

Redox-active salicylidenehydrazide analogues of vitamin E: synthesis, antioxidant and cytotoxic properties

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Salicylic aldehyde ($\geq 99\%$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid (99%), soybean lipoxygenase type 1-B (158000 units/mg), xanthine, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (USA). 2-Hydroxynaphthaldehyde (98%) was purchased from Acros Organics (Belgium). The solvents (EtOH (95%), MeOH, petroleum ether (40–70 °C), DMSO, and CH_2Cl_2) were used as supplied. Trolox hydrazide was obtained according to the known procedure.^{S1}.

Infrared spectra in the region of 4000–370 cm^{-1} were obtained using IR200 Nicolet spectrometer (KBr; Thermo Fisher Scientific, USA). The ^1H and ^{13}C NMR spectra were recorded in CDCl_3 and $\text{DMSO}-d_6$ using Avance-400 spectrometer (Bruker, Germany) operating at 400.1 MHz (^1H) and 100.6 MHz (^{13}C). Chemical shifts are given in ppm using ^1H -TMS as an internal reference. Elemental analysis was performed on 2400 Series II elemental analyzer (PerkinElmer, USA).

S1. General synthesis method of the compounds 1, 2.

Trolox hydrazide was prepared by a well-known method from Trolox methyl ester^{S2} by boiling with an excess of hydrazine hydrate and subsequent washing with water. The Trolox methyl ester, in turn, was obtained through the reaction of Trolox with methanol in the presence of a catalytic amount of concentrated sulfuric acid.

Compounds **1** and **2** were synthesized using a general procedure. Equimolar quantities of the corresponding aldehydes were added to 0.1 mole of Trolox hydrazide dissolved in methanol, and the mixture was heated vigorously for several hours. After the reaction, the mixture was evaporated *in vacuo*, washed with diethyl ether, and dried in air.

N'-((1*E*)-(2-Hydroxyphenyl)methylidene)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carbohydrazide **1**: colourless solid, yield was 75%, $T_{\text{melt}} = 185\text{--}188^\circ\text{C}$.

^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ , ppm): 1.48 (s, 3H, CH_3); 1.80 (m, 1H, CH_2); 2.00 (s, 3H, CH_3); 2.07 (s, 3H, CH_3); 2.16 (s, 3H, CH_3); 2.28 (m, 1H, CH_2); 2.55 (m, 2H, CH_2); 6.88 (m, 2H, 2CH-Ar); 7.27 (td, 1H, CH-Ar, $^3J_{\text{H-H}} = 17.2$ Hz); 7.45 (dd, 1H, CH-Ar, $^3J_{\text{H-H}} = 9.2$ Hz), 7.51 (s, 1H, OH-Tr); 8.56 (s, 1H, -N=CH-); 11.11 (s, 1H, OH-Sal); 11.15 (s, 1H, -C(O)-NH-).

^{13}C NMR (100.6 MHz, $\text{DMSO}-d_6$, δ , ppm): 11.81 (CH_3); 12.26 (CH_3); 12.81 (CH_3); 20.06 (CH_2); 23.82 (CH_3); 29.60 (CH_2); 76.98 (-O-((CH_2)C(CH_3))-C(O)NH-); 116.42 (C-Ph); 117.05 (C-Ph); 118.53 (C-Ph); 119.35 (C-Ph); 120.27 (C-Ph); 121.57 (C-Ph); 122.78 (C-Ph); 129.58 (C-Ph); 131.42 (C-Ph); 143.88 (C-Ph); 146.08 (C-Ph); 149.16 (C-Ph); 157.46 (-N=CH-); 169.91 (-C(O)NH-).

IR, cm^{-1} : 3330.5 (OH, free); 3070.6–2843.5 (CH, val); 1667.6 (C=O, val); 1616.1, 1518. (C-C, ar); 1576.0; 1487.8; 1454.6; 1445.4; 1428.5; 1416.0; 1372.6; 1364.4; 1340.8; 1269.9; 1254.9; 1235.7; 1192.3; 1154.2; 1139.2; 1112.7; 1083.8.

Found (%): C, 68.6; H, 6.46; N, 7.52. Calc. for $\text{C}_{21}\text{H}_{24}\text{N}_2\text{O}_4$. (%): C, 68.46; H, 6.58; N, 7.61.

