Supporting Information

DESIGN AND SYNTHESIS OF A MITOCHONDRIA-TARGETED MIMIC OF GLUTATHIONE PEROXIDASE, MITOEBSELEN-2, AS A RADIATION MITIGATOR

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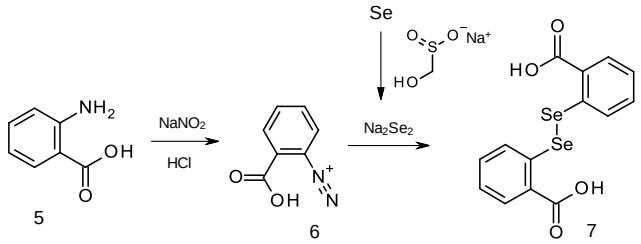
Organic synthesis

General information: Reagents and solvents were purchased from Sigma, Inc., and were used without further purification. 13S-hydroperoxy-9Z,11E-octadecadienoic acid was purchased from Cayman Chemical, Inc. HPLC separations were carried out with a Shimadzu chromatographic system equipped with an SPD-M10Avp photodiode detector. NMR spectra were obtained on a Bruker 300 MHz spectrometer. MS spectra were recorded on an LXQ ion trap mass spectrometer (ThermoFisher, San Jose, CA). Compound purity was determined by LC/MS and HPLC and carried out under the conditions listed below. All compounds reported herein were with purity $\geq$ 95% as measured in the wavelength range of 200 – 500 nm.

2-[(2-carboxyphenyl)diselanyl]benzoic acid (7)



Na_2Se_2 was prepared by stirring Se (100 mesh; 3.15 g; 40 mmol) in 20 % NaOH (35 mL) containing sodium hydroxymethanesulfinate (4.57 g; 40 mmol) for 2 hours at 40 – 45 ^{o}C. In parallel, **6** was prepared by treating a suspension of anthranilic acid (5.6 g; 40 mmol) in H_2O (6 mL) with HCl (11 mL, 32 %; 98 mmol; t = 0 – 5 ^{o}C; dropewise addition of HCl over 1 hour). The resulting diazonium salt, kept at 0 – 4 ^{o}C, was added in small portions to the solution of Na_2Se_2 (vigorous steering) while keeping the temperature and the pH of the reaction solution under 10 ^{o}C and above 10, respectively. The final reaction solution was stirred for 2 hours at 25 ^{o}C and then the pH was decreased to 8.5. The solution was filtered and the filtrate stirred with active charcoal (1 g) for 1 hour at room temperature. After filtration of the charcoal, the pH of the filtrate was first adjusted to 5 – 5.5 with HCl (upward pH drift for ~ 20 min) and then to 4.2 – 4.5. The crystals formed were filtered and dissolved in water by titration of the solution with aqueous NaOH to pH 8.5. Then, the final solution was treated for a second time with charcoal. The pH-depended precipitation of **7** was repeated and, after filtration, the product was dried in vacuum (20 mm Hg; 40 ^{o}C; yield, 5.1 g (63%)).

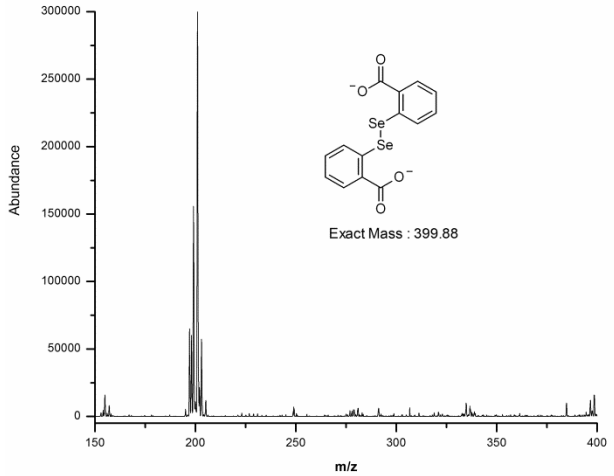
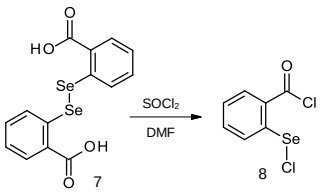


