Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Bio-specific and bio-orthogonal chemistry to switch the quenching of a FRET-based fluorescent probe: application to living-cell biothiol imaging.

Coraline Egloff, Sylvain A. Jacques, Marc Nothisen, Denis Weltin, Cynthia Calligaro, Michel Mosser, Jean-Serge Rémy and Alain Wagner*

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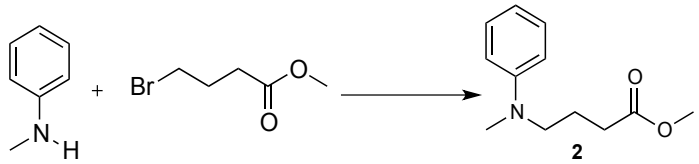
General methods

Materials: All reagents were purchased from commercial suppliers and used without further purification. Dry DMF were obtained from Aldrich. Unless otherwise indicated, reactions were carried out under an atmosphere of argon in flame-dried glassware with magnetic stirring. Air and/or moisture-sensitive liquids were transferred *via* syringe. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an ethanolic solution of cerium sulfate, followed by treatment with a heat gun. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 μM) from Macherey-Nagel.

Instrumentation: UV-Vis spectra and kinetic were recorded on Shimadzu UV-1800 spectrophotometer. Fluorescence spectra were recorded on a multilabel plate reader (Victor X2, PerkinElmer) or on a Fluorolog (Jobin Yvon, Horiba). Melting points were taken on a Stuart Scientific SMP3 apparatus from Bibby and are uncorrected. IR spectra were recorded on a Nicolet 380 FT-IR spectrometer from Thermo Electron Corporation as a CH_2Cl_2 solution or solid on a diamond plate. ^{1}H and ^{13}C NMR spectra were recorded at 23 °C on Bruker 400 spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent. Data are represented as follows: Chemical shift, mutiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, bs = broad singulet), coupling constant (*J*, Hz) and integration. High resolution mass spectra (HRMS) were obtained using a Agilent Q-TOF (time of flight) 6520 and low resolution mass spectra using a Agilent MSD 1200 SL (ESI/APCI) with a Agilent HPLC1200 SL. The semi-preparative HPLC system consisted of a Waters 600 pump, a 2487 detector (Waters), a 5 mL sample loop and a Sunfire C18 column (150 mm × 19 mm i.d., 5 μm, Waters). Flow: 17 mL/min. Injection volume = 1 mL. Eluant water/acetonitrile with 0.05% TFA. Gradient: 5% to 95% acetonitrile in 30 minutes and 10 minutes of re-equlibration. Detection: 254 nm.

Synthesis

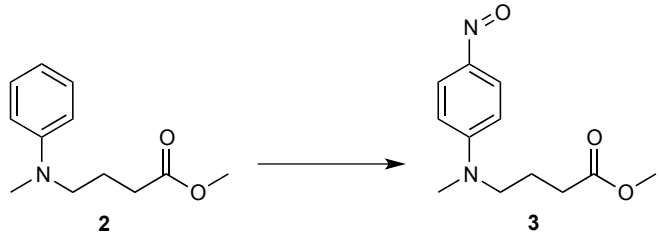
Synthesis of 2:



A solution of *N*-methylaniline (2 mL, 18.7 mmol) with methyl 4-bromobutyrate (2.8 mL, 22.4 mmol) and triethylamine (3.1 mL, 22.4 mmol) in acetonitrile (20 mL) was heated at reflux at 82 °C for 16 h. The dark purple solution was cooled to room temperature and the triethylammonium salt precipated out and was removed by filtration on celite. Diethyl ether (50 mL) was added to the filtrate and the organic layer was washed with water (3 × 50 mL), dried over Na_2SO_4, evaporated to dryness and the crude product was purified by silica gel chromatography (cyclohexane/ethyl acetate 95:5) giving the title product as a colorless oil (2.04 g, 53%).

R_f = 0.14 (cyclohexane/ethyl acetate 95:5); **^{1}H** NMR (400 MHz, $CDCl_3$) δ = 7.26-7.22 (m, 2H), 6.74-6.67 (m, 3H), 3.68 (s, 3H), 3.37 (t, *J* = 7.3 Hz, 2H), 2.93 (s, 3H), 2.37 (t, *J* = 7.3 Hz, 2H), 1.93 (quint, *J* = 7.3 Hz, 2H) ppm; **^{13}C NMR (101 MHz, $CDCl_3$)** δ = 173.8, 149.4, 129.3, 116.4, 112.4, 112.1, 52.1, 51.7, 38.4, 31.5, 22.3 ppm; **IR** ν = 2358, 1732, 1598, 1504, 1361, 1241, 1115, 990, 861, 747, 691 cm^{-1}; **ESI-MS** m/z: $[M+H]^+$ = 208.2; **HRMS** : calculated for $C_{12}H_{18}NO_2$ $[M+H]^+$ 208.1259, found 208.1328.

Synthesis of 3:

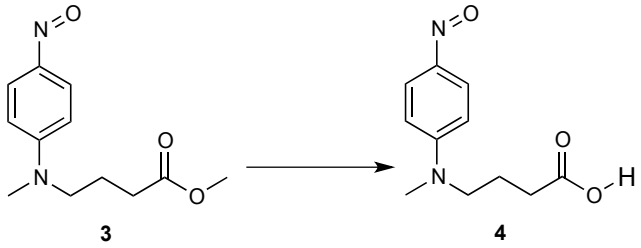


This compound was synthesized following a described procedure.[1] Compound **2** (581 mg, 2.80 mmol) was dissolved in a solution of HCl (2.6 mL) and CH_3OH (3.6 mL) at 0 °C. $NaNO_2$ (389 mg, 5.64 mmol) was added and the reaction mixture was stirred for 30 min at 0 °C, then for 30 min at room temperature and, finally, 4 h at 30 °C. The solution turned from colourless to a dark orange colour. Water (100 mL) was added until the solution turned green. The organic layer was extracted with CH_2Cl_2 (3 × 100 mL), dried over Na_2SO_4, evaporated to dryness and the crude product was purified by silica gel chromatography (cyclohexane/ethyl acetate 6:4) giving the title product as a green oil (250 mg, 38%).

R_f = 0.25 (cyclohexane/ethyl acetate 6:4); **^{1}H NMR (400 MHz, $CDCl_3$)** δ = 6.70 (d, *J* = 7.0 Hz, 2H), 3.68 (s, 3H), 3.53 (t, *J* = 7.4 Hz, 2H), 3.13 (s, 3H), 2.39 (t, *J* = 7.4 Hz, 2H), 1.97 (quint, *J* = 7.4 Hz, 2H) ppm; **^{13}C** NMR (101 MHz, $CDCl_3$) δ = 173.2,

163.2, 154.6, 110.2, 51.9, 39.0, 30.8, 22.3 ppm; IR ν = 2950, 1731, 1598, 1522, 1362, 1333, 1229, 1106, 823, 740, 634 cm^{-1}; **ESI-MS** m/z: $[M+H]^+$ = 237.0; **HRMS**: calculated for $C_{12}H_{17}N_2O_3$ $[M+H]^+$ 237.1161, found 237.1133.

Synthesis of 4:

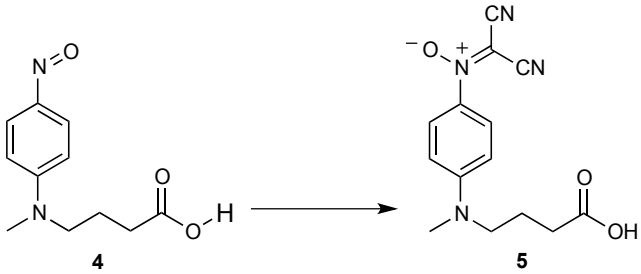


3 **4**

Compound **3** (240 mg, 1.02 mmol) was dissolved in CH_3OH and a solution of NaOH (41 mg, 1.02 mmol) in water was added to the green mixture. After stirring overnight at room temperature, the reaction mixture turned green-brown. The solvent was removed under reduced pressure to afford the title compound as a brown viscous solid (164 mg, 87%) which was used without any further purification.

R_f = 0.26 (CH_2Cl_2/CH_3OH 9:1); **m.p**. = 95 °C; **^{1}H NMR (400 MHz, CD_3OD)** δ = 6.92 (d, *J* = 5.6 Hz, 2H), 3.60 (t, *J* = 7.5 Hz, 2H), 3.20 (s, 3H), 2.23 (t, *J* = 7.5 Hz, 2H), 1.94 (quint, *J* = 7.5 Hz, 2H) ppm; **^{13}C NMR (101 MHz, CD_3OD)** δ = 181.4, 163.9, 157.2, 112.0, 53.7, 39.3, 35.7, 24.9 ppm; **IR** ν = 3389, 2928, 2359, 2341, 1598, 1568, 1402, 1364, 1293, 1231, 1109, 822 cm^{-1}; **ESI-MS** m/z: $[M+H]^+$ = 223.0; **HRMS**: calculated for $C_{11}H_{15}N_2O_3$ $[M+H]^+$ 223.1007, found 223.1079.

Synthesis of 5:

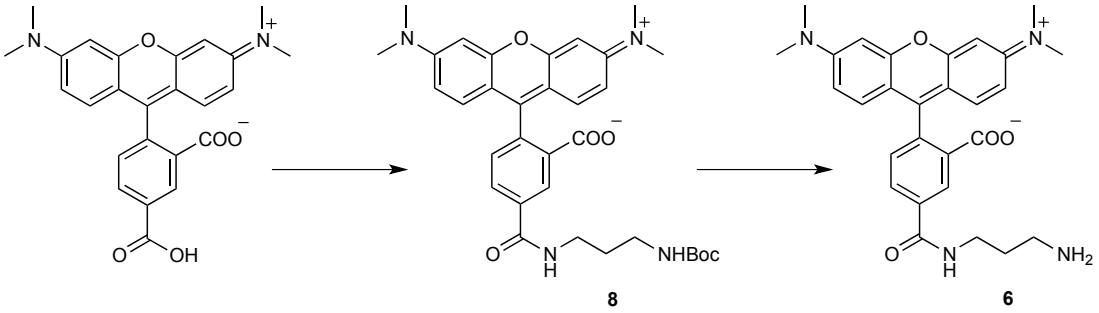


4 **5**

A brown solution of tetracyanoethylene (TCN) (284 mg, 2.22 mmol) in DMF (2 mL) was added to a green-brown solution of **4** (154 mg, 0.70 mmol) in DMF (3 mL). The reaction mixture turned dark purple and was stirred overnight at room temperature and poured into water (100 mL). The organic layer was extracted with diethyl ether (3 × 100 mL) and evaporated to dryness. The crude product was purified by preparative HPLC to afford the title compound as a dark purple solid (31 mg, 15%).

R_f = 0.24 (CH_2Cl_2/CH_3OH 9:1); **m.p**. = 78 °C; **^{1}H NMR (400 MHz, $CDCl_3$)** δ = 8.86 (bs, 1H), 7.72 (d, *J* = 9.4 Hz, 2H), 6.68 (d, *J* = 9.4 Hz, 2H), 3.51 (t, *J* = 7.3 Hz, 2H), 3.10 (s, 3H), 2.44 (t, *J* = 7.3 Hz, 2H), 1.95 (quint, *J* = 7.3 Hz, 2H) ppm; **^{13}C NMR (101 MHz, $CDCl_3$)** δ = 178.2, 153.7, 134.6, 125.9, 111.7, 111.4, 110.9, 110.8, 93.4, 51.8, 38.9, 30.8, 21.8 ppm; **IR** ν = 2917, 2216, 1705, 1592, 1519, 1392, 1325, 1146, 959, 820, 679, 531 cm^{-1}; **ESI-MS** m/z: $[M+H]^+$ = 287.0; **HRMS**: calculated for $C_{14}H_{15}N_4O_3$ $[M+H]^+$ 287.1137, found 287.1144.

