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Short communication

Investigation of the antioxidant properties of some new 4-hydroxycoumarin derivatives

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ABSTRACT

The aim of this investigation was to measure the activity of four 4-hydroxycoumarin derivatives – three of them were described before and one was newly synthesized. The substances were ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (**SS-14**), 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**), ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**) and ethyl 2-[(3,4,5-trimethoxyphenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**T-2**). The synthesis of **T-2** consists of two steps. First step was Knoevenagel reaction between 3,4,5-trimethoxybenzaldehyde and ethylacetoacetate. Ethyl 2-(3,4,5-trimethoxy)-phenylmethyleneacetoacetate was the product. Second step was Michael addition reaction between the latter product and 4-hydroxycoumarin. All the compounds were tested in vitro for antioxidant activity in hypochlorous system. The assay was based on the luminol-dependent chemiluminescence of free radicals, which decreased in the presence of 4-hydroxycoumarin derivative. Compound **SS-14** (ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate) expresses the best scavenger activity at the highest concentration (10^{-4} mol/L).

1. Introduction

4-Hydroxycoumarins are very important class of biologically active substances in nature and in medicine. They exhibit mainly anticoagulant activity and there are some drugs which are widely used as anticoagulants – Warfarin and Acenocoumarol. They exhibit cytotoxic and spasmolytic activities and also activity against HIV virus [1–5]. The mechanism of anti-HIV activity is probably blocking of HIV-1 protease and integrase [3–5].

Three of the tested 4-hydroxycoumarin derivatives in the present study – ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**), 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**) and ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (**SS-14**) – were synthesized and investigated for cytotoxic activity in two tumor cell lines – HL-60 and EJ [6]. The results are compared with the utilized anticancer drug Melphalan. **SS-21** shows comparatively good cytotoxic properties. Its activity is weaker than Melphalan.

Differently substituted 4-hydroxycoumarins are tested for free radical scavenger activity in the system of 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical [7]. Investigated 4-hydroxycoumarins are substituted with electron-donating and electron-withdrawing substituents on the third place in the coumarin ring. The principle of the method is that the solution of DPPH in ethanol is intensively colored and has an absorption maximum at 517 nm. In the presence of scavenger substance the solution becomes colorless. The results show that 4-hydroxycoumarins with electron-donating groups on the third position and on the seventh position in the coumarin ring have the best scavenger activity.

Structure–activity relationship of some coumarin antioxidants as protective agents against linoleic acid hydroperoxide (LOOH) toxicity in cultivated human umbilical vein endothelial cells was explored [8]. Coumarin, 4-hydroxycoumarin, 7-hydroxycoumarin, esculetin (6,7-dihydroxycoumarin), scopoletin (7-hydroxy-6-methoxycoumarin) and 8-acetyl-6-hydroxy-7-methoxycoumarin were used in the assay. There are two types of treatment – one is pretreatment, when after treatment with antioxidant the medium is changed with Earle solution (containing LOOH) and consequently the cells incubated for 3 h. The second type is concurrent treatment, when the cells are treated for 3 h with Earle solution containing both LOOH and antioxidant. Among the coumarins examined, esculetin has the best protective activity against LOOH-induced toxicity in both the treatments – pretreatment and concurrent treatment. Esculetin is known as a strong lipoxygenase inhibitor. The presence of *o*-phenolic hydroxyl groups can be important for the protection against LOOH-induced toxicity.

The antioxidative activity of some coumarins is investigated by measuring their protective action toward linoleic acid peroxidation

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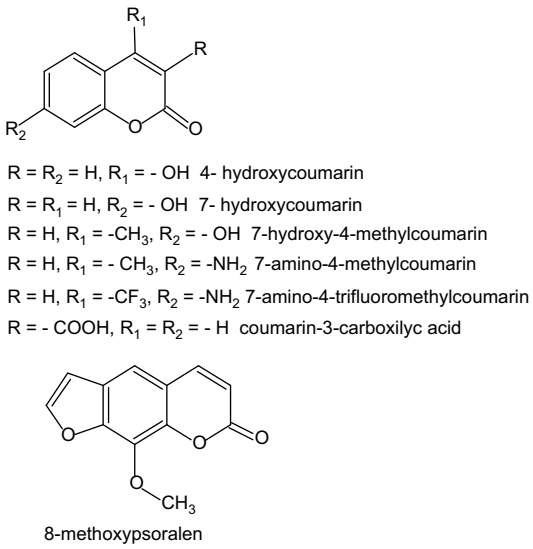

