Electronic Supplementary Information

A Generator of Peroxynitrite Activatable with Red Light

Cristina Parisi,[†,#] Mariacristina Failla,[§,#] Aurore Fraix,[†,#] Luca Menilli, [‡]Francesca Moret,[‡] Elena Reddi,[‡] Barbara Rolando,[§] Francesca Spyrakis,[§] Loretta Lazzarato,[*,§] Roberta Fruttero,[§] Alberto Gasco,[§] and Salvatore Sortino[*,†]

E-Mail: loretta.lazzarato@unito.it; ssortino@unict.it

[†]*PhotoChemLab Department of Drug Sciences and Health, University of Catania, Viale Andrea Doria 6, I-95125 Catania, Italy.*

[§]*Department of Drug Science and Technology, University of Torino, Via Pietro Giuria 9, 10125 Torino, Italy*

[‡]*Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35131 Padova, Italy*

---

* Corresponding authors
# Equally contributed

Materials

Chemicals. All chemicals were purchased by Sigma-Aldrich and used as received. Organic solvents were removed under reduced pressure at 30 °C. Synthetic-purity solvents were used. All solvents used for the spectrophotometric studies were spectrophotometric grade. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Gibco Life Technologies (Milan, Italy). BODIPY® FL C5-ceramide and ER-Tracker™ Green were from Molecular Probes (Life Technologies). The CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS) was from Promega Co. (Madison, USA).

Cell lines. HeLa (derived from a human cervix carcinoma) and MDA-MB-231 (derived from a human breast carcinoma) cells were purchased from American Type Culture Collection (ATCC, Rockville, USA). The cells were grown in DMEM with Glutamax™ supplemented with 10% FBS, 100 U/mL streptomycin and 100 μg/mL penicillin G and maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Instrumentation

^{1}H and ^{13}C-NMR spectra were recorded on a Jeol 600 at 600 and 150 MHz respectively. Chemical shifts (δ) are given in parts per million (ppm). The following abbreviations are used to designate the multiplicities: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet. Mass spectra were recorded on a Micromass Quattro microTM API (Waters Corporation, Milford, MA, USA) with electrospray ionization. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM). The progress of the reactions was followed by thin layer chromatography (TLC) on 5×20 cm plates with a layer thickness of 0.2 mm. The purity of target compounds and the monitoring of the photodegradation reactions of the compound were performed with an HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA). equipped with a quaternary pump (G1311A), a membrane degasser (G1379A), a diode-array detector (DAD) (G1315B) integrated in the HP1100 system. Data analysis were processed by HP ChemStation system (Agilent Technologies). The analytical column was a LiChrospher® 100 C18-e (250×4.6 mm, 5μm) (Merck KGaA, 64271 Darmstadt, Germany) eluted with CH_3CN/H_2O 0.1% TFA in a ratio depending on the characteristics of the compound. All compounds were dissolved in the mobile phase at a concentration of about 0.1 mg/ml and injected through a 20 μL loop. UV signals were recorded at 254, 391 and 660 nm (with 800 nm as reference wavelength) and the purity of the test samples was evaluated as a percentage ratio between the areas of the main peak and of possible impurities at the three wavelengths and also using DAD purity analysis of the chromatographic peak.

The quantitative evaluation of the photoinduced NO was performed with HP 1200 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1322A), a multiple wavelength UV detector (MWD, model G1365D), a fluorescence detector (G1321A) integrated in the HP1200 system. Data analysis was performed using an HP ChemStation (Agilent Technologies) system. The sample was eluted on a

HyPURITY Elite C18 column (250 × 4.6 mm, 5 µm, Hypersil, ThermoQuest Corporation, UK). The injection volume was 20 µl (Rheodyne, Cotati, CA).

Data analysis was performed with Agilent ChemStation.

UV-Vis spectra absorption and fluorescence emission spectra were recorded with a Perkin Elmer spectrophotometer (mod. Lambda 365) and a Spex Fluorolog-2 (mod. F-111) spectrofluorimeter, respectively, using either quartz cells with a path length of 1 cm. Fluorescence lifetimes were recorded with the same fluorimeter equipped with a TCSPC Triple Illuminator. The samples were irradiated by a pulsed diode excitation source Nanoled at 455 nm. The kinetics were monitored at 710 nm and the solvent itself was used to register the prompt at 455 nm. The system allowed measurement of fluorescence lifetimes from 200 ps. The multi-exponential fit of the fluorescence decay was obtained using equation (1):

$$I(t) = \Sigma\alpha_i \exp(^{-t/\tau i}) \quad (1)$$

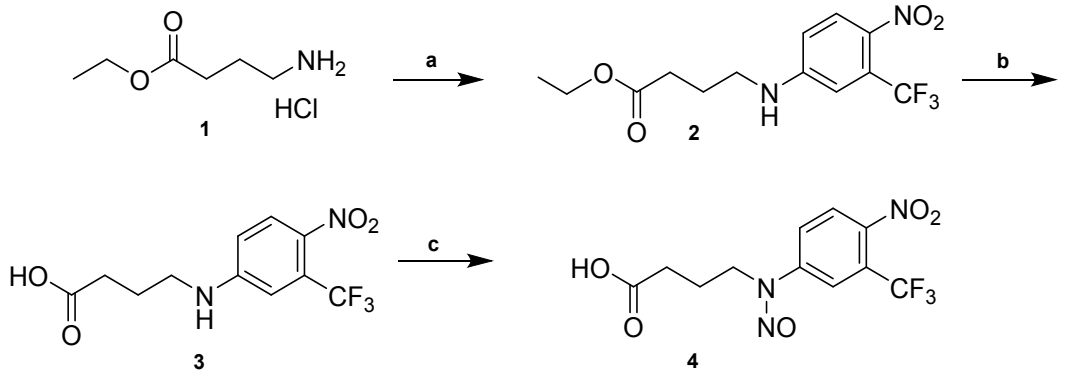
Direct monitoring of NO release for samples in solution was performed by amperometric detection with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM – 20 µM. The analog signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction (2):

$$4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2 \quad (2)$$

Synthesis and characterization

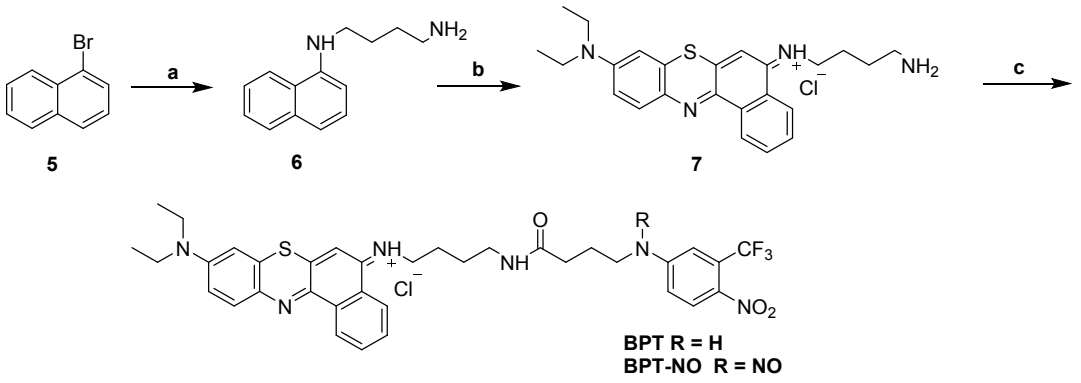
The designed **BPT-NO**, and its main model compound **BPT** are based on a benzophenotiazine scaffold which was synthesized according to literature[1S] through reaction between the Bunte salt, also syntesized according to literature,[1S] and S-arylthiosulfate, with the appropriate naphthalene derivative in the presence of $K_2Cr_2O_7$ in DMSO and MeOH with the addition of HCl 2 M. According to the synthetic routes showed in the next schemes, the benzophenotiazine ring was directly synthesized with the side chain acting as a spacer and then bound with the nitrosated or denitrosated nitro-derivative appendage. All syntheses were carried out under a low intensity level of visible light.

