Electronic supplementary materials *Mendeleev Commun.*, 2021, **31**, 507–508

Nanoparticles of lipoic acid esters: preparation and antioxidant effect

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General

The ^{1}H NMR spectra were recorded using a Bruker DPX-300 (Germany) pulse NMR spectrometer with an operating frequency of 300 MHz in deuterated solvents; the δ values of the protons were measured in relation to the ^{1}H NMR solvents ($CDCl_3$, δ = 7.26 ppm, $(CD_3)_2SO$, δ = 2.50 ppm). The mass spectrometry was performed using a Bruker autoflex speed MALDI-TOF mass spectrometer (Germany).

The TLC was performed on Sorbfil plates (ZAO Sorbpolymer, Russia) using the following solvent systems:

(a) chloroform : methanol (9.9 : 0.1)

(b) hexane : ethyl acetate (6 : 4)

(c) hexane : ethyl acetate (7 : 3)

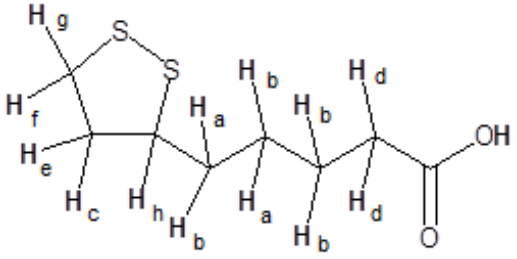
(d) chloroform

The spots on the chromatographic plates were detected using UV light, treatment with 10% H_2SO_4 in MeOH, or the [phosphomolybdic acid + Ce_2SO_4] complex with subsequent charring at 200°C. The column chromatography was performed using Silica gel (0.040–0.063 mm, Merck, Germany).

The particle sizes were determined using dynamic light scattering on a Delsa Nano C analyzer (Beckman Coulter Inc., United States); the ζ-potential, using electrophoretic light scattering on a Zetasizer Nano ZS (Malvern, United States). The nanoparticle morphology was studied using a JEM-100CX electron microscope (JEOL, Japan) at the UNIQEM Center for Collective Use (Biotechnology Federal Research Center, Russian Academy of Sciences).

The reagents used in this study were as follows: soybean phosphatidylcholine Lipoid S-100, purity 94% (Lipoid GmbH, Germany); lipoic acid, hexane-1,6-diol, Pluronic F68, 1,3-(dipalmitoylamino)propan-2-ol, luminol, phorbol-12-myristate-13-acetate (Sigma–Aldrich, United States); DMAP (Merck, Germany), DCC (Lancaster, United Kingdom); propane-1,3-diol, *myo*-inositol, glycerol, ion-exchange resin Dowex H^+ (Chimmed, Russia); Hanks' solution without phenol red, ficoll solution (PanEco, Russia); the solvents: ethanol, methanol, dichloromethane, ethyl acetate, hexane (Chimmed, Russia).

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



Methods

2-*O*-Lipoyloxy-1,3-(dipalmitoylamino)propane (3a, LADPAP**)**

A solution of LA **1** (148 mg, 0.71 mmol), 1,3-(dipalmitoylamino)propan-2-ol **2**a (200 mg, 0.35 mmol), DMAP (128 mg, 1.05 mmol) and DCC (174 mg, 0.84 mmol) in CH_2Cl_2 (30 ml) was stirred at room temperature for 24 h in the dark, filtered, and evaporated. The residue was purified by column chromatography on silica gel eluting of compound **3a** with a chloroform–methanol system (1% methanol). The yield was 240 mg (90%, oil), R_f 0.20 (a).

^{1}H NMR (300.0 MHz, $CDCl_3$): 0.90 (t, J = 6.6 Hz, 6H, $2CH_3$, Pam), 1.27 (s, 48H, $24CH_2$, Pam), 1.45–1.54 (m, 2H, 2H**a**), 1.60–1.76 (m, 8H, 4H**b**, 2β-CH_2, Pam), 1.88–1.99 (m, 1H, H**c**), 2.23 (t, J = 7.6 Hz, 4H, 2α-CH_2, Pam), 2.34 (t, J = 7.3 Hz, 2H, 2H**d**), 2.43–2.54 (m, 1H, H**e**), 3.09–3.24 (m, 2H, H**f**, H**g**), 3.26–3.45 (m, 2H, CH_2, Prop), 3.47–3.68 (m, 3H, H**h**, CH_2, Prop), 4.80–4.90 (m, 1H, CH, Prop), 6.22 (dd, J_1 = 3.2, J_2 = 6.6 Hz Hz, 2H, 2NH). MS (MALDI-TOF) m/z: calcd. for $C_{43}H_{82}N_2O_4S_2$ 755.260; found 778.684 [M+Na^+].