Synthesis of 6:



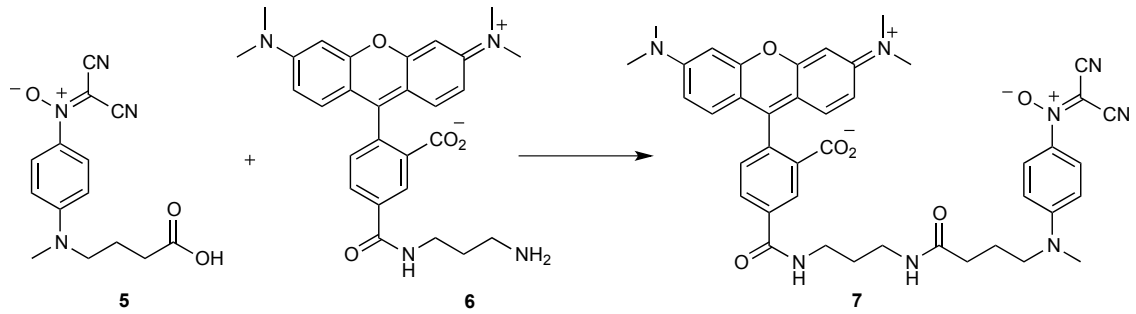
This compound was synthesized following a described procedure.[2] To a solution of tetramethylrhodamine (**5-TAMRA**) (100 mg, 0.23 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (88 mg, 0.46 mmol), 1-hydroxybenzotriazole (HOBt) (62 mg, 0.46 mmol) and triethylamine (62 μL, 0.46 mmol) in DMF was added *N*-Boc propylamine (40 mg, 0.23 mmol). This solution was stirred overnight at room temperature under Argon. The crude product was purified by preparative HPLC to afford the title compound as a dark pink solid (60 mg, 44%).

^{1}H NMR (400 MHz, CD_3OD) δ = 8.91-8.874 (m, 1H), 8.79 (d, *J* = 1.4 Hz, 1H), 8.27 (dd, *J* = 1.4, 7.9 Hz, 1H), 7.53 (d, *J* = 7.9 Hz, 1H), 7.16-6.99 (m, 6H), 3.53-3.49 (m, 2H), 3.18 (t, *J* = 6.8 Hz, 2H), 1.86-1.79 (m, 2H), 1.45 (s, 9H) ppm; **^{13}C NMR (101 MHz, CD_3OD)** δ = 159.1, 159.0, 138.1, 137.8, 132.9, 132.3, 131.2, 131.3, 115.6, 114.8, 97.5, 40.9, 38.8, 38.6, 30.8, 28.8 ppm; **ESI-MS** m/z: $[M+H]^+$ = 587.0.

Compound **8** (60 mg, 0.10 mmol) was added to a 4N HCl in dioxane (5.1 mL) and the solution was stirred for 5 h at room temperature. The solvent was removed under reduced pressure to afford the title compound **6** which was used directly in next step (48 mg, 83%).

^{1}H NMR (400 MHz, CD_3OD) δ = 8.80 (d, *J* = 1.8 Hz, 1H), 8.30 (dd, *J* = 1.8, 7.9 Hz, 1H), 7.56 (d, *J* = 7.9 Hz, 1H), 7.14-6.99 (m, 6H), 3.39-3.36 (m, 2H), 3.07 (t, *J* = 7.3 Hz, 2H), 2.08-2.01 (m, 2H) ppm; **^{13}C NMR (101 MHz, CD_3OD)** δ = 168.8, 167.3, 160.5, 159.1, 159.0, 138.3, 137.2, 132.9, 132.4, 132.0, 131.9, 131.4, 115.6, 114.7, 97.5, 64.3, 40.9, 38.5, 37.8, 28.9 ppm; **ESI-MS** m/z: $[M+H]^+$ = 487.0.

Synthesis of 7:



To a solution of **5** (10 mg, 0.035 mmol), EDC (35 mg, 0.18 mmol), HOBt (24 mg, 0.18 mmol) and triethylamine (6,2 µL, 0.046 mmol) in DMF (1 mL) was added **6** (48 mg, 0.091 mmol). This solution was stirred overnight at room temperature. The crude product was purified by preparative HPLC to afford the title compound as a dark purple solid (16 mg, 27%).

R_f = 0.63 (CH_2Cl_2/CH_3OH 9:1) neutral alumina; **m.p**. = 155 °C; **^{1}H NMR (400 MHz, dmso-*d*$_6$)** δ = 8.86 (t, *J* = 5.5 Hz, 1H), 8.68 (s, 1H), 8.28 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.91 (t, *J* = 5.5 Hz, 1H), 7.77 (d, *J* = 9.4 Hz, 2H), 7.57 (d, *J* = 7.9 Hz, 1H), 7.046-6.84 (m, 8H), 3.50-3.46 (m, 2H), 3.38-3.33 (m, 2H), 3.27 (s, 12H), 3.18-3.13 (m, 2H), 3.08 (s, 3H), 2.17 (t, *J* = 7.0 Hz, 2H), 1.82-1.75 (m, 2H), 1.75-1.68 (m, 2H) ppm; **^{13}C NMR (101 MHz, dmso-*d*$_6$)** δ = 171.5, 164.5, 153.3, 136.4, 136.0, 133.4, 130.5, 129.4, 125.9, 111.7, 110.6, 101.4, 96.3, 93.6, 74.8, 72.1, 51.3, 38.3, 37.3, 36.4, 31.9, 29.1, 22.2 ppm; **IR** ν = 3305, 2929, 2359, 2216, 1708, 1648, 1594, 1492, 1408, 1345, 1187, 1131, 928, 823, 699 cm^{-1}; **ESI-MS** m/z : $[M+H]^+$ = 755; **HRMS** : calculated for $C_{42}H_{43}N_8O_6$ $[M+H]^+$ 755.3227, found 755.3289.

Figure 2: A stock solution of cysteine (20 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). Nitrone **1** was dissolved in DMSO at room temperature to afford the stock solution (10 mM). 5 µL of stock solution of nitrone **1** was diluted in 945 µL of phosphate buffer (0.1 M, pH 7.4) at room temperature. 50 µL of stock solution Cys was added to this solution. The resulting solution was shaken well. The absorption spectra were recorded by UV spectrophotometry at 1, 5, 10, 30, 50, 80, 100 min.

Dithionite cleavage procedure for nitrone **1**: A 10 mM nitrone **1** solution was prepared in DMSO and a solution stock of sodium dithionite (20 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 5 µL of nitrone **1** solution was diluted in 945 µL of phosphate buffer (0.1 M, pH 7.4). Then, this solution was added to 50 µL of freshly prepared solution of sodium dithionite. The reaction was followed by UV spectrophotometry.

Figure 4: A solution stock of Cys (0.1 M), Hcy (0.1 M), GSH (0.1 M) and iodoacetate (0.1M) stock solutions were prepared in phosphate buffer (0.1 M, pH 7.4). Probe **7** was dissolved in DMSO at room temperature to afford the probe stock solution (200 µM). 50 µL of each analyte stock was added to a solvent mixture of 5 µL of the probe stock and 945 µL of phosphate buffer (0.1 M, pH 7.4). A solution stock of sodium dithionite (20 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 50 µL of sodium dithionite stock was added to a solvent mixture of 5 µL of the probe stock and 945 µL of phosphate buffer (0.1 M, pH 7.4). The resulting solutions were shaken well. After 30 min, fluorescence data were measured for Cys + IA, Cys, Hcy and GSH. After 1 min, fluorescence data was measured for dithionite. For all the measurements, the excitation and emission wavelength were 550/8 nm and 580/10 nm respectively. The measurements were performed with a microplate reader.

Figure 5: see biological procedures

Figure 6: see biological procedures

Figure S1: A stock solution of nitrone **1** (10 mM) and a stock solution of TAMRA (200 µM) were prepared in DMSO at room temperature. 5 µL of the nitrone **1** stock was diluted in 995 µL of phosphate buffer (0.1 M, pH 7.4) and the absorption spectrum was recorded. 5 µL of the TAMRA stock was diluted in 995 µL of phosphate buffer (0.1 M, pH 7.4) and the emission spectrum was recorded.

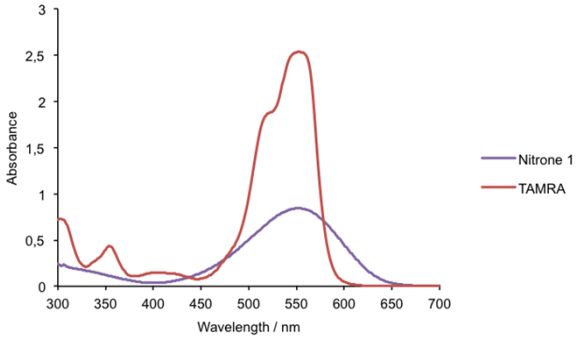


Figure S1. The absorption spectra of **1** (50 µM) and **TAMRA** (1 µM) in phosphate buffer (0.1 M, pH 7.4).

Others analytes were tested on nitrone **1**: with GSH, a thiol substrate, the same reactivity was observed but slower; with nonthiol compounds such as hydrogen peroxide (H_2O_2), arginine (Arg) and lysine (Lys) no response on UV-visible absorption was noticed (Figures S2 and S3).

Figure S2: A stock solution of glutathione (20 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 5 µL of stock solution of nitrone **1** (10 mM) was diluted in 945 µL of phosphate buffer (0.1 M, pH 7.4) at room temperature. 50 µL of stock solution GSH was added to this solution. The resulting solution was shaken well and the absorption spectra were then recorded.

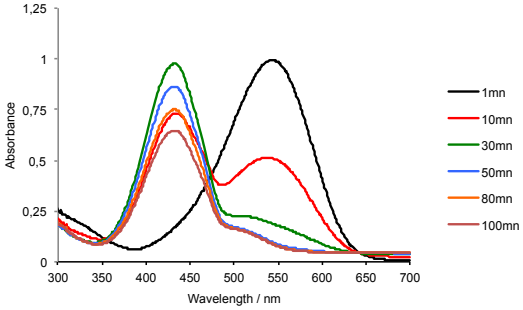


Figure S2. Absorption spectra of **1** (50 µM) upon addition of GSH (1mM) in phosphate buffer (0.1 M, pH 7.4).

Figure S3: Stock solutions of arginine (20 mM), lysine (20 mM) and hydrogen peroxide (20 mM) were prepared in phosphate buffer (0.1 M, pH 7.4) at room temperature. 5 µL of stock solution of nitrone **1** (10 mM) was diluted in 995 µL of phosphate buffer (0.1 M, pH 7.4) to afford the blank. 5 µL of stock solution of nitrone **1** (10 mM) was diluted in 945 µL of phosphate buffer (0.1 M, pH 7.4) at room

temperature. 50 µL of stock solution Arg, Lys or H_2O_2 was added to the previous solution. The resulting solutions were shaken well. The absorption spectra were then recorded at 1, 30 and 60 min.