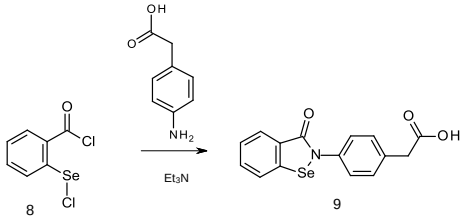
Figure 1S. MS analysis (negative ionization mode) of **7** (10 μM) in 90 % methanol containing 1 % NH_4OH.

(2-chlorocarbonylphenyl) selenohypochlorite (8)



Diselenide **7** (1 g) was refluxed for 2 hours in $SOCl_2$ (10 mL) containing DMF (0.5 mL). After evaporation of $SOCl_2$ (20 mm Hg; 40 oC), the oily residue was extracted with hexane. The suspension was filtered and evaporation of the hexane extract afforded 1.2 g of **8** (Yield, 96 %; yellow crystals, m.p. 64-66 ^{o}C.[1]

2-[4-(3-oxo-1,2-benzoselenazol-2-yl)phenyl]acetic acid (**9**)



To a stirred solution of **8** (2.54 g; 10 mmol) in dry CH_3CN (50 mL; 0 – 4 ^{o}C) was added, over 1 hour, CH_3CN (20 mL) containing anthranilic acid (1.51 g; 10 mmol) and Et_3N (2 g; 20 mmol).[2] The reaction solution was further incubated at room temperature for 2 hour and then CH_3CN was rotor-evaporated. The dry residue was re-suspended in water and titrated with HCl (20 %) to pH 4.0. The resulting suspension was vigorously extracted with AcOEt (5 x 200 mL) and the organic extract dried over Na_2SO_4. After rotor-evaporation of AcOEt, recrystallization of the product from CH_3CN afforded 2.4 g of pure **9** (yield, 73 %).

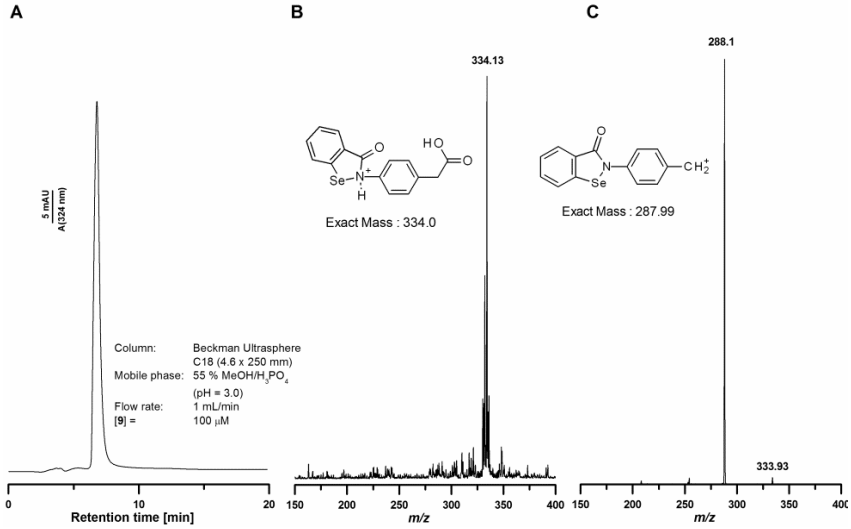
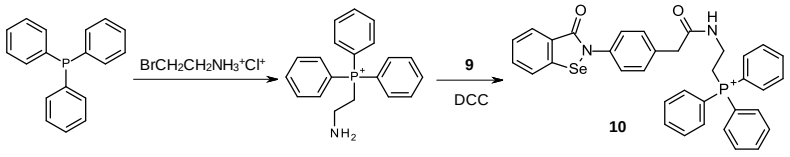


Figure 2S. HPLC (A), MS (B; (positive ionization mode)) and MS-MS (C) analysis of **9**. The analyte (A- 0.1 mM; B,C- 10 µM) was dissolved in methanol in the absence (A) or presence of trifluoroacetic acid (1%; B,C).