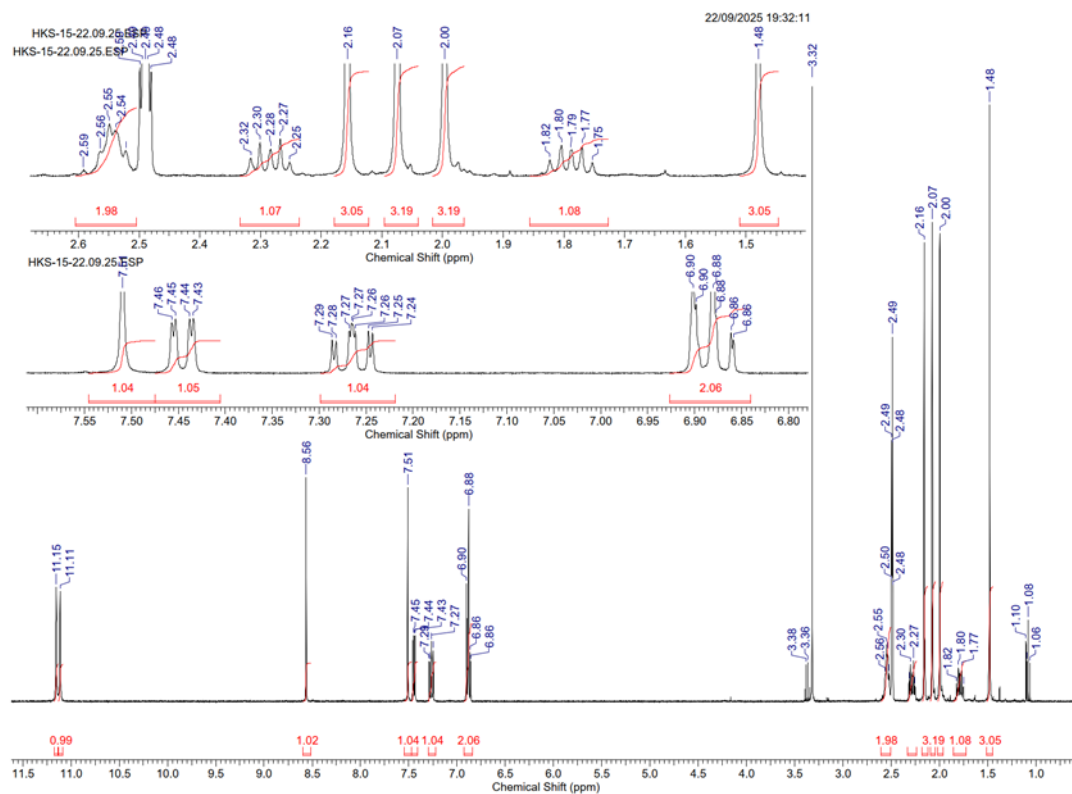


Figure S1. ^1H NMR spectrum of compound **1**.

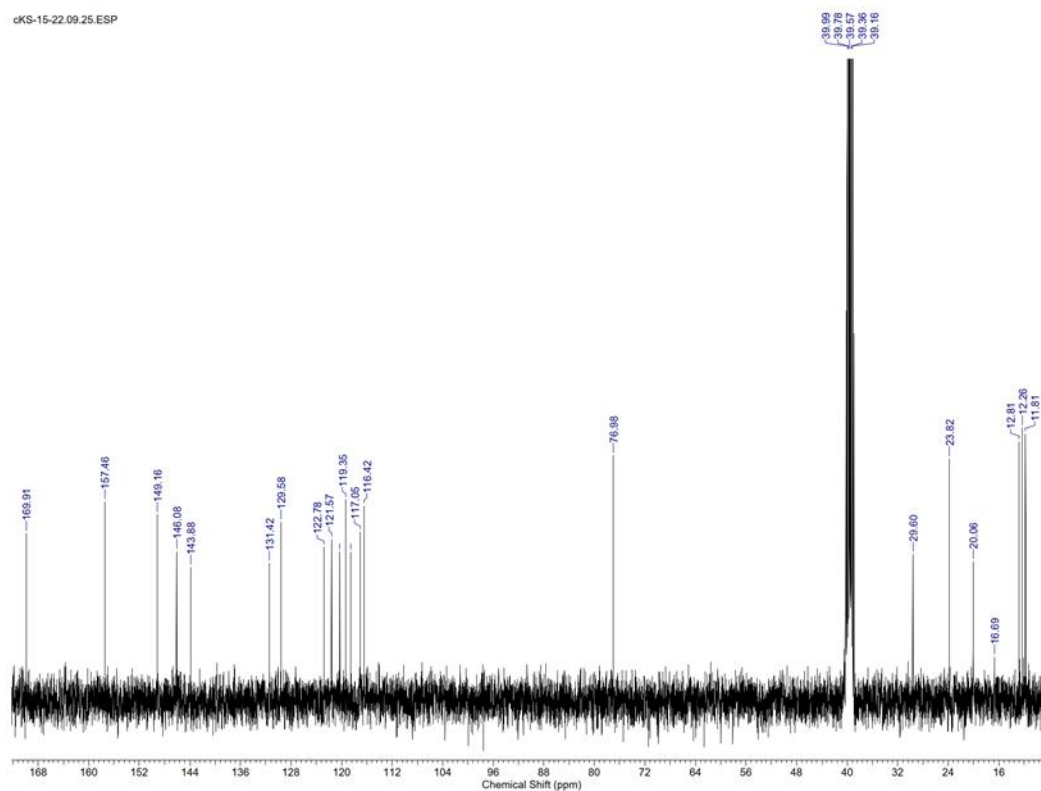


Figure S2. ^{13}C NMR spectrum of **1**.

