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Antioxidant and antiplatelet effect of nanodispersions with conjugate of glutathione and lipoic acid

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Materials

The following materials were used in the work: acetic acid, triethylamine, toluene, ethanol, methanol, dichloromethane, chloroform, ethyl acetate, hexane, tetrahydrofuran (ChemMed, Russia).

Triethylamine and toluene were distilled over calcium hydride. Ethanol and tetrahydrofuran were distilled over potassium hydroxide. Dichloromethane was distilled over phosphorus pentoxide. Ethyl acetate was distilled over potash. Chloroform was distilled over calcium chloride.

The following reagents were used: soy phosphatidylcholine Lipoid S-100, 94% purity (Lipoid GmbH, Germany), α-lipoic acid, glutathione, triphenylmethanol, boron trifluoride etherate, Pluronic F68, N-hydroxysuccinimide, triisopropylsilane, trifluoroacetic acid, luminol, phorbol-12-myristate-13-acetate (Sigma–Aldrich, USA), dicyclohexylcarbodiimide (Lancaster, England), 4-dimethylaminopyridine (Merck, Germany), Hanks' solution without phenol red, ficoll–verografin with specific densities of 1.119 and 1.078 g/cm³ (PanEco, Russia).

The N-hydroxysuccinimide ester of lipoic acid **3** was obtained using a well-known method [S1].

^{1}H-NMR spectra were recorded on a Bruker DPX-300 pulsed NMR spectrometer (Germany) with an operating frequency of 300 MHz in deuterated solvents. The δ-values of the protons were measured relative to the ^{1}HNMR solvents ($CDCl_3$, δ=7.26 ppm, $(CD_3)_2SO$=2.50 ppm, D_2O=4.79 ppm).

The morphology of the nanoparticles was determined using a Merlin electron microscope (Zeiss, Germany) with a SE2 back-scattered electron detector and a STEM transmission detector.

For venous blood sampling, standard Improvacuter® test tubes with lithium heparin (China) were used, cat. No. 652040112 and with sodium citrate 3.8%, cat. No.632452112. Cell cultures were isolated using a CM-6MT multilift centrifuge (ELMI, Latvia).

Chemiluminescence of neutrophils was recorded at room temperature using a Lum-1200 chemiluminometer (Interoptika-S, Russia). The chemiluminescence spectra were processed using the Power Graph 3.3 Professional software. Platelet aggregation was registered using a four-channel AggRAM aggregometer (Helena Laboratories Bioscience, USA).

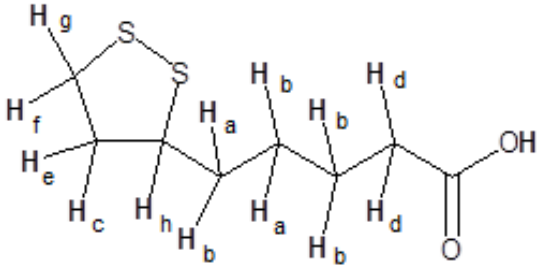
High-resolution mass spectra were recorded on an Agilent 1100 chromatograph (Agilent Technologies, USA) using an Agilent 6340 Ion Trap mass spectrometric detector with electrospray ionization and an ELS detector.

TLC was performed on Sorbfil plates (Sorbpolymer CJSC, Russia) in the chloroform:methanol:water system (6:4:0.8).

Detection of spots on chromatograms was carried out in UV light by treatment with the [FMC + Ce2SO4] complex followed by burning at 200 °C.

Column chromatography was performed on Silica gel (0.040–0.063 mm) (Merck, Germany).

We used the following designations for the hydrogen atoms in lipoic acid:



Methods

S-tritylglutathione 2

A solution of 1.0 g (3.25 mmol) of glutathione **1**, 0.85 g (3.25 mmol) of triphenylmethanol and 0.51 ml (3.56 mmol) of boron trifluoride etherate in glacial acetic acid (100 ml) was stirred at 85°C for 30 minutes. Then it was stirred for 60 minutes at room temperature, cooled water (100 ml), sodium acetate (1.0 g) and again water (50 ml) were added to the reaction mixture until a milky-white suspension appeared, the precipitated white crystals were filtered off, washed with water, acetone, diethyl ether and purified by crystallization from acetone.