2-[[2-[4-(3-oxo-1,2-benzoselenazol-2-yl)phenyl]acetyl]amino]ethyl-triphenyl-phosphonium (**10**)



A. 2-aminoethyl(triphenyl)phosphonium chloride was prepared by steering and refluxing Br-$CH_2CH_2NH_3^+Cl^-$ (7.81 g; 38.1 mmol) and Ph_3P (10 g; 38.1 mmol) in CH_3CN (50 mL) for 24 hours.[3] Thereafter, the solvent was rotor-evaporated, the remaining crystals were dissolved in minimal amount of H_2O, and the solution was titrated with concentrated NaOH to

pH 11.0. The water was evaporated (10 mm Hg; 45 ^{o}C) and the amine was extracted with methanol. After filtration of NaCl, the methanolic filtrate was triturated with diethyl ether to afford 9.6 g amine (Yield, 74 %).

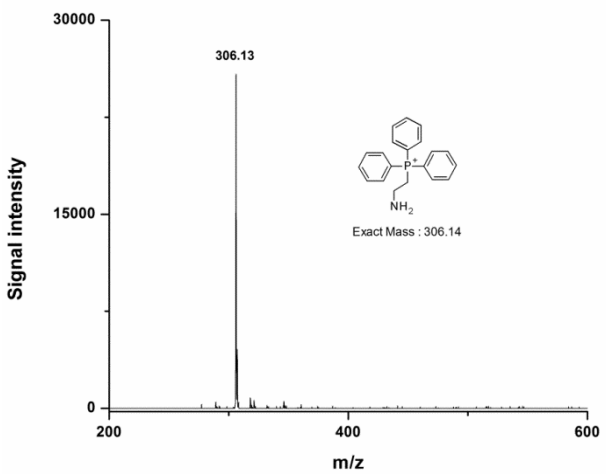


Figure 3S. MS analysis (positive ionization mode) of 2-aminoethyl(triphenyl)phosphonium chloride (10 μM; solvent- 90 % methanol).

B. To a suspension of **9** (0.66 g; 2 mmol) and 2-aminoethyl(triphenyl)phosphonium chloride (0.682 mg; 2 mmol) in ice-cold CH_2Cl_2 (200 mL) *N,N'*-dicyclohexylmethanediimine (DCC; 0.41 g; 2 mmol) was added. The reaction solution was steered for 1 hour at 0 – 4 ^{o}C and then for 2 hours at room temperature. The reaction profile was monitored by HPLC as shown in Fig. 4SA. Upon completion of the incubation, the reaction solution was filtered and the solvent from the filtrate was evaporated. From the crude reaction product, **10** was purified by normal phase chromatography (Silica Gel 60; column- 2.5 x 25 cm; isocratic elution from 0 - 30 min with CH_2Cl_2 then linear gradient from 0 – 100 % methanol over 1 hour; flow rate, 5 mL/min). In Fig. 4S both the HPLC profile and MS spectrum of the purified **10** (pale-yellow crystals; Yield, 84 %) are presented. Further structural elucidation of **10** was performed by MS-MS analysis as shown in Fig. 5S.