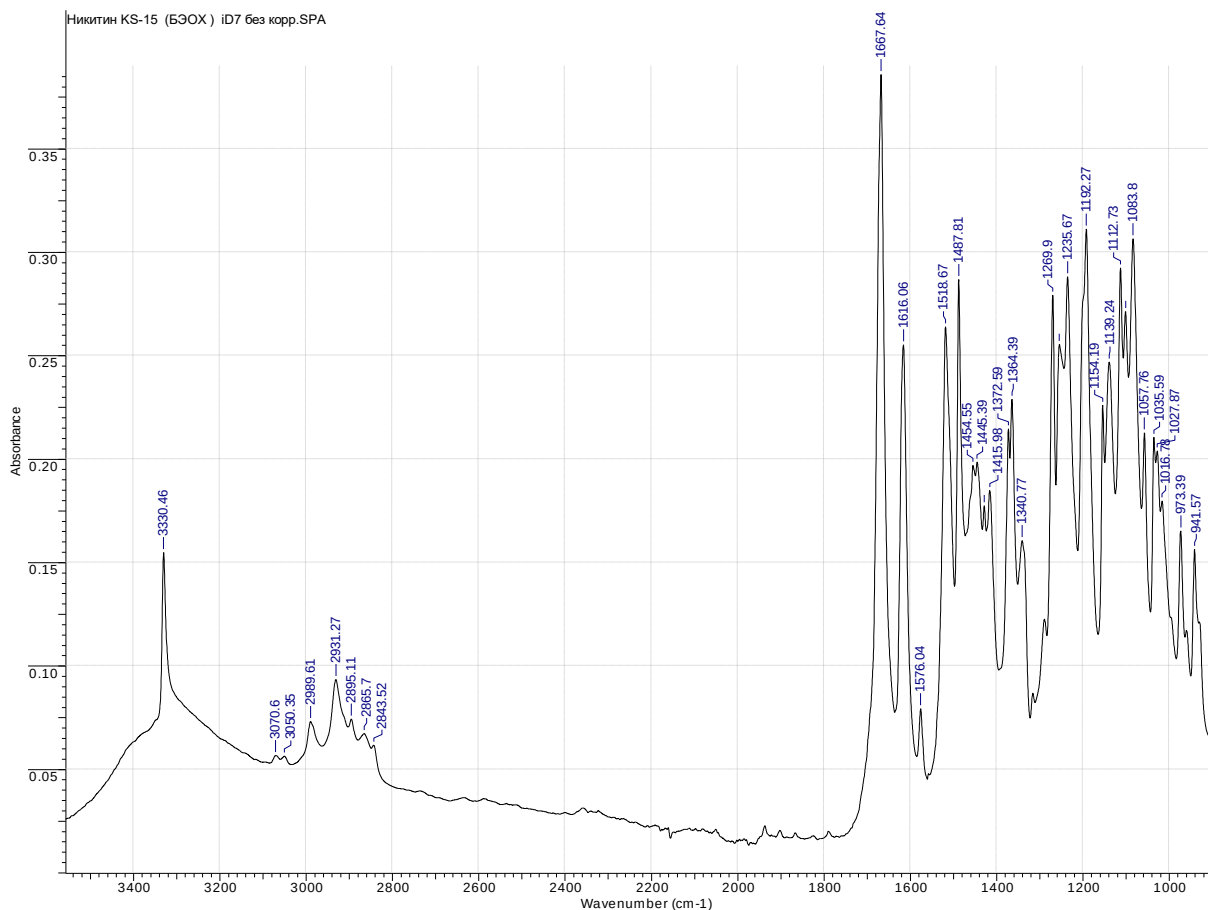


Figure S3. IR spectrum of compound **1**.

N'-((1*Z*)-(2-Hydroxynaphthalene-1-yl)methylidene)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carbohydrazide **2**: pale yellow solid, yield was 71%, $T_{\text{melt}} = 229\text{--}234^{\circ}\text{C}$.

^1H NMR (400 MHz, $\text{DMSO-}d_6$, δ , ppm): 1.51 (s, 3H, CH_3); 1.84 (m, 1H, CH_2); 2.01 (s, 3H, CH_3); 2.09 (s, 3H, CH_3); 2.20 (s, 3H, CH_3); 2.29 (m, 1H, CH_2); 2.58 (m, 2H, CH_2); 7.20 (d, 1H, CH-Ar, $^3J_{\text{H-H}} = 8.9$ Hz); 7.38 (t, 1H, CH-Ar, $^3J_{\text{H-H}} = 14.8$ Hz); 7.54 (m, 2H, CH-Ar, OH-Tr); 7.89 (m, 2H, 2CH-Ar); 8.18 (d, 1H, CH-Ar, $^3J_{\text{H-H}} = 8.6$ Hz); 9.48 (s, 1H, $-\text{N}=\text{CH}-$); 11.23 (s, 1H, OH-Naft); 12.69 (s, 1H, $-\text{C}(\text{O})-\text{NH}-$).

^{13}C NMR (100.6 MHz, $\text{DMSO-}d_6$, δ , ppm): 11.84 (CH_3); 12.35 (CH_3); 12.84 (CH_3); 20.02 (CH_2); 23.63 (CH_3); 29.73 (CH_2); 77.06 ($-\text{O}-((\text{CH}_2)\text{C}(\text{CH}_3))-\text{C}(\text{O})\text{NH}-$); 108.40 (C-Ph); 117.12 (C-Ph); 118.93 (C-Ph); 120.30 (C-Ph); 120.76 (C-Ph); 121.66 (C-Ph); 122.85 (C-Ph); 127.67 (C-Ph); 127.80 (C-Ph); 128.91 (C-Ph); 128.95 (C-Ph); 131.73 (C-Ph); 132.77 (C-Ph); 143.80 (C-Ph); 146.16 (C-Ph); 147.81 (C-Ph); 158.00 ($-\text{N}=\text{CH}-$); 169.81 ($-\text{C}(\text{O})\text{NH}-$).

IR, cm^{-1} : 3586.5 (OH, free); 3333.8 (OH, free); 3021.4–2873.9 (C-H, val); 1667.2 (C=O, val); 1624.7 (C=N, val); 1624.7, 1529.8 (C-C, ar); 1604.0; 1534.8; 1579.4; 1505.7; 1468.5; 1456.5; 1416.9; 1394.8; 1367.8; 1331.6; 1286.8; 1256.4; 1247.7; 1202.0; 1190.8.

Found (%): C, 71.74; H, 6.30; N, 6.69. Calc. for $\text{C}_{25}\text{H}_{26}\text{N}_2\text{O}_4$ (%): C, 71.75; H, 6.27; N, 6.70.