Yield of compound **2**: 1.27 g (71.1%, amorphous), R_f 0.39.

^{1}H-NMR spectrum (DMSO-d_6, δ, ppm): 1.78-1.95 (m, 2H, Gluβ), 2.26-2.35 (m, 2H, Gluγ), 2.37- 2.41 (m, 2H, Cysβ), 3.51-3.66 (m, 3H, Gluα, Glyα), 4.19-4.38 (m, 1H, Cysα), 7.18-7.35 (m, 15H, Tr), 8.33-8.50 (m, 2H, Gly-NH, Cys-NH).

N-lipoyl-S-tritylglutathione 4

A solution of 500 mg (0.91 mmol) of S-tritylglutathione **2**, 552 mg (1.82 mmol) of N-hydroxysuccinimide ester of lipoic acid **3** and 0.89 ml (6.37 mmol) of triethylamine in anhydrous methylene dichloride (100 ml) was boiled for 9 hours, evaporated and purified by column chromatography on silica gel, eluting compound **4** with chloroform:methanol system (20% methanol).

Yield of compound **4** 381 g (56.7 %, amorphous), Rf 0.71.

^{1}H-NMR spectrum (DMSO-d_6, δ, ppm): 1.21-1.39 (m, 2H, Ha), 1.41-1.59 (m, 4H, Hb), 1.74-1.95 (m, 3H, Hc, Gluβ), 2.01-2.16 (m, 5H, Gluγ, Hd, He), 2.27-2.45 (m, 2H, Cysβ), 3.02-3.33 (m, 2H, Hf, Hg), 3.46-3.61 (m, 3H, Glyα, Hh), 3.93-4.02 (m, 1H, Gluα), 4.11-4.24 (m, 1H, Cysα), 7.18-7.35 (m, 15H, Tr), 8.33-8.50 (m, 2H, Gly-NH, Cys-NH).

N-lipoylglutathione 5

A solution of 300 mg (0.41 mmol) of the protected conjugate of lipoic acid and glutathione **4**, 0.42 ml (2.04 mmol) of triisopropylsilane and 0.15 ml (2.04 mmol) of trifluoroacetic acid in methylene dichloride (50 ml) was stirred for 2 hours at room temperature, concentrated under vacuum, re-evaporated with toluene, ethanol and methylene dichloride. The residue was purified by column chromatography on silica gel in the system chloroform:methanol (30% methanol).

Yield of compound **5** 91 mg (44.6%, amorphous), Rf 0.67.

^{1}H-NMR spectrum (D_2O, δ, ppm): 1.28-1.46 (m, 2H, Ha), 1.48-1.71 (m, 4H, Hb), 1.78-1.97 (m, 2H, Gluβ), 1.99-2.13 (m, 1H, Hc), 2.17-2.48 (m, 5H, Gluγ, Hd, He), 2.79-2.87 (m, 2H, Cysβ), 3.05-3.22 (m, 2H, Hf, Hg), 3.52-3.74 (m, 3H, Glyα, Hh), 4.07-4.18 (m, 1H, Gluα), 4.44-4.53 (m, 1H, Cysα).

HRMS (ESI) calcd. for $C_{18}H_{29}N_3O_7S_3$ [$M+H^+$]: 496.4, found: 496.4

Preparation of Nanodispersions

6 mg of the Glutathione and Lipoic Acid Conjugate was dispersed in an aqueous solution of Pluronic F68 (10 mg/ml, 2 ml). The resulting nanodispersion was stirred for 10 minutes at room temperature.

Determination of Nanodispersion Morphology

Nanoparticles were visualized using scanning and transmission electron microscopy. For scanning microscopy, samples of the nanoparticle suspension were applied to a microscope slide, dried at room temperature, and then Au/Pd coating with a layer thickness of 5 nm was applied under high vacuum conditions.

For transmission microscopy, the nanoparticle suspension was applied to grids coated with formvar film, dried at room temperature, and graphite was deposited on the sample surface under

high vacuum. Deposition was performed using the Quorum Q150T ES sample preparation system (Quorum Technologies, UK).