1,3-Di-*O*-lipoyloxypropane (3b, LA_2Prop**)** was obtained similarly to compound **3a**: from LA **1** (200 mg, 0.97 mmol), propane-1,3-diol **2b** (30 mg, 0.39 mmol), DMAP (178 mg, 1.46 mmol) and DCC (239 mg, 1.16 mmol). Compound **3b** was isolated by column chromatography on silica gel eluting with a hexane–ethyl acetate system (20% ethyl acetate). The yield was 153 mg (85%, oil), R_f 0.74 (b).

^{1}H NMR (300.0 MHz, $CDCl_3$):1.42–1.55 (m, 4H, 4H**a**), 1.60–1.77 (m, 8H, 8H**b**), 1.87–2.03 (m, 4H, 2H**c**, CH_2, Prop), 2.34 (t, J = 7.4 Hz, 2H, 4H**d**), 2.43–2.54 (m, 2H, 2H**e**), 3.09–3.25 (m, 4H, 2H**f**, 2H**g**), 3.54–3.63 (m, 2H, 2H**h**), 4.17 (t, J = 6.3 Hz, 4H, $2CH_2$, Prop). MS (MALDI-TOF) m/z: calcd. for $C_{19}H_{32}O_4S_4$ 452.700; found 475.280 [M+Na^+].

1,6-Di-*O*-lipoyloxyhexane (3c, LA_2Hx**)** was obtained similarly to compound **3a** from LA **1** (200 mg, 0.97 mmol), hexane-1,6-diol **2c** (46 mg, 0.39 mmol), DMAP (178 mg, 1.46 mmol) and DCC (239 mg, 1.16 mmol). Compound **3c** was isolated by column chromatography on silica gel eluting with a hexane–ethyl acetate system (15% ethyl acetate). The yield was 169 mg (85%, oil), R_f 0.82 (c).

^{1}H NMR (300.0 MHz, $CDCl_3$): 1.39–1.42 (m, 4H, $2CH_2$, Hx), 1.45–1.56 (m, 4H, 2H**a**), 1.64–1.74 (m, 12H, $2CH_2$ Hx, 8H**b**), 1.88–1.99 (m, 2H, 2H**c**), 2.34 (t, *J* = 7.4 Hz, 4H, 4H**d**), 2.44–2.54 (m, 2H, 2H**e**), 3.10–3.25 (m, 4H, 2H**f**, 2H**g**), 3.55–3.64 (m, 2H, 2H**h**), 4.09 (t, 4H, *J* = 6.7 Hz, $2CH_2$, Hx). MS (MALDI-TOF) *m*/*z*: calcd. for $C_{22}H_{38}O_4S_4$ 494.780; found 517.270 [M+Na^+].

1,2,3-Tri-*O*-lipoylglycerol (3d, LA_3Gro**)** was obtained similarly to compound **3a** from LA **1** (200 mg, 0.97 mmol), glycerol **2d** (24 mg, 0.26 mmol), DMAP (178 mg, 1.46 mmol) DCC (239 mg, 1.16 mmol). Compound **3d** was isolated by column chromatography on silica gel eluting with a hexane–ethyl acetate system (20% ethyl acetate). The yield was 115.6 mg (67%, oil), R_f 0.78 (d).

^{1}H NMR (300.0 MHz, $CDCl_3$): 1.44–1.58 (m, 6H, 6H**a**), 1.64–1.77 (m, 12H, 12H**b**), 1.88–2.00 (m, 3H, 3H**c**), 2.37 (t, *J* = 7.3 Hz, 6H, 6H**d**), 2.44–2.54 (m, 3H, 3H**e**), 3.10–3.24 (m, 6H, 3H**f**, 3H**g**), 3.55–3.64 (m, 3H, 3H**h**), 4.14–4.22 (m, 2H, CH_2, Gro), 4.32–4.40 (m, 2H, CH_2, Gro), 5.26–5.34 (m, 1H, CH, Gro). MS (MALDI-TOF) *m*/*z*: calcd. for $C_{27}H_{44}O_6S_6$ 657.000; found 680.556 [M+Na^+].

The NMR spectra for compounds **3b-d** are in agreement with the literature [S1].

1(3),4(6)-Di-*O*-lipoyl-2,3(1);5,6(4)-di-*O*-isopropylidene-*sn*-*myo*-inositol (3e) was obtained similarly to compound **3a** from LA **1** (200 mg, 0.97 mmol), *myo*-inositol derivative **2e** (101.4 mg, 0.40 mmol), DMAP (178 mg, 1.46 mmol) and DCC (239 mg, 1.16 mmol). Compound **3e** was purified by column chromatography on silica gel eluting with a hexane–ethyl acetate system (20% ethyl acetate). The yield was 198 mg (80%, amorphous), R_f 0.85 (d).