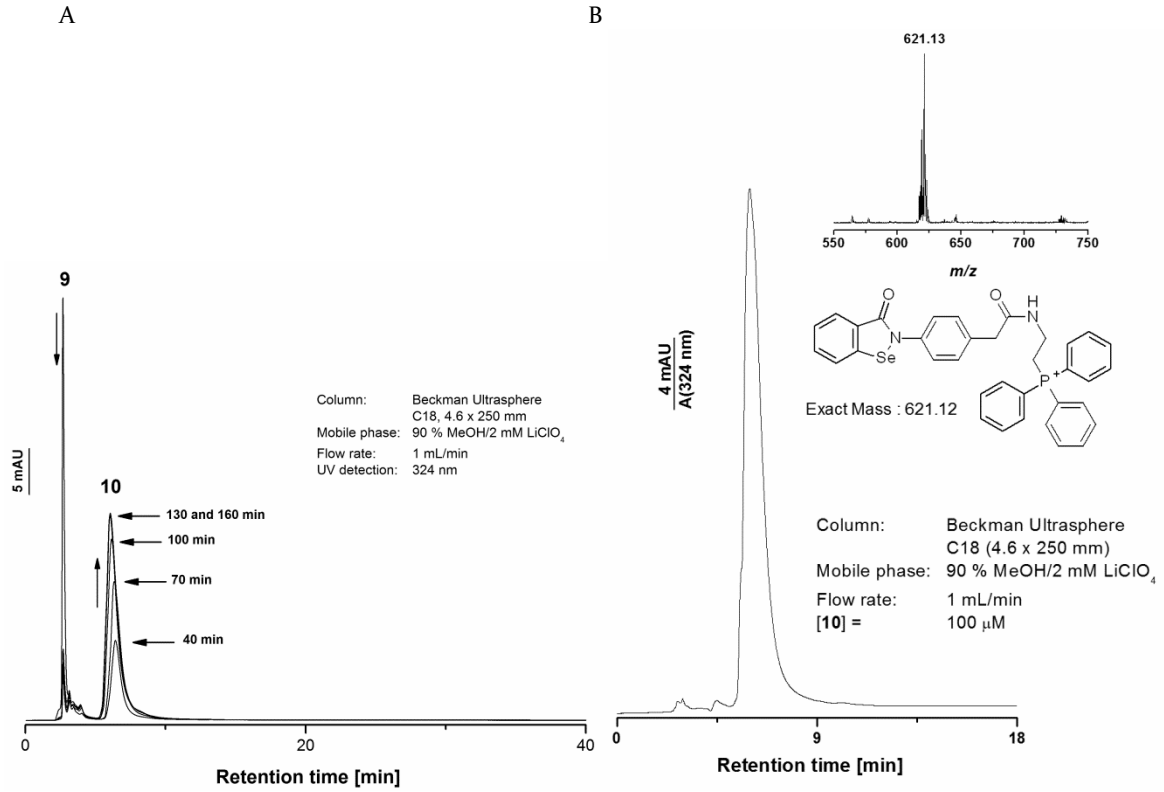
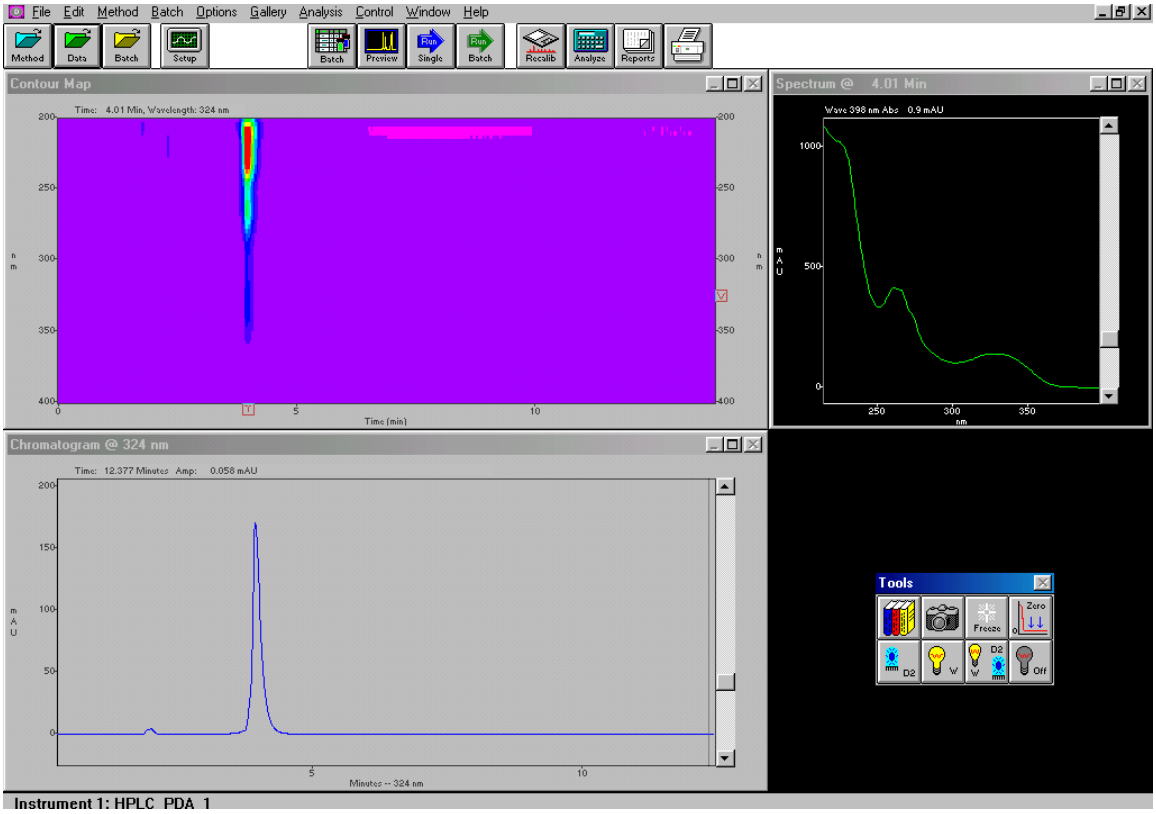
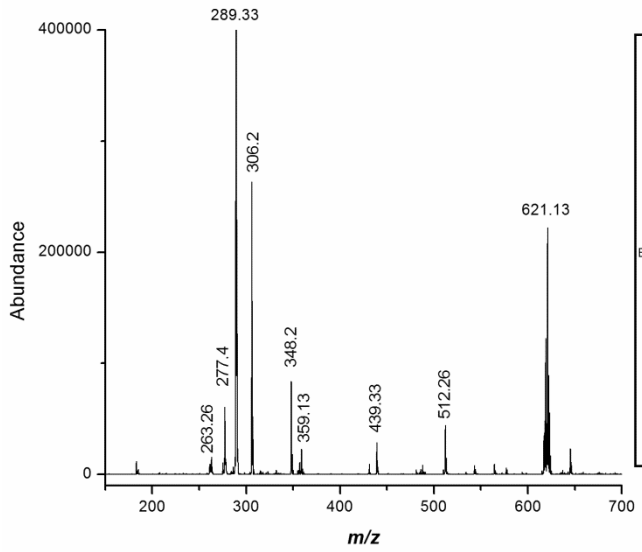