Isolation of Neutrophils from Blood

To isolate neutrophils, we used blood from apparently healthy donors (male and female) aged 20–30 years (n = 10). For venous blood sampling, standard Improvacuter® test tubes with lithium heparin were used. Neutrophils were isolated from the leukocyte suspension in a double density gradient of ficoll-verografin solutions. Ficoll-verografin with specific densities of 1.119 and 1.090 g/cm³ was carefully layered into sterile polystyrene tubes, followed by venous blood in a ratio of 1.5 : 1.5 : 2. After centrifugation for 45 minutes at 135 g, the formed blood elements were divided into three fractions: red blood cells, neutrophils, and monocytes. The neutrophil fraction was washed from ficoll-verografin with a sterile Hanks' solution (pH 7.4) and centrifuged for 10 minutes at 135 g. If red blood cells were found in the neutrophil fraction, the red blood cells were lysed by adding distilled water (1 ml) to the precipitate. After all washes, the cells were carefully resuspended in Hanks' solution and the number of neutrophils was determined in a Goryaev chamber. The cells were stored at 4 °C for no more than 4 hours [S2].

Chemiluminescence of neutrophils

To the suspension of neutrophils (1.5×10^5 cells), nanodispersions with GSH-LA (0.75, 1, 1.2 or 1.5 mM in the sample) were added and incubated for 10 minutes at T=37°C. To activate the luminescence, luminol (0.01 mM in the sample) was introduced into the system. The initiation of luminescence was caused by the introduction of PMA solution (1.6×10^{-6} mM in the sample), and the chemiluminescence kinetics were recorded for 30 minutes (T=37 °C). The chemiluminescence spectra were processed using the Power Graph 3.3 Professional software.

Isolation of platelets from blood

Platelets were isolated from blood samples obtained from apparently healthy donors (male and female) aged 20–30 years (n = 10). Standard Improvacuter® tubes with an anticoagulant (sodium citrate, 3.8%) were used for venous blood sampling. Platelet-rich plasma (PRP) was obtained by centrifuging the blood for 10 minutes at 135 g at room temperature.

Investigation of the platelet aggregation process

Samples with PRP were supplemented with nanodispersions containing a conjugate of glutathione and lipoic acid (0.75, 1, 1.2 or 1.5 mM in the sample) and incubated for 10 minutes at T = 37 °C. Then, an inducer of platelet aggregation — ADP (0.23 mM in the sample), was added to the samples. The aggregatogram was recorded for 10 minutes.

Statistical analysis

The statistical processing of the results was carried out using the STATISTICA 6 application software package (StatSoft Corporation, USA). The Kruskal–Wallis ANOVA statistical criterion was used to analyze differences in quantitative traits in three or more unrelated groups. Differences were considered significant at $p<0.05$. The results in Table 1 are presented as mean values and standard deviations.