^{1}H NMR (300.0 MHz, $(CD_3)_2SO$): 1.38–1.82 (2m, 12H, 4H**a**, 8H**b**), 1.33–1.60 (4s, 12H, $2CMe_2$), 1.86–2.05 (m, 2H, 2H**c**), 2.40–2.55 (m, 6H, 4H**d**, 2H**e**), 3.08–3.23 (m, 4H, 2H**f**, 2H**g**), 3.46–3.64 (m, 3H, 2H**h**, H_5 Inos), 4.09–4.18 (m, 2H, H_3, H_6 Inos), 4.60–4.64 (t, *J* = 4.5 Hz, 1H, H_2 Inos), 5.09–5.18 (m, 1H, H_1 Inos), 5.27–5.33 (m, 1H, H_4 Inos).

1(3),4(6)-Di-*O*-lipoyl-*sn*-*myo*-inositol (4, LA_2Inos**)**

The mixture of compound **3e** (271 mg, 0.26 mmol) and Dowex H^+ resin (1.1 g) in methanol (20 ml) was stirred at 50°C for 2 h; then the resin was removed by filtration, and the filtrate was concentrated under reduced pressure. The yield was 203.6 mg (75%, amorphous), R_f 0.72 (d).

^{1}H NMR (300.0 MHz, $(CD_3)_2SO$): 1.31–1.45 (m, 4H, 4H**a**,), 1.50–1.73 (m, 8H, 8H**b**), 1.81–1.94 (m, 2H, 2H**c**), 2.27–2.40 (m, 6H, 2H**e**, 4H**d**), 3.07–3.21 (m, 4H, 2H**f**, 2H**g**), 3.52-3.84 (2m, 5H, 2H**h**, H_3, H_5, H_6 Inos), 4.48 (dd, J_1 = 9.2, J_2 = 7.1 Hz, 1H, H_2 Inos), 4.73 (d, *J* = 6.4 Hz, 1H, OH), 4.92–5.02 (m, 4H, H_1, H_4, 2OH), 5.10 (d, *J* = 4.0 Hz, 1H, OH). MS (MALDI-TOF) *m*/*z*: calcd. for $C_{22}H_{36}O_8S_4$ 556.670; found 579.139 [M+Na^+].

The preparation of the nanoparticles

(1) Phosphatidylcholine nanoparticles (NPs). Distilled water was injected into the solution of phosphatidylcholine (PC, 30 mg) in methanol (2 ml) under intense stirring (pH 6.0, 18 ml); then the mixture was stirred for 15 min (T = 25°C), and the organic solvent and the excess water were removed at low pressure, with periodic treatment in an ultrasonic bath (2 times for 2–3 min). The final volume of the nanodispersion (10 ml) was stirred at room temperature for 10 min.

(2) Pluronic F68–based nanodispersions with LA esters. The aqueous solution of Pluronic F68 (10 mg ml^{-1}, 18 ml) was injected to the solution of derivative **3a-d (**5 or 10 mg) in methanol (1-2 ml) under intense stirring; then the mixture was stirred for 15 min, and the organic solvent and the excess water were removed under reduced pressure. The final volume of the nanodispersion (10 ml) was stirred at room temperature for 10 min.

(3) The phosphatidylcholine nanosuspensions containing LA_2Inos were obtained similarly to the method described in (1): injection of distilled water (pH 6.0, 18 ml) into the solution of LA_2Inos **4** (2.5 mg) with PC (30 mg) in methanol (2 ml).

The isolation of PMNs from blood

To isolate the neutrophils (PMNs), the blood of nominally health donors aged from 20 to 30 was used. All the volunteer donors gave their voluntary consent to the use of their biological samples in the research. The neutrophils were isolated from the suspension of leukocytes in a double ficoll-verographin density gradient. Ficoll-verographin with the densities 1.119 and 1.090 g cm^{-3} and venous blood, in the 1.5 : 1.5 : 2 ratio, were accurately layered in sterile polystyrene test tubes. After centrifuging at 135 *g* for 45 min, the blood cells were found to be separated into three fractions: erythrocytes, neutrophils, and monocytes. The neutrophil (PMN)–containing fraction was isolated, transferred to sterile test tubes, washed with Hanks' sterile solution (pH 7.4) three times to remove the gradient, and centrifuged at 135 *g* for 10 min. If necessary, the erythrocytes were lysed by adding distilled water to the pellet (1 ml). After all the washings, the cellular pellet was carefully resuspended in Hanks' solution.

