Theonellasterone, a steroidal metabolite isolated from a Theonella sponge, protects Peroxiredoxin-1 from oxidative stress reactions.

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Experimental Section

Theonellasterone modification by biotin linker:

100 µl of THS at 50 µg/µl in MeOH reacted with 100 µl of β-mercapto-ethyl-amine at 100 µg/µl in MeOH in a final volume of 1 mL with 0.5% tri-ethyl-amine for 30 min at 40°C under stirring. Product formation has been monitored by RP-HPLC-UV at 220 nm using Agilent 1100 binary pump and Phenomenex column (Jupiter C4 50X2 mm 5 µ) at 0.2 mL/min. Gradient was from 5% to 95% buffer B (A= 100% H_2O and 0.1% TFA and B= 95% ACN, 5% H_2O and 0.1% TFA) in 25 min. Mass spectra were acquired on LTQ XL mass spectrometry system (Thermo-Scientific) equipped with an ESI source. The product has been purified by RP-HPLC.

500 µg of THS-β-mercapto-ethyl-amine has been conjugated with 2.5 mg of NHS activated S-S biotin in 1 mL of PBS buffer for 2h at 37°C under stirring. Product formation has been monitored by RP-HPLC-UV at 220 nm using Agilent 1100 binary pump and Phenomenex column (Jupiter C4 50X2 mm 5 µ) at 0.2 mL/min. Gradient was from 5% to 95% buffer B (A= 100% H_2O and 0.1% TFA and B= 95% ACN, 5% H_2O and 0.1% TFA) in 25 min. Mass spectra were acquired on LTQ XL mass spectrometry system (Thermo-Scientific) equipped with an ESI source. The product has been purified by RP-HPLC.

As control, 500 µg of β-mercapto-ethyl-amine has been conjugated with 2.5 mg of NHS activated S-S biotin in 1 mL of PBS buffer for 2h at 37°C under stirring and the product has been characterized and purified as previously reported.

Theonellasterone targets specifically Peroxiredoxin 1:

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, at 37°C in a 5% CO_2 atmosphere (all reagents were from Sigma–Aldrich). Cells were collected by centrifugation (600*g*, 5 min), washed twice with PBS and resuspended in 1X ice cooled PBS containing Igepal (0.1%), supplemented with a protease inhibitor cocktail, and lysed by Dounce manual homogenizations. Cellular debris were removed by centrifugation at 10000*g* for 10 min at 4 °C. Protein concentration was determined using Bradford assay and adjusted to 1 mg/ml. THS-biotin adduct (100 nmol) and the same amount of the control linker were separately incubated with 1 mg of HeLa total proteins extracts under continuous shaking (1 h, 4°C) and then, 20 µL of streptavidin-modified beads (Pierce) were added and left for 1h at 4°C. The beads were collected by centrifugation (865*g*, 1 min, 4°C) and washed six times with PBS (pH 7.4). The bound proteins were eluted by adding 35 µL of DTT 40 mM for 30 min at 30°C, separated on 12% SDS-PAGE and stained with Coomassie G-250 (Bio-Rad, Hercules, CA). SDS-PAGE gel lanes were cut and digested. The experiment has been repeated twice using an opportune control matrix bearing the linker without any metabolite. Each piece was washed with ultrapure water and CH_3CN and subjected to *in situ* protein digestion as described by Shevchenko.[20] Briefly, each slice was reduced with 10 mM 1,4-dithiothreitol (DTT) and alkylated with 54 mM iodoacetamide, then washed and rehydrated in trypsin solution (12 ng/mL) on ice for 1 h. After the addition of ammonium bicarbonate (30 µL, 50 mM, pH 7.5), proteins digestion was allowed to proceed overnight at 37°C. The supernatant was collected and peptides were extracted from the slice using 100% CH_3CN and both supernatants were combined. The peptide sample was dried and dissolved in formic acid (FA, 10%) before MS analysis. The peptide mixture (5 µL) was injected into a nano-ACQUITY UPLC system (Waters). Peptides were separated on a 1.7 mm BEH C18 column (Waters) at a flow rate of 400 nL/min. Peptide elution was achieved with a linear gradient (solution A: 95 % H_2O ,5 % CH_3CN, 0.1% FA; solution B: 95 % CH_3CN, 5 % H_2O, 0.1 % FA); 15–50% B over 55 min). MS and MS/MS data were acquired on a LTQ XL high-performance liquid chromatography mass spectrometry system (ThermoScientific). The five most intense doubly and triply charged peptide ions were chosen and fragmented. The resulting MS data were processed by Xcalibur software to generate peak lists for protein

identifications. Database searches were carried out on the Mascot server (http://www.matrixscience.com/). The SwissProt database (release 2014_07, 14 July 2014, 546,439 entries, 194,445,396 residues) was employed (settings: two missed cleavages; carbamidomethyl (C) as fixed modification and oxidation (M) and phosphorylation (ST) as variable modifications; peptide tolerance 80 ppm; MS/MS tolerance 0.8 Da).