Figure 4S. HPLC and MS analysis of **10**. *A*- Aliquots from the reaction solution consisting of 2-aminoethyl(triphenyl)phosphonium chloride and DCC and **9** were taken at the specified time points during the incubation at room temperature and subjected to HPLC analysis. *B*- HPLC tracing and MS spectrum (positive ionization mode) of purified **10** (flow rate, 1 mL/min). *C*- 3D HPLC analysis (flow rate, 2 mL/min) of **10** in the wavelength range of 200 – 400 nm.

C



A



B

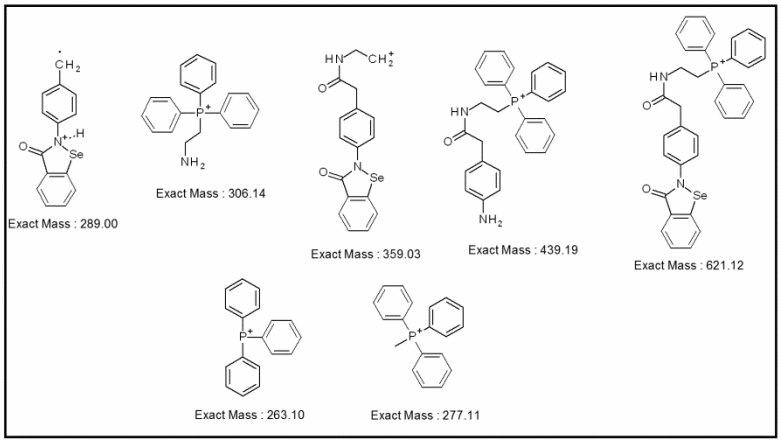


Figure 5S. MS-MS analysis of 10 (A) and structural assignment of the observed fragments (B).

Figure 6S. MS-MS analysis (negative ionization mode) of ROOH (A) and ROH (B) and structural assignment of the observed fragments (B,C). All spectra were recorded in negative ion mode.

