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Research Article

Synthesis of New Lipoic Acid Conjugates and Evaluation of Their Free Radical Scavenging and Neuroprotective Activities

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A series of new lipoic acid derivatives were designed and synthesized as multitarget ligands against Alzheimer's disease. In particular, analogues combining both lipoic acid and cysteine core structures were synthesized. The antioxidant properties of these compounds were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation scavenging assays and ferrous ion chelation. The antioxidant potential of the synthesized compounds was also evaluated in a cellular context and compared to α-lipoic acid and its reduced form, dihydrolipoic acid. The antioxidant effects observed for these compounds *in vitro* confirmed the importance of free thiol functions for effective antioxidant capacities. However, these promising *in vitro* results were not mirrored by the antioxidant activity in T67 cell line. This suggests that multiple factors are at stake and warrant further investigations.

Key words: Alzheimer's disease, antioxidant, ferrous chelation, lipoic acid, multitarget ligands

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Alpha-lipoic acid (LA) is a clinically safe supplement exhibiting significant antioxidant and cell regulatory properties (1). Exogenously supplied LA is transported to tissues, taken up by cells and reduced by lipoamide dehydrogenase to the corresponding dihydrolipoic acid (DHLA; Figure 1). Both LA and DHLA induce a variety of major effects, including direct radical scavenging and redox modulation of cell metabolism, and potentially contrast oxidatively induced injuries (2,3). While DHLA is considered the active species, the antioxidant activity of LA and DHLA is attributed to their ability to regenerate other antioxidants, such as vitamins C and E, to induce a variety of antioxidant enzymes, but also to serve as a proglutathione agent (4–6). These properties prompted the medicinal chemistry community to investigate the therapeutic potential of LA and DHLA derivatives in various oxidative stress-related disorders (7,8). In most of these studies, the lipoyl fragment has been linked to an amine-type partner through an amide bond, giving raise to hybrid molecules with an improved biological profile with respect to LA itself (9–18). Focusing on neurodegenerative disorders, LA has demonstrated a variety of beneficial properties that can positively modulate disease pathogenesis and progression. On this basis, some of us have proposed LA as a privileged structure in the search for novel ligands against neurodegeneration and have validated this concept through the development of several series of LA-based neuroprotective agents (19–22). It was argued that the incorporation into a single molecular entity of a lipoyl fragment and a second one with an alternative mechanism of action could potentially lead to compounds retaining the pharmacological properties of the two starting fragments and consequently showing a synergistic and more effective mechanism of action. In other words, the resulting hybrid molecules act as multitarget-directed ligands to combat the neurotoxic network underlying Alzheimer's disease (AD) (23).

N-Acetylcysteine (NAC) is another sulfur-containing natural antioxidant, which has been addressed as patient supplementation in several diseases. *In vivo*, NAC is readily hydrolyzed to cysteine. Indeed, NAC has been reported to be beneficial in a number of oxidative stress models, such as systemic sclerosis, HIV infection, hypertension, radiation injury, and others. Recently, we reported that various NAC analogues and notably *N*-(*N*-acetyl-L-cysteinyl)-*S*-acetylcysteamine increased intracellular GSH levels in various cell lines and displayed interesting antiviral and immunomodulatory activities (24–28). These compounds were designed to liberate after metabolization, NAC and 2-mercaptoethylamine (MEA), two potential pro-GSH compounds, which act as part of the glutathione (GSH/GSSG) redox system (29,30). Notably, this glutathione enhancing effect, which is peculiar to the mechanism of

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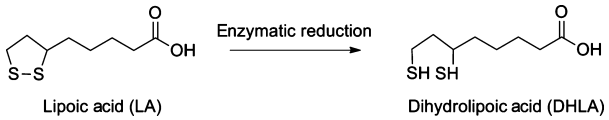


Figure 1: Dihydrolipoyl dehydrogenase reduces lipoic acid (LA) to dihydrolipoic acid (DHLA) in the presence of NADH.

antioxidant action of NAC, has been recently proposed as particularly effective in contrasting oxidative stress in AD (31).

Even more interestingly, it has been reported that a cocktail containing both LA and NAC improves cognitive performance while decreasing AD neuropathology in a transgenic mouse model, and may thus represent a safe, natural treatment for AD (32). Inspired by these findings and building on the hybrid molecules approach, we envisioned that merging the structural features of LA and DHLA with mercaptoamine-derived units (mimicking the NAC) could provide ligands with improved antioxidant and neuroprotective properties. Herein, the design, synthesis, and preliminary examination of the *in vitro* antioxidant and neuroprotective activities of the novel LA-NAC conjugates will be discussed.

