740 nm, 100 mW cm^{-2} for 1 h) in HT-29 cells in the presence of indicated photosensitizers under normoxia (**a**) and hypoxia (**b**) measured using Dihydroethidium staining (DHE, 10 µM for 0.5 h). Data expressed as mean ± SD from three independent experiments and represented as normalized DHE fluorescence. Statistical significance with respect to control (0µM) based on *p<0.05 and **p<0.01 and ***p<0.001 using 2way ANOVA test.

Figure S15. Hypoxia chamber (Plas labs 856-Series hypoxia chamber glove box) containing the irradiation setup.

5. ^{1}H and ^{13}C NMR spectra and HR ESI-MS of the compounds

Compound 2

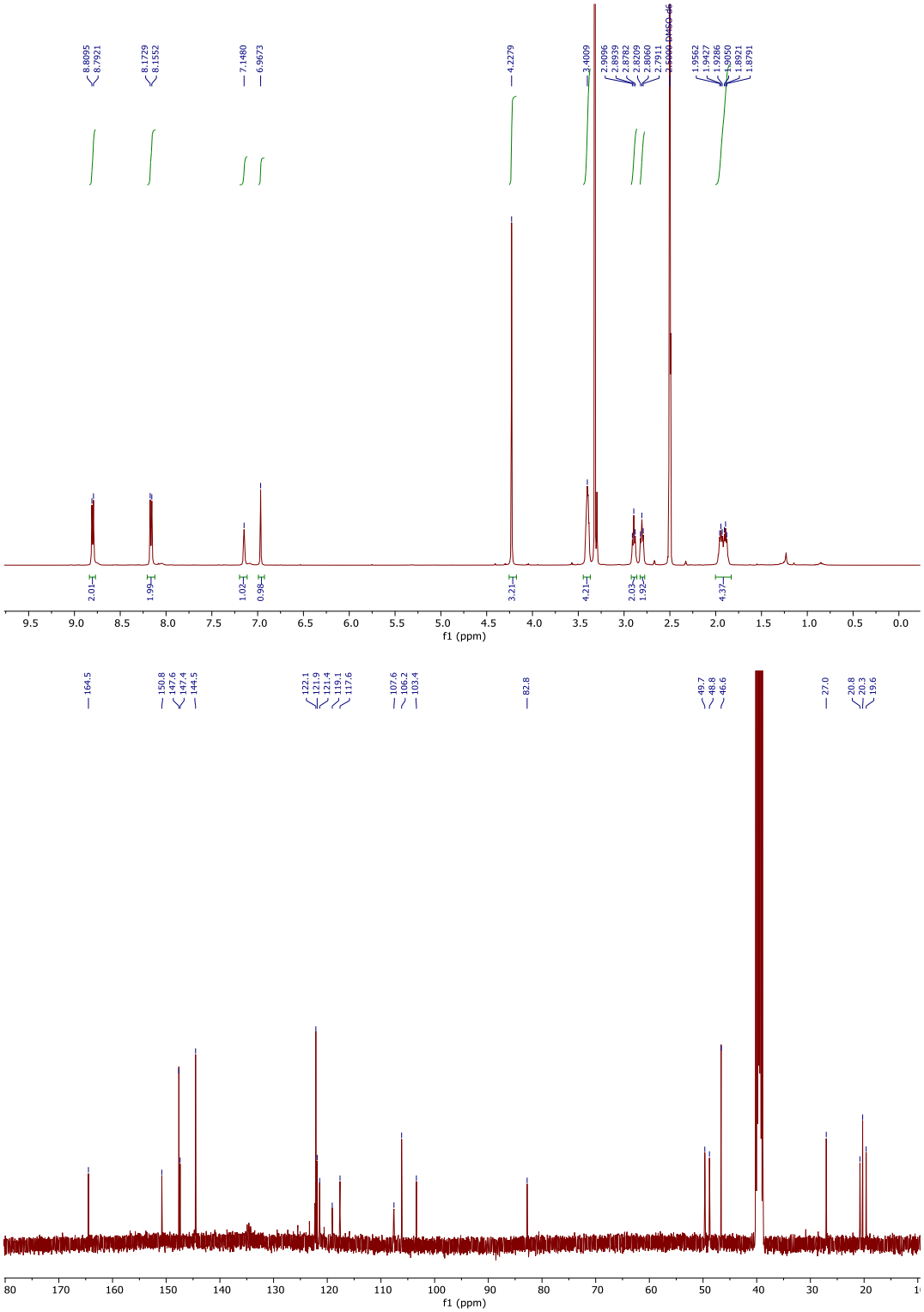


Figure S16. ^{1}H and ^{13}C NMR spectra of compound **2** in DMSO-d_6.