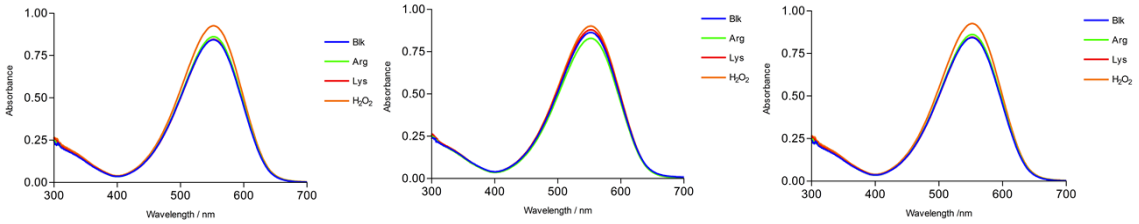


Figure S3. Absorption spectra of **1** (50 µM) upon addition of Arg, Lys and H_2O_2 (1 mM) at 1 min, 30 min and 60 min.

Figure S4: A stock solution of cysteine (20 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 5 µL of stock solution of nitrone **1** (10 mM) was diluted in 995 µL of phosphate buffer (0.1 M, pH 7.4) at room temperature (a). 5 µL of stock solution of nitrone **1** (10 mM) was diluted in 945 µL of phosphate buffer (0.1 M, pH 7.4) at room temperature. 50 µL of stock solution Cys was added to the previous solution (b, c, d, e).

(a) (b) (c) (d) (e)

Figure S4: Photos of **1** (50 µM) (a) upon addition with cysteine (1 mM) at different times (b) 1 min ; (c) 5 min ; (d) 10 min ; (e) 90 min in phosphate buffer (0.1 M, pH 7.4).

Figure S5: See biological procedures.

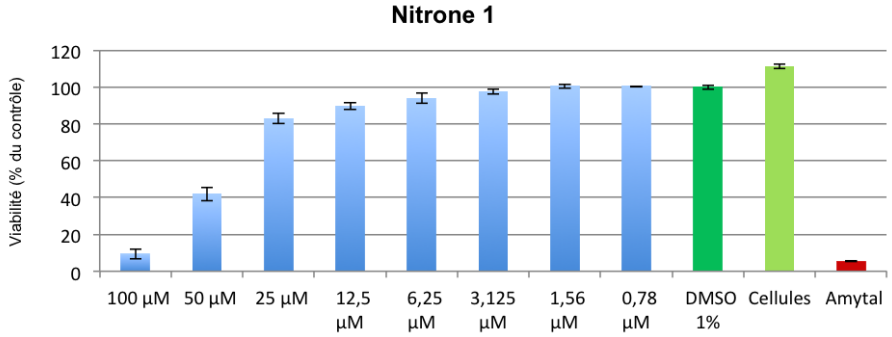
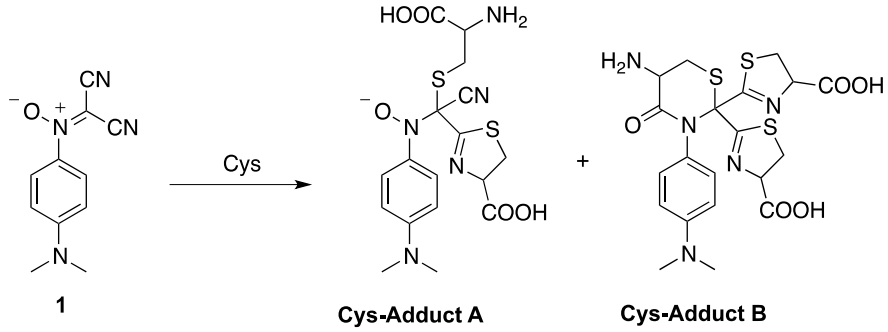


Figure S5: Cell viability measured by the MTT assay. Results are expressed as percent relative to untreated HaCaT cells (mean ± SD).

To explain the reactivity, we anticipated either the Michael addition of the thiols to the N=C double bond or the addition to the cyano group to form a 4,5-dihydrothiazole derivative. This was confirmed by a LC-MS analysis of the reaction mixture containing **1** (1 mM) and Cys (10 equivalents) in phosphate buffer (0.1 M, pH 7.4).

The mass spectral revealed two main signals (m/z = 439.0 and 510.0). The two species can result from a cyclization and an addition **Cys-Adduct A** and three cyclizations **Cys-Adduct B**.



Scheme 1. Reaction of **1** with Cys.

Figure S6: ESI-LC/MS of nitrone **1** (1 mM) in phosphate buffer (0.1 M, pH 7.4) with 1% DMSO. The main peak is at t_r = 3.72 with *m/z* = 215.0.

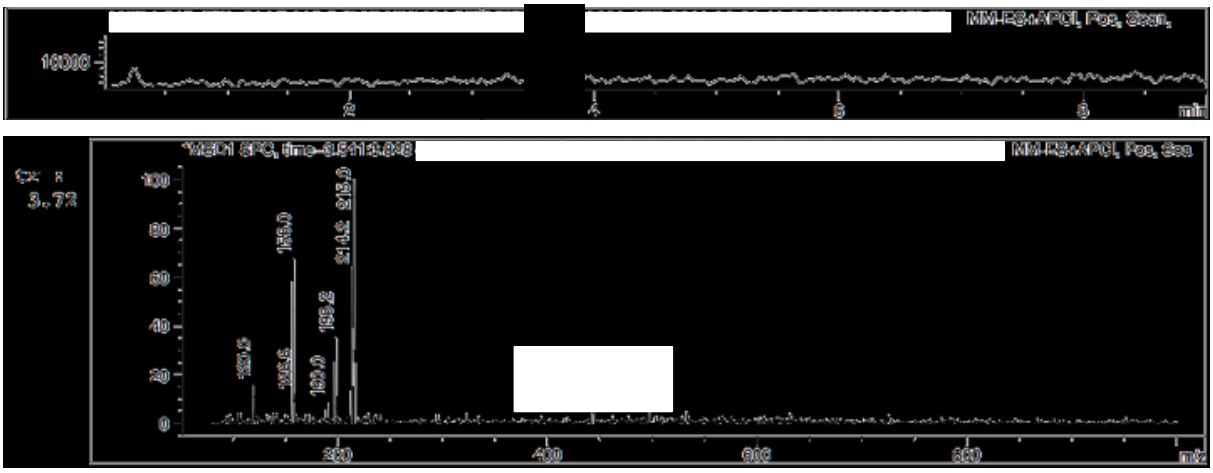


Figure S7: Nitrone **1** (1 mM) was dissolved with Cys (10 eq.) in phosphate buffer (0.1 M, pH 7.4) with 1% DMSO. After 1 h, the reaction mixture was analyzed by ESI-LC/MS. Two main peaks at t_r = 2.23 and 3.61 were observed with *m/z* = 439.0 and 510.0 respectively.

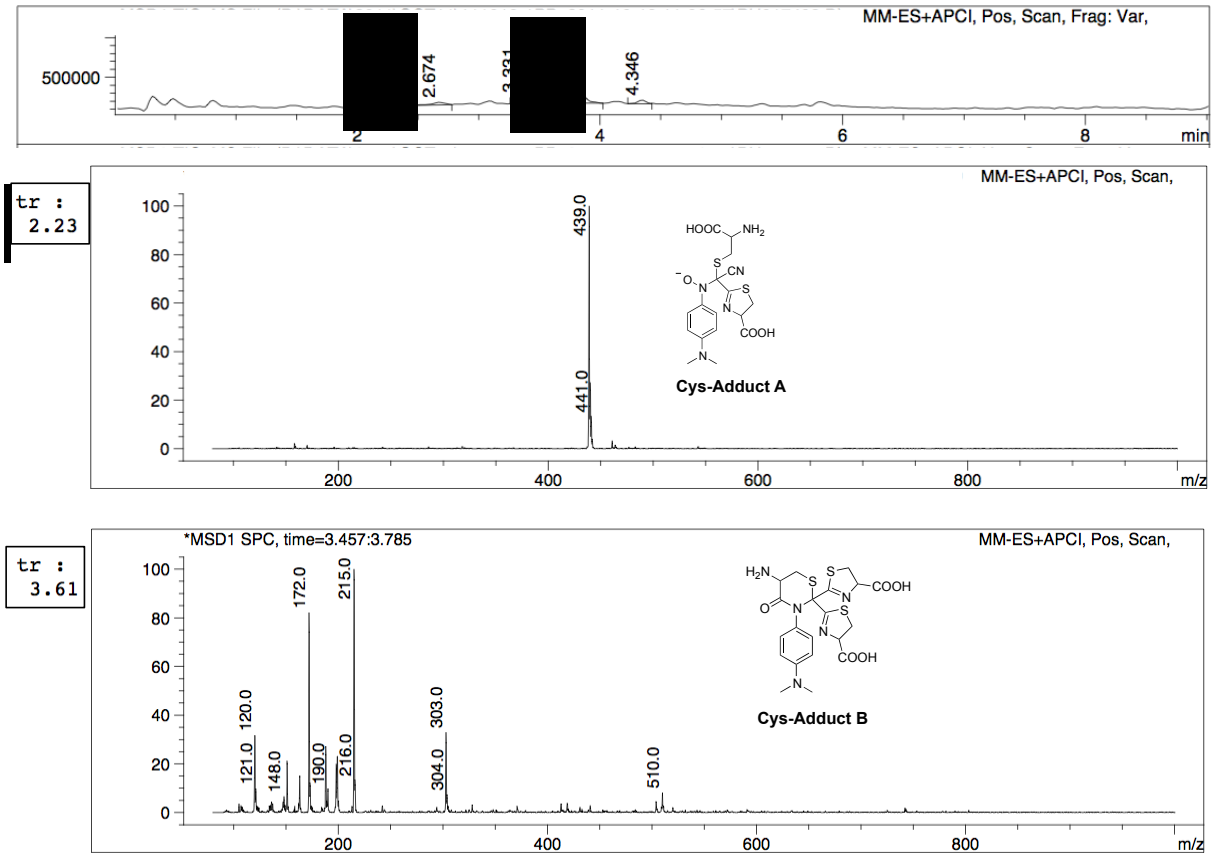


Figure S8: A stock solution of cysteine (10 mM) was prepared in PBS buffer (10 mM, pH 7.4). Probe **7** was dissolved in DMSO at room temperature to afford the probe stock solution (200 µM). 5 µL of the probe stock was diluted with appropriate aliquots of cysteine stock solution (0, 4, 8, 10, 20, 40, 60, 80, 100 µL) in different volumes of PBS buffer (to have a final volume of 1 mL). Increases in fluorescence intensity were followed by fluorescence spectrophotometry. The excitation wavelength was 557 nm and the emission wavelength was 580 nm. Excitation and emission slit widths were 5 nm. The emission spectra were recorded after 30 min.