Fig. 1. Chemical structure of the investigated compounds.

in micelles of sodium dodecylsulfate in buffer solution (pH = 7.4) [9]. Results are expressed as relative antioxidant efficiency (RAE), defined as the ratio of the antioxidant efficiency (AE) of the tested compound to that of α-tocopherol. The coumarins tested are 4-hydroxycoumarin, 7-hydroxycoumarin (umbelliferone), scopoletin, 5,7-dihydroxy-4-methylcoumarin, 6,7-dihydroxy-4-methylcoumarin, 7,8-dihydroxycoumarin (daphnetine) and 4-methyldaphnetine. The best RAE was established for daphnetine (7,8-dihydroxycoumarin). 4-Methyldaphnetine and 6,7-dihydroxy-4-methylcoumarin also displayed a very good relative antioxidant efficiency. The reason for that is probably the presence of *o*-phenolic (catechol) hydroxyl groups. The activity of such compounds can be connected with the different capabilities to transfer hydrogen atom to LOO•. Hydrophilicity also played a role in penetration through sodium dodecylsulfate micelles and antioxidative activity.

The chemiluminescence characteristics of seven different coumarins were studied in detail in peroxyoxalate–hydrogen peroxide system [10]. Their structure is shown in Fig. 1.

The fluorescence and chemiluminescence spectra were compared and all used coumarins were found to be good fluorescers. The intensity and kinetic parameters of the chemiluminescent systems were evaluated from computer fitting of the resulting intensity–time plots. Among different coumarins, 7-amino-4-trifluoromethylcoumarin revealed the most promising characteristics as an efficient blue fluorescence emitter.

The aim of the present investigation was to test some new 4-hydroxycoumarin derivatives for antioxidative (scavenger) activity in model system, which generates free radicals.

2. Results and discussion

2.1. Chemistry

Compound **T-2** was synthesized in a two-step synthetic scheme. First step was a Knoevenagel reaction between 3,4,5-trimethoxybenzaldehyde and ethylacetoacetate. The product was ethyl 2-(3,4,5-trimethoxy)-phenylmethyleneacetoacetate (Fig. 2).

The second step was Michael reaction between 4-hydroxycoumarin and ethyl-2-(3,4,5-trimethoxy)-phenylmethyleneaceto acetate. Arylmethylene-β-ketoester fragment was added to the third position in the coumarin ring, because of the lowest electronic density on that position. The reason for this was the electron-withdrawing effects of carbonyl and hydroxyl groups and the p,π-conjugation between lone pair electrons of the hydroxyl oxygen atom and π-electrons of the C=C bond (Fig. 3).

The other investigated compounds have chemical structures as shown in Fig. 4:

Ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl) methyl]-3-oxobutanoate (**SS-21**) [6],
4-[1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl] benzoic acid (**SS-17**) [6],
Ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl) methyl]-3-oxobutanoate (**SS-14**) [6].

These compounds were synthesized in the same way as **T-2** [6] (Fig. 5 and Fig. 6).

First step was Knoevenagel reaction between differently substituted aromatic aldehydes and ethylacetoacetate at room temperature in the presence of piperidine. The products of this type of reaction were substituted in the aromatic ring arylmethylene-β-ketoesters.

The second step was Michael reaction between the arylmethylene-β-ketoesters and 4-hydroxycoumarin [6] (Fig. 5).

The products were 4-hydroxycoumarin derivatives, substituted on the third place with ethyl 2-acetyl-3-phenylbutanoate fragment.