PMNs chemiluminescence. The nanoparticles of LA derivatives **3a-d** or **4** ($C_{\mathbf{3a}}$ = 0.27 мМ; $C_{\mathbf{3b}}$ = 0.22 mM; $C_{\mathbf{3c}}$ = 0.40 mM; $C_{\mathbf{3d}}$ = 0.30 mM; $C_{\mathbf{4}}$ = 0.08 mM) were added to the suspension of PMNs in Hanks' solution ($1.5*10^5$ cells ml^{-1}) and incubated at T = 37°C for 10 min. The reference samples were NDs without LA esters or the solution of LA in the PBS (pH 7.4). To activate the luminescence, luminol was added to the system (0.01 mM in sample). The luminescence was initiated by the addition of phorbol-12-myristate-13-acetate (PMA) solution ($1.6*10^{-6}$ mM in sample), and the chemiluminescence kinetics was recorded for 30 min (T = 37°C) using a Lum-1200 chemiluminometer (Russia).

The determination of the NADPH oxidase activity in PMNs

The activity of NADPH oxidase was determined using a $NADP^+$/NADPH Assay Kit MAK312 (Sigma–Aldrich, United States) by the rate of the NADPH substrate oxidation [S2,S3]. We added the NPs with LA esters **3a**,**c,d** ($C_{\mathbf{3a}}$ = 0.27 mM; $C_{\mathbf{3c}}$ = 0.40 mM; $C_{\mathbf{3d}}$ = 0.30 mM) and PMA ($1.6*10^{-6}$ mM) to the neutrophil suspension in Hanks' solution ($1*10^5$ cells ml^{-1}) and incubated the mixture at T = 37°C for 30 min. Then the samples were centrifuged at 55 *g* for 5 min and the supernatant was collected. The activity of NADPH oxidase was determined using a Hitachi F 7000 (Japan) plate spectrofluorimeter according to the instructions for Assay Kit NADP+/NADPH MAK312 (Sigma–Aldrich, United States). The reference samples were nanodispersions without LA esters or the solution of LA in PBS (pH 7.4).

Statistical analysis

The results were statistically processed using a STATISTICA 6 applied software package (StatSoft Corporation, United States). To analyze the differences between the quantitative characteristics in three or more independent groups, we used the Kruskal-Wallis ANOVA statistical criterion. The differences with $p < 0.05$ were regarded as significant. The results in Table 1 (main text) and Figure S3 are represented in the form of mean values and standard deviations from the mean.

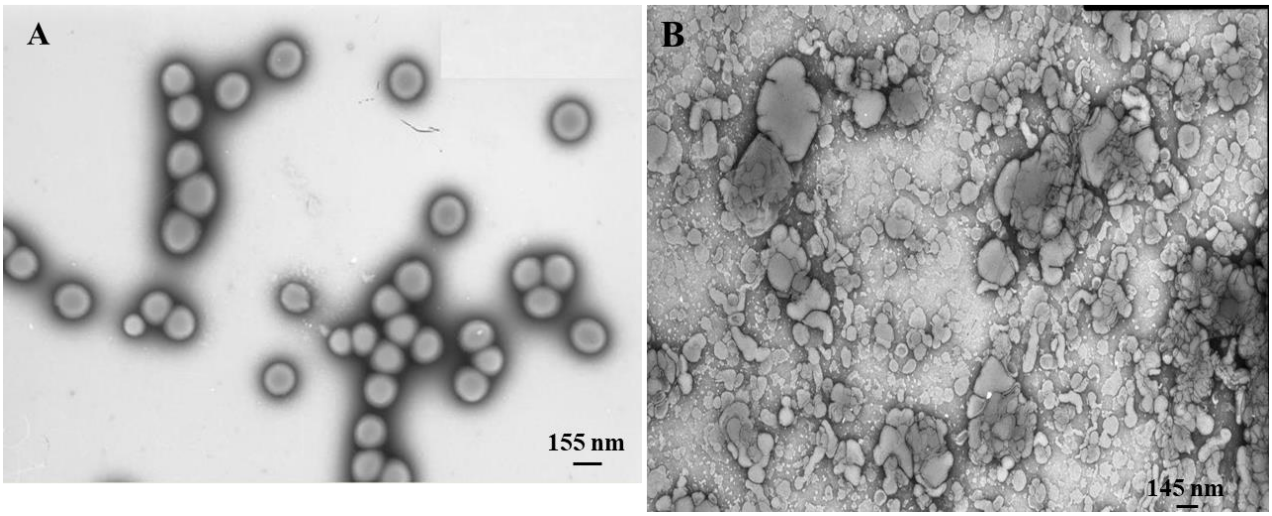


Figure S1. Electron micrographs of the LA ester nanoparticles: (**A**) compound **3c**; (**B**) compound **4**.