In vitro and _in silico_ validation of Theonellasterone binding to Peroxiredoxin 1:

Proteins eluted from the above-described experiments, carried out both on HeLa cell lysates, were separated on 12% SDS-page and transferred to a nitrocellulose membrane. The membrane was incubated for 1 h in a blocking solution containing 25 mM Tris pH 8, 125mM NaCl, 0.05% Tween-20, 5% non-fat dried milk prior of exposition, primary antibodies raised against PRX-1 (1: 500; Novus Biologicals). The recognition of specific epitopes was favorite overnight, at 4°C. Then, membrane was incubated for 1 h with an anti-rabbit peroxidase-conjugated secondary antibody (1:5000) (Sigma-Aldrich). PRX-1 was detected by a chemo-luminescence detection system.

PRX-1 and PRX-6 were immobilized onto two different flow cells of a CM5 sensor chip using standard amine coupling procedures. Phosphate–buffered saline, which consisted of 10 mM Na_2HPO_4 and 150 mM NaCl, pH 7.4, was used as running buffer. The carboxymethyl dextran surface was activated as already described and both proteins were diluted to a final concentration of 30 ng/µl in 10 mM sodium acetate, pH 4.5 and injected separately onto the two flow cells. 100 µl prior the injection onto the activated chip surface at flow rate of 5 µl/min. After protein injections a RU of 9000 for PRX-1 and a RU of 14000 for PRX-6 was reordered. The activated carboxymethyl dextran surface was finally blocked with a 7-min injection of 1.0 M ethanolamine-HCl, pH 8.5, at 5 µl/min. THS solutions (0.01-10 µM), were prepared in running buffer containing 1% of DMSO and injected at least three times. Since the dissociated back to baseline within a reasonable time frame, no regeneration has been required. The interaction experiments were carried out at a flow rate of 10 µl/min, employing a 3 min injection time. The dissociation time was set at 600 seconds. Rate constants for associations (k_a) dissociations (k_d) and the dissociation constants (K_D) were obtained by globally fitting data from injections of all concentrations, using the BIAevaluation software, using the simple 1:1 Langmuir binding model.

1 µL of PRX-1 at 10 µM treated by phosphine at 5 mM was mixed with 1 µL of THS at 100 µM or 1 mM for 30 min at 37°C in a final volume of 10 µL PBS at pH 7.4. MALDI spectra has been recorded loading 1 µl of each solution mixed with 1 µL of sinnapinic acid as matrix on a MALDI

96 well plate. Mass spectra were acquired on a MALDI micro-MX (Waters, Co., Milford Massachusetts, USA) in linear positive ion mode in a m/z range of 10 000–50 000.

The *in silico* dissection of the THS/PRX1 complex was performed according to a molecular docking procedure using the Autodock Vina software [22] on an Intel Core i7/Mac OS X 10.9 - based platform. The three-dimensional structure of the PRX 1 monomer was obtained from a human PRX 1 crystallographic assembly (pdbID: 3HY2 in [23]), available in the Protein Data Bank, and minimizing the subunit A using the Discovery module of InsightII R2005 software (Accelrys Ltd., Cambridge, U.K.), the consistent valence force field (CVFF), a water solvation shell of 5 Å and the conjugate gradients algorithm to a rms derivative of 0.001 kcal/mol. The three-dimensional structure of THS was constructed and minimized (with a universal force field, UFF, and a conjugate gradient algorithm until a dE lower than 0.001 kJ/mol was achieved) using Avogadro software [Avogadro: an open-source molecular builder and visualization tool. Version 1.1.0. http://avogadro.openmolecules.net and 24]. The molecular docking was performed setting the pdbqt file (polar hydrogens were considered) of THS as ligand and of PRX1 as receptor, and setting a docking zone of 37×36×42 Å around the entire PRX1. All modeling complexes and images were rendered using PyMOL software (Python Molecular Graphics – version 1.3). Energy contributions for the resulted binding affinity (-6.89 kcal/mol) were: gauss1 = 58.6, gauss2 = 1151.65, repulsion = 0.85, hydrophobic = 57.1 and hydrogen = 0.

Theonellasterone protects Peroxiredoxin 1 from oxidation *in vitro* and in live cells:

60 µg of HeLa cell lysates were treated with 5 mM DTT for 45 min at 30°C and incubated or not with THS at different concentration as 50 and 250 µM for 30 min at 30°C and H_2O_2 at 5 and 50 mM for 15 min at r.t. Samples were loaded on 15% SDS-PAGE and revealed using anti-PRX-1-SO_3 antibody (1:500, AbCam). The recognition of specific epitopes was favorite overnight, at 4°C. Then, membrane was incubated for 1 h with an anti-rabbit peroxidase-conjugated secondary antibody (1:5000) (Sigma-Aldrich). PRX-1-SO_3 was detected by a chemo-luminescence detection system.

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 24 h of THS treatment in a concentration range from 1 µM to 100 µM, the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to assess the effect of the molecule.

