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Supporting Information

DOI: 10.1002/cbic.200900159

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for

Small-Molecule Targeting to the Mitochondrial Compartment by an Endogenously Cleaved Reversible Tag

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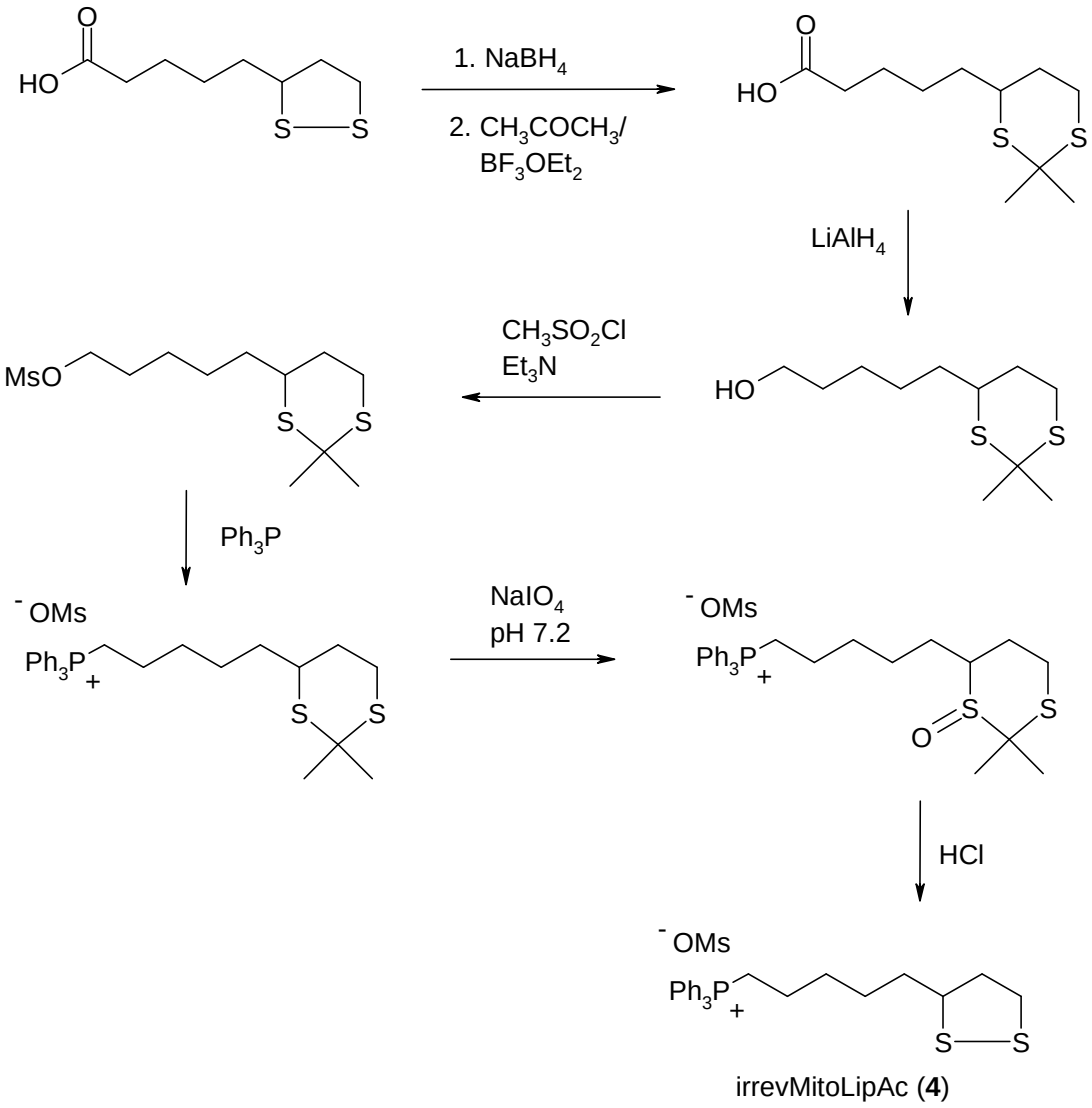
General Information