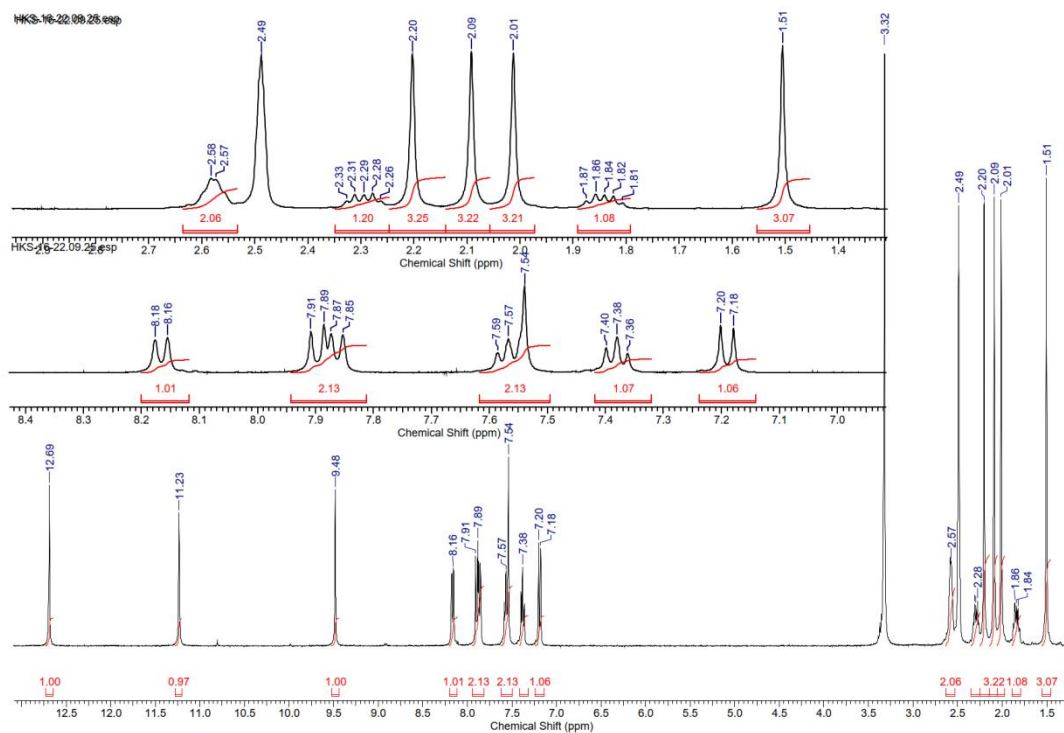


Figure S4. ^1H NMR spectrum of 2.

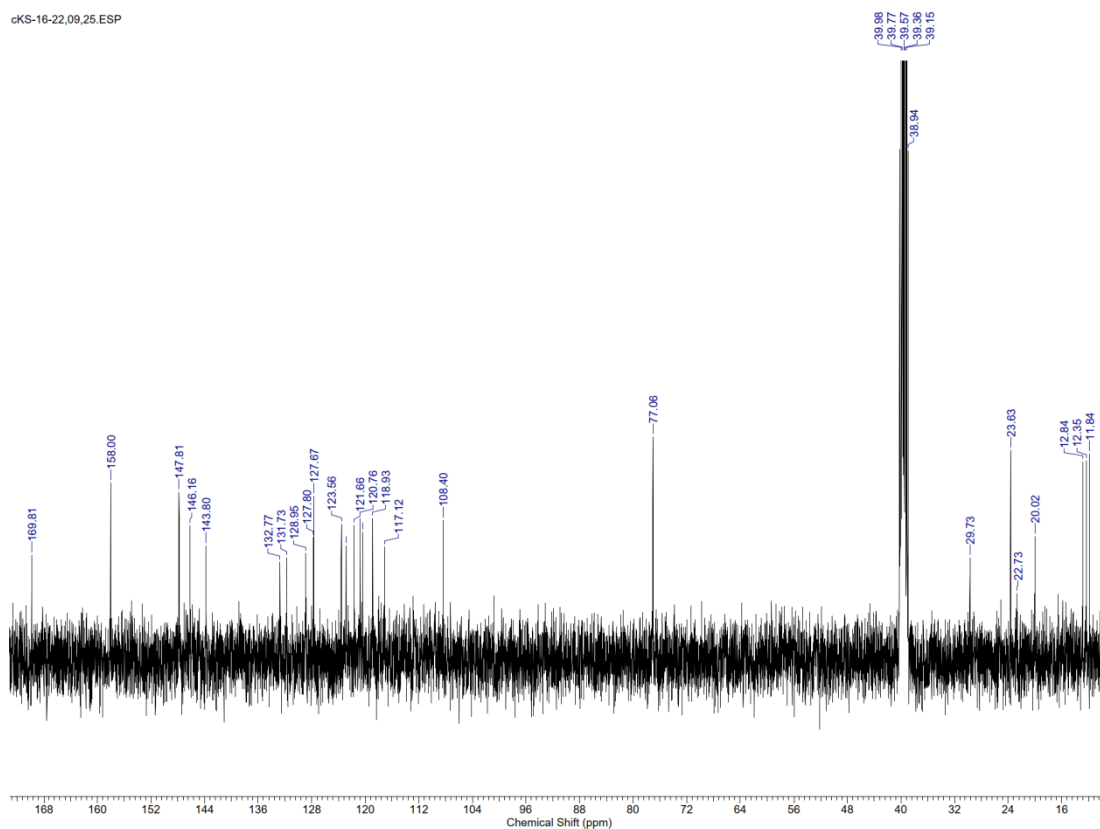


Figure S5. ^{13}C NMR spectrum of 2.