The direct coupling of commercial ethyl 4-aminobutyrate hydrochloride **1** with 5-fluoro-2-nitrobenzotrifluoride in acetonitrile at room temperature gave rise to **2**, which, in turn, yielded the acid **3** when treated with sodium hydroxide in dioxane. The nitroso component **4** was obtained through nitrosation of **3** with $NaNO_2$ and CH_3COOH (Scheme S1).



Scheme S1. Synthesis of nitroaniline acid derivative **3** and **4** a) 5-fluoro-2-nitrobenzotrifluoride, K_2CO_3, CH_3CN, r.t. 79%; b) NaOH, dioxane, r.t. 95%; c) $NaNO_2$, CH_3COOH/THF 1/1, r.t. 86%.

The reaction of 1-bromonaphthalene **5** with butane-1,4-diamine in 2-methoxyethanol solution at reflux, afforded the naphthyl derivative **6**, which, after reaction with Bunte salt, in presence of $K_2Cr_2O_7$ in DMSO and MeOH gave rise to the amino benzophenotiazine derivative **7**. The reaction of **7** with compound **3** in presence of EDC•HCl as coupling agent, HOBt and DMAP in dry DMF gave the model compound **BPT**. The related nitroso-amide **BPT-NO**, was obtained in analogous way from nitroso compound **4** (Scheme S2).



Scheme S2. Synthesis of amido NOPD-PS **1** and its main stable photoproduct **1a.** a) butane-1,4-diamine, CuI, Cs_2CO_3, 2-methoxyethanol, reflux, 33%; b) Bunte salt, $K_2Cr_2O_7$, DMSO, CH_3OH, HCl, r.t. 23%; c) **4** or **5**, EDC•HCl, HOBt, DMAP, DMF dry, r.t., 16%.

*Ethyl 4-((4-nitro-3-trifluoromethylphenyl)amino)butanoate (**2**)*. A solution of ethyl 4-aminobutyrate hydrochloride (**1**, 2 g, 12 mmol) and 5-fluoro-2-nitrobenzotrifluoride (1.3 g, 6 mmol) in CH_3CN (50 mL) was stirred at room temperature in the presence of K_2CO_3 (3.4 g, 12 mmol). The reaction mixture was stirred for 72 h and the solvent removed under reduced pressure. The residue was dissolved in EtOAc (20 mL) and washed with water (3 × 25 mL), dried over Na_2SO_4 and concentrated to dryness to give a yellow compound that was treated with PE, filtered and the filtrate concentrated to dryness to give the target compound as a yellow solid (1.5 g, 79%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01 (m, 1H), 6.87 (bs, 1H), 6.66 (dd, J = 9.1, 2.6

Hz, 1H), 4.16 (q, J = 7.1 Hz, 2H), 3.30 (m, 2H), 2.47 (t, J = 6.6 Hz, 2H), 2.00 (qi, J = 6.6 Hz, 2H), 1.27 (t, J = 6.6 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.5, 151.8, 136.5, 129.3, 126.7 (q, ^2J_F = 33 Hz), 122.2 (q, ^1J_F = 271.5 Hz), 112.8, 111.2, 60.9, 43.2, 31.9, 23.9, 14.2. ESI-MS $[M+H]^+$: m/z 321.3.

*4-((4-Nitro-3-trifluoromethylphenyl)amino)butanoic acid (**3**).* To a solution of **2** (1.5 g, 4.67 mmol) in 1,4-dioxane (10 mL) NaOH 1 M (23 mL) was added and the resultant mixture was stirred at room temperature for 5 h. The reaction mixture was acidified to pH 3 with 7% sulphuric acid and the product was extracted with EtOAc (20 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to obtain the target compound as a yellow solid (1.29 g, 95%). ^{1}H NMR (600 MHz, CD_3OD) δ 8.02 (d, J = 9 Hz, 1H), 7.00 (s, 1H), 6.80 (dd, J = 9.1, 2.5 Hz, 1H), 3.27 (t, J = 7.2 Hz, 2H), 2.43 (t, J = 7.2 Hz, 2H), 1.93 (quint, J = 7.2 Hz, 2H). ^{13}C NMR (150 MHz, CD_3OD) δ 175.8, 153.5, 134.9, 129.0, 126.0 (q, ^2J_F 34.5 Hz), 122.7 (q, ^1J_F = 270 Hz), 117.2, 110.8, 42.0, 30.5, 24.0. ESI-MS $[M-H]^-$: m/z 291.2.

*N-Nitroso-4-((4-nitro-3-trifluoromethylphenyl)amino)butanoic acid (**4**).* To a solution of **4** (685 mg, 2.34 mmol) in a mixture THF/CH_3COOH (1/1 v/v; 22 mL) cooled at 0 °C with an ice bath, sodium nitrite (65 mg, 9.37 mmol) was added; the reaction mixture was stirred for 1 h. When the reaction was completed (TLC), the obtained mixture was diluted with EtOAc (20 mL) and washed with water (3 × 20 mL), dried over Na_2SO_4 and concentrated to dryness to obtain the target compound as a dark yellow solid (644 mg, 86%). ^{1}H NMR (600 MHz, CD_3OD) δ 8.30 (s, 1H), 8.19 (d, J = 9 Hz, 1H), 8.15 (d, J = 9 Hz, 1H), 4.16 (t, J = 9 Hz, 2H), 2.34 (t, J = 7.2 Hz, 2H), 1.78 (quint, J = 7.8 Hz, 2H). ^{13}C NMR (150 MHz, CD_3OD) δ 176.2, 146.7, 146.4, 128.5, 125.9 (q, ^2J_F 34.5 Hz), 123.3 (q, ^1J_F = 271.35 Hz), 122.7, 118.1, 42.8, 31.2, 22.2. ESI-MS $[M-H]^-$: m/z 320.3.

*N^1-(naphthalen-1-yl)butane-1,4-diamine (**6**).* To a solution of 1-bromonaphthalene (**5**, 2 g, 9.7 mmol) and butane-1,4-diamine (4.87 mL g, 48.5 mmol) in 2-methoxyethanol (20 mL), CuI (92 mg, 0.49 mmol) and Cs_2CO_3 (2.5 g, 7.6 mmol) were added and the mixture was refluxed at 125 °C for 24 h. After completion of the reaction (TLC), the mixture was allowed to reach room temperature and filtered off to yield a pale brown solution. The filtrate was diluted with EtOAc (100 mL) and washed with saturated NaCl solution (3 × 20 mL), dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by silica gel column chromatography with CH_2Cl_2/MeOH (NH_3 sat) (95/5 v/v) and gets the desired dark orange oil product (660 mg, 33%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.79 (d, J = 8.4 Hz, 2H), 7.47 – 7.34 (m, 3H), 7.25 (m, 1H), 6.54 (m, 1H), 4.57 (bs, 1H), 3.16-3.02 (m, 2H), 2.67-2.43 (m, 2H), 1.63-1.50 (m, 2H), 1.47-1.33 (m, 4H).^{13}C NMR (150 MHz, $CDCl_3$) δ 143.0, 133.6, 127.8, 126.1, 125.1, 123.8, 122.7, 119.5, 116.0, 103.4, 43.1, 41.1, 30.4, 25.8. ESI-MS $[M+H]^+$: m/z 215.2.