All chemicals were used as received from the respective supplier. Tetrahydrofuran, 4-dimethylaminopyridine, dicyclohexylcarbodiimide and triphenylphosphonium hydrobromide were obtained from Sigma-Aldrich (Schnelldorf, Germany). Diethylether and methanol were obtained from Roth (Karlsruhe, Germany). DL-alpha lipoic acid was obtained from Fluka (Schnelldorf, Germany). irrevMitoLipAc has been synthesized according to published procedures.[1] TLC analysis was performed on Silica Gel 60 F254 coated plates from Merck (Darmstadt, Germany). 1H NMR and 13C NMR spectra were recorded at 300 and 75.47 MHz, respectively, on Bruker (Bremen, Germany) AMX 300 with CDCl3 as solvent and TMS as internal standard. Electrospray ionisation mass spectrometry (ESI-MS) was performed in positive ionisation mode on a MAT 95 XL-Trap (Thermoquest Finnigan, Bremen, Germany).

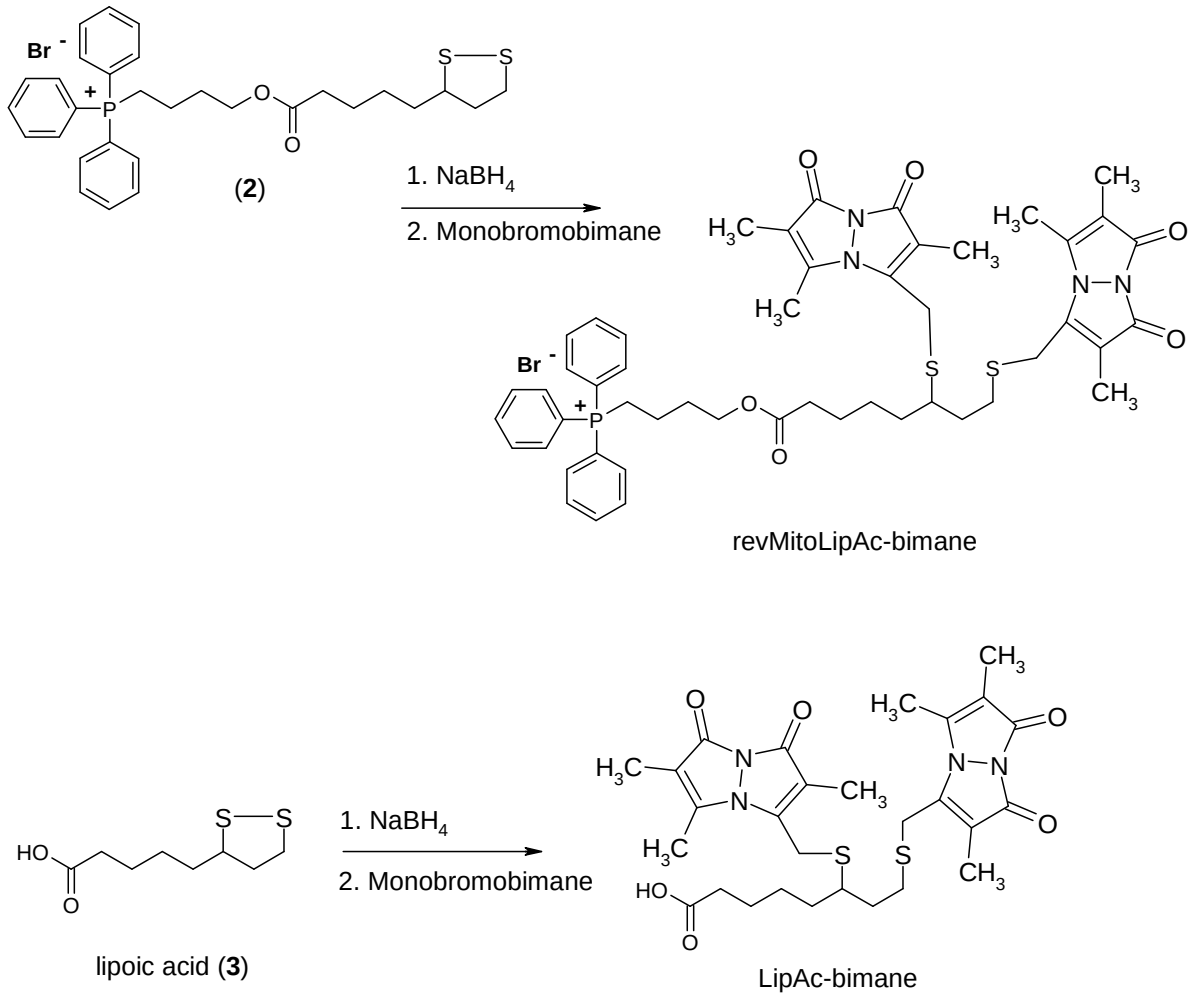
Synthesis of irreversibly tagged lipoic acid described by Brown et al.[1] In brief, lipoic acid was reduced and thiol-protected with aceton and BF_3*OEt_2. The carboxy-residue was reduced by $LiAlH_4$ and the alcohol was sulfonated by methylsulfonylchloride. The sulfonyl-group was then substituted by triphenylphosphine to yield the triphenylphosphonium salt. The thiol-protecting group was removed by a two step procedure. The yield of each step was comparable to the original published synthesis (Scheme S1). IrrevMitoLipAc (**4**) has been identified by its mass spectrum. IrrevMitoLipAc: (ESI+)-MS; *m/z* calcd for $C_{26}H_{30}PS_2$: 437.2 $[M+H]^+$; found: 437.2.