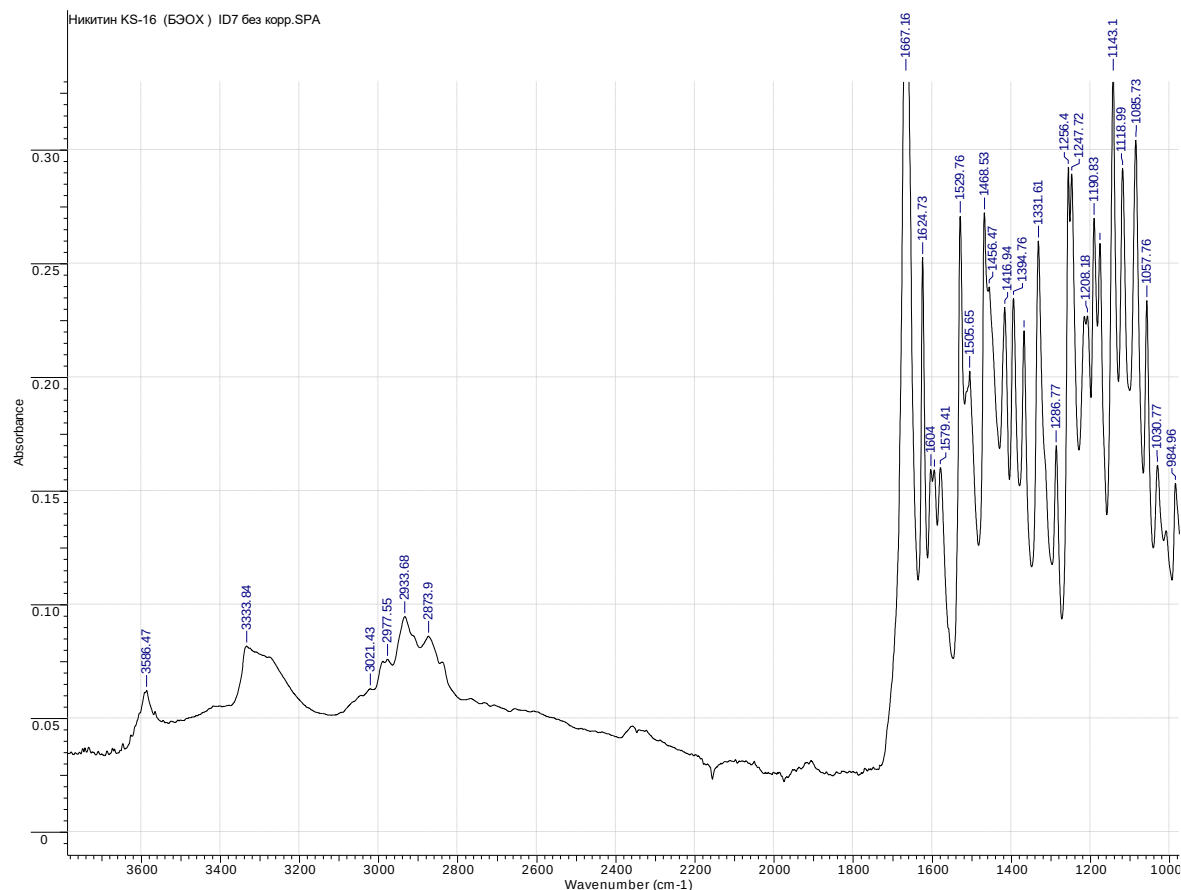


Figure S6. IR spectrum of **2**

S2. Crystallographic data collection and structure determination

X-ray diffraction analysis was performed on a STOE StadiVari Pilatus 100 K diffractometer ($\lambda(\text{CuK}\alpha) = 1.5418 \text{ \AA}$, ω -scan at 295 K). CCDC 2386368 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

S3. Measurement of antioxidant activity

The activity of the obtained compounds in single-electron transfer using the CUPRAC test was determined on a spectrophotometer by the increase in the optical density of the complex solution at a wavelength of $\lambda_{\text{max}} = 450 \text{ nm}$.^{S3} The reaction mixture (0.2 ml) consisted of: 0.05 ml of acetate buffer, 0.05 ml of $10^{-2} \text{ mol L}^{-1} \text{ CuCl}_2$ solution, 0.05 ml of $7.5 \cdot 10^{-3} \text{ mol L}^{-1}$ neocuproine solution and 0.05 ml of $0.5 \cdot 10^{-3} \text{ mol L}^{-1}$ solution of the studied compounds in methanol. The experimental temperature was 25 °C, the time was 30 minutes. An increase in the optical density of the reaction mixture (A_1) compared to the control (A_0) indicated the reducing ability of the studied compound. The experiments were performed in triplicate and expressed in Trolox equivalents (TEAC) and calculated as:

$$\text{TEAC} = A_1/A_0. \quad (1)$$

The DPPH test consists of the ability of the obtained compounds to reduce the stable radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) by transferring a hydrogen atom and is carried out spectrophotometrically by measuring the optical density of the solution at $\lambda_{\text{max}} = 517 \text{ nm}$.^{S4,S5} The reaction mixture consisted of: DPPH (0.75 ml, 0.2 mM) and a solution of the test compound in EtOH (0.75 ml, 0.2 mM). Antioxidant activity was calculated as the percentage of reduced DPPH according to formula (2):

$$I, \% = \frac{(A_0 - A_1)}{A_0} \cdot 100 \quad (2)$$

where A_0 is the optical density of the control solution of 0.1 mM DPPH in EtOH ($\epsilon_{\text{DPPH}} = 1.15 \cdot 10^4$), A_1 is the optical density of the reaction mixture in the presence of the test compound.