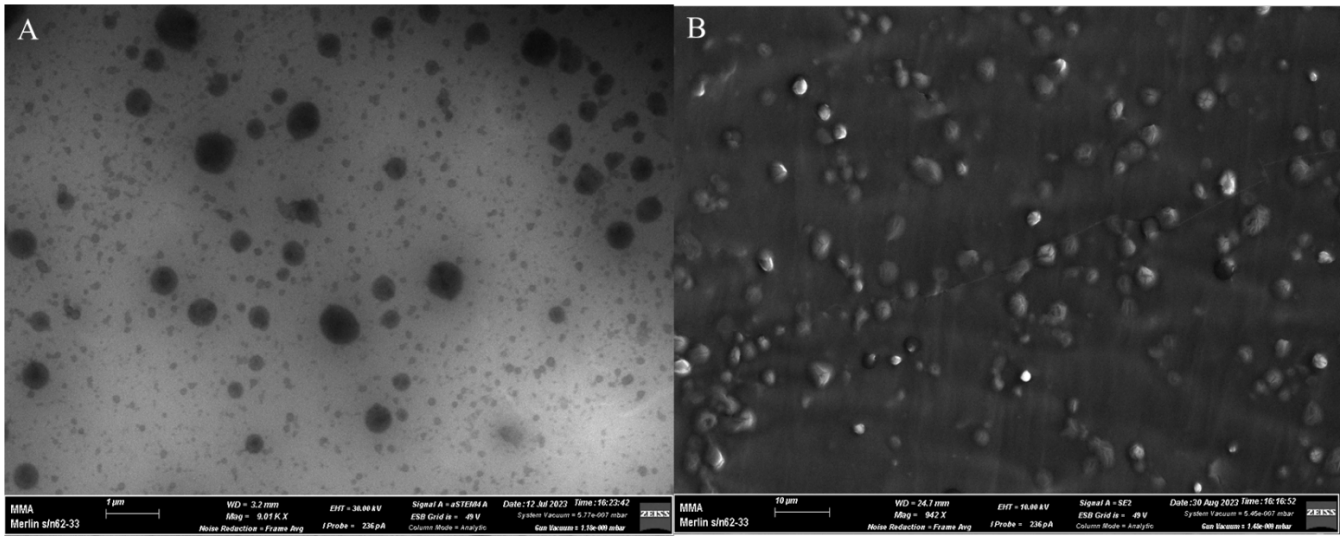


Figure S1. Electron micrographs of nanodispersions with GSH-LA: **A** - TEM, **B** - SEM.