Scheme S1: Synthesis of irrevMitoLipAc as performed by Brown et al.

*General procedure for the synthesis of bimane labeled lipoic acid (**1**) and bimane labeled revMitoLipAv (**2**)*: To visualize the intracellular distribution of free and targeted lipoic acid we synthesized two bimane-derivatives of **2** and **3** (Scheme S2). In general, lipoic acids were reduced by 2 equivalents of sodium borohydride in chloroform under argon atmosphere at room temperature, according to a well known procedure.[2] After 4h the reaction was quenched by the addition of 1 M HCl. The reduced thiols were extracted by $CHCl_3$ (2 x) and the organic solvent was dried with sodium sulfate. The solvent was evaporated and the residue was used without further purification. The thiols were dissolved in a 1 M Na_2CO_3 solution and 2 equiv monobromobimane (Sigma, Schnellendorf, Germany) was added. The mixture was stirred in the dark for 12 h. Bimane derivatives were purified from unreacted monobromobimane

by reversed phase column chromatography. In brief, the reaction mixtures were applied to pre-conditioned RP-Phase cartridges (Baker-bond RP-18, 3ml, 500 mg), the columns were washed with water and bimanes were eluted by increasing the amount of methanol in the eluent. Bimanes elute at a methanol concentration of 50-60 % (*v/v*). Methanol was evaporated under vacuum at 40 °C. B imane derivatives showed an intense fluorescence under UV-light. High resolution mass spectra were recorded for each bimane derivative. LipAc-bimane: HRMS (ESI+): *m/z* calcd for $C_{28}H_{36}NaN_4O_6S_2$: 611.1971 $[M+H]^+$; found: 611.1974 $[M+H]^+$. RevMitoLipAc-bimane: HRMS (ESI+): *m/z* calcd for $C_{50}H_{58}N_4O_6PS_2$: 905.3535 $[M+H]^+$; found: 905.3524 $[M+H]^+$.



Scheme 2: Synthesis of bimane labeled revMitoLipAc and lipoic acid.

Cleavage of mitochondrially targeted lipoic acid (revMitoLipAc): Our approach suggests the fast accumulation of revMitoLipAc (**2**) within the mitochondria matrix, which is followed by an enzymatic hydrolysis of the lipoyl ester by mitochondrial aldehyde dehydrogenase (EC1.2.1.3, ALDH-2). RevMitoLipAc was incubated with energized

mitochondria from pig liver and enzymatic hydrolysis was monitored by TLC. After incubation the mitochondria were extracted by organic solvents ($CHCl_3$/MeOH 8:2 *v/v*) and applied to TLC. The cleavage reaction was completed within 60 min (Figure S1A). No cleavage took place when the incubation was performed in the presence of benomyl, an inhibitor of mitochondrial aldehyde dehydrogenase (Figure S1B).