Methods and Materials

Synthesis

Reactions were conducted in oven-dried glasswares. All reagents were purchased from Aldrich or local suppliers and used without purification. All reactions were monitored by TLC, and visualization was effected by UV and/or by developing in vanillin. Chromatography refers to open column chromatography on silica gel (Merck, 40–63 μm). Melting points were determined on a SMP3 Stuart Scientific apparatus and are uncorrected; NMR spectra were recorded on a Bruker DRX-300 MHz spectrometer at 300 (^{1}H) and 75 (^{13}C) MHz at 298 K. Chemical shifts are reported in ppm relative to the solvent residual peak $CDCl_3$ as internal standards (^{1}H and ^{13}C). Data for ^{1}H are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constants (*J*) in Hz, and integration. All compounds were further characterized by mass spectrometry (Q-TOF; Waters, Milford, MA, USA) and high-resolution mass spectrometry using an ESI/Q-TOF Micromass spectrometer.

General procedure for the coupling of LA activated N-hydroxysuccinimide

To a solution of the appropriate amine (1 eq, 0.5 mmol) in dioxane (cystamine for **2a**, L-cystine diethyl ester for **2b**, and butanediamine for **4)** were added *N*-ethyldiisopropylamine (5 eq, 2.5 mmol) and LA activated *N*-hydroxysuccinimide (33) (2.5 eq, 1.25 mmol), and the mixture was stirred at room temperature overnight. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous NaCl, dried over sodium sulfate, and the solvent was evaporated in vacuo. The crude product was purified by flash column chromatography.

N,N'-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(5-(1,2-dithiolane-3-yl)pentanamide) (2a)

Yield: 62%, white solid, mp: 85–87 °C. ^{1}H-NMR δ 6.3 (sb, 2H), 3.42–3.55 (m, 6H), 2.96–3.18 (m, 4H), 2.76 (t, J = 6.4 Hz, 4H), 2.41 (m, 2H), 2.17 (t, J = 7.4 Hz, 4H), 1.85 (m, 2H), 1.52–1.63 (m, 8H), 1.32–1.48 (m, 4H); ^{13}C-NMR δ 173.1, 56.5, 40.3, 38.5, 37.8, 36.2, 34.6, 30.9, 28.9, 25.4; MS (ESI$^+$) *m/z*: 529.12 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{20}H_{37}N_2O_2S_6$ [M + H]$^+$ 529.1179 Found 529.1176.

Diethyl-3,3'-disulfanediylbis(2-(5-(1,2-dithiolane-3-yl)pentanamido))propanoate (2b)

Yield: 60%, yellow solid, mp: 63 °C; ^{1}H-NMR δ 6.38 (d, J = 7.31 Hz, 2H), 4.85 (m, 2H), 4.25 (q, J = 8.2 Hz, 4H), 3.53–3.62 (m, 2H), 3.06–3.24 (m, 8H), 2.42–2.55 (m, 2H), 2.28 (t, J = 7.35 Hz, 4H,), 1.88–1.96 (m, 2H), 1.62–1.77 (m, 8H), 1.43–1.56 (m, 4H), 1.32 (t, J = 8.2 Hz, 6H); ^{13}C-NMR δ 172.6, 170.4, 62.1, 56.4, 51.7, 40.8, 40.2, 38.5, 36.1, 34.6, 28.8, 25.2, 14.1; MS (ESI$^+$) *m/z*: 673.16 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{26}H_{45}N_2O_6S_6$ [M + H]$^+$ 673.1602 Found 673.1599.

N,N'-(Butane-1,4-diyl)bis(5-(1,2-dithiolane-3-yl) pentanamide) (4)

Compound **4** is obtained directly by filtration of the reaction mixture.

Yield: 86%, white solid, mp: 164 °C. ^{1}H-NMR δ 7.8 (sb, 2H), 3.56 (m, 2H), 2.99–3.19 (m, 8H), 2.42 (m, 4H), 2.08 (t, J = 6.90 Hz, 4H), 1.83–1.90 (m, 2H), 1.22-1.50 (m, 14H). ^{13}C-NMR δ 171.7, 55.9, 39.6, 38.07, 37.9, 34.5, 33.9, 28.1, 26.4, 24.8.MS (ESI$^+$) *m/z*: 465.17 ([M + H]$^+$ 80%), 487.16 ([M + Na+], 100%; HRMS-ESI$^+$ *m/z* calcd for $C_{20}H_{37}N_2O_2S_4$ [M + H]$^+$ 465.1738 Found 465.1734.

N,N'-(((Disulfanediylbis(ethane-2,1-diyl))bis (azanediyl))bis(2-oxoethane-2,1-diyl))bis(5-(1,2-dithiolane-3-yl)pentanamide) (7)

Yield: 45%, white solid, mp: 224 °C; ^{1}H-NMR δ 7.95 (s, 2H), 6.95 (s, 2H), 3.96 (d, J = 5.33 Hz, 4H), 3.55–3.63 (m, 2H), 3.06–3.23 (m, 8H), 2.75 (t, J = 6.29 Hz, 4H), 2.42–2.53 m (2H), 2.21 (t, J = 7.40 Hz, 4H), 1.84–1.96 (m, 2H), 1.62–1.76 (m, 8H), 1.421.53 (m, 4H); ^{13}C-NMR δ 174.2, 170.8, 56.4, 43.3, 40.2, 38.9, 38.6, 38.5, 35.9, 34.6, 28.9, 25.3; MS (ESI$^+$) *m/z*: 643.16 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{24}H_{43}N_4O_4S_6$ [M + H]$^+$ 643.1609 Found 643.1612.