*(Z)-4-Amino-N-(9-(diethylamino)-5H-benzo[a]phenothiazin-5-ylidene)butan-1-aminium chloride (**7**).* To a solution of Bunte salt (605 mg, 2.19 mmol) and **6** (670 mg, 3.13 mmol) in DMSO (12 mL), potassium dichromate (757 mg, 2.57 mmol) was added. The mixture was stirred at room temperature for 20 min, then methanol (120 mL) and HCl 2M (12 mL) were added. The resulting mixture was stirred for further 40 min at

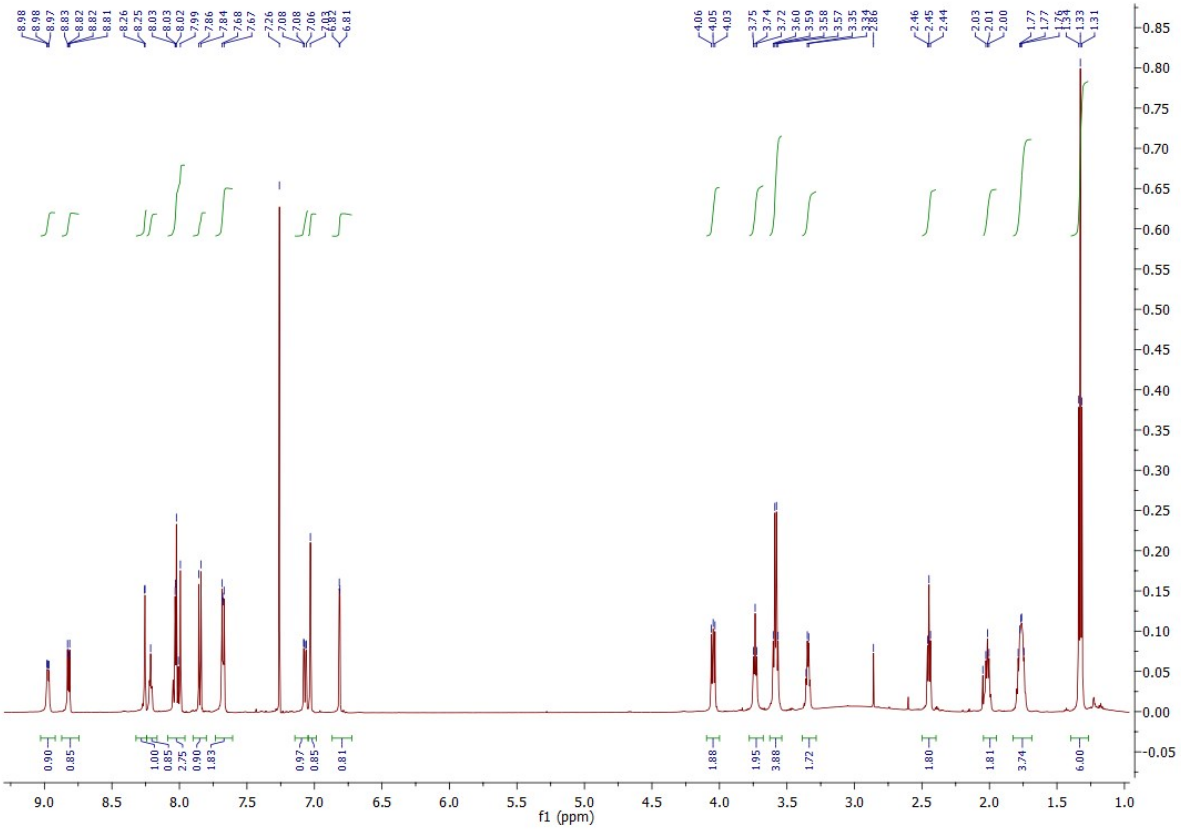
r.t., and the reaction progress was monitored by TLC. When the reaction was completed, methanol and water were removed under reduced pressure, and the remaining solution was slowly poured into a saturated NaCl solution (60 mL). The blue precipitate was filtered, collected and dried. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (NH_3 sat) (90/10 to 80/20 v/v) as the eluent. The target compound was obtained as a dark blue solid (223 mg, 23%). ^{1}H NMR (600 MHz, CD_3OD) δ 8.20 (d, J = 7.8 Hz, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.44 (m, 2H), 7.30 (d, J = 9.2 Hz, 1H), 6.97 (dd, J = 9.3, 2.2 Hz, 1H), 6.73 – 6.67 (m, 2H), 3.49 (q, J = 7.0 Hz, 4H), 3.36 (t, J = 6.7 Hz, 2H), 3.02 (t, J = 7.0 Hz, 2H), 1.85 – 1.76 (m, 4H), 1.23 (t, J = 7.2 Hz, 6H). ^{13}C NMR (150 MHz, CD_3OD) δ 153.7, 152.3, 140.5, 138.1, 134.4, 134.0, 133.6, 132.8, 131.8, 130.4, 125.8, 125.0, 123.7, 118.5, 105. 9, 103.0, 46.9, 44.8, 40.4, 26.8, 26.2, 13.3. ESI-MS $[M]^+$: m/z 405.6.

*General procedure for the synthesis of **BPT-NO** and **BPT**.* Under N_2 atmosphere, a mixture of **7** (1 mmol), **3** or **4** (2 mmol), EDC•HCl (1.95 mmol), HOBt (2.25 mmol), and DMAP (1.95 mmol) were dissolved in dry DMF (50 mL), and the mixture was stirred at r.t. for 24 h. After the reaction was complete, the resulting solution was diluted with saturated NaCl solution (50 mL) and extracted with EtOAc (3 × 20 mL), dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (95/5 to 90/10 v/v) as the eluent. All operations were carried out under a low intensity level of visible light.