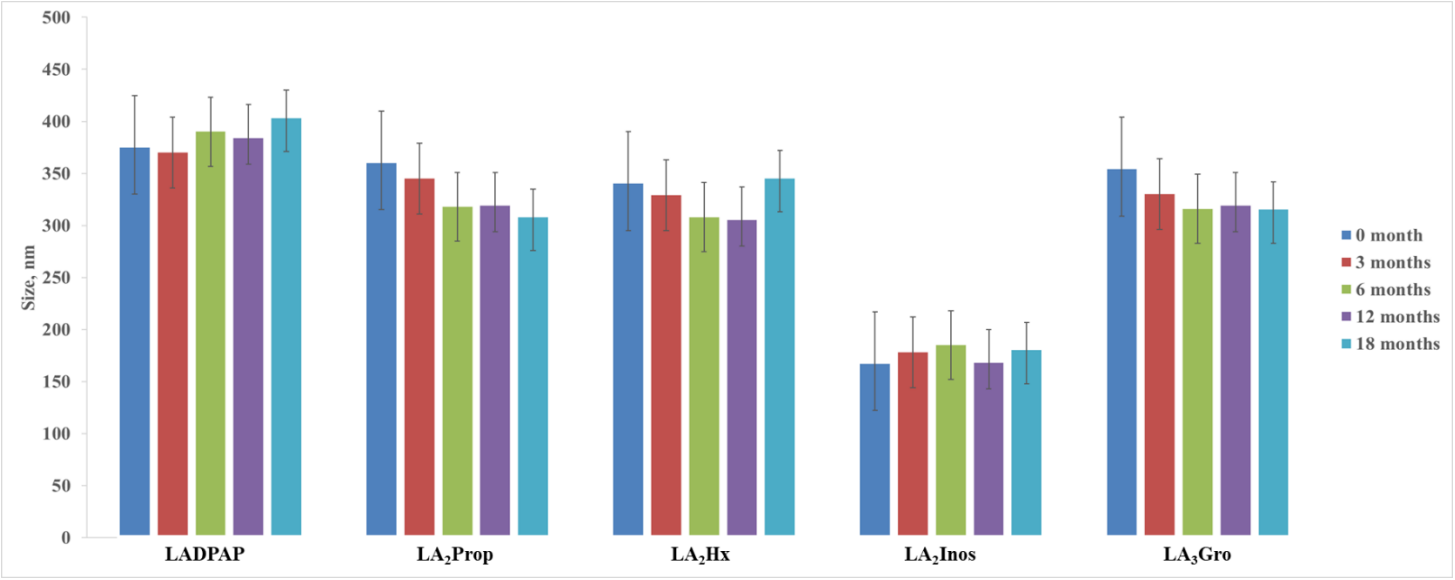


Figure S2. The stability of the LA ester nanoparticles during long-term storage (18 months, T = 25°C).