General procedure for the coupling of LA

Methyl 2-(5-(1,2-dithiolane-3-yl)pentanamido) acetate (5)

To a solution of LA (1.5 eq, 3.6 mmol) in 10 mL of dry DMF under N_2 at 0 °C were added successively 0.5 mL of Et_3N, 1 eq of glycine methyl ester hydrochloride (2.40 mmol), and 1.5 eq (3.6 mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI•HCl). The mixture was stirred at 0 °C for further 15 min and at rt until completion (TLC monitoring). The mixture was then diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated under vacuum. The crude product was purified by flash column chromatography. Yield 63%, colorless oil; ^{1}H-NMR δ 6.02 (bs, 1H), 4.05 (d, J = 6.0 Hz, 2H), 3.76 (s, 3H), 3.57 (m, 1H), 3.07–3.19 (m, 2H), 2.41–2.49 (m, 1H), 2.26 (t, J = 7.5 Hz, 2H), 1.74–1.97 (m, 1H), 1.64–1.72 (m, 4H), 1.44–1.53 (m, 2H); ^{13}C-NMR δ 172.8, 170.5, 56.4, 52.4, 41.2, 40.2, 38.5, 36.0, 34.6, 28.8, 25.2; MS (ESI$^+$) *m/z*: 278.09 ([M + H]$^+$ 50%), 300.07 ([M + Na]$^+$ 50%); HRMS-ESI$^+$ *m/z* calcd for $C_{11}H_{20}NO_3S_2$ [M + H]$^+$ 278.0885 Found 278.0885.

2-(5-(1,2-Dithiolane-3-yl)pentanamido)acetic acid (6)

To a solution of 1.77 g. (6.4 mmol) of **5** in 24 mL of methanol/THF (1/1) was added dropwise 12 mL of a 1.0 *N* sodium hydroxide solution. The resulting mixture was stirred at rt overnight and then neutralized with a 1.0 *N* hydrochloric acid solution. The mixture was then diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated under vacuum. The crude product was purified by flash column chromatography. Yield 65%, yellow solid, mp: 206 °C; ^{1}H-NMR δ 8.09 (bs, 1H), 3.73 (d, J = 5.85 Hz, 2H), 3.63 (m, 1H), 3.12–3.26 (m, 2H), 3.39–3.49 (m, 1H), 2.16 (t, J = 7.15 Hz, 2H), 1.87–1.92 (m, 1H), 1.52–1.72 (m, 4H), 1.41 (m, 2H); ^{13}C-NMR δ 172.3, 171.3, 66.3, 56.1, 41.4, 38.0, 34.8, 34.0, 28.2, 24.9; MS (ESI$^+$) *m/z*: 286.05 ([M + Na]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{10}H_{17}NO_3S_2Na$ [M + Na]$^+$ 286.0548 Found 286.0549.

General procedure for the reduction in lipoic derivatives

To a solution of the corresponding lipoic derivatives (0.54 mmol) in 15 mL abs, EtOH was added at 0 °C $NaBH_4$ (0.08 g, 2.2 mmol), and the mixture was stirred at room temperature overnight. The reaction was quenched with H_2O, and EtOH was evaporated in vacuo. The residue was extracted with CH_2Cl_2 and H_2O. The organic layer was washed with saturated aqueous NaCl, dried over sodium sulfate, and the solvent was evaporated in vacuo. The residue was purified by flash column chromatography.

6,8-Dimercapto-*N*-(2-mercaptoethyl)octanamide (3a)

Yield 81%: colorless viscous oil. ^{1}H-NMR δ 5,81 (bs, 1H), 3.31–3.43 (m, 2H), 2.80–2.91 (m, 1H), 2.53–7.72 (m, 4H), 2.15 (t, J = 7.33 Hz, 2H), 1.75–1.80 (m, 1H), 1.33–1.60 (m, 7H), 1.22–1.33 (m, 3H); ^{13}C-NMR δ 172.8, 42.8, 42.2, 39.3, 38.7, 36.5, 26.6, 25.2, 24.7, 22.3; MS (ESI$^+$) *m/z* : 268.09 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{10}H_{22}NOS_3$ [M + H]$^+$ 268.0864 Found 268.0862.