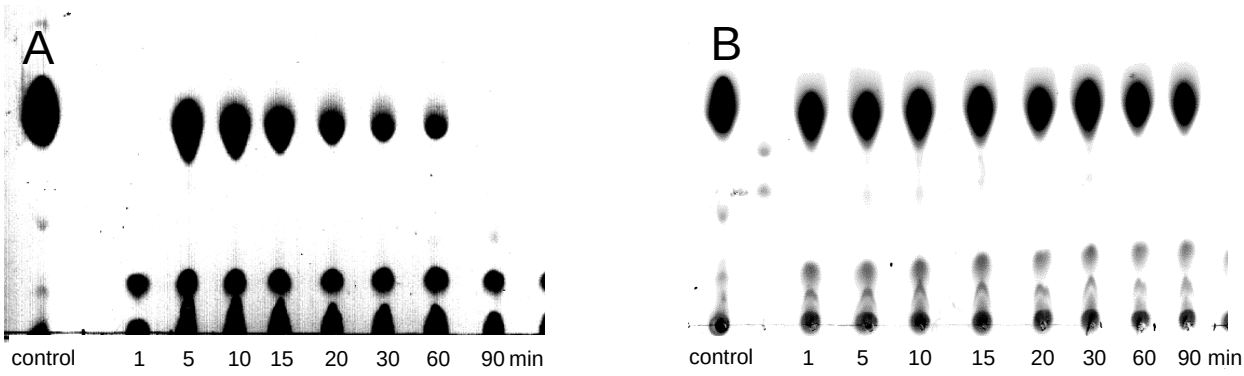


Figure S1: A) Thin layer chromatography (TLC) of extracts obtained from incubations of isolated mitochondria with revMitoLipAc (2). Control lane depicts revMitoLipAc. B) TLC of extracts obtained from incubations of isolated mitochondria with revMitoLipAc in the presence of benomyl (an ALDH-2-inhibitor). Control lane depicts revMitoLipAc.

Glucose and lactate assays: Lipoic acid is a known cofactor retaining PDH activity. Since we forced the mitochondrial import of LipAc, we expect to observe a shift in oxidative glycolysis depicted as a positive ratio of consumed glucose vs. generated lactate in HepG2 cells. Glucose and lactate levels has been determined as previously described.[3] At all time points observed (Figure S2), we show a significant increase in the glucose to lactate ratio when cells were incubated with revMitoLipAc (10 μM). The pronounced increase of this ratio was attributed solely to revMitoLipAc, since LipAc or HBTPP alone did not change the ratio.

HepG2 cells were handled as described and incubated with compounds to be tested. Glucose and lactate assays were performed as described previously.[3] In brief, to monitor glucose utilization after various time intervals, medium supernatants were collected from each sample and transferred to a 96-deep well plate. To assess the actual glucose content at the beginning of experiments, a volume of fresh medium was also collected. Supernatants were subsequently diluted in deionized water (1:30) and analysed using the glucose (GO) assay kit (Sigma Aldrich) that provided a convenient photometric determination of glucose concentration.

Diluted supernatants (60 µl) were mixed with 120 µl assay reagent and incubated for 30 min at 37 ℃. The assay principle bases on the o xidation of D-glucose to gluconic acid by glucose oxidase. Absorbance was measured using a microplate fluorometer NOVOstar (BMG Labtech, Offenburg, Germany) at 540 nm and is proportional to the glucose concentration of samples.

Glucose concentrations of supernatants were calculated according to a standard plate that was prepared and measured in parallel to sample plates. The difference in glucose quantity between fresh and used medium was then regarded as the amount of substrate consumed by cells.

Quantities of lactate, generated by cells and released into the medium, were determined with the L-lactate test kit (R-Biopharm, Darmstadt, Germany) by using a photometric approach. Lactate is converted to pyruvate by lactate dehydrogenase (LDH) at the expense of NAD^+. Reduced NADH is measured at 340 nm and the absorption is proportional to the amount of lactate converted.