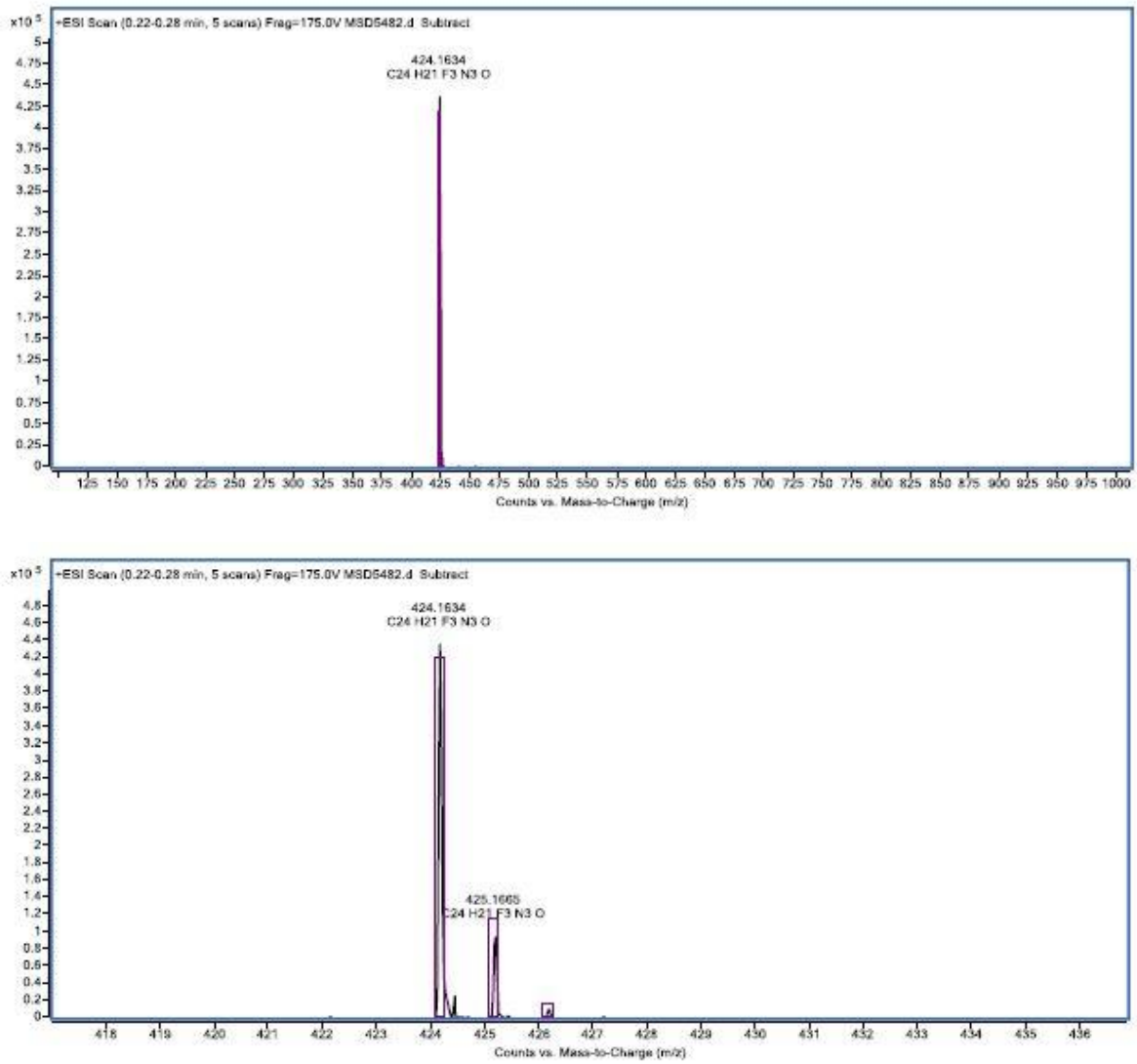


Figure S17. HR ESI-MS spectrum of compound **2**.

Compound 6

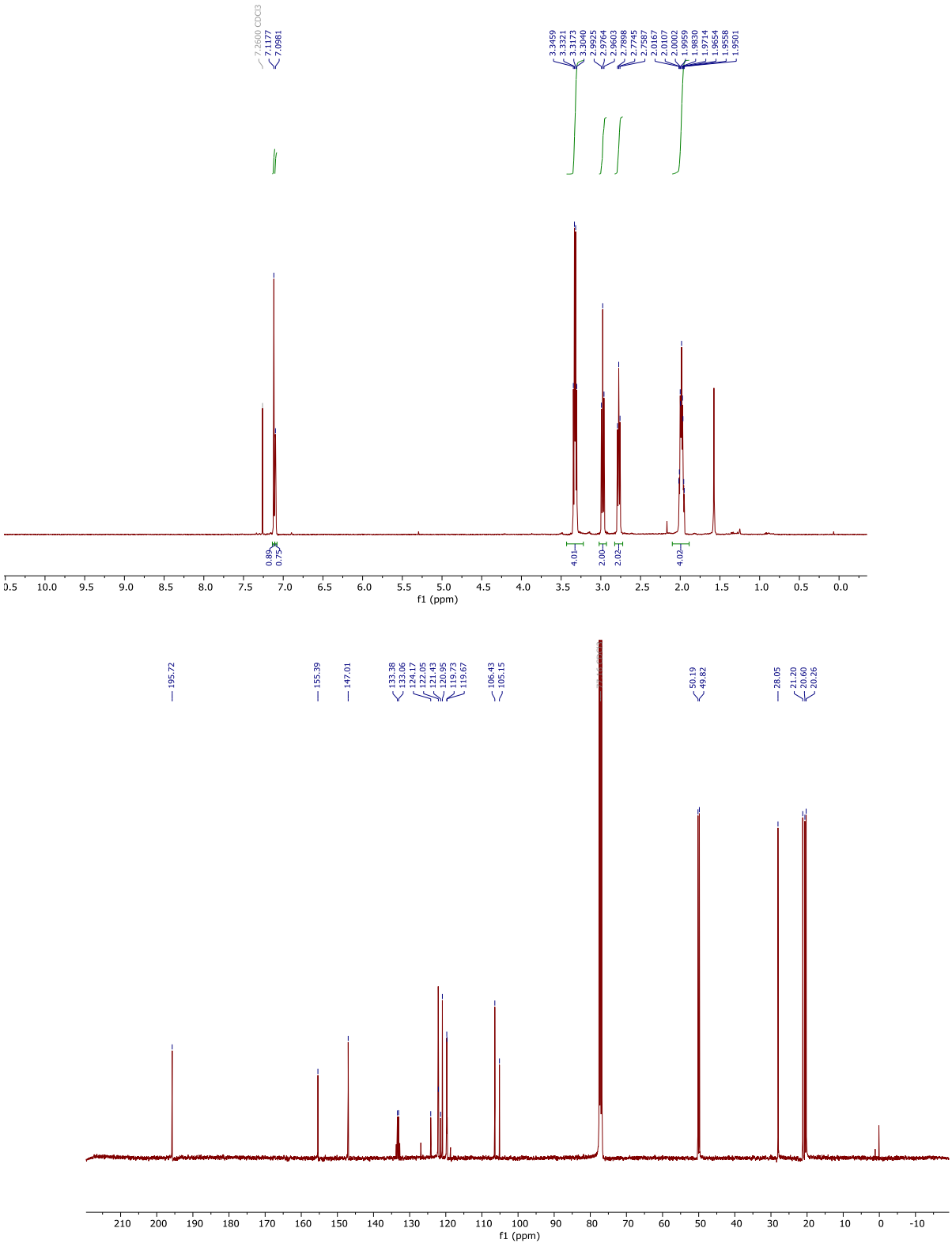


Figure S18. ^{1}H and ^{13}C NMR spectra of compound **6** in $CDCl_3$.