Ethyl 2-(6,8-dimercaptooctanamido)-3-mercaptopropanoate (3b)

Yield: 90%, colorless viscous oil; ^{1}H-NMR δ 6.28 (bs, 1H), 4.82-4.92 (m, 1H), 4.23-4.32 (m, 2H), 3.05 (dd, J = 8.0 Hz, 2H), 2.85–2.96 (m, 1H), 2.63–2.74 (m, 3H), 2.28 (t, J = 7.7 Hz, 2H), 1.82–1.92 (m, 1H), 1.43–1.78 (m, 6H), 1.26–1.47 (m, 6H); ^{13}C-NMR δ 172.5, 170.1, 62.1, 56.3, 53.4, 42.7, 39.3, 38.7, 36.2, 34.6, 26.9, 25.1, 14.2; MS (ESI$^+$) *m/z*: 340.11 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{13}H_{26}NO_3S_3$ [M + H]$^+$ 340.1075 Found 340.1079.

6,8-Dimercapto-*N*-(2-((2-mercaptoethyl)amino)-2-oxoethyl)octanamide (8)

Yield 81%, white solid, mp: 108 °C; ^{1}H-NMR δ 6.36 (s, 1H), 6.12 (s, 1H), 3.95 (d, J = 5.26 Hz, 2H), 3.42–3.51 (m, 2H), 3.88–3.95 (m, 1H), 2.62–2.78 (m, 4H), 2.28 (t, J = 7.3 Hz, 2H), 1.83–1.98 (m, 1H), 1.62–1.81 (m, 4H), 1.42–1.53 (m, 3H), 1.25–1.42 (m, 3H); ^{13}C-NMR δ 173.5, 169.0, 43.4, 42.8, 42.4, 39.3, 38.7, 36.1, 26.6, 25.1, 24.5, 22.3; MS (ESI$^+$) *m/z*: 325.11 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{12}H_{25}N_2O_2S_3$ [M + H]$^+$ 325.1078 Found 325.1081.

Methyl 2-(6,8-dimercaptooctanamido)acetate (9)

Yield 74%, colorless oil; ^{1}H-NMR δ 5.88 (bs, 1H), 4.03 (d, J = 5.1 Hz, 2H,), 3.77 (s, 3H), 2.83–2.96 (m, 1H), 2.62–2.78 (m, 2H), 2.27 (t, J = 7.3 Hz, 2H), 1.86–1.96 (m, 1H), 1.63–1.82 (m, 4H), 1.42–1.53 (m, 3H), 1.28–1.38 (m, 2H); ^{13}C-NMR δ 173.2, 170.2, 52.4, 42.8, 41.2, 39.3, 38.7, 36.1, 26.6, 25.1, 22.3; MS (ESI$^+$) *m/z*: 280.10 ([M + H]$^+$ 100%); HRMS-ESI$^+$ *m/z* calcd for $C_{11}H_{22}NO_3S_2$ [M + H]$^+$ 280.1041 Found 280.1045.

Antioxidant activity

UV spectra were recorded on a Varian Cary 300 Bio UV/Vis spectrometer.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The scavenging effect of the synthesized compounds on the DPPH radical was evaluated according to previously

published methods (34,35). Various concentrations of the test compounds were incubated in 1 mL of a 60% ethanolic solution containing the DDPH radical (60 μM). The mixture was shaken vigorously and allowed to stand for 30 min; absorbance at 517 nm was determined, and the percentage of activity was calculated by the following equation:

$$\% \text{ inhibition} = \left(\frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}}\right) \times 100.$$

All tests and analyses were undertaken in triplicate and the results averaged.

ABTS$^{\bullet+}$ radical cation scavenging assay

The scavenging effect of the synthesized compounds on the ABTS radical cation (ABTS$^{\bullet+}$) was evaluated according to previously published methods (36). The radical cation was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate (final concentration). This mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The ABTS$^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. Diluted ABTS$^{\bullet+}$ solution (0.9 mL) and 100 μL of various concentrations of the test compounds were mixed and measured immediately after 6 min at 734 nm. The antioxidant activity was calculated using the previous equation.

Chelation activity on Fe^{2+}

The synthesized compounds were evaluated for their ability to compete with ferrozine for iron (II) ions in solution according to previously published methods (37). Various concentrations of the test compounds (1 mL in water) were added to a solution of 2 mM $FeCl_2.4H_2O$ (0.4 mL). The reaction was initiated by the addition of 2.5 mM ferrozine (0.1 mL); the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured at 562 nm against the blank performed in the same way using $FeCl_2$ and water. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following equation:

$$\% \text{ Chelating activity} = \left(\frac{1 - A_{\text{test}}}{A_{\text{control}}}\right) \times 100.$$

Biological evaluation

Reagents

All chemicals used throughout this study were of the highest analytical grade, purchased from Sigma Chemical Company, Milan, Italy, unless otherwise specified.