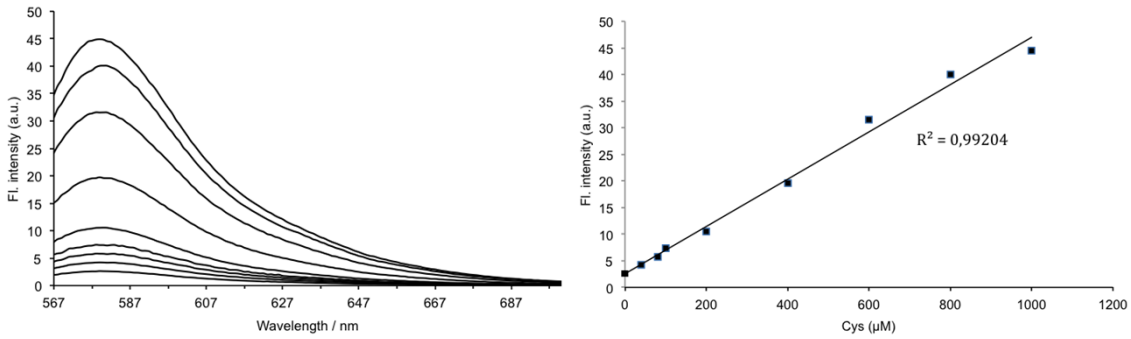
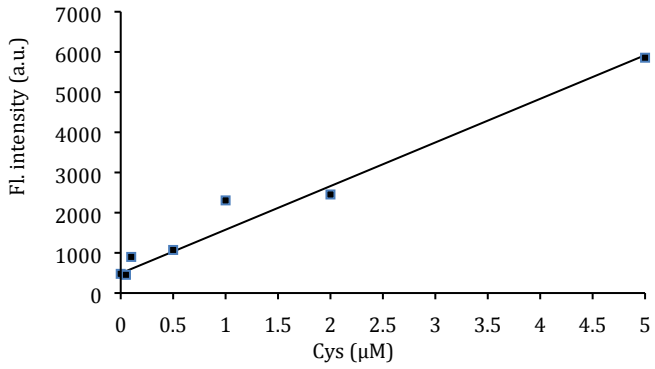


Figure S8: Fluorescence of **7** (1 µM) upon addition of increasing concentrations of Cys (0, 40, 80, 100, 200, 400, 600, 800, 1000 µM) in PBS buffer. Each spectrum was acquired 30 min after Cys addition.



Fluorescence of **7** (1 µM) upon addition of increasing concentration of Cys (0.05, 0.1, 0.5, 1, 2 and 5 µM) in PBS buffer. Each spectrum was acquired 30 min after Cys addition. The detection limit was determined based on a fluorescence titration. The emission intensity of probe **7** (1 µM) in PB buffer (10 mM, pH 7.4) was measured without Cysteine 5 times and the standard deviation of blank measurements was determined. The probe was then treated with cysteine (0.05, 0.1, 0.5, 1, 2 and 5 µM) and a nearly linear relationship was observed (R^2 = 0.969). The detection limit was then calculated with the following equation: detection limit = 3σ/m, where σ is the standard deviation of blank measurements and m is the slope between fluorescence intensity versus sample concentration. The detection limit was measured to be 0.05 µM.

Figure S9: Absorption spectra of probe **7** alone (1 µM) and towards cysteine (5 mM) and dithionite (1 mM). The absorption spectra were recorded after 5 min for the probe alone; after 30 min after treatment of the probe with cysteine and 1 min after treatment with dithionite.

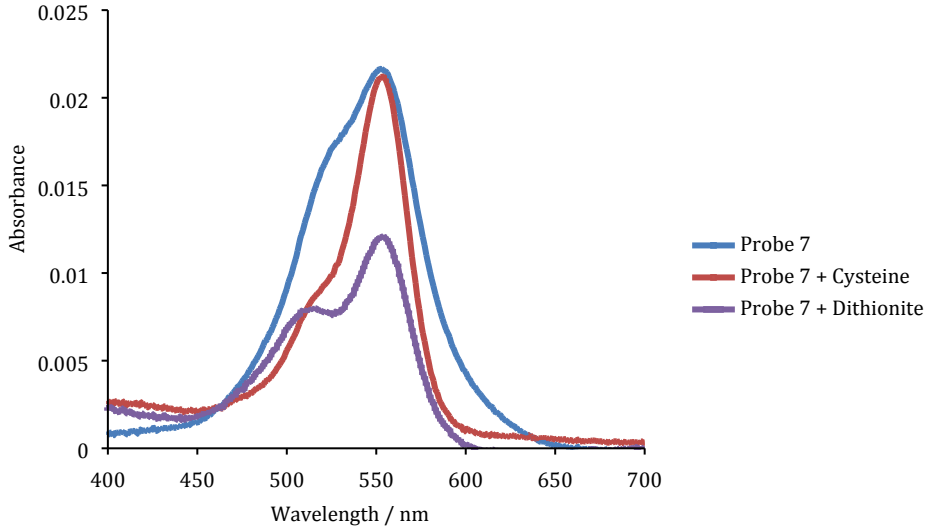


Figure S10: The amino acids (Ala, Arg, Asp, Glu, Gly, His, Lys, Met, Pro, Ser, Val, Cys, Hcy,), GSH, metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+}), oxidizing agents (sodium hypochlorite, hydrogen peroxide), reducing agent (NADPH), glucose, nucleoside (uridine), ascorbic acid stock solutions (0.1 M) were prepared in phosphate buffer (0.1 M, pH 7.4). Probe **7** was dissolved in DMSO at room temperature to afford the probe stock solution (200 µM). 50 µL of analyte stock was added to a solvent mixture of 5

μL of the probe stock and 945 μL of phosphate buffer (0.1 M, pH 7.4). For all the measurements, the excitation and emission wavelengths were 550/8 nm and 580/10 nm respectively. The fluorescence data were performed with a microplate reader.

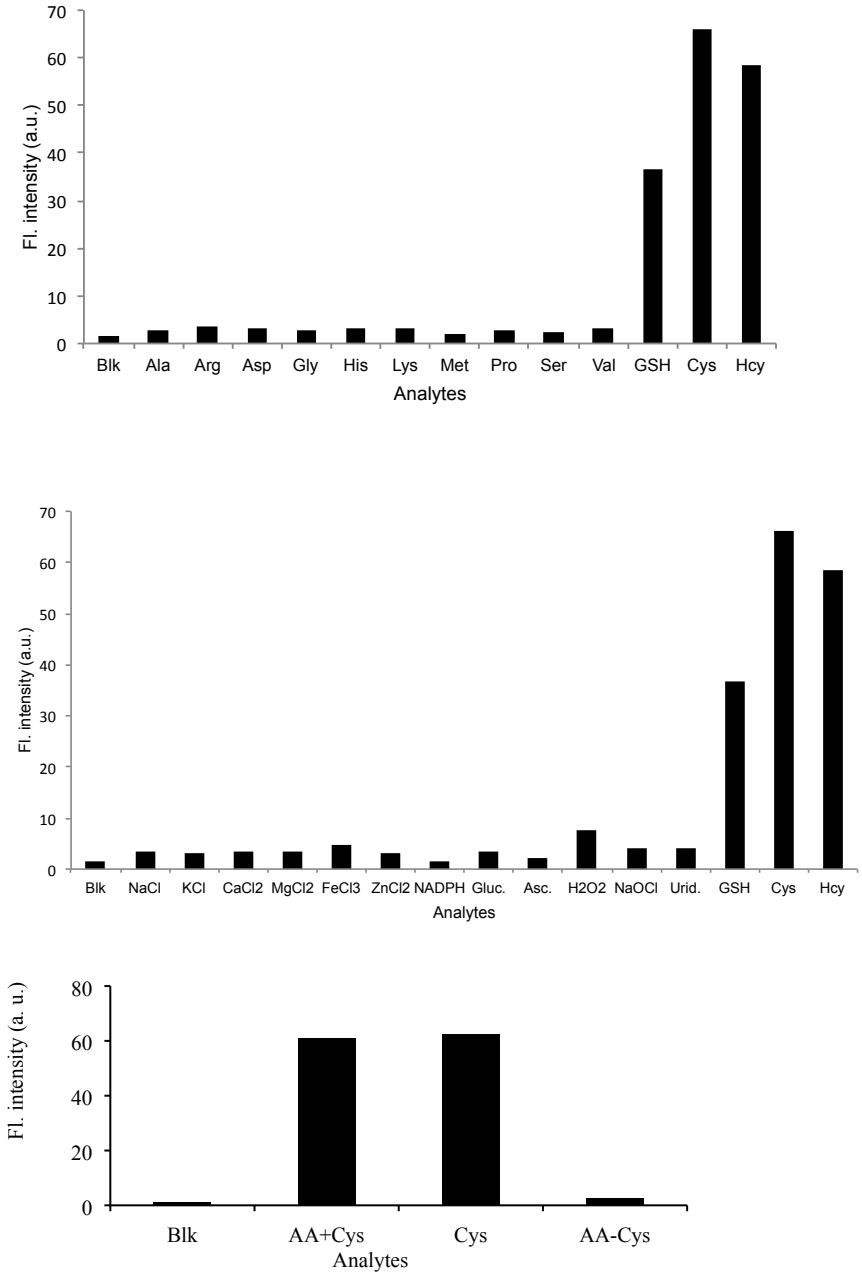


Figure S10: Fluorescence response of **7** (1 μM) towards various analytes (5 mM) in phosphate buffer (0.1 M, pH 7.4) (gluc. for glucose, asc. for ascorbic acid, urid. for uridine, AA+Cys: All amino acids and cysteine, AA-Cys: All amino acids without cysteine).

Figure S11: Fluorescence response of probe **7** (1 μM) towards different analytes (5 mM) and dithionite (1 mM). The data were collected 30 min (except dithionite 1 min) after incubation of probe **7** with analytes. Fluorescence data were measured with fluorescence plate reader with excitation filter 550/8 nm and emission filter 580/10 nm.

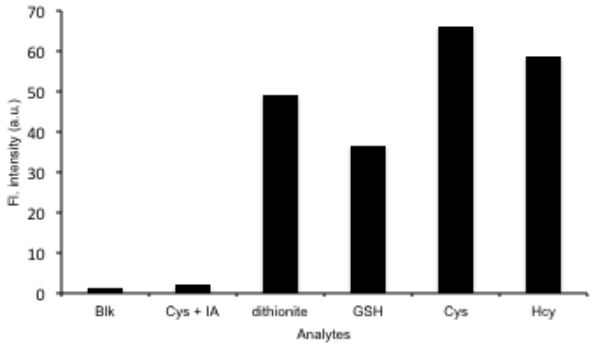
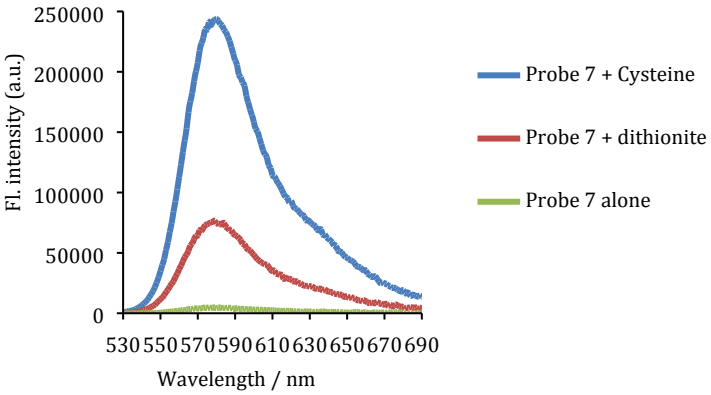


Figure S12: Probe 7 was dissolved in DMSO at room temperature to afford the probe stock solution (200 µM). A stock solution of cysteine (20 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). 50 µL of analyte stock was added to a solvent mixture of 5 µL of the probe stock and 945 µL of phosphate buffer (0.1 M, pH 7.4). The emission spectra were recorded after 5 min for the probe only, 30 min after treatment of the probe with cysteine and 1 min after treatment with dithionite.