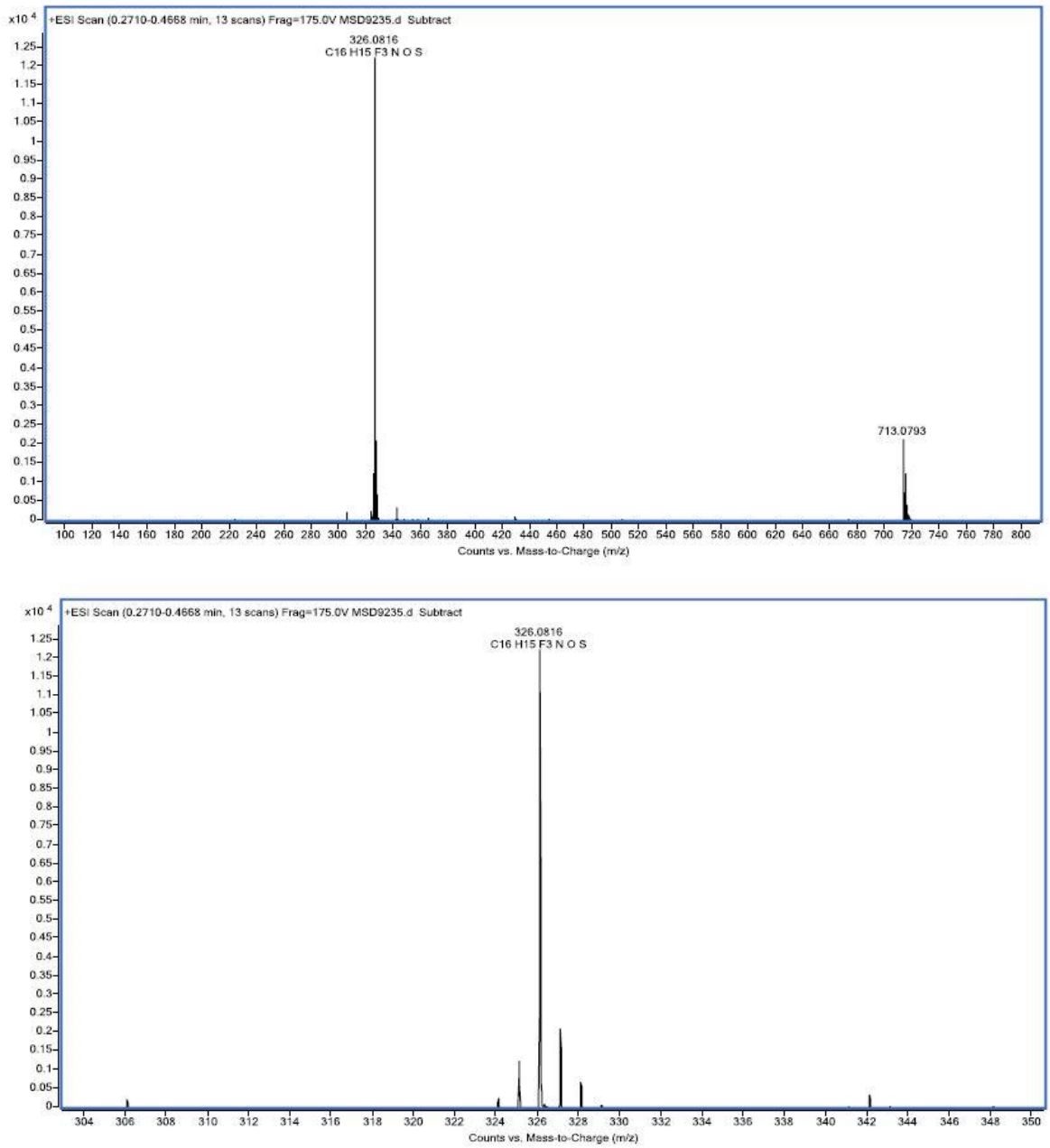


Figure S19. HR ESI-MS spectrum of compound **6**.