The NBT test is an assessment of the ability of the obtained compounds to neutralize the superoxide anion radical O_2^- , formed in the xanthine-xanthine oxidase system by reaction with tetrazolium blue (NBT) dye.⁵⁶ The experiment was carried out at $\lambda_{\text{max}} = 560$ nm using a ThermoScientific Multiskan Go plate spectrophotometer for 600 s. The reaction mixture consisted of: carbonate buffer (270 μL , 40 mM, pH 10.0) containing EDTA (0.1 mM), xanthine solution (6 μL , 10 mM) in carbonate buffer, 0.5% bovine serum albumin (3 μL) in water, NBT chloride (3 μL , 2.5 mM) in water and a solution of the test compound in DMSO (6 μL , 5 mM). The volume of xanthine oxidase in the buffer was 12 μL , 0.004 units. The control experiment was carried out in DMSO solution (6 μL) in the absence of the studied compounds. All experiments were carried out in triplicate. Inhibition was determined by formula (3):

$$I, \% = 1 - \frac{A_i}{A_0} \cdot 100\% \quad (3)$$

where A_i is the optical density in the presence of the test compound (600 s), A_0 is the optical density of the blank solution.

The activity of the obtained compounds in the process of lipoxygenase inhibition was determined spectrophotometrically at $\lambda_{\text{max}} = 234$ nm (characteristic absorption band of linoleic acid hydroperoxides) using a Multiskan Go well microplate spectrophotometer (Termo Scientific, USA).⁵⁷ The results of this method are expressed as a percentage of the slowing down of the enzymatic oxidation reaction. The composition of the test solution: 30 μL of borate buffer (pH 9.0), 100 μL of linoleic acid (0.45 mM) in borate buffer, 3 μL of a 1 mM solution of the analyzed compound in DMSO. To initiate the reaction, 17 μL of lipoxygenase (500 units) in borate buffer were added. The degree of inhibition I (%) of lipoxygenase was determined by formula (7):

$$I, \% = \left(\frac{v_0}{v'_0} \right) \cdot 100\% \quad (4)$$

where v_0 and v'_0 are the initial rates of the enzymatic reaction in the presence and absence (control) of the test compounds, respectively. The initial rate (v_0 and v'_0) was calculated using formula (5):

$$v_0 = \frac{\Delta C}{\Delta t} = \frac{\Delta A}{\Delta t \cdot \epsilon} = tg\alpha / (\Delta t \cdot \epsilon) \quad (5)$$

where $\Delta A = A_1 - A_0$, A_0 is the optical density of the control solution, and A_1 is the optical density of the reaction mixture in the presence of the test compound 5 min after the start of the reaction. All experiments were carried out in triplicate.

ABTS assay was carried according to following procedure. Phosphate Buffered Saline (PBS), was prepared as follows: 0.1 M KH_2PO_4 (1.36 g/100 mL), Na_2HPO_4 (1.41 g/100 mL) dissolved in 1 L distilled water, titrated with HCl to pH 6.8. Hemoglobin (Hb) was prepared *ex tempore*, 1 mg Hb dissolved in 1 mL phosphate buffer. 0.2 mg ABTS dissolved in 1 mL PBS (450 μM concentration, 150 μM final concentration in cuvette). Hydrogen peroxide (H_2O_2) was diluted 1:400 overnight, stored in dark at room temperature. Test compounds were dissolved in DMSO to obtain a stock solution with a concentration of 40 mM. Then, 10 μL of the resulting solution was transferred to 390 μL of EtOH (concentration - 1 mM). Before use, serial dilutions were performed to achieve final concentrations in the reaction mixture of 1000 μM , 333 μM , 100 and 33 μM . ABTS concentration was determined spectrophotometrically at 734 nm using a TecanInfinite M200 PRO multi-plate reader. The target compound activity was determined using the formula (6):

$$\% = (\text{AUC}(\text{Exp}) \cdot 100 / \text{AUC}(\text{Contr})) - 100 \quad (6)$$

where $\text{AUC}(\text{Exp})$ is the area under the curve of the test samples and $\text{AUC}(\text{Contr})$ is the area under the curve of the control samples.

Method for Determining Antiglycation Activity Using Glucose.

The glycation reaction was carried out in a 0.05 M phosphate buffer solution, pH 7.4. The reaction medium contained 0.36 M glucose and 1 mg/ml BSA (fraction V). The test compounds were dissolved in 99% DMSO (final concentration in the reaction medium ~3%), and dissolution was performed *ex tempore*. Compound activity was tested at concentrations of 1000, 333, 100, 33, 10, 3, 1 and 0.3 μ M. Control samples contained an equivalent volume of solvent. The samples were incubated for 24 hours at 60°C. Data were recorded spectrofluorimetrically, determining APG by specific fluorescence at excitation/emission wavelengths of 370/440 nm (M 200 PRO spectrofluorimeter, TECAN, Austria). To eliminate false-positive results for compounds that suppress APG fluorescence due to interference, logarithmic normalization of the obtained data was performed using the formula (7):

$$\text{Flu}(\log) = 10(\log_{10}(\text{Exp}) - \log_{10}(\text{Blank})) - 1 \quad (7)$$

where Flu(log) is the normalized APG fluorescence intensity, $\log_{10}(\text{Exp})$ and $\log_{10}(\text{Blank})$ are the decimal logarithms of the actual fluorescence levels of glycated and corresponding non-glycated samples (both containing the test compound and controls).