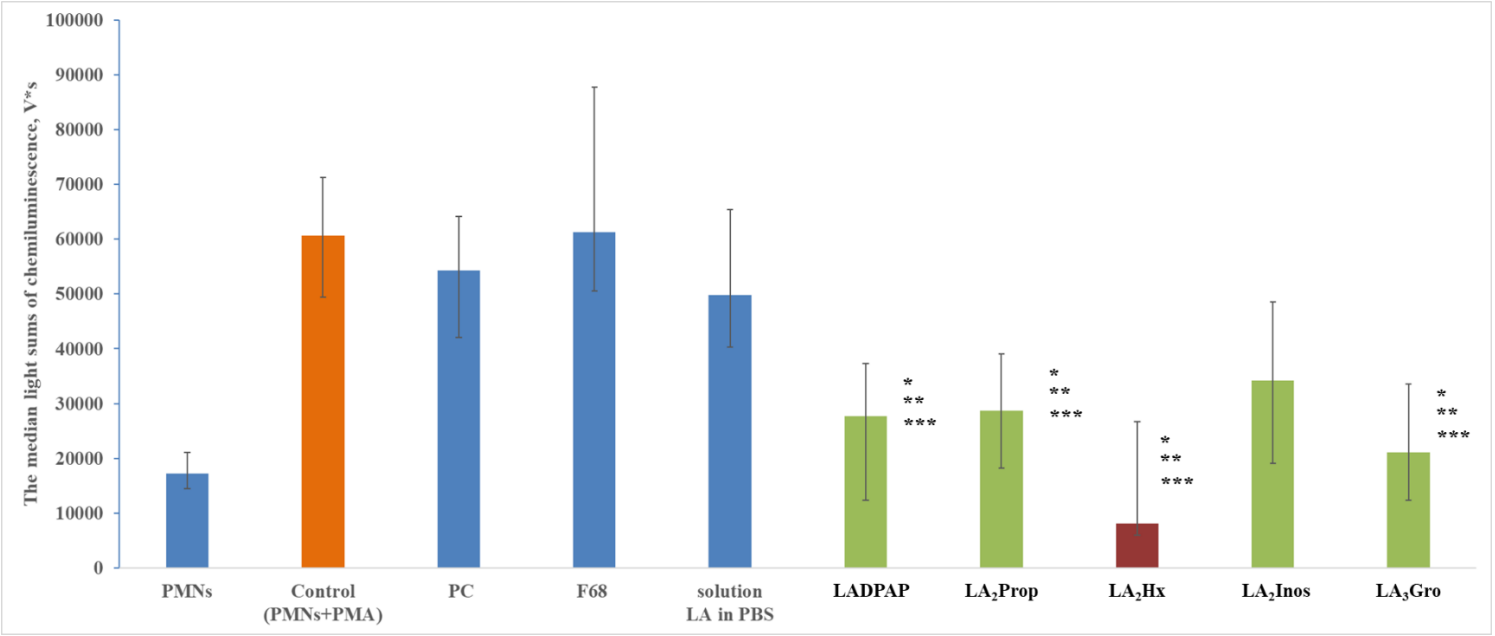


Figure S3. The effect of the LA ester nanoparticles on the oxidative burst in PMA-activated PMNs (n = 15, $1.7*10^5$ cells ml^{-1}).

Solution LA in PBS: C_{LA} = 0.8 mM;
PC: C_{PC}=0.70 mM;
F68: C_{F68}=0.5 mM;
F68-LADPAP: C_{LADPAP} = 0.27 mM;
F68-LA_2Prop: $C_{LA2Prop}$ = 0.22 mM;
F68-LA_2Hx: C_{LA2Hx} = 0.40 mM;
F68-LA_3Gro: C_{LA3Gro} = 0.30 mM, C_{F68} = 0.43 mM;
PC-LA_2Inos: $C_{LA2Inos}$ = 0.08 mM, C_{PC}= 0.70 mM.
Note: * $p < 0.05$ relative to the control (PMNs + PMA);
** $p < 0.05$ relative to the nanoparticles without LA esters;
*** $p < 0.05$ relative to the LA solution in PBS.