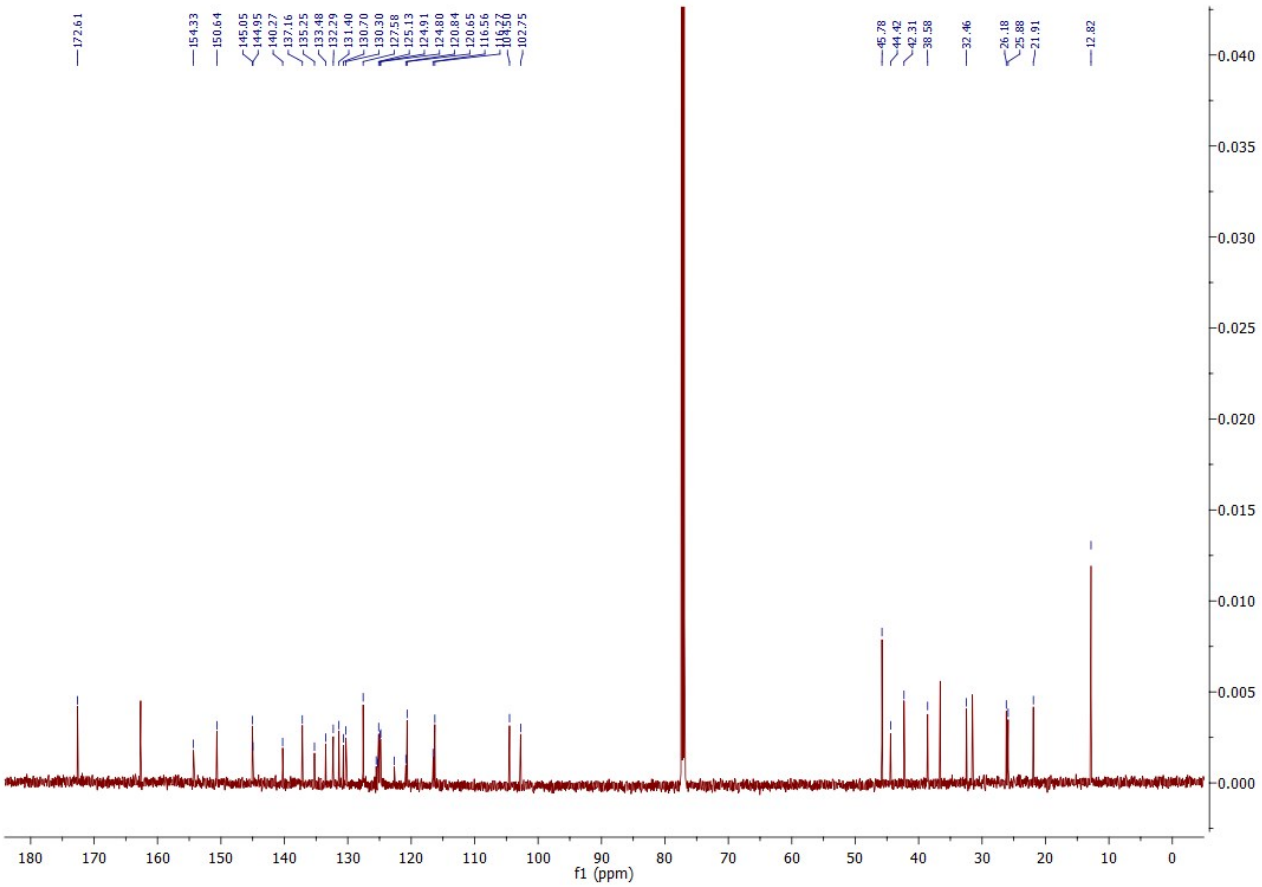
*(Z)-N-(9-(Diethylamino)-5H-benzo[a]phenothiazin-5-ylidene)-4-(4-((4-nitro-3-(trifluoromethyl)phenyl)amino)butanamido)butan-1-aminium chloride (**BTP**).* Yield 16%, blue/green solid. Purity: 100%. This compound exists as a mixture of rotamers in a 72:28 ratio as determined by ^{1}H NMR. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.94 (d, J = 8.1 Hz, 1H), 8.81 (d, J = 8.2 Hz, 1H), 8.25 and 8.16 (bs, 1H), 7.92 (d, J = 9.4 Hz, 1H), 7.81 – 7.71 (m, 2H), 7.68 (m, 2H), 7.10 (dd, J = 9.4, 2.7 Hz, 1H), 7.03 (s, 1H), 6.88 (bs, 1H), 6.94 (bs, 1H), 6.83 (d, J = 2.6 Hz, 1H), 6.59 (d, J = 10.0 Hz, 1H), 3.69 (bs, 2H), 3.59 (q, J = 7.2 Hz, 4H), 3.32 and 3.27 (m, 2H), 3.14 and 3.04 (m, 2H), 2.45 and 2.39 (t, J = 7.0 Hz, 2H), 2.00 – 1.87 (m, 4H), 1.70 and 1.54 (m, 2H), 1.34 (t, J = 7.2 Hz, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 174.1, 154.1, 152.6, 150.7, 140.3, 137.2, 135.2 and 134.8, 133.5, 132.5, 131.4, 131.0, 130.2, 129.2 and 129.0, 126.3 (q, ^2J_F = 27.2 Hz), 125.7, 125.2, 124.6, 124.0, 122.4 (q, ^1J_F = 271.5 Hz) 117.5, 116.4, 111.0, 104.3, 102.4, 45. 7, 44.4, 42.9 and 42.2, 39.2 and 38.7, 33.8 and 33.7, 26.8 and 26.2, 26.1 and 25.8, 24.6 and 24.2, 12.7. ESI-MS $[M]^+$: m/z 679.8.

*(Z)-4-(4-(((3-Trifluorometil-4-nitro)phenyl)(nitroso)amino)butanamido)-N-(9-(diethylamino)-5H-benzo[a]phenothiazin-5-ylidene)butan-1-aminium chloride (**BTP-NO**).* Yield 16%, dark blue solid. Purity: 97%. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.99 (dd, J = 6.0, 3.2 Hz, 1H), 8.81 (dd, J = 6.2, 3.4 Hz, 1H), 8.26 (d, J = 1.8 Hz, 1H), 8.22 (t, J = 5.3 Hz, 1H), 8.05 – 7.97 (m, 3H), 7.84 (d, J = 9.4 Hz, 1H), 7.66 (m, 2H), 7.08 (dd, J = 9.4, 2.7 Hz, 1H), 7.01 (s, 1H), 6.81 (d, J = 2.7 Hz, 1H), 4.04 (m, 2H), 3.74 (t, J = 7.0 Hz, 2H), 3.58 (q, J = 7.2 Hz, 4H), 3.33 (dd, J = 11.8, 5.7 Hz, 2H), 2.45 (t, J = 6.7 Hz, 2H), 2.01 (m, 2H), 1.77 (m, 4H), 1.32 (t, J = 7.2 Hz, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.6, 154.3, 150.6, 145.0, 145.0, 140.3, 137.2, 135.2, 133.5,

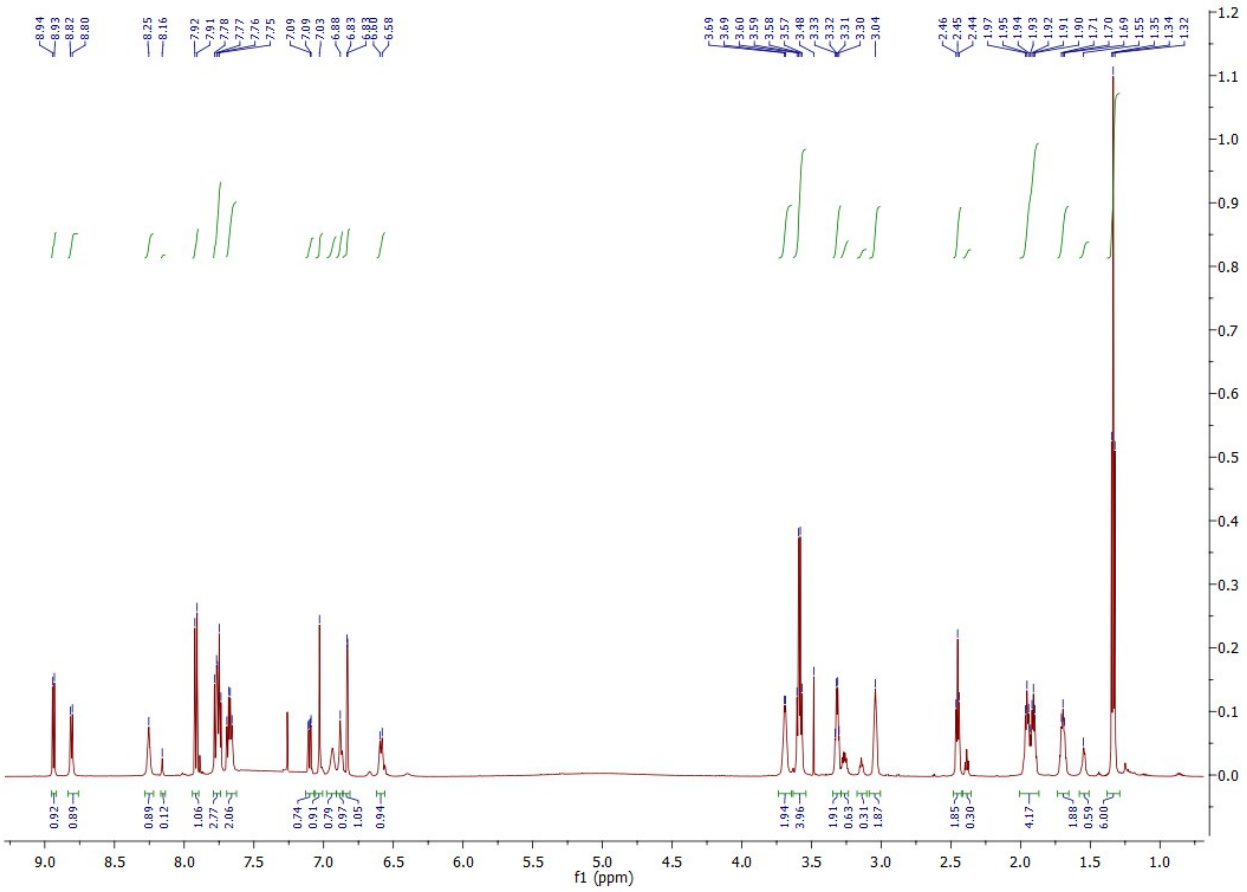
132.3, 131.4, 130.7, 130.3, 127.6, 125.4 (q, $^{2}J_{F}$ = 34.4 Hz), 125.1, 124.9, 124.8, 121.8 (q, $^{1}J_{F}$ = 273 Hz), 120.6, 116.6, 116.3, 104.5, 102.8, 45.8, 44.4, 42.3, 38.6, 32.6, 26.2, 25.9, 21.9, 12.8. ESI-MS $[M]^{+}$: m/z 708.6.