2.2. Chemiluminescent antioxidant assay

The measurements of the luminol-dependent CL in the presence of OCl^- showed that **SS-14** has significant antioxidant effect at concentration 10^{-4} M. In Fig. 7 are shown typical kinetic curves for such systems, for control (phosphate buffer solution) and for **SS-14**.

The corresponding CL-SI values are presented in Fig. 8.

There is a very good linearity between the concentration and chemiluminescence's scavenger index (Fig. 9) ($R^2 = 0.95$–1.00), so the decreasing CL-SI is proportional to the increasing concentration of the tested 4-hydroxycoumarin derivative. The most significant standard deviation was measured for **SS-21** at concentration 5×10^{-5} mol/L and also for **T-2** at concentration 10^{-5} mol/L.

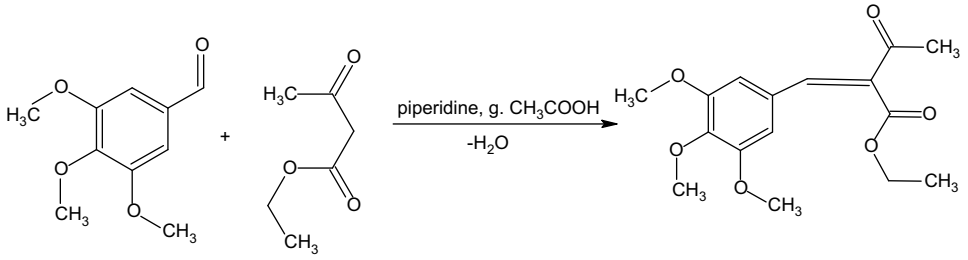

Fig. 2. Knoevenagel interaction between 3,4,5-trimethoxybenzaldehyde and ethylacetoacetate.

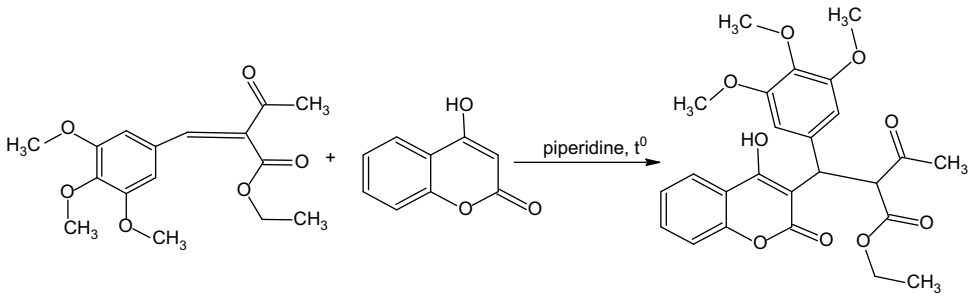

Fig. 3. Michael addition reaction between ethyl 2-(3,4,5-trimethoxy)-phenylmethyleneacetoacetate and 4-hydroxycoumarin.

It can be seen from Fig. 8 that a strong scavenger activity for **SS-14**, **SS-17** and **T-2** is found at 10^{-4} mol/L.

The worst radical capturing activity was found for **SS-21**. At the highest tested concentration (10^{-4} mol/L) CL-SI had the value only 7% of the control (control sample has 100%). At the lowest concentrations there was lack of antioxidant effects.

From all of the tested compounds, **SS-14** showed the strongest scavenger activity at the concentration 10^{-4} mol/L, followed by **T-2** and **SS-17**.

The chemical structure–scavenger activity relationship can be made. The antioxidant activity in general of 4-hydroxycoumarins can be explained with the presence of enolic hydroxyl group at the fourth position in coumarin ring. The hydroxyl group, which is also present at *p*-position in the aromatic ring in **SS-14**, has high electron-releasing properties (positive mesomeric effect is higher than negative inductive effect) and it activates aromatic ring. The same is valid for methoxy groups at positions 3, 4 and 5 in aromatic ring in a molecule of **T-2**. These groups have a bit higher electron-releasing activity than hydroxyl group and also activate the aromatic ring. Hydroxyl and methoxy groups have a good capability to catch free radicals by themselves. Carboxyl and nitro groups are electron-withdrawing substituents, they deactivate aromatic ring and have no capability to bind the free radicals. The better scavenger activity of **SS-17** is probably due to the lower electron-withdrawing effect of carboxyl group, than that of nitro group. So, that could be the probable explanation of the good antioxidative activity of **SS-14** and **T-2**, and the worst activity of **SS-21**.