Cell culture

The T67 human glioma cell line was derived by Lauro *et al.*(38) from a World Health Organization Grade III gemistocytic astrocytoma. T67 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Cambrex BioScience, Belgium), supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 100 UI/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL gentamicin, in a 5% CO_2 atmosphere at 37 °C, with saturating humidity. Cell stocks were cryopreserved by standard methods and stored in liquid nitrogen. Cell viability was measured by trypan blue exclusion (21).

To evaluate the antioxidant activity of the compounds, T67 cells were seeded in 24-well plates at 1 × 105 cells/well. Experiments were performed after 24 h of incubation at 37 °C in 5% CO_2. After this time cells were washed and treated for 24 h with the compounds, the antioxidant activity was evaluated after 30 min of incubation with 10 μM fluorescent probe (2′,7′-dichlorofluorescein diacetate, DCFH-DA) in DMEM, by measuring the intracellular ROS formation evoked by 30-min exposure of T67 cells to 100 μM tert-butyl hydroperoxide (*t*-BuOOH) in PBS. The fluorescence increase in the cells from each well was measured (λ_{exc} = 485 nm; λ_{em} = 535 nm) with a spectrofluorometer (Wallac Victor multilabel S9 counter; Perkin-Elmer Inc., Boston, MA, USA). Data are reported as the mean ± standard deviation of at least three independent experiments (21).

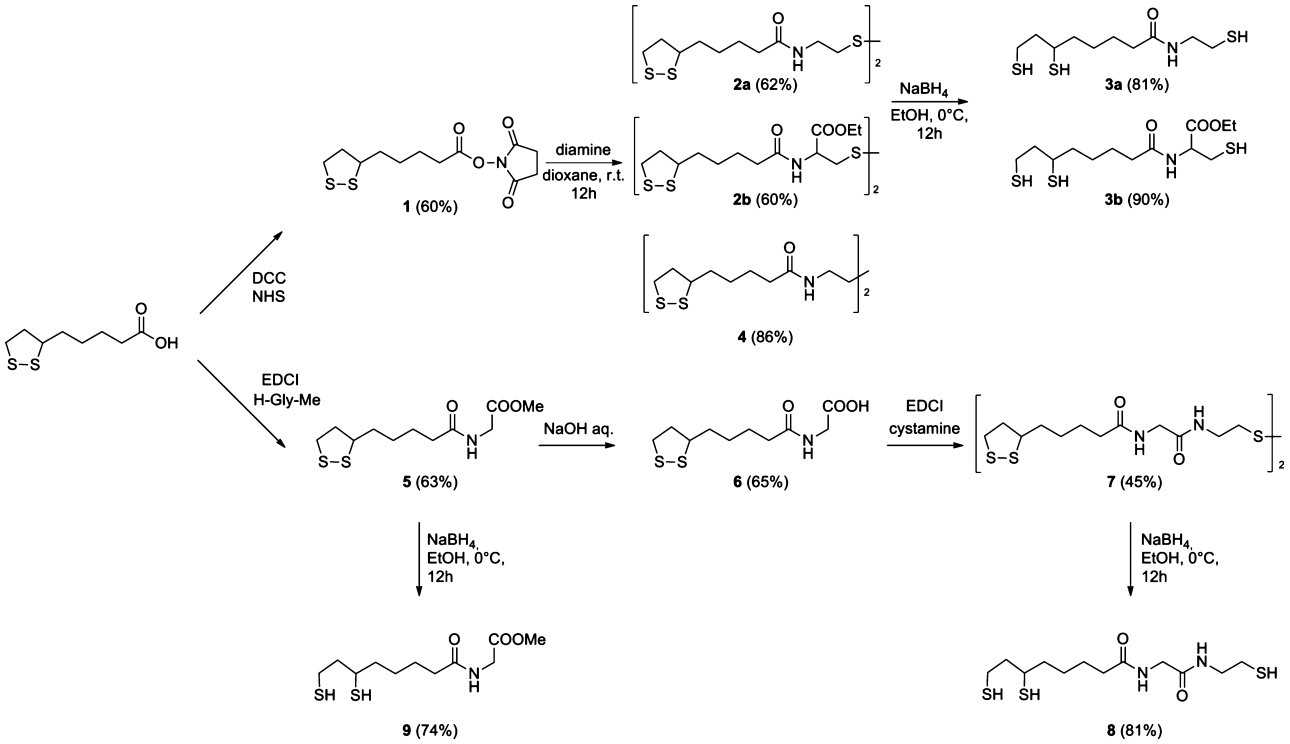
Results and discussion

Chemistry

The syntheses of the target compounds are shown in Scheme 1. In details, the carboxylic acid functionality of LA was coupled to different sulfur-containing amines. Hybrid structures **2a**, **2b,** and **4** were prepared from *N*-hydrosuccinimide-activated LA (39) and cystamine, L-cystine diethyl ester, and butanediamine, respectively. Reduction in the disulfide bonds in **2a** and **2b** using $NaBH_4$ yielded the corresponding trithiols **3a** and **3b**. Compound **3b** thus features an unprecedented LA-cysteine core structure. To obtain a better understanding of the structure–activity relationship and to probe the influence of the added disulfide unit, LA was also coupled with glycine methyl ester upon activation of LA in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI). The corresponding ester **5** was then hydrolyzed in the presence of sodium hydroxide and further coupled with cystamine leading to the original bis-lipoyl derivative **7**. Compounds **5** and **7** were then reduced using $NaBH_4$ affording the desired free thiols **9** and **8**, respectively (Scheme 1).