The quantum yield of fluorescence for probe **7** was calculated following the equation

$$Quantum\ yield_{Probe7}\ (\Phi) = \frac{A(Fluo_{Probe7})}{A(Fluo_{Ref})} \cdot \frac{Abs_{Ref}}{Abs_{Probe7}} \cdot Quantum\ yield_{Ref}$$

where $A(Fluo_{Probe7})$ is the area under the fluorescence curve of probe **7**

where $A(Fluo_{Ref})$ is the area under the fluorescence curve of the reference

where Abs_{Ref} is the absorbance of the reference measured at 520 nm (excitation wavelength of the fluorophore)

where Abs_{Probe7} is the absorbance of the probe **7** measured at 520 nm (excitation wavelength of the fluorophore)

Rhodamine 101 was used as the reference and has a quantum yield of 90%[3]

$\Phi_{(probe\ alone)} = 0.14\%$ $\Phi_{(probe\ 7 + Cysteine)} = 15\%$ $\Phi_{(probe\ 7 + Dithionite)} = 5\%$

Figure S13: Probe **7** (40 µM) was dissolved in Dulbecco's Modified Eagle Medium (DMEM) without cells in presence or not of ALA (500 µM), NMM (500 µM) and BSO (100 µM). For all the measurements, the excitation and emission wavelengths were 530/25 nm and 590/35 nm respectively. The fluorescence data were recorded using a microplate reader after 1 h.

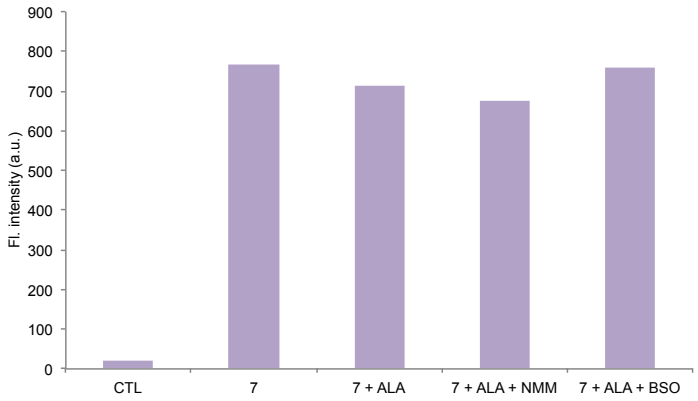


Figure S13: Fluorescence response of **7** (40 µM) in DMEM with ALA (500 µM), NMM (500 µM), BSO (100 µM) after 1 hour.

Figure S14: Dithionite (10 mM) in DMEM was added to each previous condition (Figure S13). For all the measurements, the excitation and emission wavelength were 530/25 nm and 590/35 nm respectively. The fluorescence data were performed with a microplate reader after 1 minute.

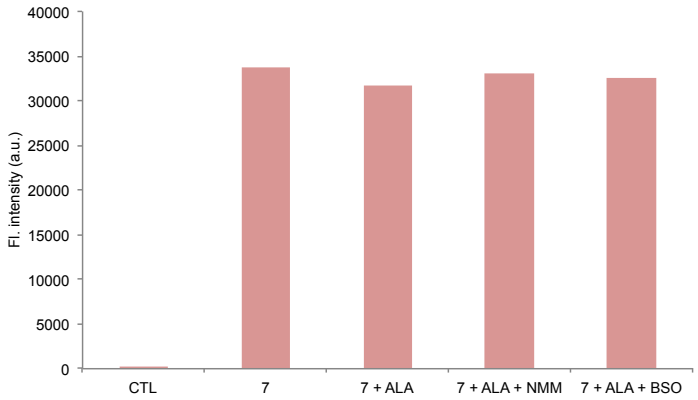


Figure S14: Fluorescence response of **7** (40 µM) in DMEM with ALA (500 µM), NMM (500 µM), BSO (100 µM) 1 minute after addition of dithionite (10 mM).

Biological procedures

MTT assay: In vitro cytotoxicity was measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The experiments were performed in 96-well plates with HaCaT cells grown to confluence in cell culture media (RPMI 1640 media supplemented with 10% fetal calf serum and 1 mM Glutamin, 200 µL per well). Cells were incubated with chemical reagent at different concentrations (100 µM to 10 mM) at 37 °C for 24 hours. After incubation, the supernatant was replaced with fresh culture media containing MTT (300 µg/mL). After 2 hours of incubation at 37 °C, the media was carefully removed and 100 µL of DMSO were added to solubilize the formazan crystals generated by mitochondrial enzymes-induced reduction of the MTT. The absorbance was measured at 595 nm using a microplate reader (Biotek, Synergy HT). The cell viabilities were expressed as percent of untreated control cells.

Cell Culture: 3T3 fibroblast cells were grown in Dulbecco's Modified Eagle Medium with 4.5 g/L glucose (IGBMC, illkirch) supplemented with 10% newborn calf serum (Invitrogen, Saint Aubin), 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Eurobio). Cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

Fluorescence intensities measurement using a 96-well microplate: 3T3 fibroblast cells were cultured in glass bottom, 96-well black plates for imaging experiment, 48 hours prior to conduction of experiments. Fluorescence intensity was measured using a microplate reader device (Biotek, Synergy HT) with excitation filter 530/25 nm and emission filter 590/35 nm.

Non-treated cells: After 48 hours, fluorescent probe (40 µM) was diluted in Dulbecco's Modified Eagle Medium (DMEM) and was then added onto the cells for 1 hour at 37 °C.

With ALA treatment: 3T3 cells were first supplemented with alpha lipoic acid (ALA, 500 µM) for 48 hours at 37 °C in Dulbecco's Modified Eagle Medium (DMEM). Fluorescent probe (40 µM) diluted in DMEM was then added onto the ALA-pretreated cells for 1 hour at 37 °C.

With ALA treatment and NMM (500 µM): 3T3 cells were first supplemented with alpha lipoic acid (ALA, 500 µM) for 48 hours at 37 °C in Dulbecco's Modified Eagle Medium (DMEM). NMM (500 µM) was diluted in DMEM and then added onto the cells for 20 min at 37 °C. Fluorescent probe (40 µM) diluted in DMEM was then added onto the cells for 1 hour at 37 °C.

With ALA treatment and BSO (500 µM): 3T3 cells were supplemented with alpha lipoic acid (ALA, 500 µM) and BSO (100 µM) for 48 hours at 37 °C in Dulbecco's Modified Eagle Medium (DMEM). Fluorescent probe (40 µM) diluted in DMEM then added onto the cells for 1 hour at 37 °C.

Treatment with dithionite: each previous condition (*Non-treated cells*, *With ALA treatment*, *With ALA treatment and NMM (500 μM)*, *With ALA treatment and BSO (100 μM)*) were incubated with 10 mM of dithionite in DMEM.

Cell Culture: Mice liver BNL CL.2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 1 g/L glucose (Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (Perbio, Brebieres, France), 2 mM L-Glutamine, 100 U/mL penicilin, 100 μg/mL streptomycin (Eurobio). Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Fluorescence imaging: 24 hours prior to experiment, 2.5×10^4 BNL CL.2 cells were seeded per well in 8-well Lab-Tek II Chambered coverglass plates (ref 155409, Nunc, Naperville, IL, USA). The fluorescent probe was diluted up to 300 μL in Minimum Essential Media (MEM) complete medium and then added onto the cells for a defined amount of time. After experiment completion the cells were washed twice with MEM and were incubated with a 5 μg/mL of Hoechst 33258 (ref H1399, Invitrogen) solution. After washing with 10% FBS red phenol free Eagle's MEM medium, cell images were acquired on a confocal Leica TSC SPE II microscope (405, 488 or 561 nm) and controlled by image acquisition software (Leica confocal LAS AF, Leica). Two different objectives were used: HXC PL APO 20×/0.7 CS and HXC PL APO 63×/1.40 OIL CS.

Non-treated cells: fluorescent probe (1 μM) was diluted in Minimum Essential Media (MEM) and then added onto the cells for 1 hour at 37°C.

With ALA treatment: BNL CL.2 cells were first supplemented with alpha lipoic acid (ALA, 500 μM) for 48 hours at 37°C in Minimum Essential Media (MEM). Fluorescent probe (1 μM) diluted in Minimum Essential Media (MEM) was then added onto the ALA-pretreated cells for 1 hour at 37°C.

With ALA treatment and NMM (500 μM): BNL CL.2 cells were supplemented with alpha lipoic acid (ALA, 500 μM) for 48 hours at 37°C in Minimum Essential Media (MEM). NMM (500 μM) was diluted in MEM and then added onto the cells for 20 min at 37°C. Fluorescent probe (1 μM) diluted in MEM was then added onto the cells for 1 hour at 37°C.