Compound 7

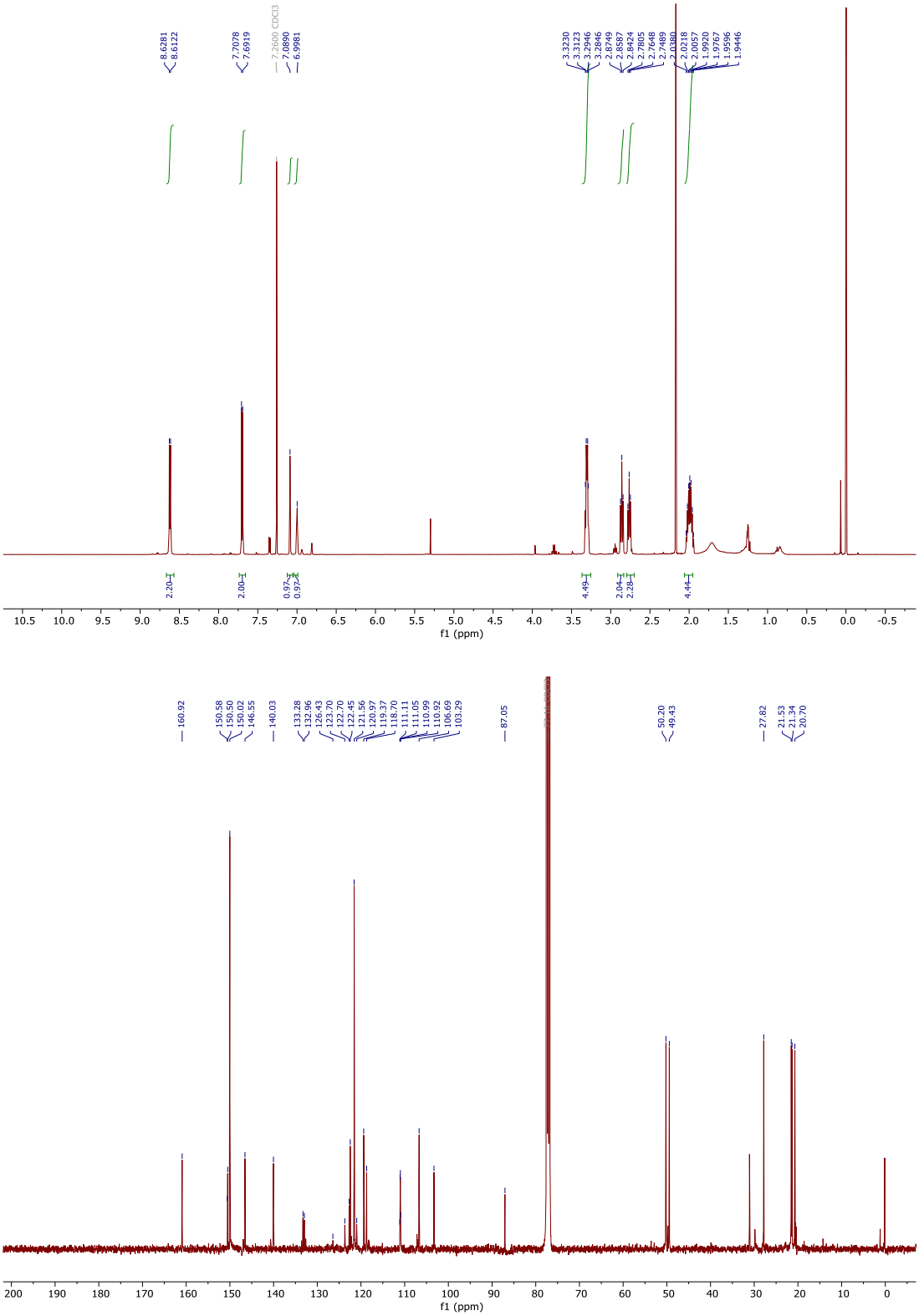


Figure S20. ^{1}H and ^{13}C NMR spectra of compound **7** in $CDCl_3$.

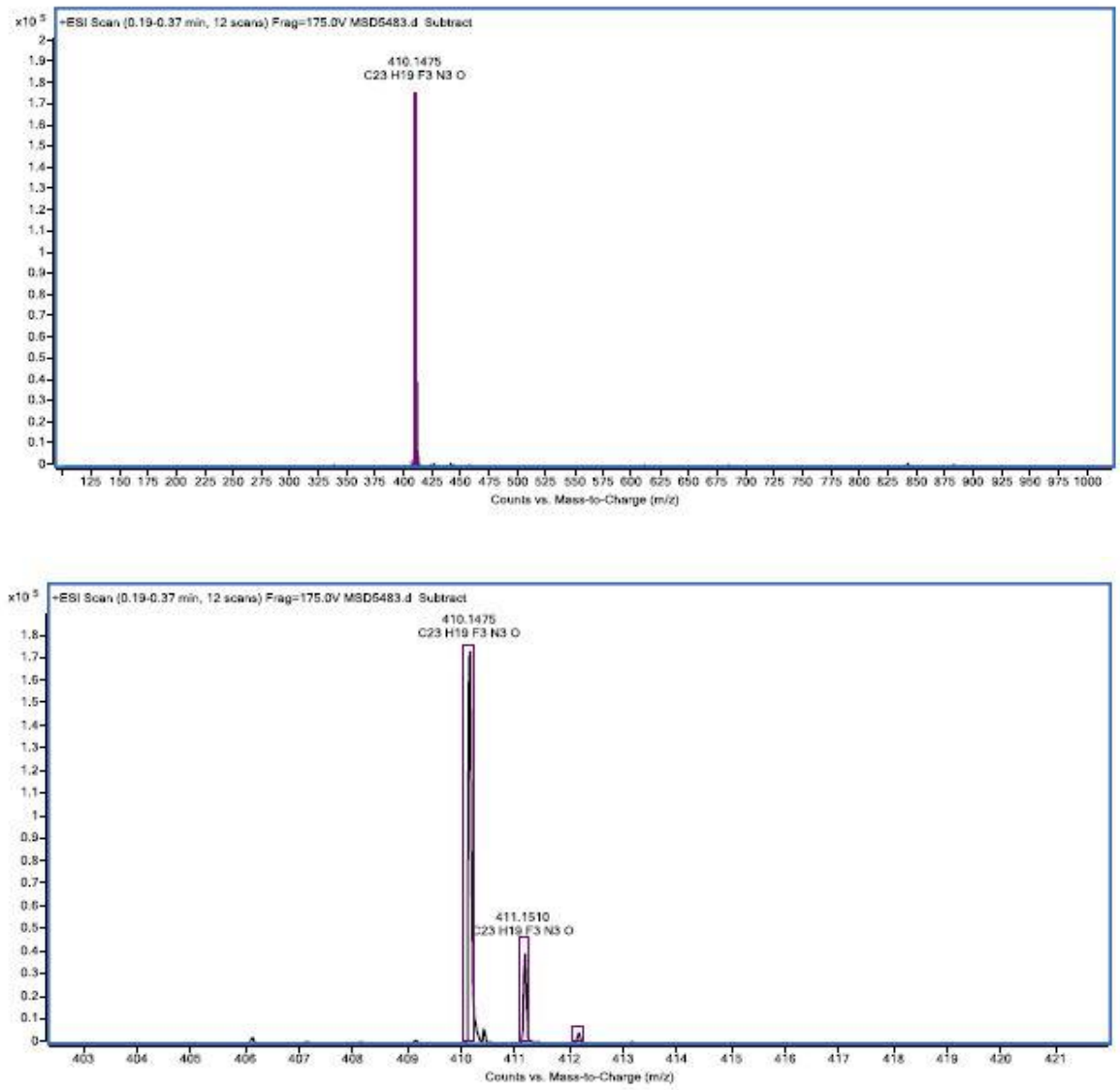


Figure S21. HR ESI-MS spectrum of compound **7**.