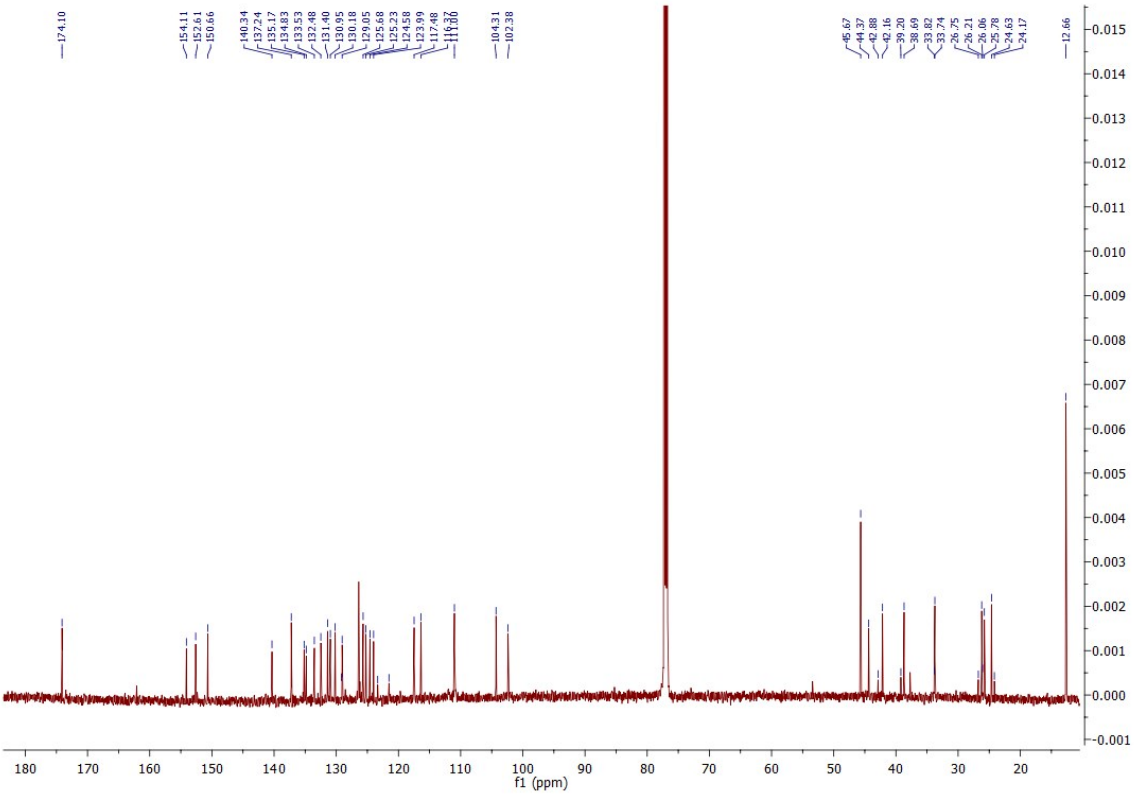
^{1}H-NMR spectrum of **BPT-NO**



^{13}C-NMR spectrum of **BPT-NO**



^{1}H-NMR spectrum of **BPT**



^{13}C-NMR spectrum of **BPT**

Experimental procedures

Photolysis experiments. Irradiation was performed in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) under gentle stirring. The photolysis experiments under anaerobic conditions were performed deoxygenating the solution by bubbling with a vigorous and constant flux of pure nitrogen (previously saturated with solvent). A 200 mW continuum red laser (λ_{exc} = 670 nm) having a beam diameter of ca. 1.5 mm or a LED 90 W lamp (Led Engin, San Jose, CA, USA) equipped a Long pass filter (LP460HT, Schneider, Kreuznach, Germany), to eliminate the light component below 460 nm, with an irradiance of 10 mW/cm^2 were used as the light sources. The reverse-phase HPLC analyses that allowed separation and quantitation of compounds were performed with an analytical column Synergi polar-RP 80A Phenomenex (150 x 2.0 mm ID, 4 μm). The mobile phase consisting of CH_3CN/water (80/20 v/v) with 0.1% TFA and the flow-rate was 0.5 mL/min. The injection volume was 20 μL (Rheodyne, Cotati, CA). The column effluent was monitored at 660 mn referenced against at 800 nm wavelength. The attribution of the chromatographic peaks to the structures **BPT-NO** and **BPT** was carried out by comparison with the reference products based on the retention times and the analysis of the UV spectrum. The values obtained from integration of the peaks of BTP-NO and BTP were interpolated in calibration curves obtained using standard solutions of BTP-NO and BTP from 1 to 50 μM (r^2 =0.999).

Fluorescence quantum yields. Fluorescence quantum yields were determined using optically-matched solutions at the excitation wavelength of **BPT-NO** and **BPT**, and a solution of Methylene Blue in EtOH as standard (Φ_f = 0.04) [2S] through equation (2):

$$\Phi_f = \Phi_{f(s)} (In^2/I_{(s)}n^2_{(s)}) \qquad (2)$$

where $\Phi_{f(s)}$ is the fluorescence quantum yield of the standard; I and $I_{(s)}$ are the areas of the fluorescence spectra of compounds and standard, respectively; n and $n_{(s)}$ are the refraction index of the solvents used for compounds and standard. Absorbance at the excitation wavelength was less than 0.1 in all cases.