Free radical scavenging activities

First, we evaluated the antioxidant activity of these compounds using the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) (34) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$) (36) antioxidant assays. For that purpose, various concentrations of the test compounds were



Scheme 1: Synthesis of the target compounds

incubated for 30 min in a solution containing the stable free radical. The antioxidant activities shown in Table 1 are expressed as the 50% inhibitory concentration (IC_{50}) based on the amount of compound required for a 50% decrease in the initial free radical concentration. The results obtained were compared with LA, DHLA, and two well-known antioxidants, Trolox (a polar analogue of vitamin E) and ascorbic acid. Both assays are valuable for ranking the potency of an antioxidant. In this context, we observed that the relative IC_{50} values obtained agreed well with one another. Overall, it was found that while free thiols are necessary for maximal free radical scavenging efficacy, a third sulphydryl group does not seem to be crucial. Indeed, IC_{50} values of compounds **3a, 3b,** and **8** are comparable with those obtained with DHLA. However, while almost no difference was observed depending on the nature of the *N*-amido group inside this series, we found that coupling the carboxylic acid moiety present in DHLA with glycine methyl ester (**9**) induces a slight decrease in activity. In general, the results indicate that free sulphydryl groups confer to these molecules interesting free radical scavenging properties similar to those of DHLA, Trolox, and ascorbic acid, thus validating a multi-antioxidant approach with compounds covering both lipophilic and hydrophilic areas of the cell.

Table 1: Antioxidant activities of synthesized product derivatives **2a**–**9** in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging assays

Compounds	$DPPH^{\bullet}$ IC_{50} (μM)	$ABTS^{\bullet+}$ IC_{50} (μM)
2a	>100	>100
2b	80	>100
3a	23	10
3b	29	9.4
4	>100	>100
5	>100	>100
6	>100	>100
7	>100	>100
8	26	8.9
9	33	15.2
Lipoic acid	>100	>100
Dihydrolipoic acid	22	11
Trolox	20	6.2
Ascorbic acid	19	8.8

Iron chelating assay

To further explore the potential neuroprotective properties of these compounds, we investigated their ability to chelate ferrous ions. These ions play a critical role in the free radicals formation, as they can convert hydrogen peroxide to hydroxyl radicals through the Fenton reaction and many drug discovery programs are devoted to the development of efficient iron chelators (40). Thus, the ability of molecules to chelate Fe^{2+} ions is a practical parameter to assess their antioxidant effects. The Fe^{2+} chelating activities of the synthesized compounds were assessed by differences in absorbance at 562 nm, with the appearance of an absorption peak upon reaction of Fe^{2+} with ferrozine and compared with the results obtained

EDTA. However, none of the lipoyl derivatives exhibited a chelating capacity comparable to the positive control, EDTA-2Na (EC_{50} = 32 μM). Observed values ranged from 300 to 3000 μM without any indication of structure–activity relationships (data not shown). These results were rather surprising as iron(II) is a medium soft Lewis acid and prefers ligation to soft Lewis base donors like thiols. While complexes of iron(II) and iron(III) with LA and DHLA have been reported, iron binding is not as effective as iron reduction (41). Ferrozine is acknowledged as an effective chelator of ferrous iron and has been used for the determination of iron in biological samples and none of the synthesized compounds was able to compete with the ferrozine–Fe^{2+} preformed complex.

Biological activities

To explore the antioxidant potential of the synthesized compounds in a cellular context, cell viability and neuroprotective activity against an oxidative insult were assayed in the human astrocytoma cell line T67, in comparison with LA as reference compound.

Cytotoxic effects on T67 cells

To assess the potential cytotoxicity, we used the MTT assay to examine the viability of T67 cells treated with three doses of the compounds (0.5, 1, and 5 μM). Treatment concentrations of 0.5 μM had no effects on cell viability, while the viability of T67 cells treated with 5 μM was decreased by 40–70% (Figure 2). Notably, LA was not toxic to the same cell line up to a concentration of 50 μM (data not shown).