The basic part of the phagocyte bactericidal effect is due to the ROS (reactive oxygen species) produced by them. The phagocytosis signal activates one cascade of biochemical reactions, which gives hypochloric acid as product. The latter is an extremely strong oxidative agent, which oxidizes biogenic amines, thioles and proteins. The reaction in which superoxide anions are formed from oxygen at the beginning and they cause a subsequent cascade of processes in which other ROS are obtained is called respiratory burst. It can be seen in Fig. 10.

Superoxide and H_2O_2 are weak reactive species, but the latter dismutates spontaneously, because it is the substrate of myeloperoxidase (MPO). Hypochloric acid can be formed as a product, which reacts rapidly with biological macromolecules. Finally hypochloric acid can interact with biogenic amines and form product chloramines, which are weak, but long lasting oxidants. The stationary concentrations of superoxide and hydrogen peroxide are very small, because of the spontaneous dismutation of H_2O_2. The basic oxidizing agent in phagocytes is HOCl. The action of the phagocytic HOCl concerns both pathogenic microorganism and the human body, because it causes oxidative damage in the surrounding tissues. Substances which can scavenge HOCl could decrease the effect of the action of the phagocytes [11].

3. Conclusions

The four tested 4-hydroxycoumarin derivatives, 2-[(4- hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (**SS-14**) [6], 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**) [6], ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**) [6] and ethyl 2-[(3,4,5-trimethoxy-phenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**T-2**), were tested for antioxidant activity in a model system of hypochlorous anion. **SS-14** has the best hypochlorous scavenging activity at 10^{-4} mol/L. The reason for higher scavenger activity of this compound is the presence of electron-donating group as substituent in the aromatic ring in the side chain.

T-2 was a brand new synthesized compound. It was synthesized by a two-step method. The first step was Knoevenagel reaction between 3,4,5-trimethoxybenzaldehyde and ethylacetoacetate. The product of this reaction was ethyl 2-(3,4,5-trimethoxy)-phenylmethyleneacetoacetate. That product was reacted with 4-hydroxycoumarin according to Michael addition reaction. The arylmethylene-β-ketoester added on the third position in coumarin

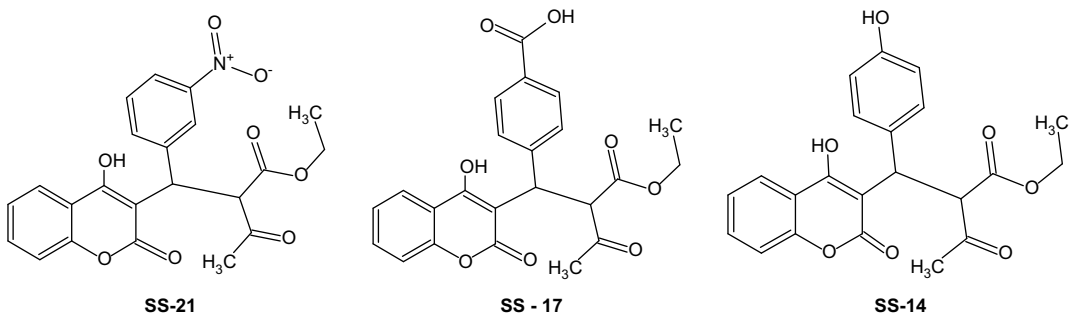

Fig. 4. Chemical structures of the investigated compounds.

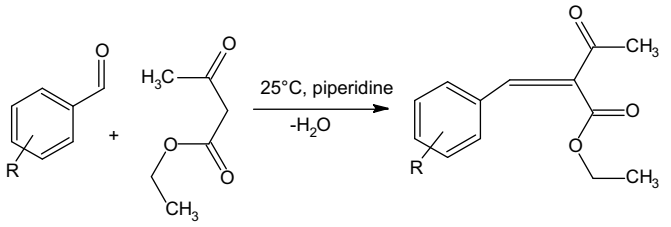

Fig. 5. Knoevenagel reaction between substituted benzaldehydes and ethylacetoacetate.