HOO^- assay. HOO^- were revealed by using the very sensitive and selective method recently developed by Radi et al.[3S] A 25 μM solution of the compounds **BPT-NO** or **BPT** were irradiated in the presence of equimolar amount of Fl-B with the red continuum laser at different times. Afterwards the fluorescence emission and excitation spectra were recorded. Quantitative comparison between **BPT** and the fluorescein generated after 30 minutes of irradiation of a 25 μM solution of **BPT-NO** in the presence of equimolar amount of Fl-B using the white LED. The irradiated solution was analyzed with RP-HPLC to detect the fluoresceine and **BPT** obtained. The sample was eluted on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm, Agilent Technologies). The injection volume was 20 μL (Rheodyne, Cotati, CA). The mobile phase consisting of CH_3CN 0.1% TFA (solvent A) and water 0.1% TFA (solvent B) at flow-rate = 1.0 mL/min with gradient conditions: 30% A until 5 min, from 30 to 90% A between 5 and 15 min, 90% A between 15 and 17 min, and

from 90 to 30% A between 17 and 20 min. The signals were obtained on fluorescence using an excitation and emission wavelength of 492 nm and 515 nm, respectively (gain factor = 10). The values obtained from integration of the peak of Fluoresceine were interpolated in a calibration curve obtained using standard solutions of Fluoresceine from 1 to 25 μM (r^2 =0.999).

$O_2^{\bullet-}$ assay. Dihydrorhodamine123 (DHR123) was used as the superoxide anion radical probe.[4S] **BPT** and DHR123 were prepared as 10 μM in PB:MeOH (1:1 v/v). Then the cuvette was exposed to 670 nm monochromatic light for different time and the fluorescence spectra were recorded immediately after each irradiation. As control, DHR123 in the same solvent without **BPT** was used and no increase of fluorescence was observed upon the same irradiation times.

NO_2^- assays. NO_2^- were first evaluated by means of the well-known, highly sensitive (detection limit on the order of the picomoles) fluorimetric bioassay of Misko et al.[5S] based on the ring closure of the nonfluorescent 2,3- diaminonaphthalene (DAN) with nitrite to form the highly fluorescent product 2,3-diaminonaphthotriazole (DANT). Aliquots of 2 mL of solutions of **BPT-NO** were irradiated with the red laser or kept in the dark. Afterwards, the samples were dried under vacuum, resuspended in 2 mL of water and filtered with a 0.2 μm GHP Acrodisc membrane filter. The filtered solution was added of 200 μL of DAN solution (DAN 0.30 M in 0.62 M HCl) and stirred for 20 min at room temperature. 300 μL of NaOH 3 M was then added to the previous solution and stirred for 20 min at room temperature. The resultant solution was put into the fluorescent cuvette and the fluorescence emission and excitation spectra were recorded.
NO_2^- were quantitatively evaluated by the Griess assay.[6S] A 50 μM solution of **BPT-NO** in MeOH/PB 1/1 was prepared and this solution was irradiated for 30 minutes using the white LED. After 30 minutes 0.5 mL aliquots of the irradiated solution were treated with 125 μL of the Griess reagent (4% w / v of sulphanilamide, 0.2% w / v of N-naphthylenylenediamine dihydrochloride, 1.47 M of phosphoric acid); after 10 minutes at room temperature, the sample was analyzed with RP-HPLC to detect the azo dye. The sample was eluted on a HyPURITY Elite C18 column (250 × 4.6 mm, 5 μm, Hypersil, ThermoQuest Corporation, UK). The injection volume was 20 μL (Rheodyne, Cotati, CA). The mobile phase consisting of acetonitrile 0.1% TFA (solvent A) and water 0.1% TFA (solvent B) at flow-rate = 1.0 mL/min with gradient conditions: 50% A until 4 min, from 50 to 90% A between 4 and 8 min, 90% A between 8 and 12 min, and from 90 to 50% A between 12 and 15 min. The column effluent was monitored at 540 mn referenced against a 800 nm wavelength. The values obtained from integration of the peak of azo dye were interpolated in a calibration curve obtained using standard solutions of sodium nitrite at 0.5 μM to 50 μM (r^2 =0.996). The yield in nitrite was expressed as percent NO_2^- (mol/mol, relative to the initial compound concentration) ± SEM. The percentage of NO_2^- released was evaluated with respect to the amount of compound initially present in the irradiated solution.
The total amount of NO_2^- and NO_3^- to nitrite was evaluated with a procedure adapted from the method of Miranda *et al*.[7S] A 50 μM solution of **BPT-NO** in MeOH/PB 1/1 was prepared and this solution was irradiated for 30 minutes using the white LED. After 30 minutes aliquots of 0.5 mL of the irradiated solution was treated

with125 μL of VCl_3 solution (400 mg of VCl_3 dissolved in 50 mL of 1 M HCl) and 125 μL of the Griess reagent; after 30 minutes of stirring at room temperature the sample was analyzed with RP-HPLC to detect the azo dye. The values obtained from integration of the peak of azo dye were interpolated in a calibration curve obtained using standard solutions of sodium nitrite at 0.5 μM to 50 μM (r^2 =0.996). The yield in nitrite was expressed as percent NO_2^- (mol/mol, relative to the initial compound concentration) ± SEM. The percentage of NO_2^- released was evaluated with respect to the amount of compound initially present in the irradiated solution.

Molecular modelling. The compound was parametrized with the ab initio RESP charges fitting methodology.[8S] The MD simulation setup was performed with BiKi and Gromacs 4.6.1 was used to run MD simulations.[9S] The water model employed was TIP3P. The solvated system was preliminary minimized by 5000 steps of steepest descent. The integration step was equal to 1 fs. The Verlet cutoff scheme, the Bussi−Parrinello thermostat, LINCS for the constraints (all bonds), and the particle mesh Ewald for electrostatics, with a short-range cutoff of 11 Å, were applied. The system was equilibrated in four steps: 100 ps in NVT ensemble at 100 K, 100 ps in NVT at 200 K, 100 ps in NVT at 300 K. 1 ns long NPT simulation was subsequently run to reach the pressure equilibrium condition. No restraint was applied. The production was carried out in the NVT ensemble at 300 K for 20 ns. Three replicas were simulated upon velocity reassignment.
To investigate if the compound can assume a U-shaped we performed two plain molecular dynamics (MD) replicas of 20 ns each. All the trajectories were cleaned, analyzed and clustered, automatically generating 10 cluster for each. In the two trajectories, in 7/10 and 6/10 clusters, respectively, the compound assumed a U-shaped conformation as reported in Figure XX. This conformation is strongly stabilized by π-π stacking and hydrophobic interactions.

Intracellular uptake and localization studies. The extent of uptake of **BPT-NO** and its intracellular localization were studied by flow cytometry (FACS) and confocal microscopy, respectively. For FACS analysis, 5×10^4 cells were grown in 24-well plates for 24 h and incubated for 2 h with increasing concentrations of **BPT-NO** diluted in cell culture medium added with 10% serum. At the end of the incubation time, the cells were washed twice with Versene, detached from the plates with trypsin that was neutralized by the addition of FBS. Cells were centrifuged and resuspended in Versene before measuring phenothiazine fluorescence using a BD LSRFortessa™ X-20 flow cytometer (Becton Dickinson, San Jose, USA). A blue laser at 488 nm was used to excite the compound and its fluorescence was detected at wavelengths longer than 685 nm (PerCP-Cy5-5 channel); for each sample 10^5 events were acquired and analyzed using the FACSDiva software. For confocal microscopy analysis, 1 x 10^5 cells were grown in 35 mm imaging dishes (Eppendorf AG, Hamburg, Germany) for 24 h and incubated for 2 h with 0.5 μM of **BPT-NO**. Fifteen minutes before completing the incubation, cells were stained BODIPY® FL C5-ceramide (15 μM), or ER-Tracker™ Green (1 μM), used as a marker for Golgi apparatus and endoplasmic reticulum, respectively. Cells were then washed twice with HBSS and observed with a Leica SP5 confocal microscope; acquired images were analyzed using ImageJ software.