The activity of other compounds (both non-fluorescent and fluorescent at the wavelengths used) was expressed using the formula (8):

$$\text{Flu}(\text{lin}) = \text{Exp} - \text{Blank} \quad (8)$$

where Flu(lin) is the APG fluorescence intensity, and Exp and Blank are the actual fluorescence levels of the glycated and corresponding non-glycated samples (both containing the test compound and the control).

Activity, expressed as % APG fluorescence suppression, was determined using the formula (9):

$$\% = 100 - (\text{Flu}(\text{Exp}) * 100 / \text{Flu}(\text{Contr})) \quad (9)$$

where Flu(Exp) and Flu(Contr) are the APG fluorescence intensities of the test and control samples, respectively (log-normalized or non-log-normalized). Mathematical data processing was performed using Microsoft Excel.

S4. Cytotoxicity Measurement

Cytotoxicity studies of the synthesized compounds were performed using the standard MTT-test.^{S8} Solutions of the studied compounds were prepared in concentrations of 1 mM, 0.25 mM, 0.0625 mM, 0.015 mM, 0.00375 mM in DMEM nutrient medium. The substances were preliminarily dissolved in DMSO (the DMSO concentration was no more than 0.5% of the final solution volume). The prepared solutions of the compounds were added to a sterile flat-bottomed plate with cell cultures (human cancer cell lines HCT116, MCF7 and A549) using 5 and 10 μ l micropipettes so that the final concentration of the substances in the wells was 50 μ M, 25 μ M, 12.5 μ M, 6.4 μ M, 3.2 μ M, 1.6 μ M, 0.8 μ M, 0.4 μ M, 0.2 μ M, 0.1 μ M. Then the plate with cells and substances was placed in a CO₂ incubator for 72 h. Then 10 μ l of MTT solution with a concentration of 5 μ g/ml were added to each well of the plate and incubated for 2 h at 37 °C in a humidified atmosphere with 5% CO₂. After 2 h of exposure, living cells reduced MTT to dark purple formazan granules. Formazan beads were dissolved in 150 μ l DMSO and the amount of recovered product was measured spectrophotometrically on a Zenyth 2000rt plate reader at 570 nm.

Primary dermal fibroblasts from outbred white rats (RDF) were used to assess in vitro cytotoxicity in non-tumor cells. Cells were isolated from skin explants obtained from 20-day-old rats after euthanasia by cervical dislocation under chloral hydrate anesthesia. Skin samples were treated with trypsin for 1 h at 37 °C and cultured in complete DMEM supplemented with 10% fetal bovine serum, L-glutamine, antibiotics, sodium pyruvate, and essential amino acids. Fibroblasts were maintained in a CO₂ incubator

at 37 °C and 5% CO₂ and passaged using trypsin-EDTA. Cytotoxicity in non-tumor cells was evaluated using the same standard MTT-test with final compounds concentrations of 0.1 - 100 µM. Cell viability was expressed as a percentage of the control. Dose-response curves were generated using nonlinear four-parameter regression in GraphPad Prism v.8.0, and the concentration inducing a 50% cytostatic effect (IC₅₀) was calculated.

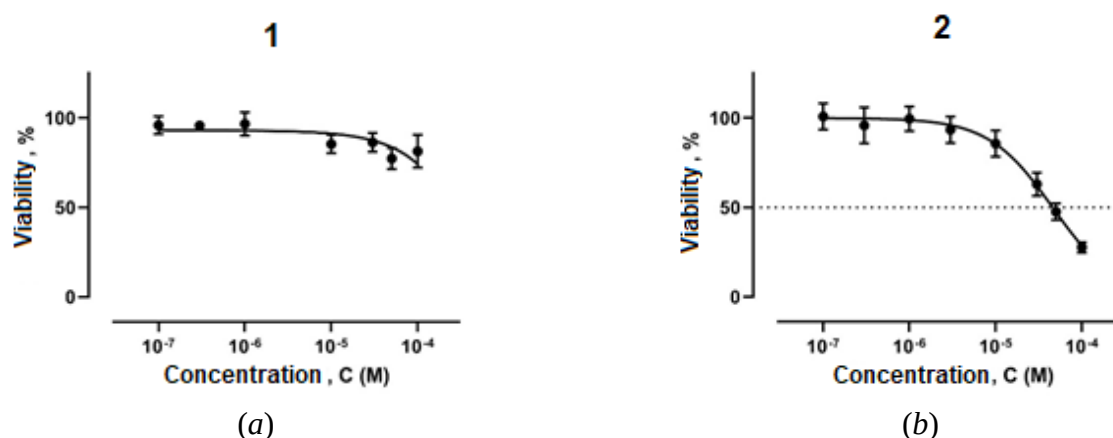


Figure S7. (a) The effect of the compound **1** at concentrations of 0.1-100 µmol/L on the metabolic activity (viability) of primary rat dermal fibroblasts during 48-hour incubation in the MTT test. (b) The effect of the compound **2** at concentrations of 0.1-100 µmol/l on the metabolic activity (viability) of primary rat dermal fibroblasts after 48 hours of incubation in the MTT test.

S5. Redox-activity Measurement

All measurements were carried out under argon at room temperature. Cyclic voltammetry experiments were performed in the three-electrode cell in DMF solution with 0.05 M Bu₄NClO₄ as a supporting electrolyte using an IPC-Win potentiostat. The number of transferred electrons was determined by comparing with the height of Fc²⁺/Fc³⁺ wave for the same concentration and by the rotating disk electrode method. A glassy carbon served as the working electrode. Ag/AgCl/KCl (aq., sat.) as reference electrode and a platinum plate as an auxiliary electrode were used. The working electrode surface was polished by alumina powder with a particle size of less than 0.5 µm (Sigma-Aldrich). In the CV measurements the potential sweep rate was 100 mV·s⁻¹. In studies with the rotating disk electrode (RDE) method the potential sweep rate 20 mV·s⁻¹. The potentials are presented with iR-compensation. Freshly opened DMF of 99.5% purity was purchased from Macklin (Belgium) and was used with no further purification.