Compound 8

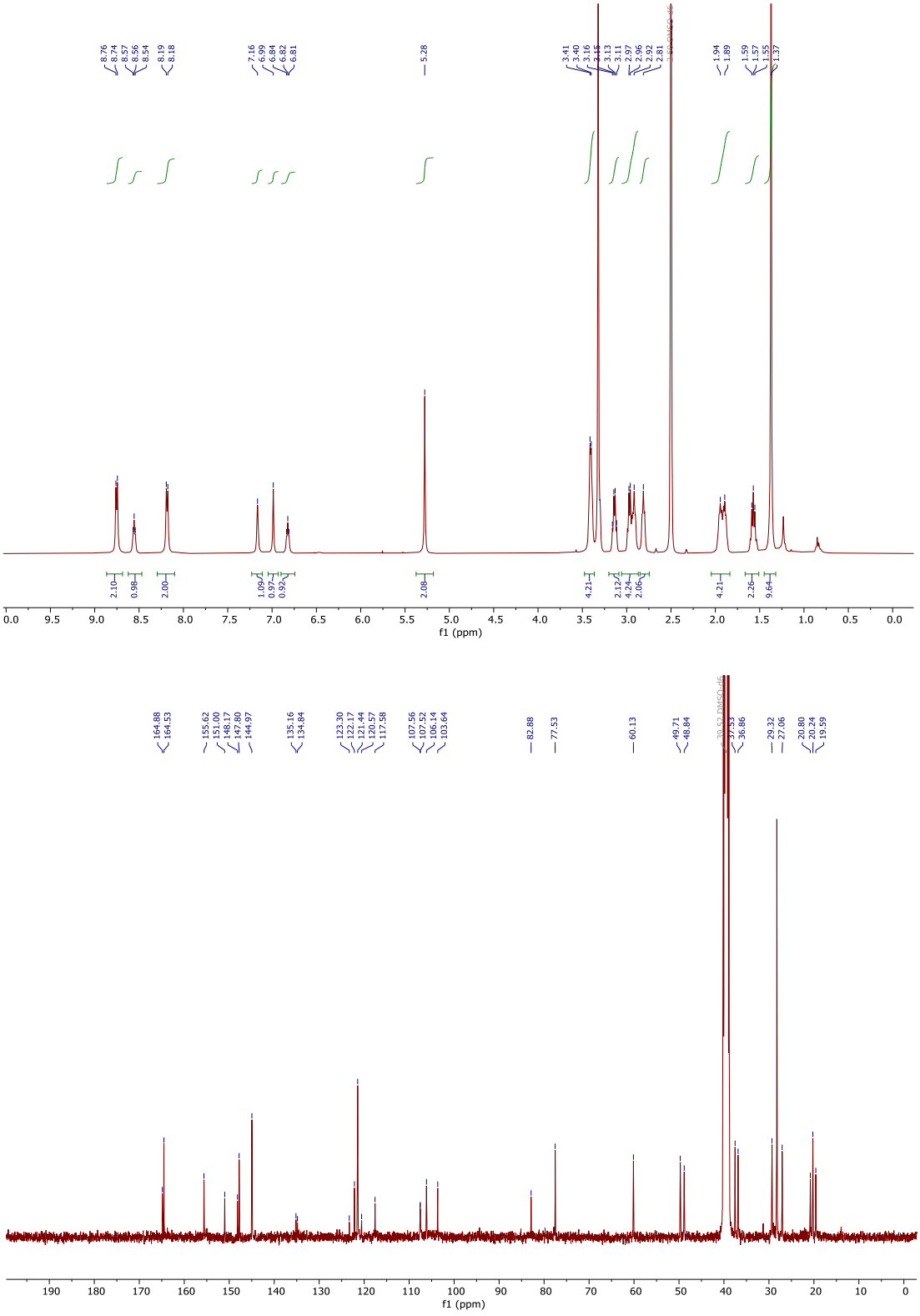


Figure S22. ^{1}H and ^{13}C NMR spectra of compound **8** in DMSO-d_6.