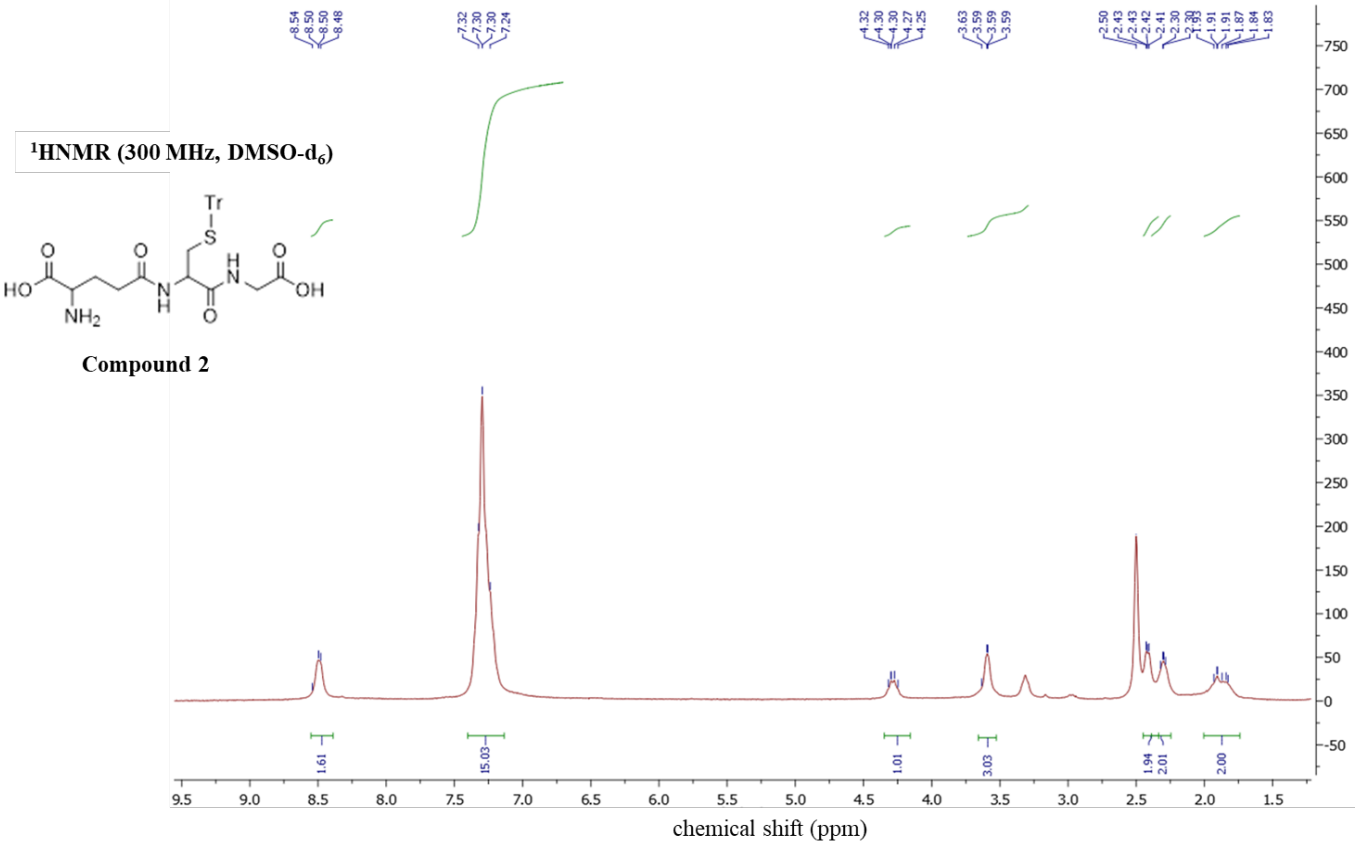


Figure S2. ^{1}H NMR of compound **2**

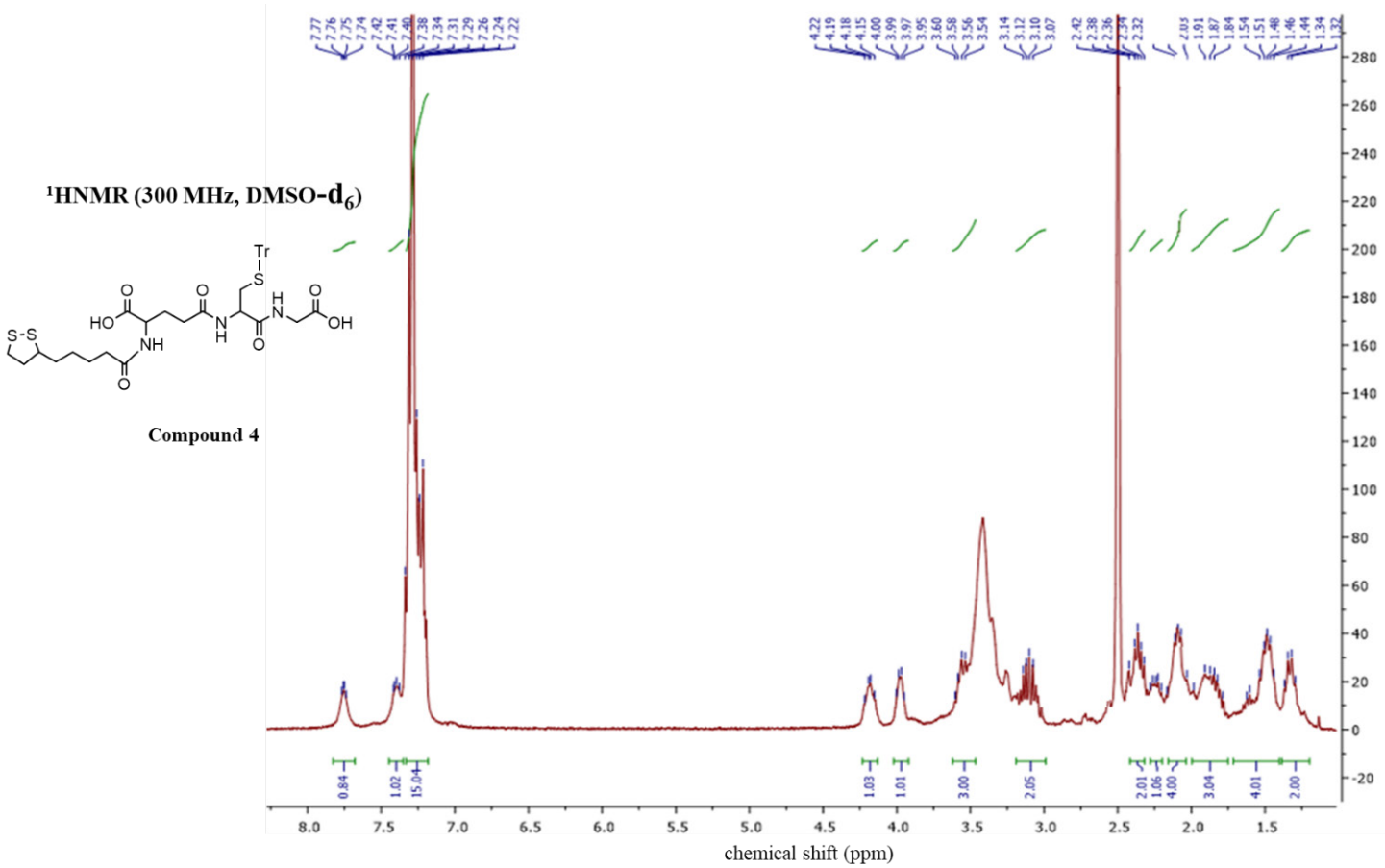


Figure S3. ^{1}H NMR of compound **4**

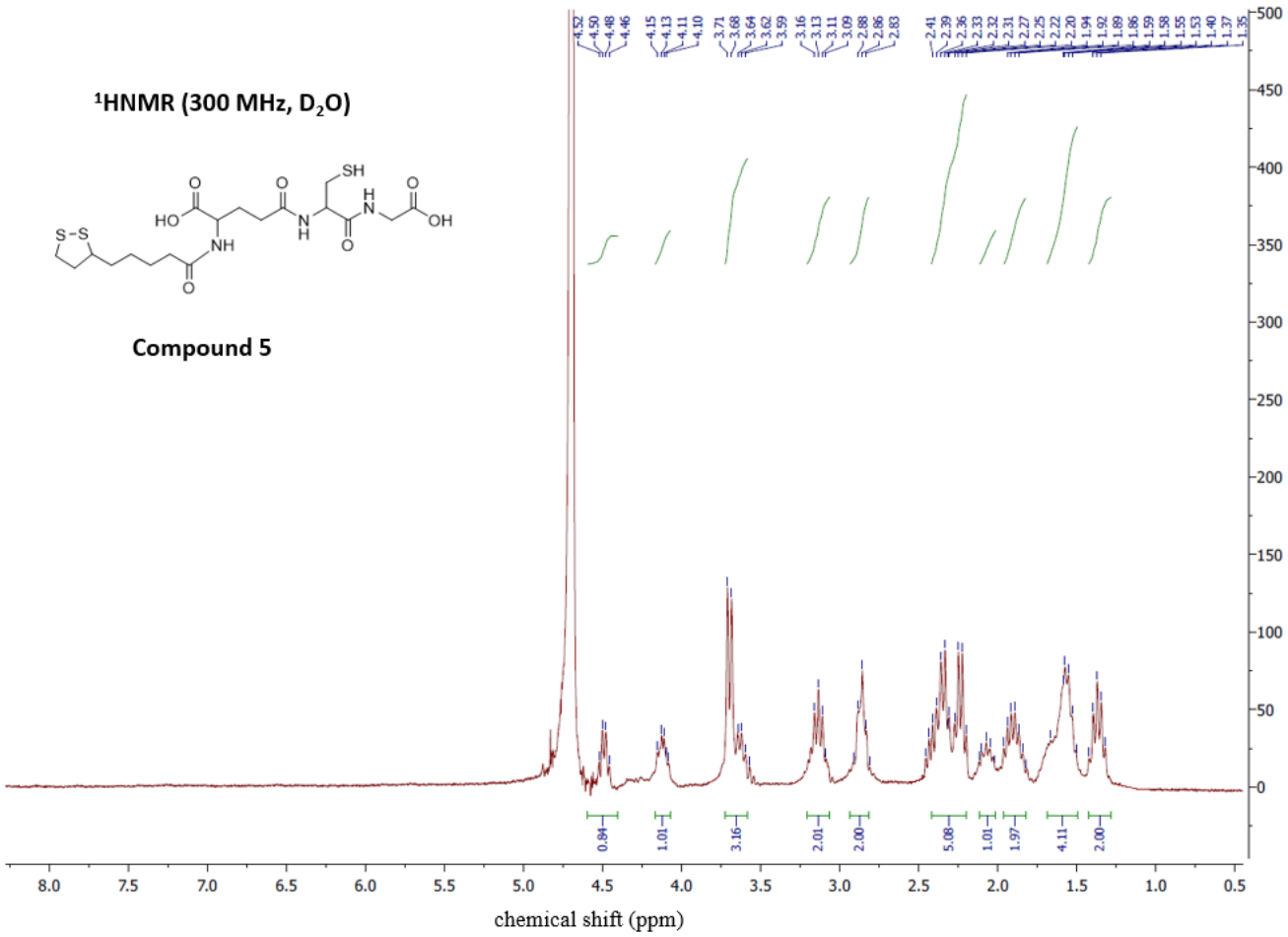