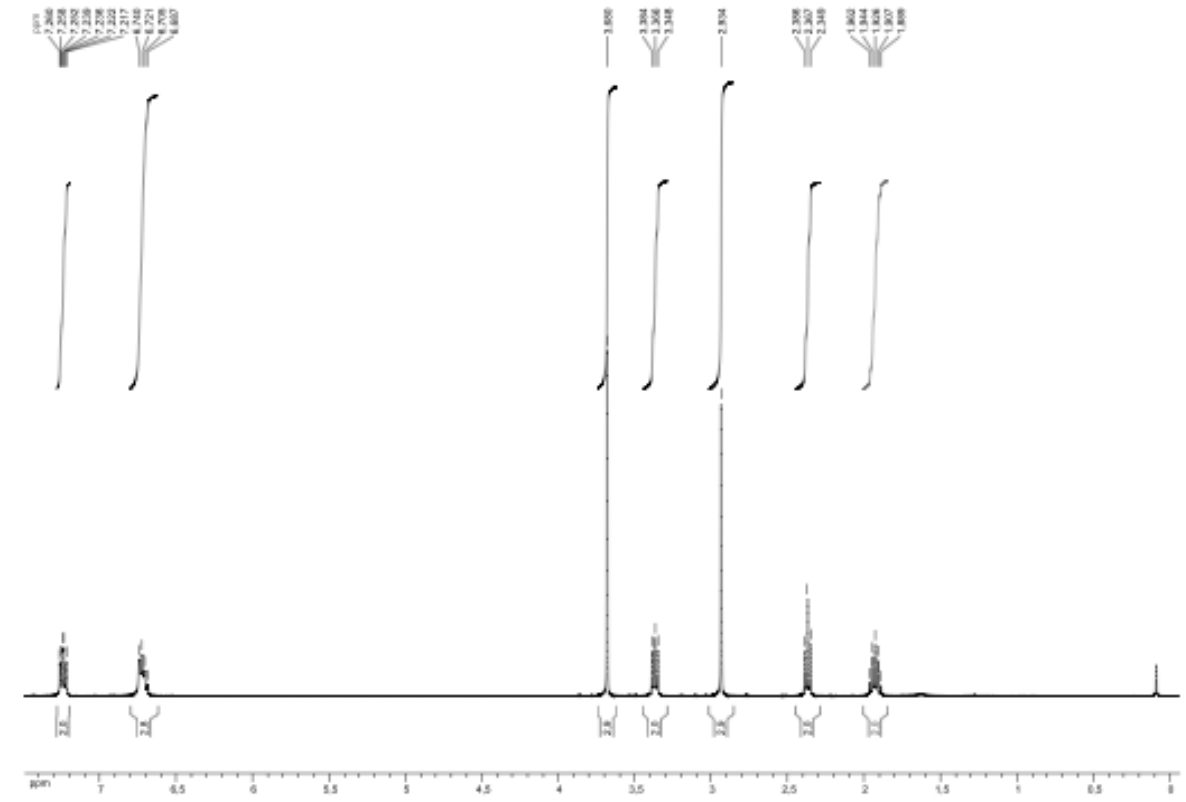
References

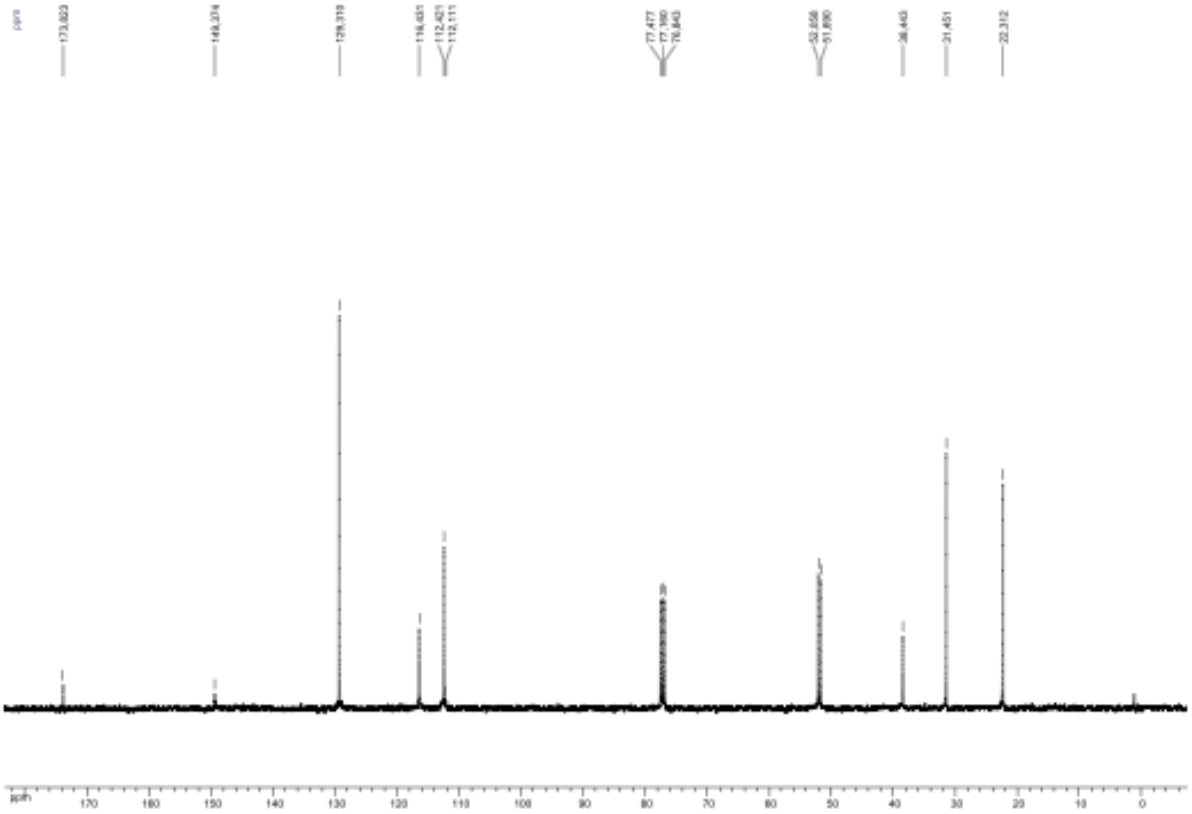
1) J. J. D'Amico, C. C. Tung, L. A. Walker, *J. Am. Chem. Soc.* 1959, **81**, 5957. (Method XLIII)

2) M. A. Brun, K.-T. Tan, E. Nakata, M. J. Hinner, K. Johnsson, *J. Am. Chem. Soc.* 2009, **131**, 5873.

3) C. Würth, M. Grabolle, J. Pauli, M. Spieles, U. Resch-Genger , *Anal. Chem.* 2011, **83**, 3431.

Spectra of compound **2**:





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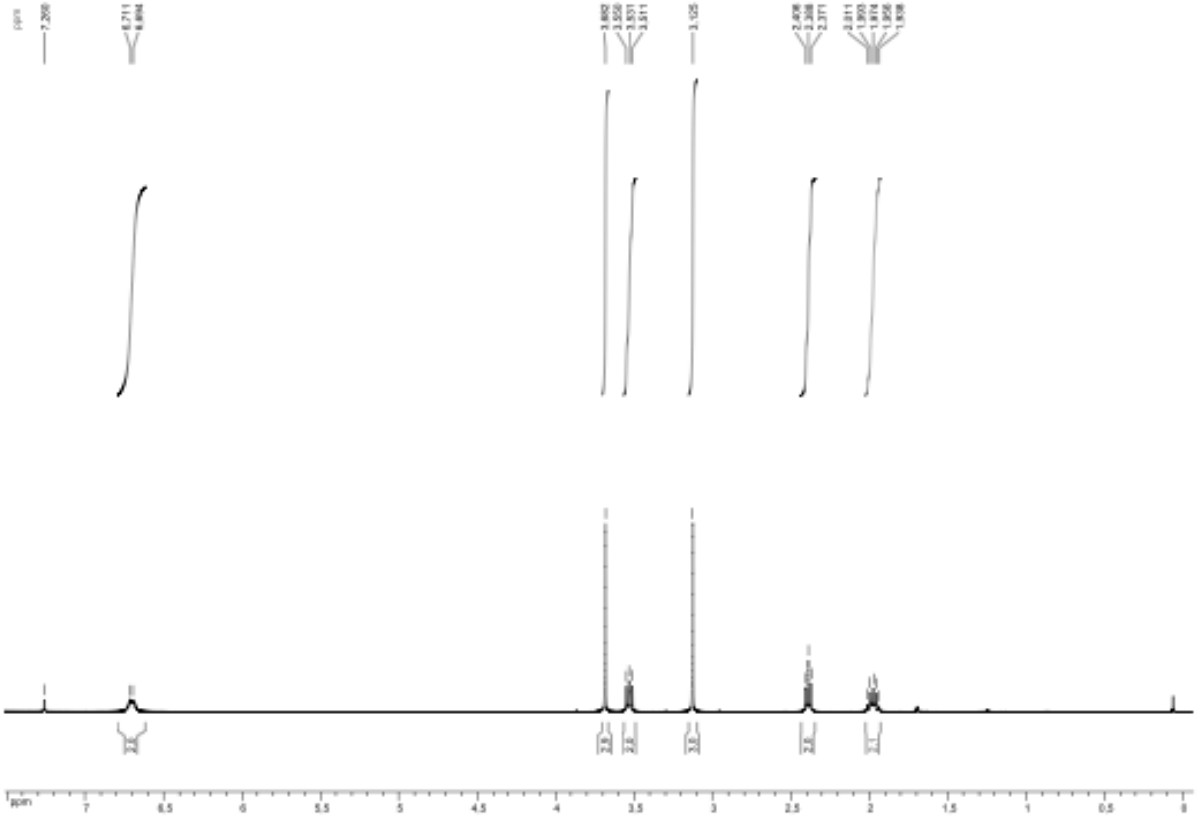

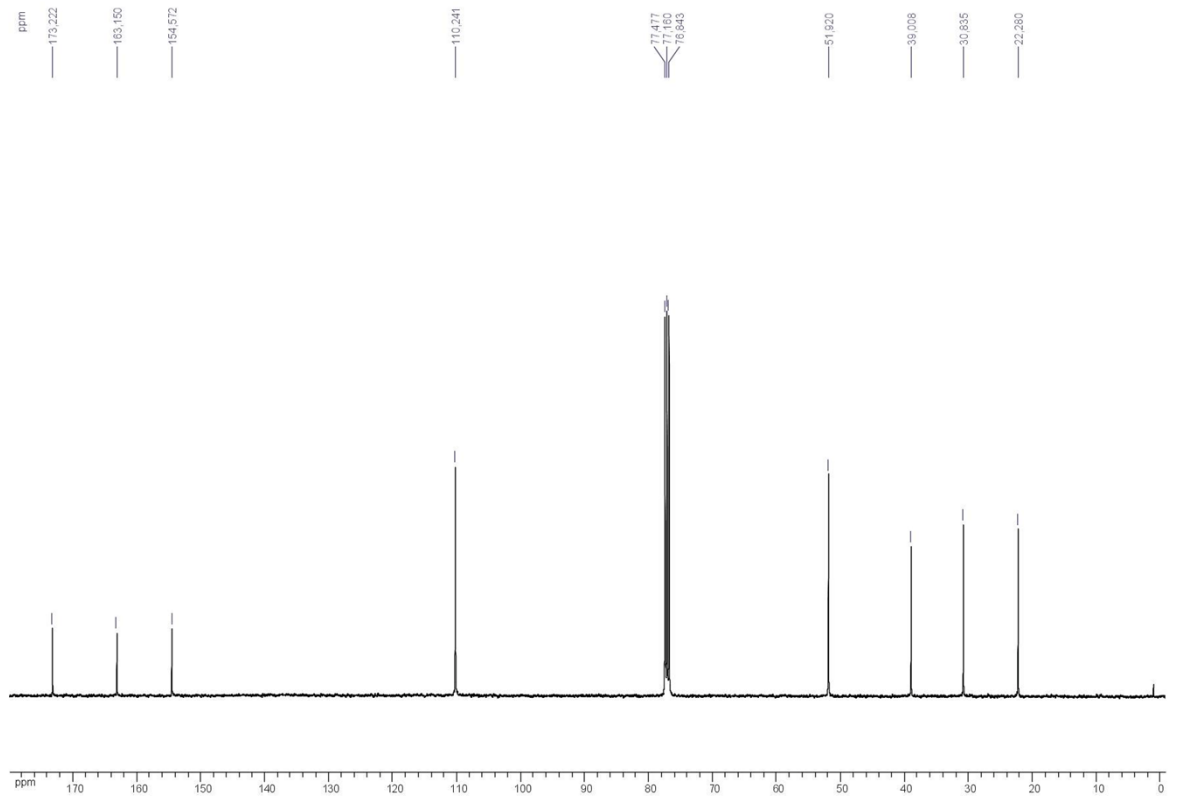

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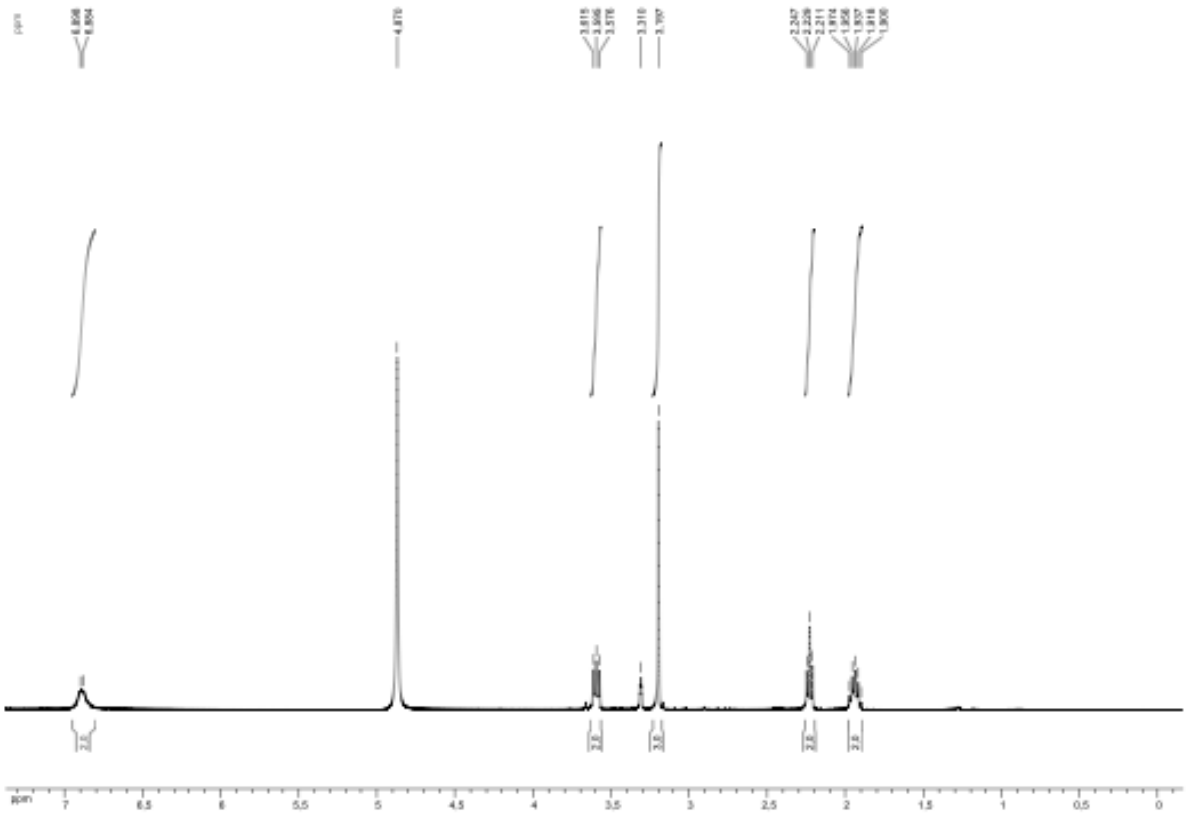