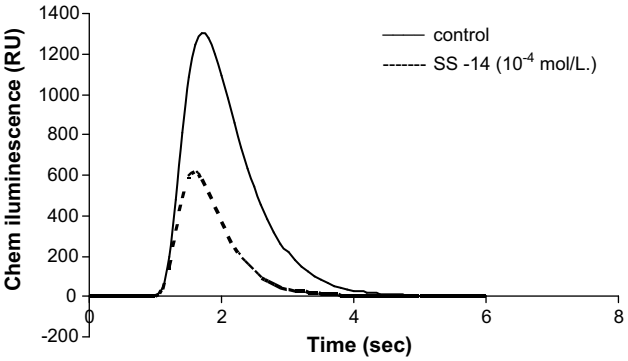

Fig. 7. Chemiluminescence kinetic curves of **SS-14** and control.

ring. The product was characterized by UV–vis, ^{1}H NMR and mass spectrometry.

4. Experimental part

4.1. Chemical part

All starting materials were purchased from Merck (Germany), Sigma–Aldrich (St. Louis, USA) and Fluka (Switzerland). They were used without further purification. Melting points were measured in open capillary tubes on a Büchi 535 melting point apparatus.

^{1}H NMR spectra were recorded in Brucker 250 MHz in DMSO-d_6 or acetone using TMS as an internal standard (chemical shifts are reported in ppm units). Abbreviations were as follows: s – singlet, d – doublet, dd – double doublet, t – triplet, m – multiplet, br – broad.

Mass-spectral analysis was performed by electron ionization on mass spectrometer Hewlett–Packard 5973 at 70 eV.

4.1.1. General procedure for the preparation of arylmethylene-β-ketoesters

Aromatic aldehyde and ethylacetoacetate in equimolar quantities were mixed in round-bottomed flask. Piperidine (0.03 mol) and glacial acetic acid (0.04 mol) were also added to the reaction mixture. The latter was stirred at room temperature for 90 min. After 20 ml ether and/or 150 ml distilled water was added to the reaction mixture, crystals with different colors were formed. These crystals were filtered off and washed. Then they were dried at room temperature and were recrystallized from appropriate solvents – mainly alcohols (ethanol, 1-propanol or 2-propanol).

4.1.1.1. Ethyl 2(4-hydroxyphenylmethylene)-3-oxobutanoatee (**SS-4**).

Yellow crystals, m.p. 141–143 °C. The substance crystallizes from ether. Purified after recrystallization from 2-propanol. Yield: 51%; UV–vis: $\lambda_{max} = 206$, 224, 286 nm; FTIR (nujol): 3325.7, 1732.3, 1641.6, 1597.3, 1462.2, 1205.7, 819.8 cm^{-1}; ^{1}H NMR (acetone, 200 MHz): $\delta = 1.3$ (t, 3H) (aliphatic), 2.3 (s, 3H) (aliphatic), 4.3 (q, 2H) (aliphatic), 6.9 (dd, 2H) (aromatic), 7.4 (dd, 2H) (aromatic), 7.6 (s, 1H) (aliphatic), 10.5 (s, 1H) (hydroxyl); ^{13}C NMR (acetone, 67 MHz): $\delta = 30$, 110, 135, 140, 160, 190; EIMS: m/z (%) = 234 (100, M^+), 233 (57), 220 (10), 219 (69), 217 (17), 205 (15), 191 (25.4), 189 (38.25), 187 (11.3), 175 (8.7), 163 (11.3), 161 (11.3), 160 (28.7), 151 (28.7), 147 (68.7), 146 (11.3), 145 (37.4), 131 (7), 123 (30.4), 120 (9.6), 119 (20), 118 (19.1), 115 (2.6), 107 (6), 91 (20), 89 (19.1), 77 (7), 65 (11.3), 63 (12.1), 53 (5), 45 (0.9); TLC: $R_f = 0.39$ (hexane/acetone = 2:1); Anal.: $C_{13}H_{14}O_4$ (234), (C, H) = (calcd/found): %C, 66.66/66.50; %H, 6.02/5.94.