Dark and photo-toxicity studies. For measuring the cytotoxicity of the compounds in the absence of light irradiation (dark cytotoxicity), cells were seeded in 96-well plates (8000 cells/well for MDA-MB-231; 6000 cells/well for HeLa) in DMEM supplemented with 10% FBS. After 24 h of cell growth, the culture medium was replaced for 2 h with fresh medium containing increasing concentrations of **BPT-NO** (range 0.1-2 μM). After this incubation time, cells were kept in drug-free medium for additional 24 h before measuring cell viability with the MTS assay. For photo-toxicity experiments, cells were seeded and incubated as described above. At the end of the 2 h of incubation with **BPT-NO**, the cells were washed twice with PBS containing Ca^{2+} and Mg^{2+} and irradiated in PBS with a total light fluence of 1 J/cm^2 of red light (600–800 nm) emitted from a Waldmann PDT 1200L lamp (Waldmann Medizintechnik, Germany). The power density was 40 mW/cm^2 as measured with a radiometer and the irradiation time was 25 seconds. Immediately after irradiation, the cells were brought back to the incubator after the replacement of PBS with fresh complete medium for 24 h. For the MTS assay, the cell medium was replaced with 100 μL of serum-free DMEM and 20 μL of CellTiter 96 Reagent and the samples were incubated for 60 min at 37 °C in the dark. Afterward, the absorbance at 492 nm was measured with a Multiskan Go plate reader (Thermo Fisher Scientific, Waltham, USA) and the viability of treated cells was expressed as percentage of the absorbance of control cells that was taken as 100% viability.

Statistical analysis. The Primer software for biostatistics (McGraw-Hill, Columbus, USA) was used for statistical analysis of the data. The data are expressed as means ± standard deviations (SD) for at least 3 independent experiments. The difference between groups was evaluated with the Student's t-test and was considered significant for $p < 0.05$.

References

1S. S. Verma, U. W. Sallum, H. Athar, L. Rosenblum, J. W. Foley and T. Hasan, *Photochem. Photobiol.*, **2009**, *85*, 111–118.

2S. J. Olmsted, *J. Phys. Chem.* **1979**, *83*, 2581-2584.

3S. N. Rios, L. Piacenza, M. Trujillo, A. Martinez, V. Demicheli, C. Prolo, M. N. Alvarez, G. V. Lopez, R. Radi, *Free Radical Biology and Medicine*, **2016**, *101*, 284–295.

4S. a) M. Li, J. Xia, R. Tian, J. Wang, J. Fan, J. Du, S. Long, X. Song, J. W. Foley and X. Peng, *J. Am. Chem. Soc*. **2018**, *140*, 14851–14859; b) J. S. Nam, M.-G. Kang, J. Kang, S.-Y. Park, S. J. C. Lee, H.-T. Kim, J. K. Seo, O.-H. Kwon, M. H. Lim, H.-W. Rhee and T.-H. Kwon, *J. Am. Chem. Soc*. **2016**, *138*, 10968-10977.

5S. Misko, T. P.; Schilling, R. J.; Salvemini, D.; Moore, W. M.; Currie, M. G., *Anal. Biochem.* **1993**, *214*, 11-16.

6S. N. S. Bryan and Matthew B. Grisham, *Free Radic Biol Med*. **2007** *43*, 645–657

7S. K. M. Miranda, M. G. Espey and David A. Wink, *Nitric Oxide: Biology and Chemistry*, **2001**, *5*, 62–71.

8S. a) http://www.bikitech.com; b) S. Sciabola, P. Benedetti, G. D'Arrigo, R. Torella, M. Baroni, G. Cruciani and F. Spyrakis, ACS Med. Chem. Lett., **2019**, *10*, 487-492.

9S. B. Hess, C. Kutzner, D. van der Spoel and E. Lindahl, *J. Chem. Theory Comput.*, **2008**, *4*, 435−447.

Figure S1. Absorption spectrum of a solution of **BPT** (solid line) and, for comparison, that of **BPT-NO** (Dash-dottet line) in the same solvent. PB:MeOH (1:1 v/v) T = 25 °C

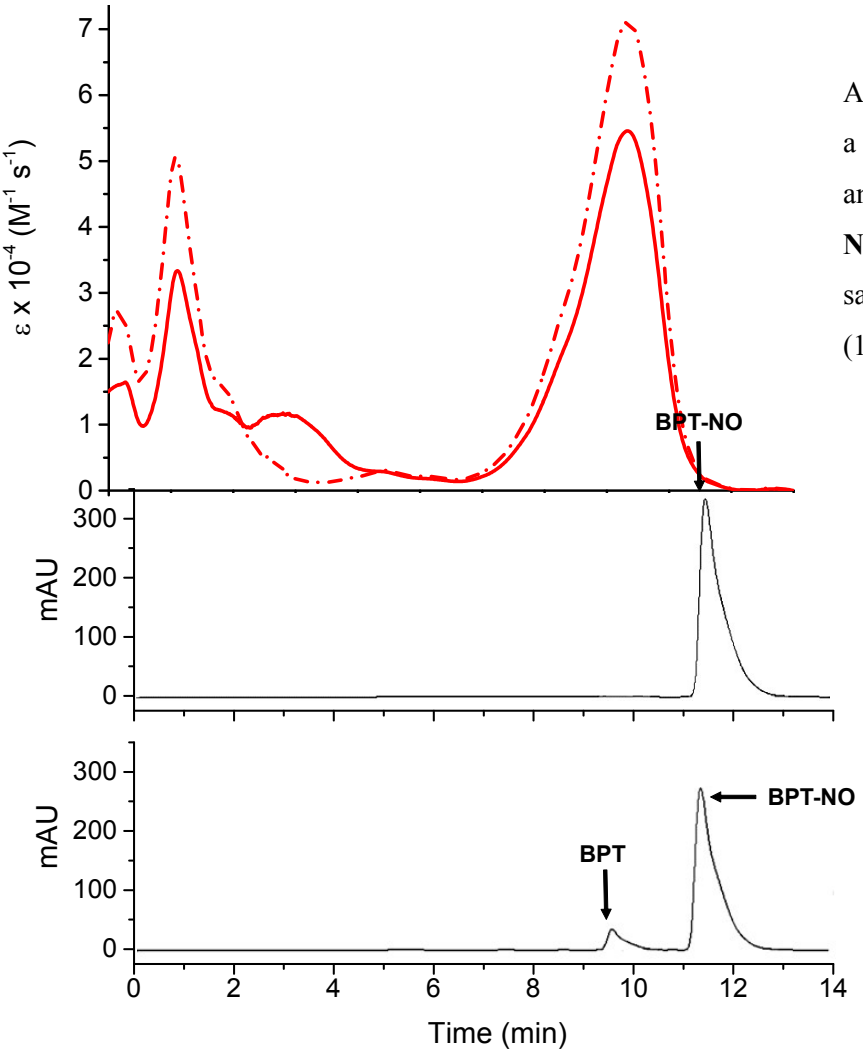


Figure S2 HPLC traces related to solutions of **BPT-NO** (50 μM) before (top) and after 30 min irradiation with red light (bottom) with red light n times with green light (top). PB:MeOH (1:1 v/v) T = 25 °C