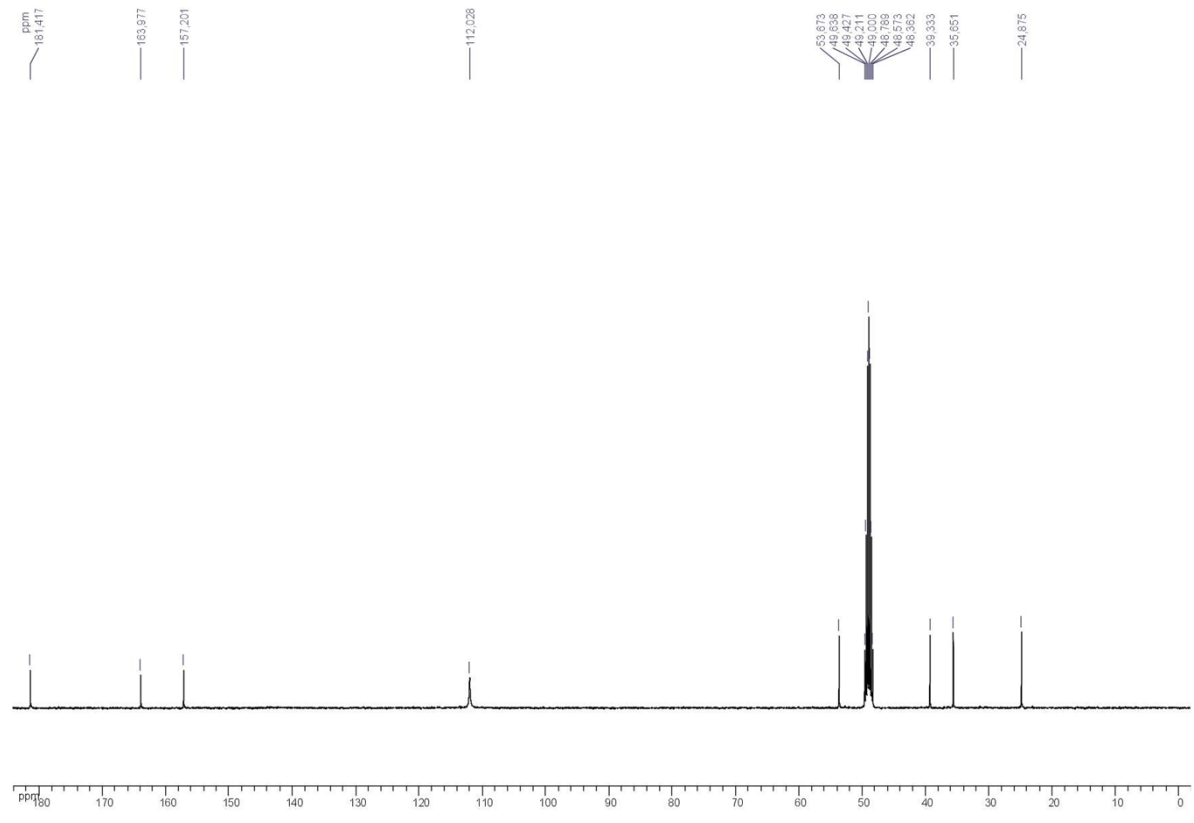

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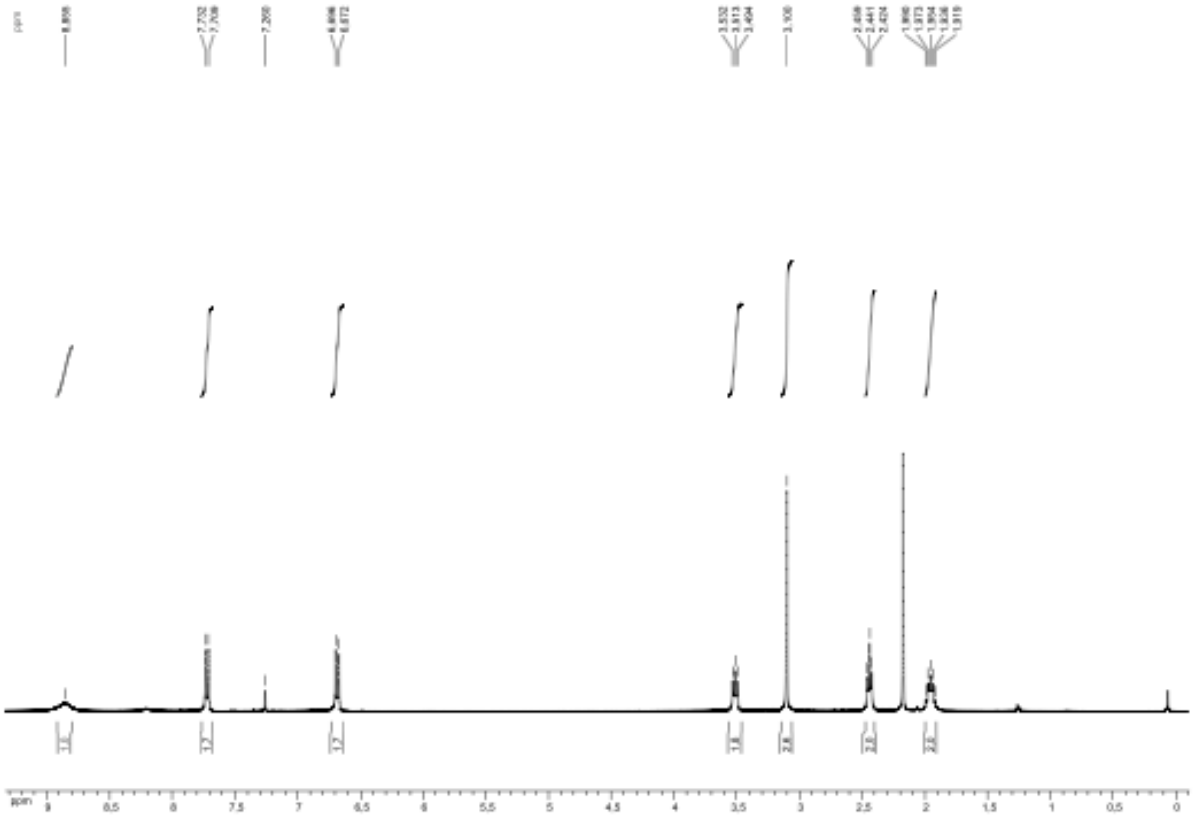