S6. ¹H NMR spectra correlations

In the presented fragments of the ¹H NMR spectra (Figures S8, S9), it is important to pay attention to the highlighted signals. These signals can provide valuable information about the structure of the molecules. The signals of the hydroxyl (OH) groups of the trolox fragment are shown in green. They are separated from the aldehyde groups and therefore have similar chemical shifts (7.54 and 7.52 ppm). The signals of the protons in the azomethine group are highlighted in black and have different chemical shifts due to the influence of the salicylic and naphthalene groups. Additionally, the signals of the aldehyde OH groups are indicated in blue. Based on X-ray diffraction data, it can be seen that in compound **1**, the hydroxyl group is bonded to the nitrogen atom of the imine group. Based on the chemical shift of the OH group in compound **2**, we can assume that it is also connected by a hydrogen bond with the "amide" nitrogen - C(O)-NH-. The proton of this nitrogen, judging by the presence of a signal in the very low field region (12.69 ppm), is also involved in the formation of this hydrogen bond. In contrast, for compound **1**, there is no hydrogen bond for the "amide" proton -C(O)-NH- due to which the signal for this proton appears in the higher field region at 11.15 ppm.

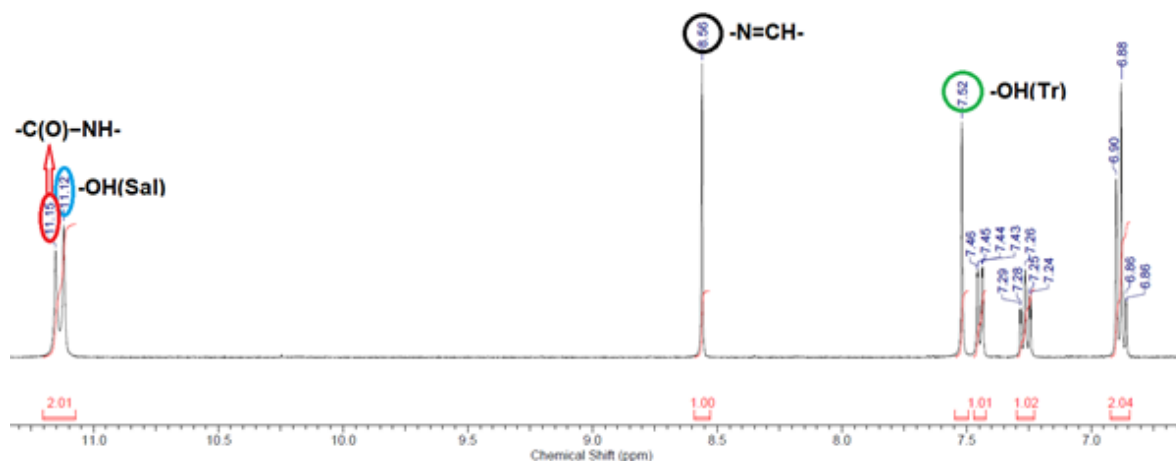


Figure S8. Part of the ^1H NMR spectrum of compound 1.

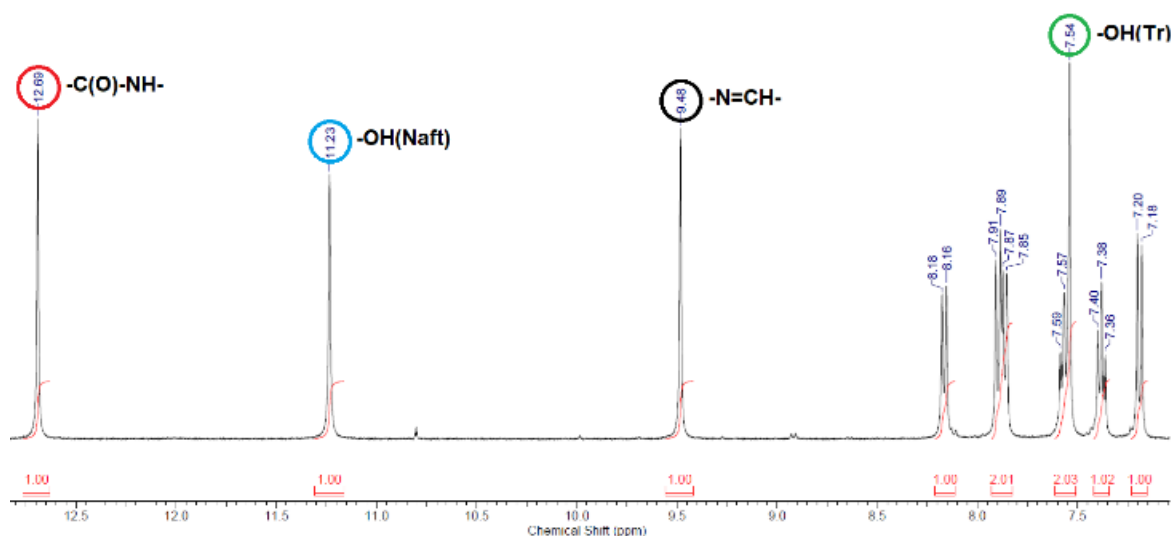


Figure S9. Part of the ^1H NMR spectrum of compound 2.

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