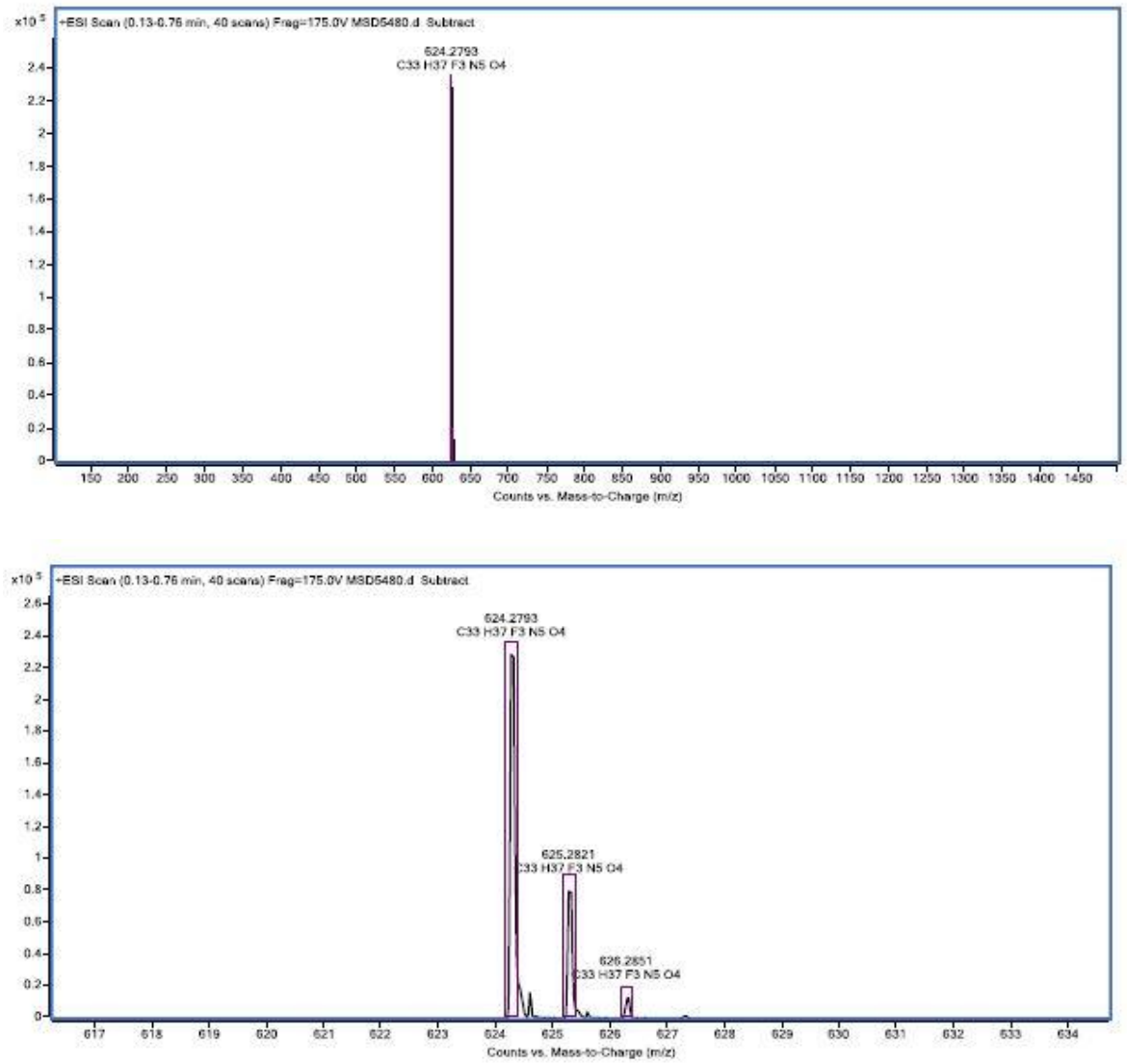


Figure S23. HR ESI-MS spectrum of compound **8**.

Compound 9

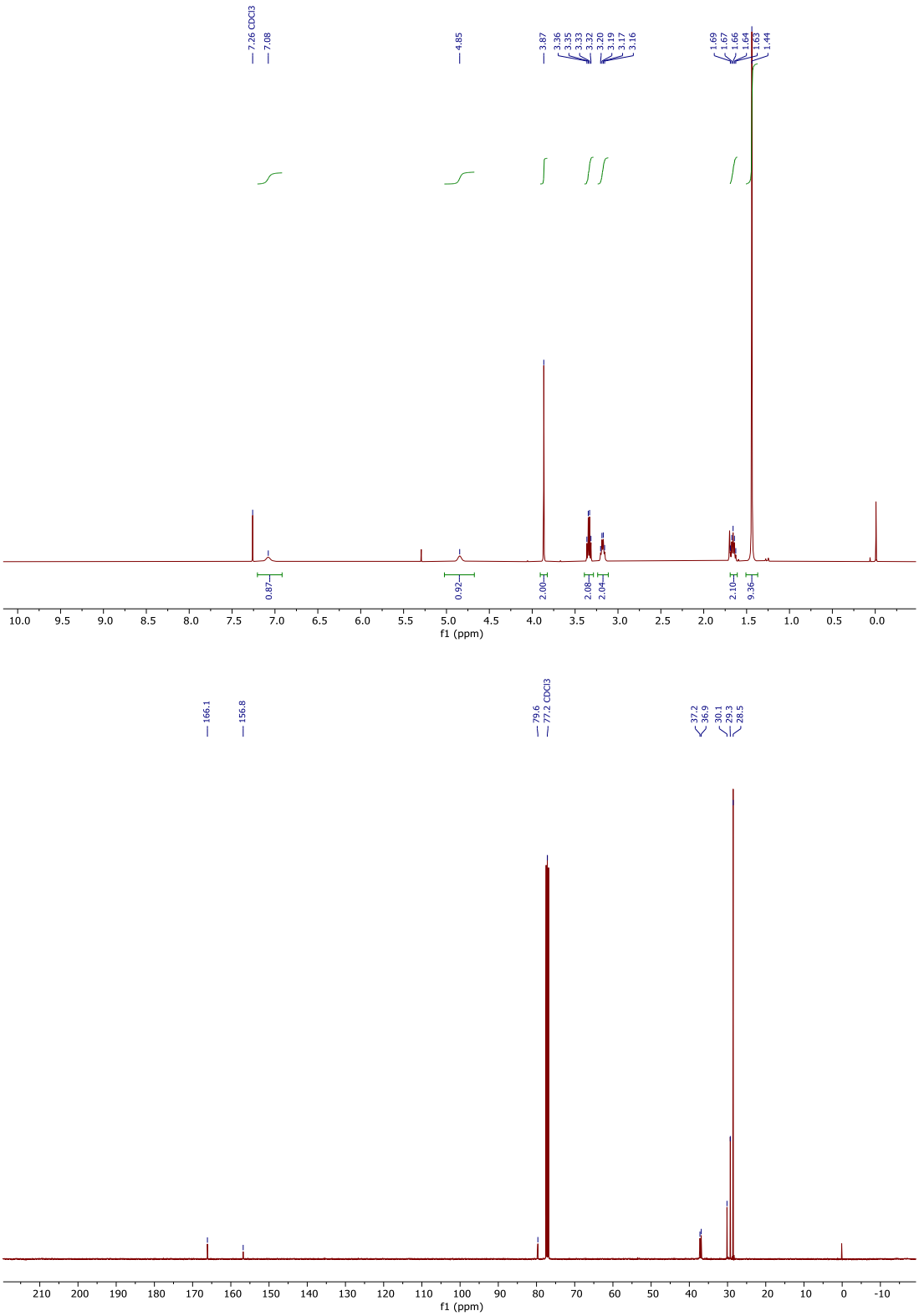


Figure S24. ^{1}H and ^{13}C NMR spectra of compound **9** in $CDCl_3$.