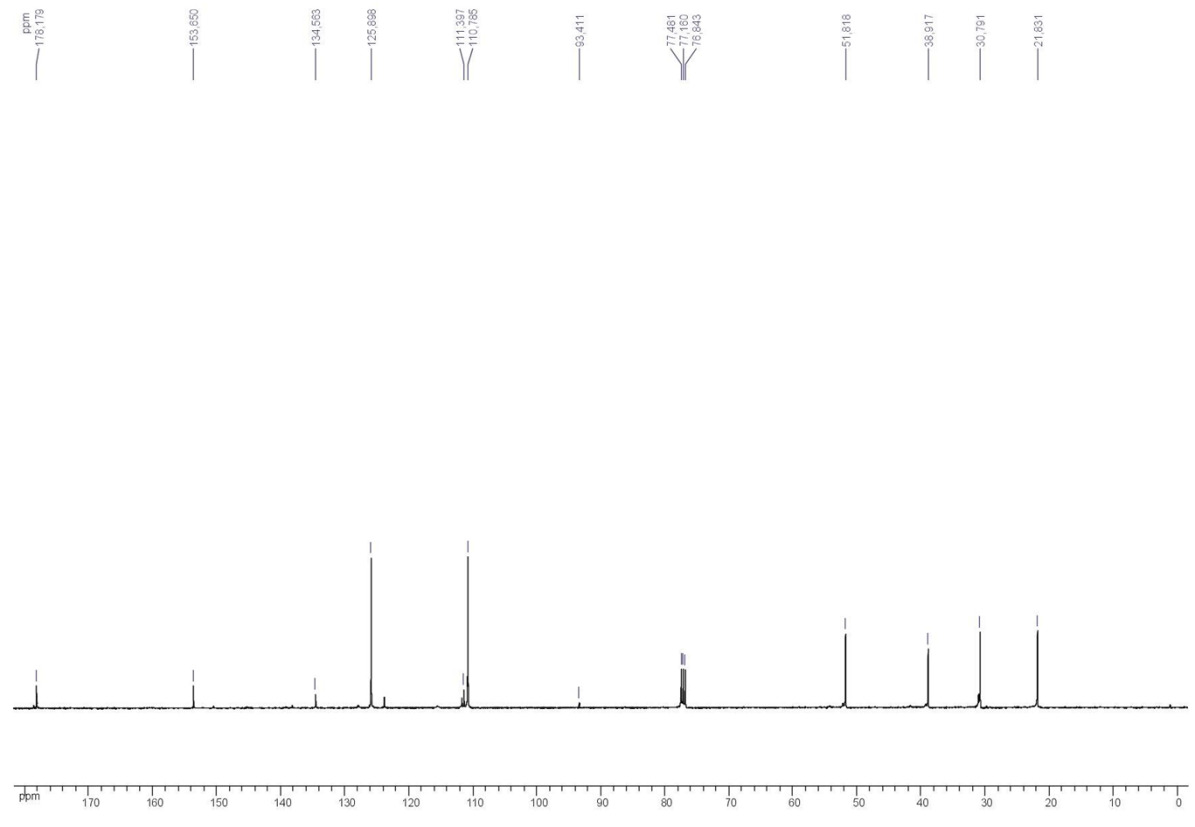

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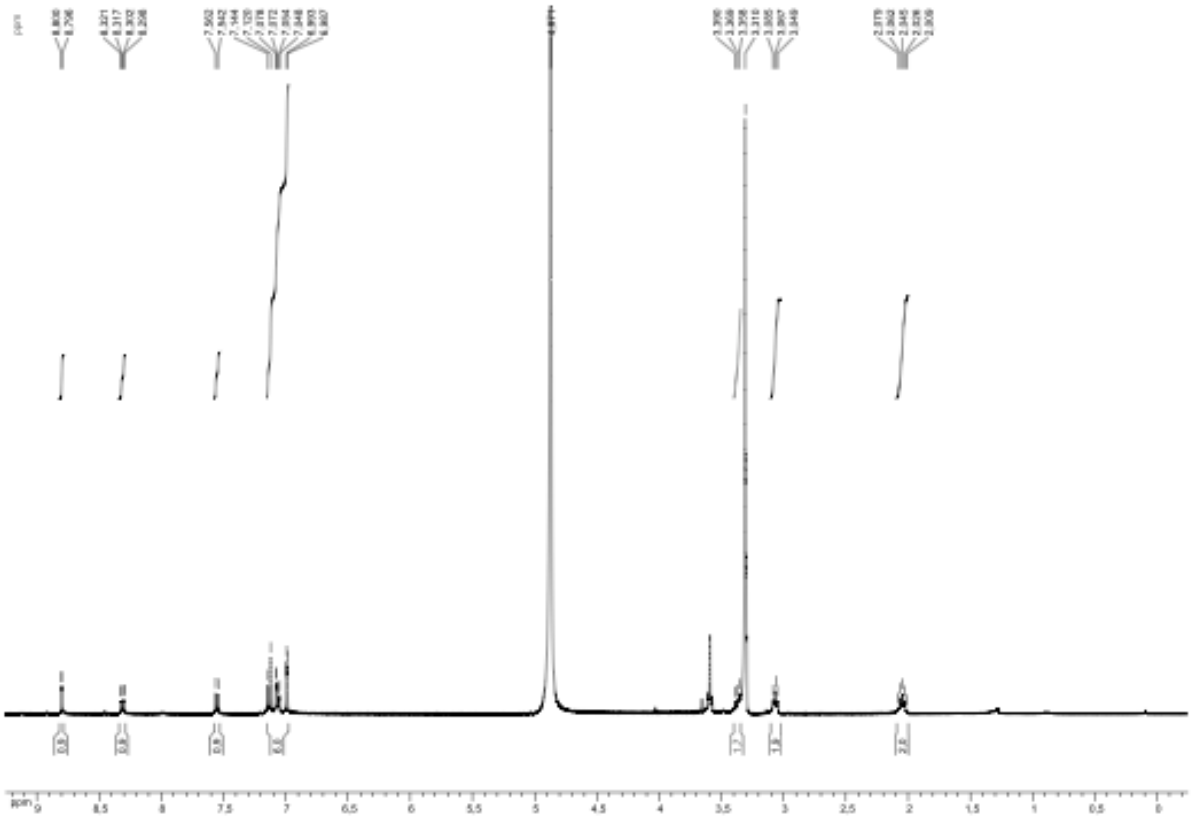

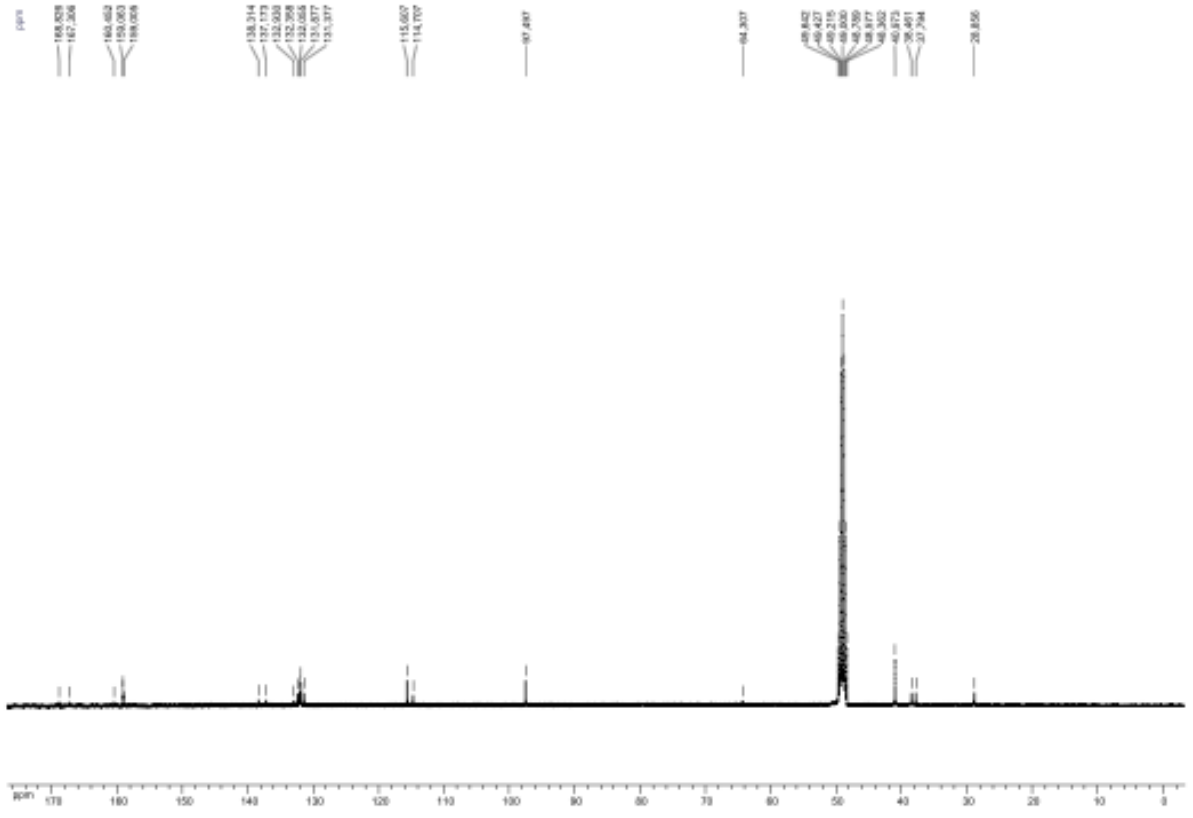

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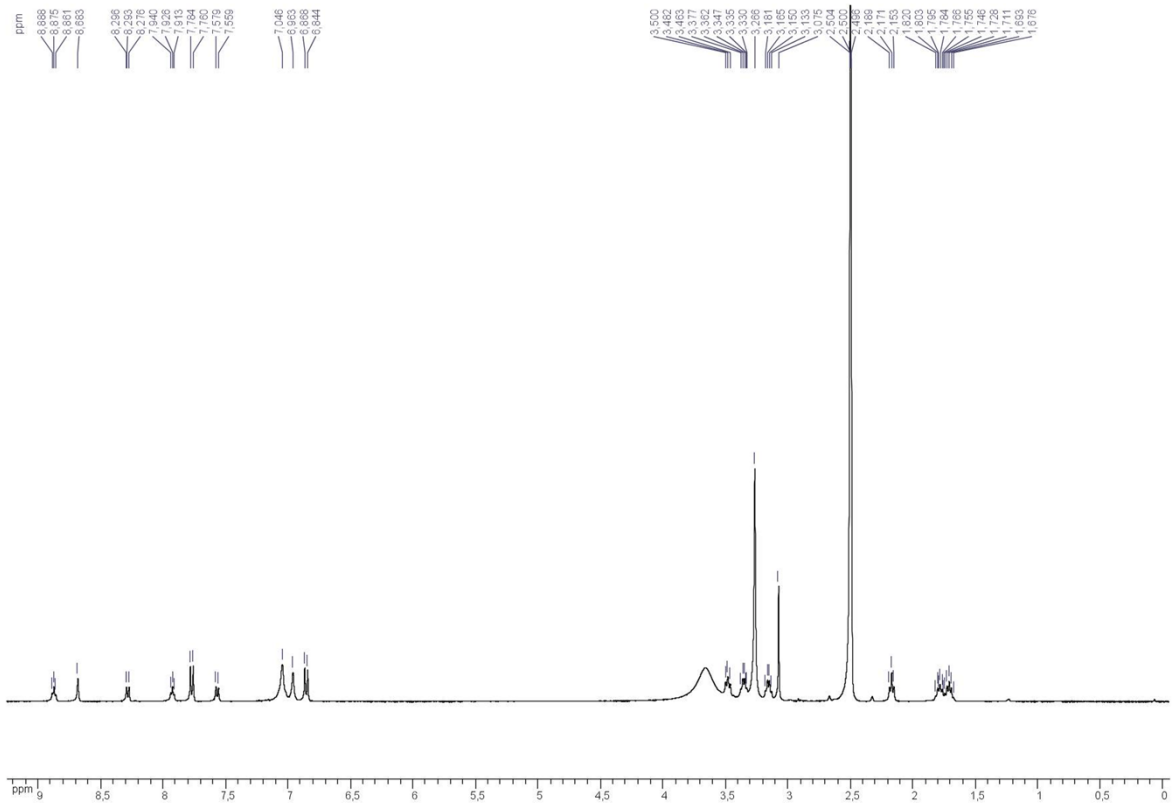

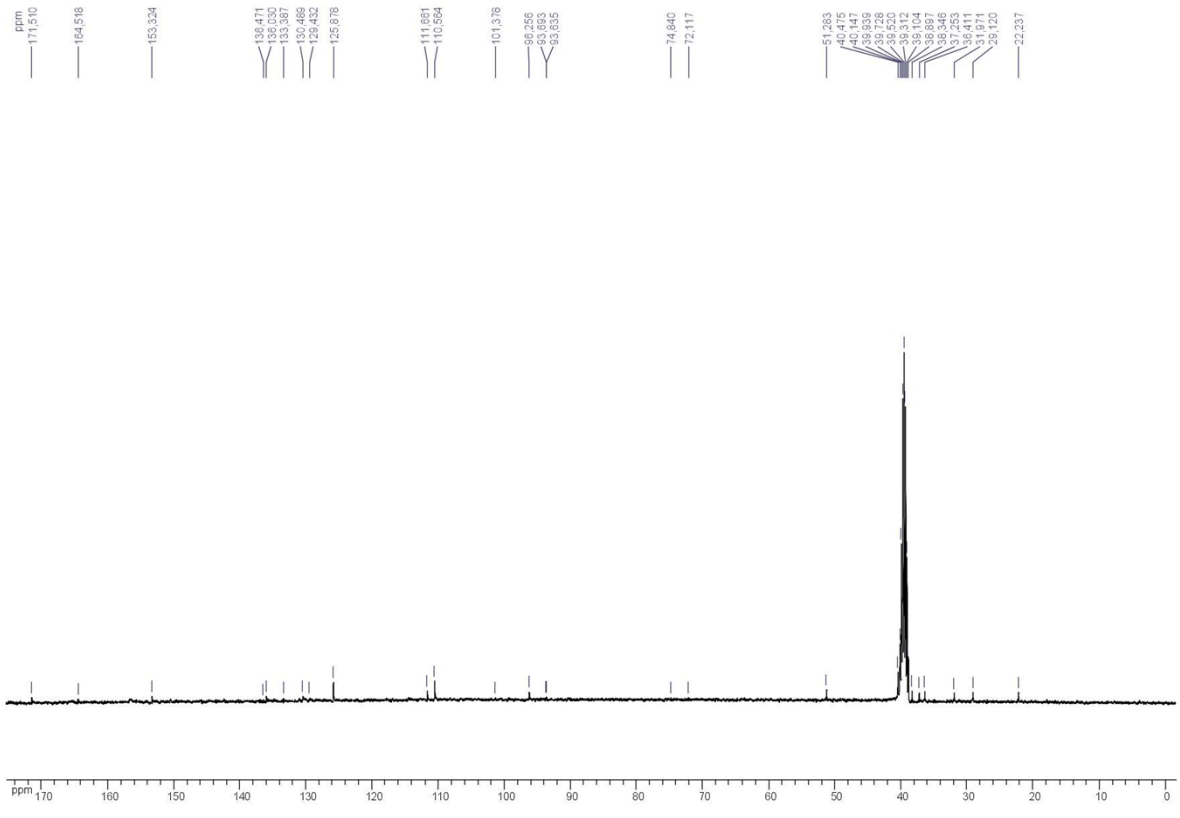

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