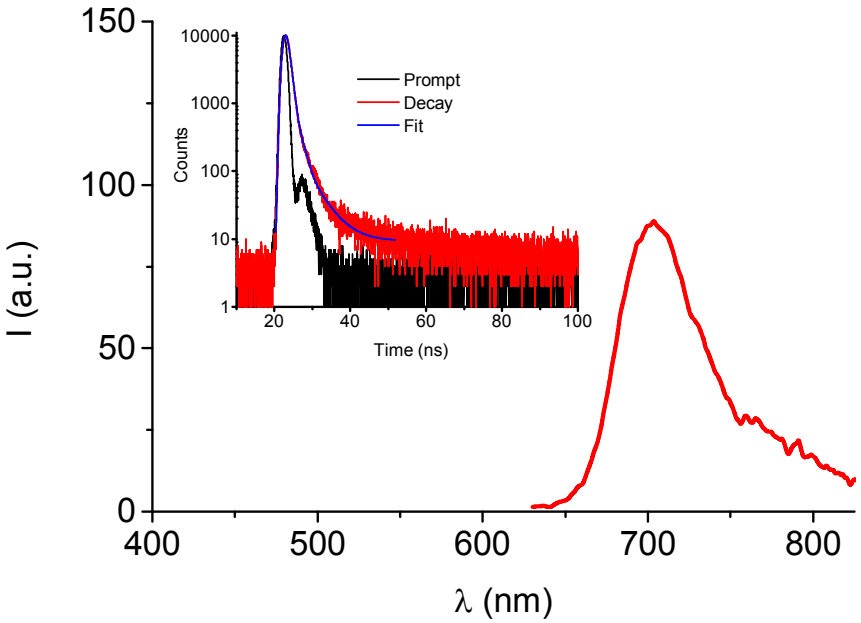


Figure S3. Fluorescence emission spectrum (λ_{exc} = 620 nm) of a solution of **BPT**. The inset shows the fluorescence decay and the related fitting of the same solutions recorded at λ_{exc} = 455 nm and λ_{exc} = 710 nm. PB:MeOH (1:1 v/v), T = 25 °C.

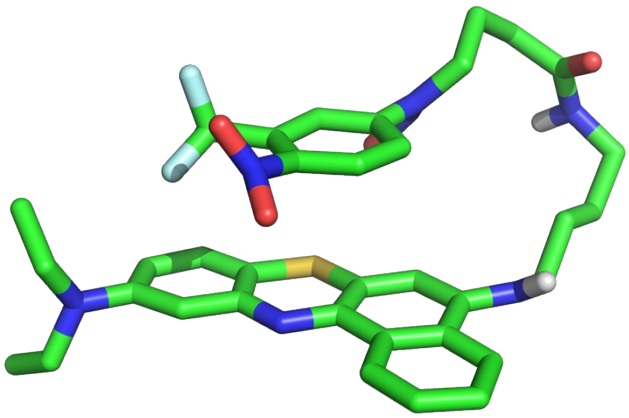


Figure S4 Collapsed U-shaped conformation assumed by compounds **BPT-NO** during the MD simulations.

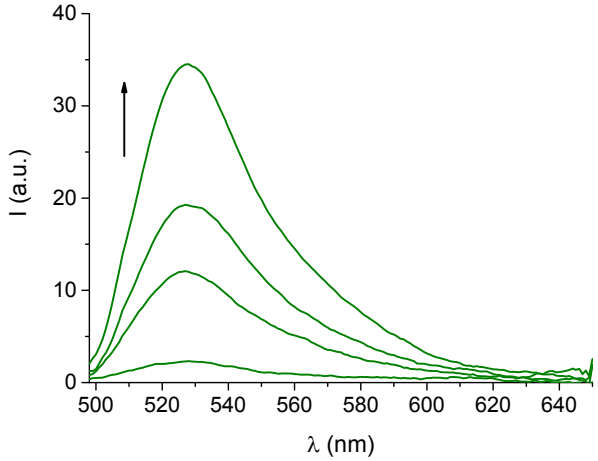


Figure S5. Fluorescence emission spectra recorded at λ_{exc} = 488 nm, observed upon irradiation with red light at λ_{exc} = 670 nm of an air-equilibrated solution **BPT** in the presence of DHR123. The arrow indicates the course of the spectral profile with the illumination time (0, 2, 4 and 8 min). [**BPT**] = [DHR123] = (10 μM); PB:MeOH (1:1 v/v), T = 25 °C.

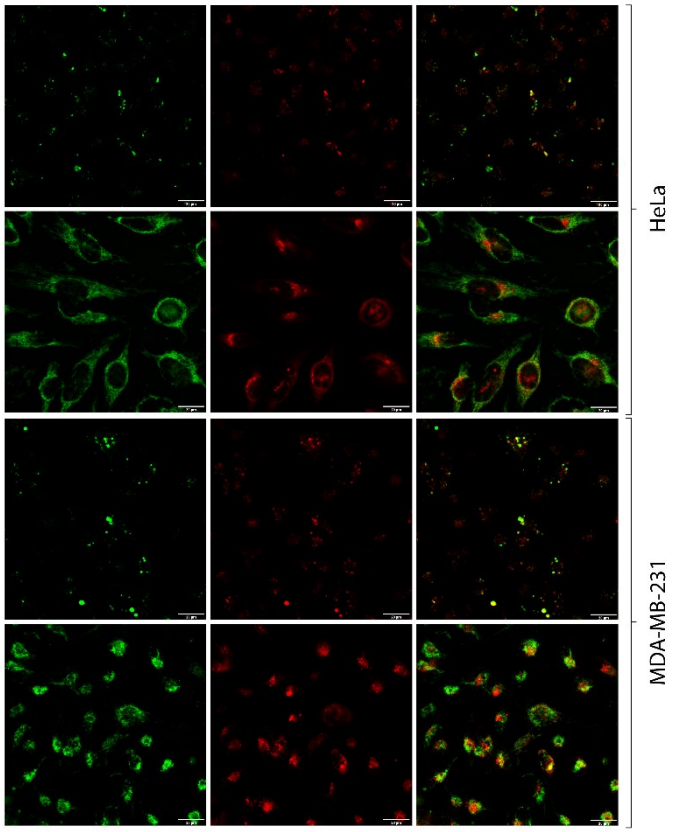


Fig. S6 Confocal images of cancer cells incubated for 2h with 0.5 μM **BPT-NO** (red fluorescence). Cells are stained with LysoTracker Green (top panels) or MytoTracker Green (bottom panels) (green fluorescence). Overlays of red and green fluorescence are showed in the right of each panel. Scale bar = 20 μm.

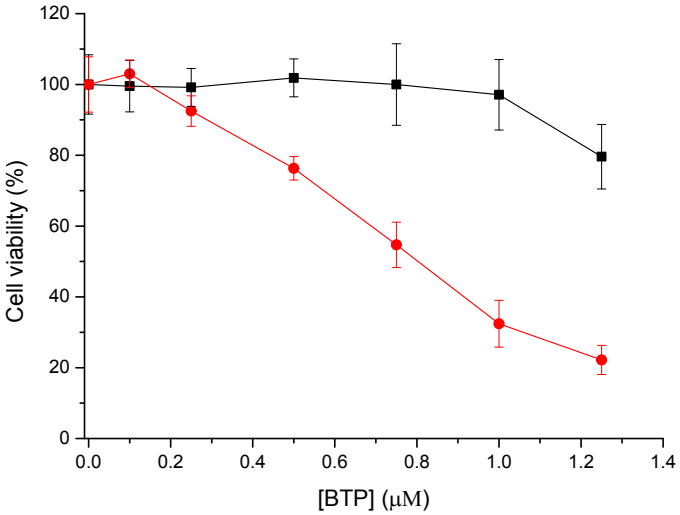


Fig. S7 Cell viability of HeLa cancer cells incubated 2h with **BPT** and either kept in the dark (square) or irradiated (circles) with red light at a fluency of 1 J cm^{-2} (40 $mWcm^{-2}$ for 25 s).