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, at 37°C in a 5% CO_2

atmosphere (all reagents were from Sigma–Aldrich). Then, HeLa cells were treated or not with THS at 100 µM for 12 h and then oxidized by H_2O_2 at 100 µM for 5 min. Cells were collected by centrifugation (600*g*, 5 min), washed twice with PBS and resuspended in 1X ice cooled PBS containing Igepal (0.1%), supplemented with a protease inhibitor cocktail, and lysed by Dounce manual homogenizations. Cellular debris were removed by centrifugation at 10000*g* for 10 min at 4 °C. Protein concentration was determined using Bradford assay and adjusted to 1 mg/ml. 12 µL of each sample was loaded on 12% SDS-PAGE and revealed using anti-PRX-1-SO_3 antibody (1:1000, AbCam). The experiments were conducted in triplicate and GADPH antibody was used for normalization.

Figure S1: MS and MSMS spectra for the characterization of the adduct between THS and β-mercapto-ethyl-amine at m/z of 502.81. The fragment at 457.23 is compatible with the loss of ethyl-amine.

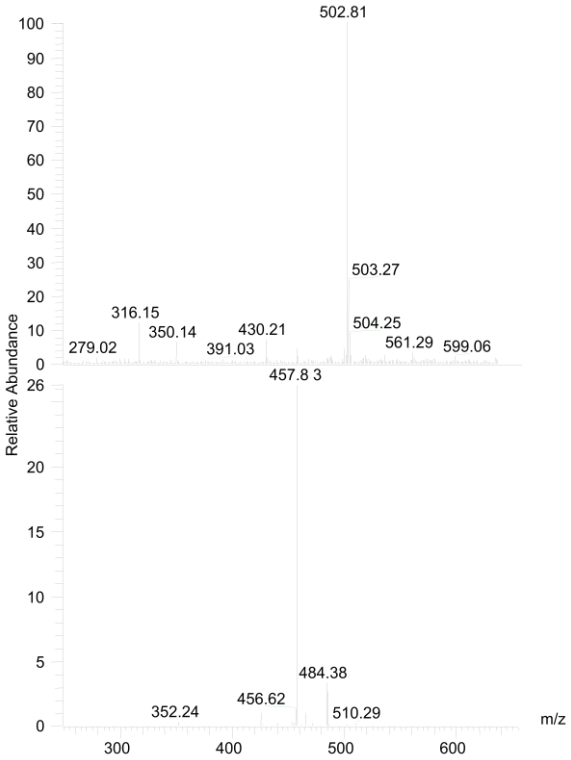

Figure S2: MS spectra for the characterization of the adduct between THS- β-mercapto-ethyl-amine and biotin at m/z of 891.48.

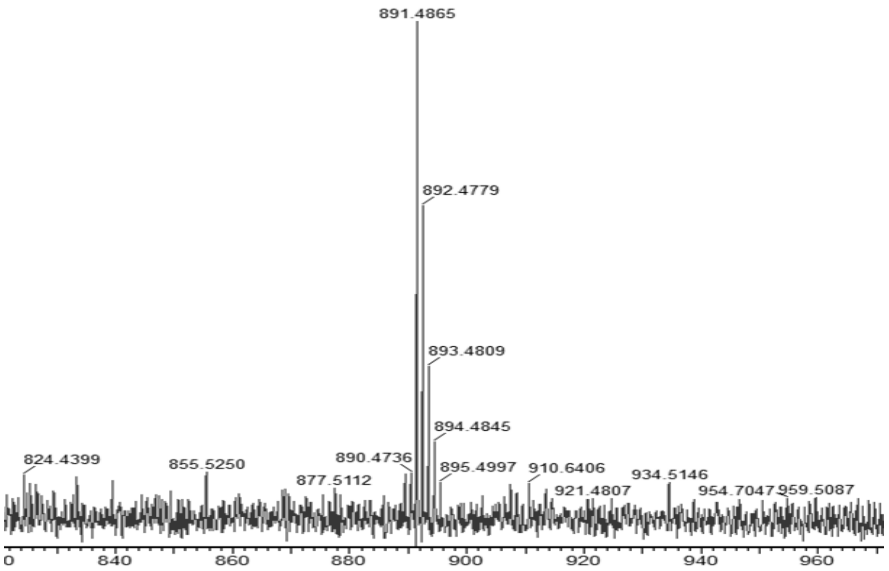

Figure S3: Structure of the adduct between Biotin and β-mercapto-ethyl-amine used as an opportune control.

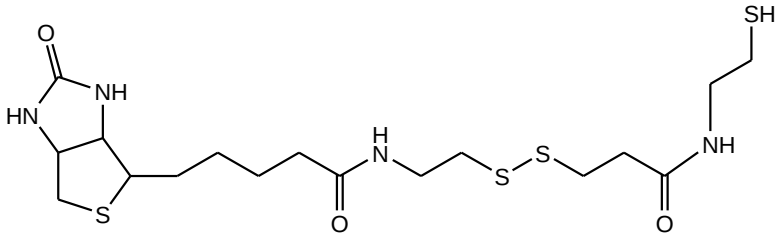

Figure S4: SPR sensorgrams obtained on PRX-6 modified sensor chip with different concentration (0.01-10 µM) of free THS.