Neuroprotective effects on T67 cells

To mimic oxidative stress conditions, ROS production in T67 cells pretreated with the test compound has been stimulated by treatment with the radical initiator tert-butyl hydroperoxide (TBHP). Then, to measure the intracellular ROS levels, the DCFDA fluorescent probe was used. Initially, all the synthesized compounds were used at a concentration that did not affect cell viability (i.e., 500 nM), and the antioxidant profile is reported in Figure 3A. Surprisingly, at this concentration, nearly all compounds only slightly counteracted the evoked oxidative stress. For the DHLA derivatives **3b** and **9**, even an increase in ROS formation was evident. This behavior and the high standard deviations made us postulating that some toxic effects could take place and mask the antioxidant activity. Thus, to rule out this possibility, we repeated the same experiment, but using a lower (less toxic) concentration of tested compounds (100 nM). However, in this case, no significant protection against oxidative stress by the synthesized conjugates was detected (Figure 3B). This outcome was better appreciated for compound **6**, which was tested in a wider range of concentrations: at 125 and 250 nM a dose-dependent effect could be seen, but at the higher concentrations it is highly feasible that toxic effects may counterbalance the beneficial ones (Figure 4A).

Actually, these findings are not surprising, albeit disappointing. In fact, we evaluated LA antioxidant capacities under the same experimental conditions. As evident from the graph of Figure 4B, LA starts to display an antioxidant effect at a concentration of 5 μM, which is a tenfold higher concentration than that used for our compounds (500 nM). A 50% reduction in ROS formation was achieved only at a LA concentration of 50 μM. Of course, this was possible thanks to the minimal cytotoxicity shown by LA in this cell line.

Thus, we can conclude that in the tested conditions, **2a**–**9** were found to be weak antioxidants. In this respect, it should be noticed that, despite it has been clearly demonstrated that LA/DHLA supplementation decreases oxidative stress and restores reduced levels of other antioxidants *in vivo*, there is also evidence indicating that they may exert pro-oxidant properties *in vitro* (42). The ability of LA and/or DHLA to act as either anti- or pro-oxidants, at least in part, has been deemed dependent on

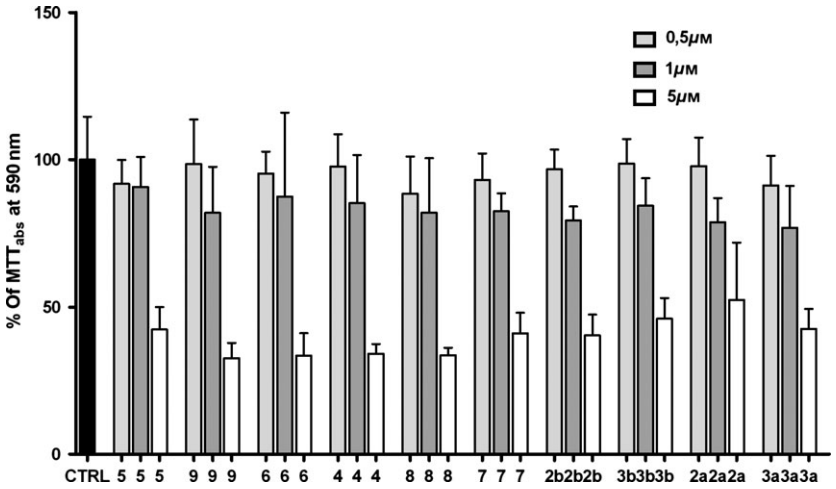


Figure 2: Effects of compounds on cell viability in T67 cells. The cell viability was determined by the MTT assay (as described in Methods and Materials) after 24 h of incubation with compounds' concentrations of 0.5, 1, and 5 μM. The results were expressed as a percentage of control cells. Values are reported as the mean ± SD of three independent experiments.