Figure S4. ^{1}H NMR of compound **5**

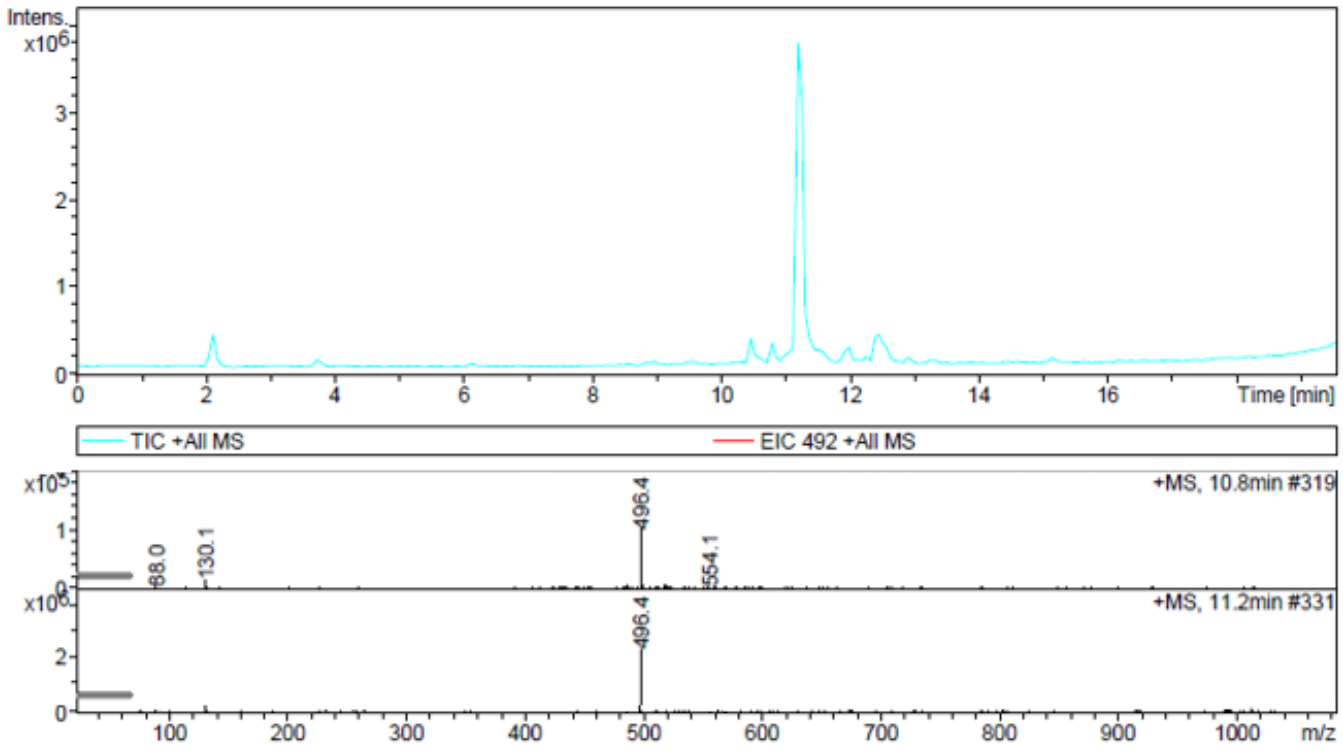


Figure S5. Chromato-mass spectrum of GSH-LA **5**.

References

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S2. V. A. Shchelkonogov, A. M. Inshakova, E. S. Darnotuk, A. V. Shipelova, A. V. Chekanov, O. A. Baranova, N. S. Shastina, E. Yu. Solov'eva and A. I. Fedin, *Russ. J. Bioorganic Chem*., 2023, **6**, 319; https://doi.org/10.1134/S1068162023060122.