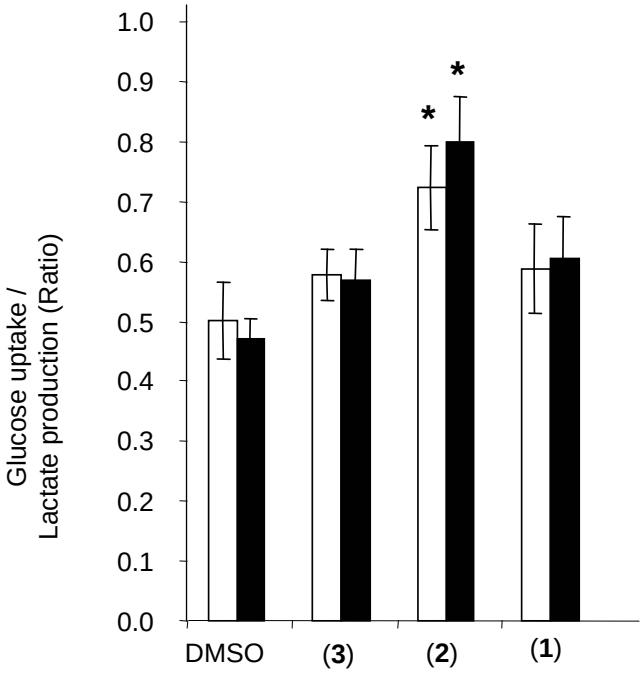


Figure S2: Values show the ratio of the amount of glucose consumed (nmol/well) and lactate generated (nmol/well). HepG2 cells has been incubated for depicted time periods prior determination of glucose and lactate. Black bars represent 12-h treatment and white bars 48-h treatment. Data are expressed as mean of n=8, * p<0.05. (**3**) LipAc; (**2**) revMitoLipAc; (**1**) HBTPP = triphenylphosphonium-tag.

Growth characteristics of HepG2-cells exposed to reversibly tagged lipoic acid: Protein content of HepG-2 cells reflecting cell growth was monitored for 12, 24 and 48 h, respectively (Figure S3). Protein content has been measured by the method of Bradford.[4] Free lipoic acid (**3**) had no effect at 1 µM concentration and slightly decreased cell growth at 10 µM after 12 h. However, cells recover from initial stress after 48 h. RevMitoLipAc (**2**) significantly induced cell growth at 1 µM for 24 and 48h. Higher concentrations (10 µM) initially reduced protein content but cells did recover after 48 h treatment. Similar results were observed with the isolated tag (HBTPP (**1**)) in 10 µM concentration, where cells did recover from an initial stress phase after 48 h.

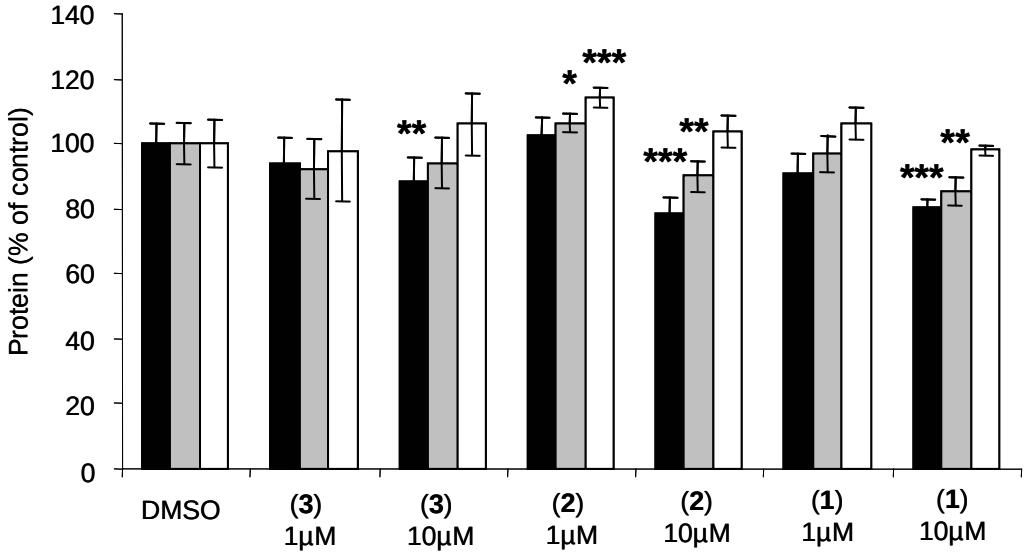


Figure S3. Protein content of HepG2 cells after different incubation time intervals. Black bars represent 12-h treatment, grey bars 24-h treatment and white bars 48-h treatment, respectively. Data are represented as mean of n=8 ± SD, * p<0.05, ** p<0.01, *** p<0.001 compared with DMSO.

References:

[1] S. E. Brown, M. F. Ross, A. Sanjuan-Pla, A. R. Manas, R. A. Smith, M. P. Murphy, *Free Radic Biol Med* **2007**, *42*, 1766.

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