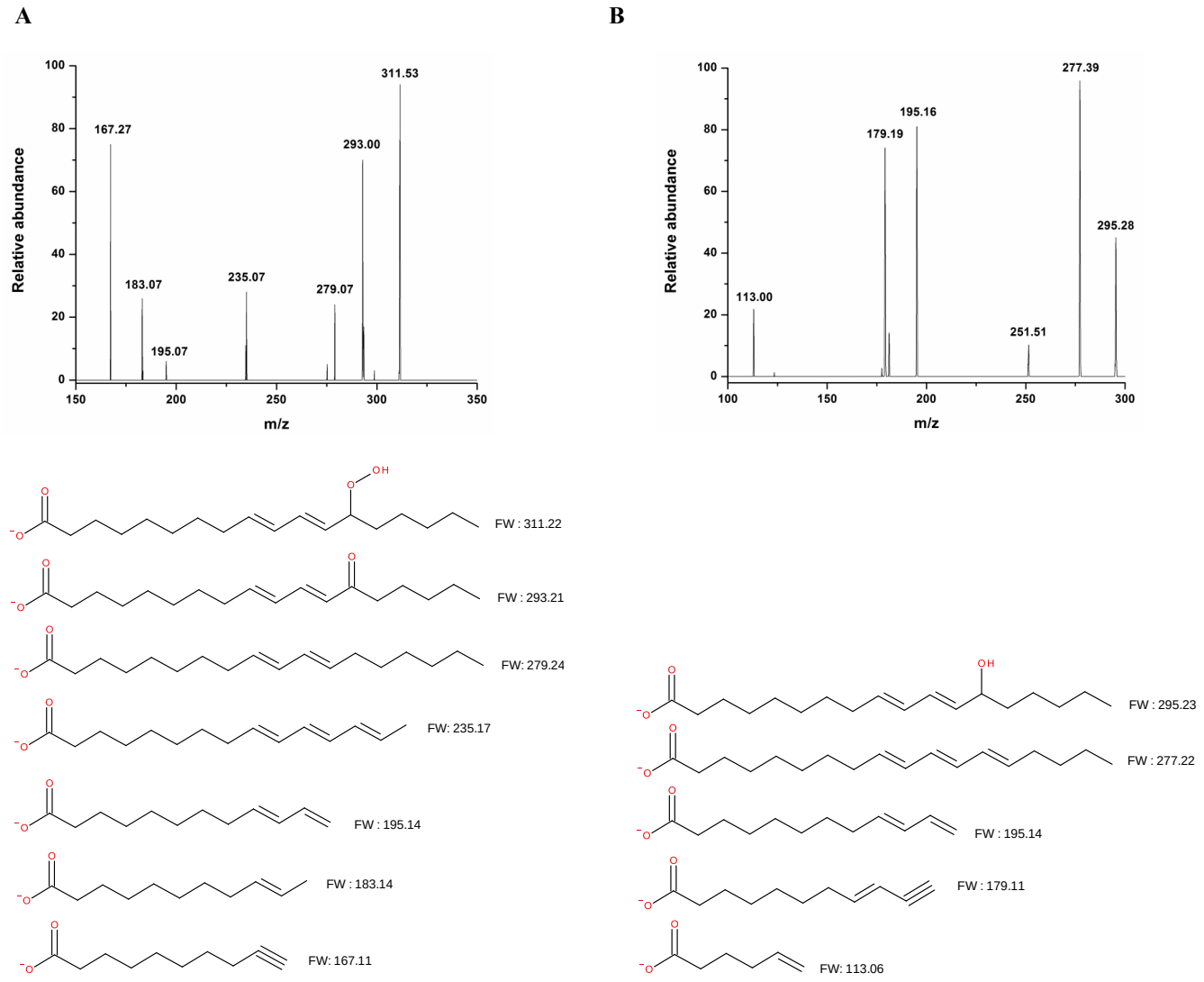
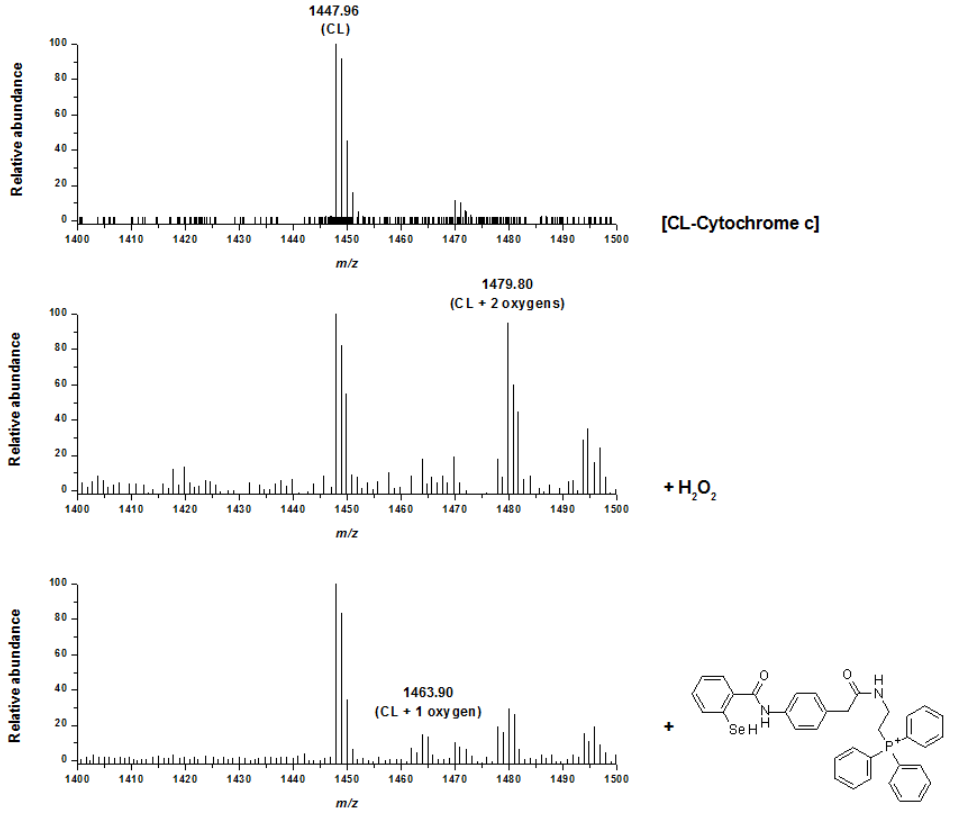