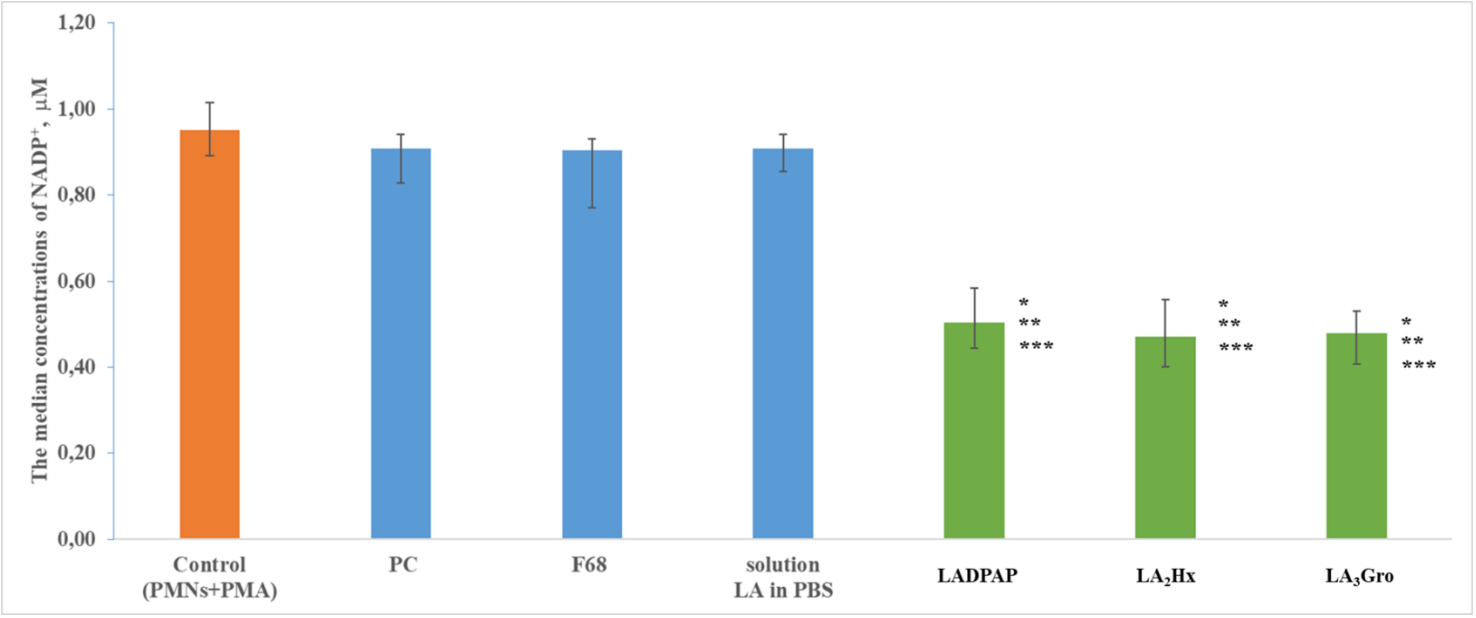


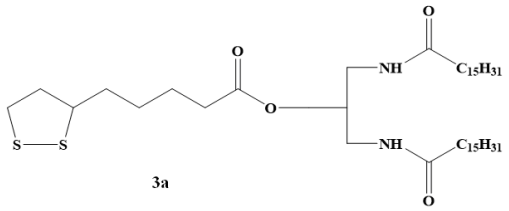
Figure S4. The effect of the LA ester nanoparticles on the activity of the NADPH oxidase in PMA-activated PMNs (n = 15, $1.7*10^5$ cells ml^{-1}).

Solution LA in PBS: C_{LA} = 0.8 mM;
PC: C_{PC} = 0.70 mM;
F68: C_{F68} = 0.5 mM;
F68-LADPAP: C_{LADPAP} = 0.27 mM;
F68-LA_2Hx: C_{LA^2Hx} = 0.40 mM;
F68-LA_3Gro: C_{LA^3Gro} = 0.30 mM, C_{F68} = 0.43 mM.
Note: * $p < 0.05$ relative to the control (PMNs + PMA);
** $p < 0.05$ relative to the nanoparticles without LA esters;
*** $p < 0.05$ relative to the LA solution in PBS.

References

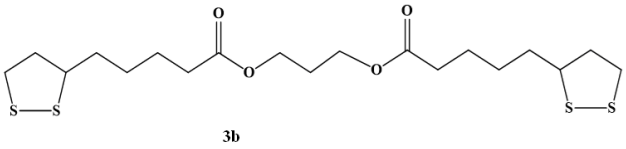
S1. B. S. Lee, X. Yuan, Q. Xu, F. S. McLafferty, B. A. Petersen, J. C. Collette, K. L. Black, and J. S. Yu, *Bioorg. Med. Chem. Lett*., 2009, **19**, 1678.
S2. R. Pinton, I. Cakmak, and H. Marschner, *J. Exp. Bot.*, 1994, **45**, 45.
S3. O. A. Gizinger, S. V. Moskvin, O. R. Ziganshin, and M. A. Shemetova, *Laser Med.*, 2016, **20**, 46.

^{1}HNMR (300 MHz, $CDCl_3$)