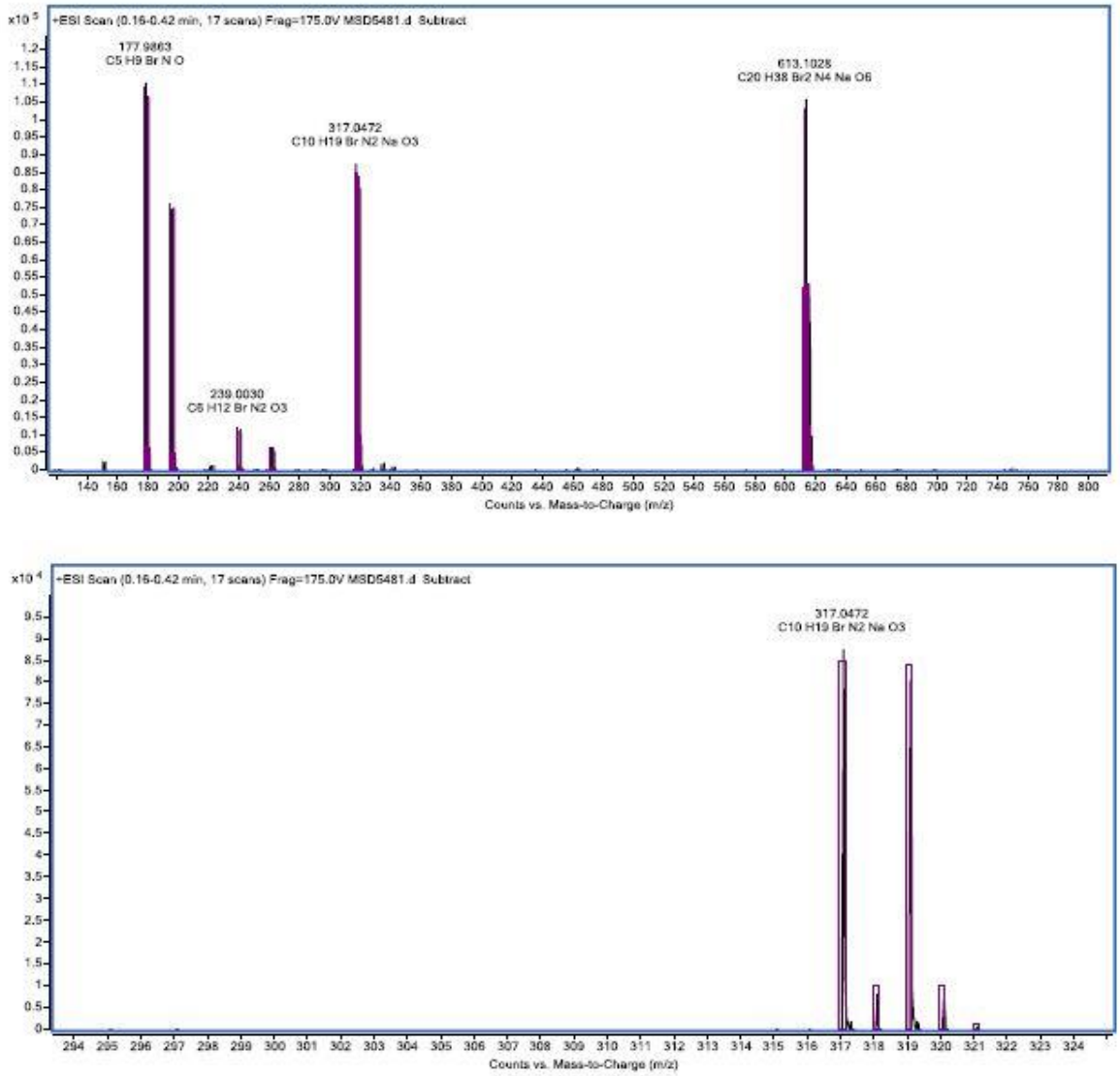


Figure S25. HR ESI-MS spectrum of compound **9**.