Figure 7S. MS analysis of the thiol-dependent reduction of hydroperoxides of CL by MitoPeroxidase 2. Reactions were carried out with CL-containing phosphatidylcholine (PC) liposomes for 30 min at 25 °C in 0.1 M Hepes (pH 7.4; 0.1 mM EDTA). Liposomes were obtained by sonication of phospholipids with ultra sound (10 x 5 sec; 30 seconds relaxation; ice bath), whereby PC, CL, cytochrome c and H_2O_2 were used at concentrations of 100, 25, 2 and 50 µM, respectively. After completion of the incubation, CL was extracted from the reaction suspension following Folch's protocol ($CHCl_3$: MeOH : H_2O = 2 : 1 : 1), the solvent was evaporated under nitrogen, the dry reside redissolved in methanol and subjected to MS analysis prior to or after addition of 20 µM selanyl-benzamide (obtained by reduction of MitoPeroxidase with DHLA as indicate in Figure 1).



Cell experiments

Mouse embryonic cells (courtesy of Dr. X. Wang, University of Texas, Dallas) were cultured in DMEM supplemented with 15% FBS, 25 mM HEPES, 50 mg/L uridine, 110 mg/L pyruvate, 2 mM glutamine, 1 × nonessential amino acids, 50 µM β-mercaptoethanol, 0.5 × 10^6 U/L mouse leukemia inhibitory factor, 100 U/L penicillin, and 100 mg/L streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C. For radiation exposure, cells were γ-irradiated (10 Gy) using a Shepherd model 143-45A irradiator (J. L. Shepherd & Associates, CA) at a dose rate of ~4 Gy/min. Ebselen and its derivatives were added to cells 30-min before or after γ-radiation exposure and removed after 4 hours of incubation. Cells were collected for further analysis after 48 hours post-radiation incubation.

Phosphatidylserine (PS) externalization: At the end of incubation, adherent cells were trypsinized and pooled with cells that had already been detached. The externalization of PS was determined by flow cytometry using an Annexin-V-FITC/Propidium iodide (PI) kit (Biovision, Mountain View, CA). Cell debris as represented by distinct low forward and side scatter were gated out for analysis. Ten thousand events were collected on a FACScanto II flow cytometer (Becton-Dickinson, Ruther-ford, NJ) equipped with Diva software. Percentages of Annexin V-positive cells were calculated by combining Annexin V+/PI- (early apoptotic) and Annexin V+/PI+ (late apoptotic or necrotic) cells.

Statistical Analysis: Data are expressed as means ± standard deviation of at least triplicate determinations. Statistical analysis was performed by either paired or unpaired Student's *t*-test. Differences were considered significant (*) at $p < 0.05$.

Animal experiments

MitoPeroxidase 2 was loaded into an emulsion formulation (F14) that consisted of soy phosphatidylcholine, sesame seed oil and PEG2000-Fmoc2-OA2 (2:4:1 w/w) with a final drug concentration of 4 mg/ml in PBS. All ingredients were dissolved in chloroform and mixed well. The chloroform was then removed by nitrogen flow. The residual solvent was further removed under vacuum for 2 h. The oily residues were hydrated with PBS and sonicated in an ice bath for 30 min. The size of drug-loaded emulsion was ~ 100 nm. The experiments were approved by the institutional Animal Care and Use Committee at the University of Pittsburgh.

C57BL/6NTac female mice were irradiated to 9.1 or 9.5 Gy total body irradiation. The mice were divided into two groups of 15 mice (irradiation only or MitoPeroxidase 2). Twenty four hours after irradiation the mice were injected IV with 100 µl of MitoPeroxidase 2.

References

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(3) Mavanoff, B. E.; Reitz, A. B.; Duhl-Emswiler, B. A. Stereochemistry of the Wittig Reaction. Effect of Nucleophilic Groups in the Phosphonium Ylide. *Journal of the American Chemical Society* **1984**, *107*, 1984.