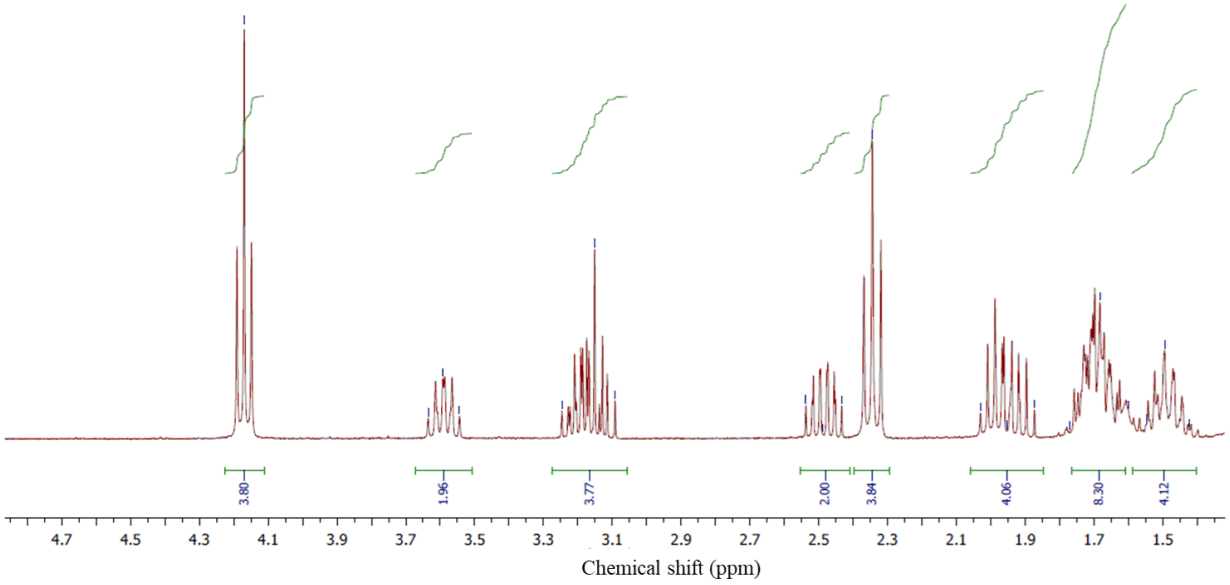


3a

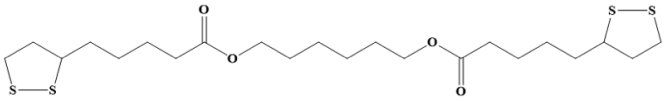
^{1}HNMR (300 MHz, $CDCl_3$)



3b

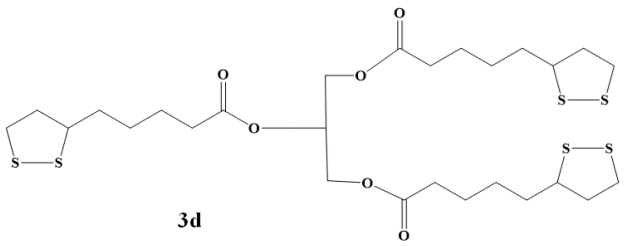


^{1}HNMR (300 MHz, $CDCl_3$)



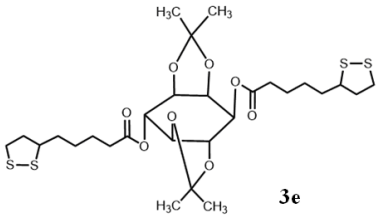
3c

^{1}HNMR (300 MHz, $CDCl_3$)

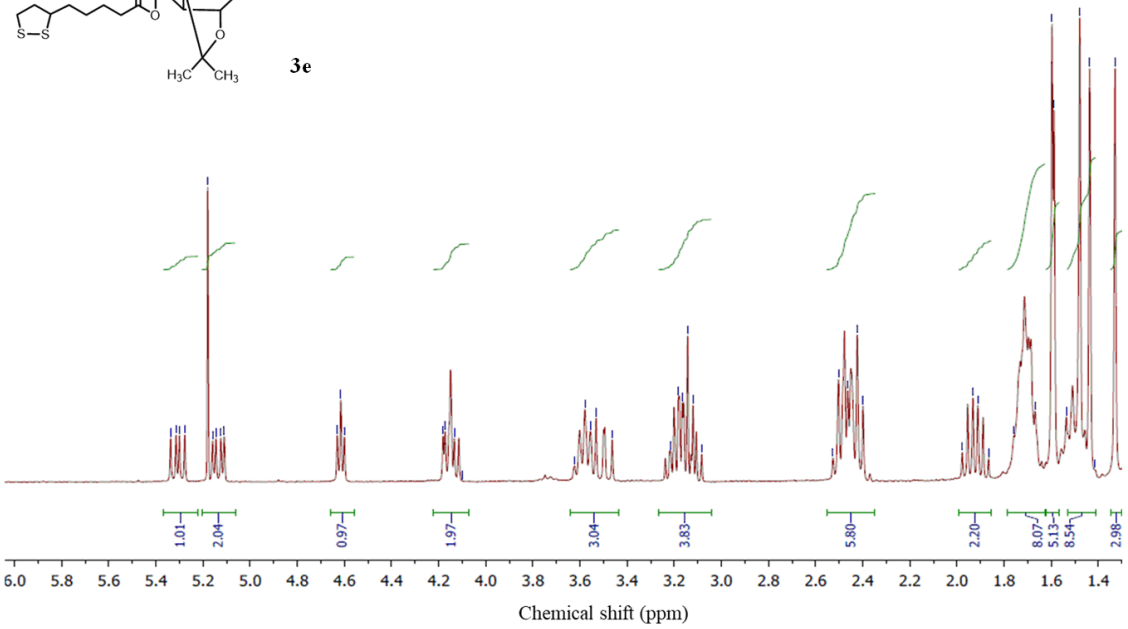


3d

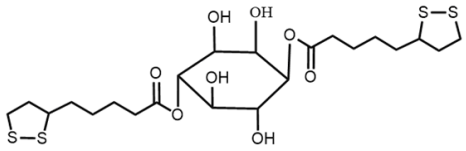
^{1}HNMR (300 MHz $(CD_3)_2SO$)



3e



^{1}HNMR (300 MHz $(CD_3)_2SO$)



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