Compound 3

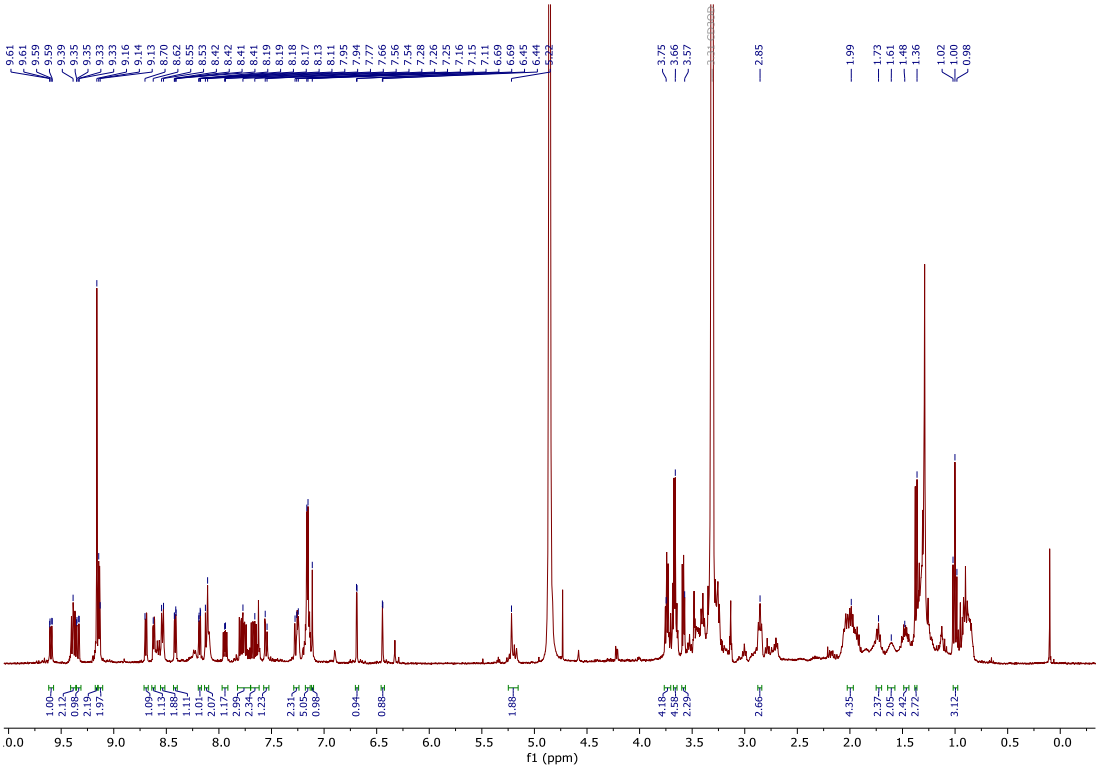


Figure S26. ^{1}H NMR spectrum of compound **3** in CD_3OD.

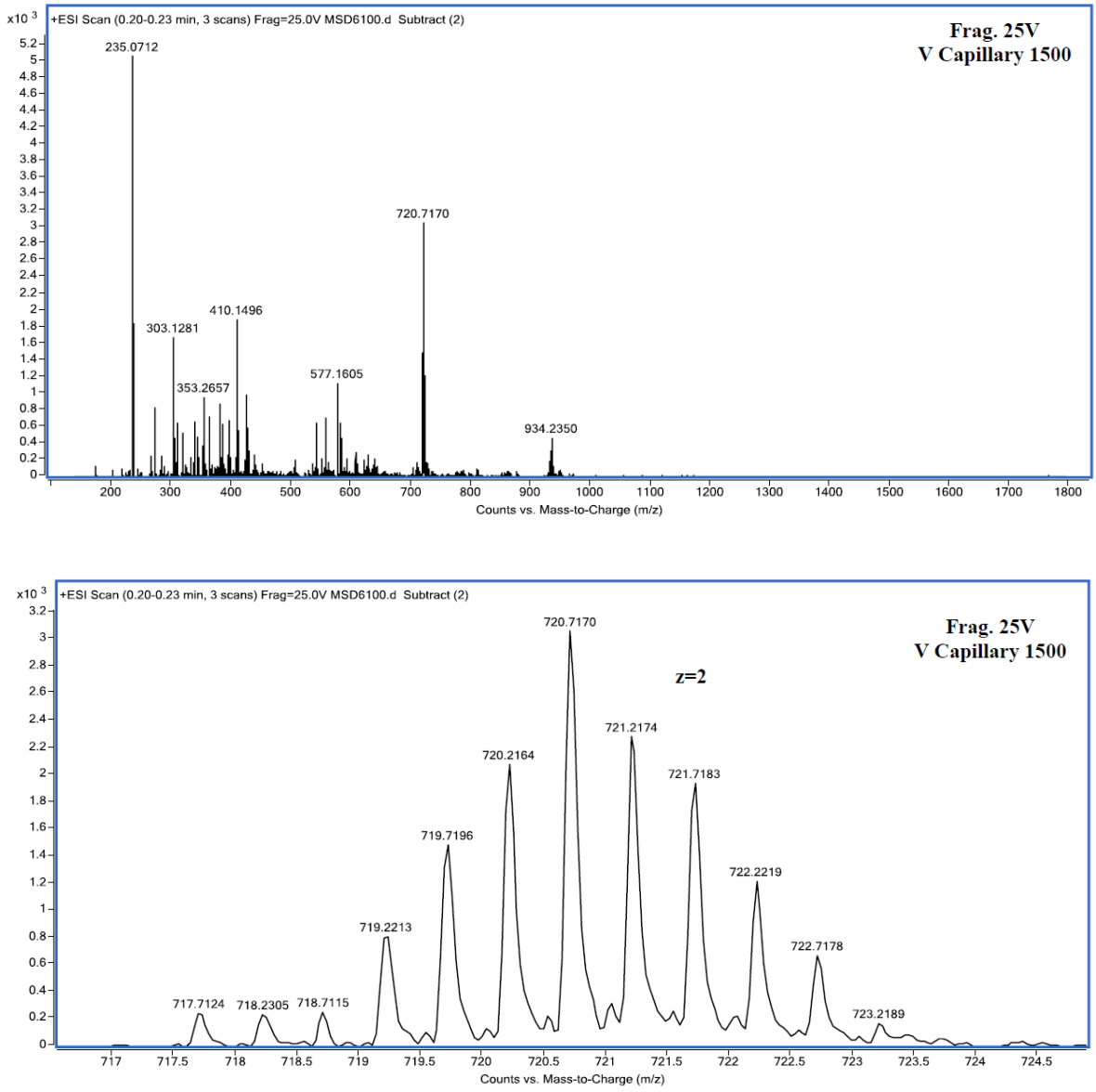


Figure S27. HR ESI-MS spectrum of compound **3**.

References

[i] Magde, D.; Brannon, J. H.; Cremers, T. L.; Olmsted, J. Absolute luminescence yield of cresyl violet. A standard for the red. *J. Phys. Chem*. **1979**, *83*, 696-699

[ii] Johannes Karges, Franz Heinemann, Marta Jakubaszek, Federica Maschietto, Chloé Subecz, Mazzarine Dotou, Robin Vinck, Olivier Blacque, Mickaël Tharaud, Bruno Goud, Emilio Viñuelas Zahínos, Bernhard Spingler, Ilaria Ciofini, and Gilles Gasser *Journal of the American Chemical Society* **2020** *142* (14), 6578-6587