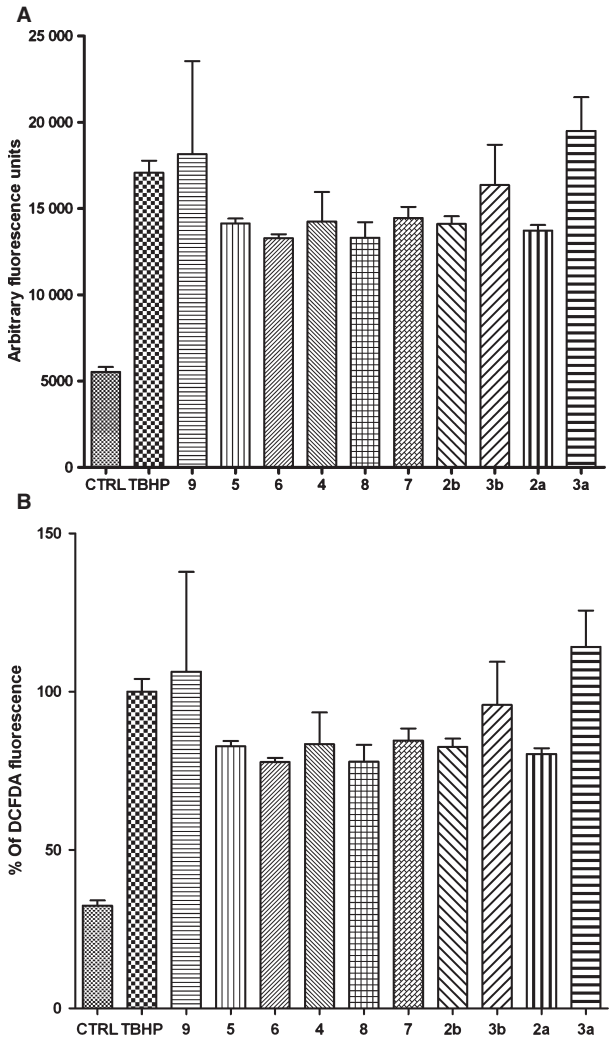


Figure 3: Effects of the lipoic acid conjugate on intracellular ROS formation in T67 cells at two different concentrations: 500 nM in (A) and 100 nM in (B). The results are expressed as percentage increase in intracellular ROS evoked by exposure to tert-butyl hydroperoxide (TBHP) for 30 min. Values are the mean ± SD of three independent experiments.

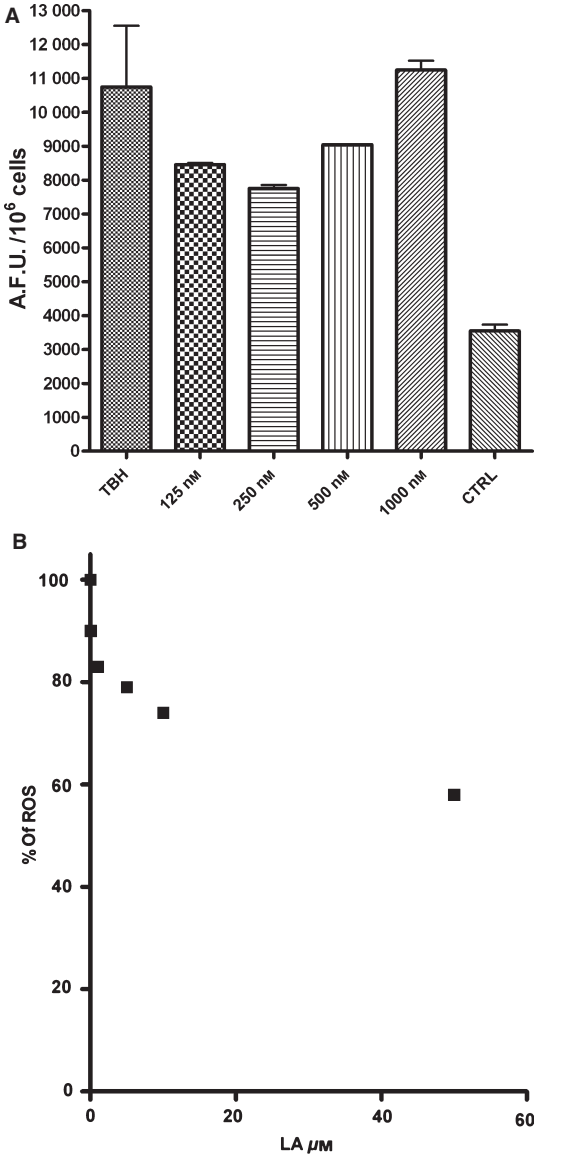


Figure 4: (A) Effects of different concentrations of **6** on ROS production in T67 cells exposed to 100 μM TBHP. The results, expressed as percentage of the control, are means ± SD of three independent experiments. (B) Dose-dependent ROS scavenging effects of lipoic acid (LA) in T67 cells exposed to 100 μM TBHP. The results, expressed as percentage of the control, are means ± SD of three independent experiments.

the type of oxidant stress and the physiological circumstances. These pro-oxidant actions suggest that LA and DHLA act by multiple mechanisms, many of which are only recently being explored. Thus, it has been proposed that the antioxidant properties of LA and DHLA should be tested experimentally in different oxidative stress conditions. The same might apply to our compounds, and this point will be taken into due consideration in the next endeavors on these and analogous compounds.

Conclusion

In conclusion, a new series of LA derivatives were synthesized and evaluated as potential antioxidants. *In vitro*, these analogues showed remarkable free radical scavenging abilities, similar to those of well-known antioxidants such as Trolox and ascorbic acid. The results obtained in the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays highlighted the importance of sulphydryl functional groups for maximal free radical scavenging efficacy. However, this promising *in vitro* profile was not mirrored by the antioxidant activity in T67 cell line. In fact, all compounds when tested at a concentration of 500 nM displayed marginal antioxidant capacities, while they seemed to develop some cytotoxicity.

We suggest that these results do not discard the idea of combining the LA moiety with those of other therapeutically relevant antioxidants. Conversely, they might serve as the basis for developing new LA-based hybrids that could exert beneficial effects against the oxidative processes underlying AD, following the fine optimization of their toxicity properties.

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Conflict of Interest

The authors declare no conflict of interest.

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