4.1.1.2. 4-[2-(Ethoxycarbonyl)-3-oxobut-1-en-1-yl]benzoic acid (**SS-6**).

Yellow crystals, m.p. 148–150 °C. The substance crystallizes from water. Purified after recrystallization from ethanol. Yield: 57%; UV–vis: $\lambda_{max} = 204$, 292 nm; FTIR (nujol): band 3300–2400, 1736.1, 1689.8, 1608.8, 1460.3, 848 cm^{-1}; ^{1}H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H) (aliphatic), 2.4 (s, 3H) (aliphatic), 4.2 (q, 2H) (aliphatic), 7.4 (s, 1H) (aliphatic), 7.6 (s, 1H) (aromatic), 7.8 (s, 1H) (aromatic), 8.0 (d, 2H) (aromatic), 13.23 (s, 1H) (carboxyl group); EIMS: m/z (%) = 262 (64, M^+), 261 (16.7), 247 (18.4), 233 (8), 218 (18.4), 217 (100), 191 (10), 189 (14.9), 179 (9.6), 175 (26.3), 173 (27.2), 171 (11.4), 155 (14.9), 151 (16.7), 147 (7.9), 131 (9.6), 129 (17.5), 115 (7.9), 103 (18.4), 101 (14.9), 91 (5.3), 77 (11.4), 75 (8.7), 63 (3.5), 51 (2.6), 45 (1.8); TLC: $R_f = 0.33$ (hexane/chloroform/acetone/methanol = 5:3:2:1); Anal.: C_{14} H_{14} O_5 (262), (C, H) (calcd/found): %C, 64.12/64.44; %H, 5.38/5.26.

4.1.1.3. Ethyl 2-(3-nitrophenylmethylene)-3-oxobutanoate (**SS-19**).

White crystals, m.p.100–103 °C. The substance crystallizes from water. Purified after recrystallization from 2-propanol. Yield: 17%; UV–vis: $\lambda_{max} = 210$, 266 nm; FTIR (nujol): 1728.4, 1660.9, 1628.1, 1529.7, 780, 735 cm^{-1}; ^{1}H NMR (DMSO, 250 MHz): $\delta = 0.9$ (t, 3H) (aliphatic), 1.6 (s, 3H) (aliphatic), 4.8 (q, 2H) (aliphatic), 7.4 (s, 1H) (aliphatic), 7.6 (t, 1H) (aromatic), 7.9 (d, 1H) (aromatic), 8.1 (d, 1H) (aromatic), 8.3 (s, 1H) (aromatic); EIMS: m/z (%) = 263 (65.2, M^+), 262 (20), 248 (99.1), 246 (100), 234 (19.1), 220 (32.1), 218 (51.8), 216 (15.7), 202 (35.7), 200 (24.3), 192 (13), 180 (18.3), 176 (66.09), 174 (27), 160 (10.4), 152 (21.7), 146 (13), 130 (20.9), 129 (36.5), 120 (17.4), 115 (19.1), 102 (35.7), 101 (47.8), 89 (13.9), 75

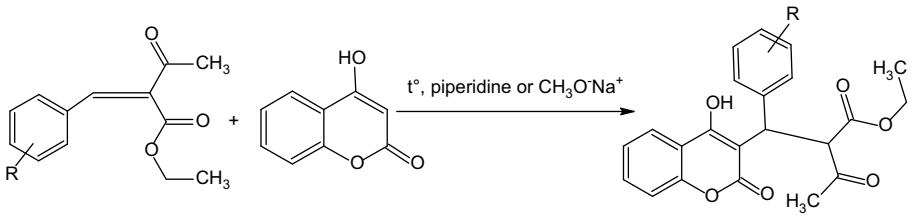

Fig. 6. Michael addition of arylmethylene-β-ketoester to 4-hydroxycoumarin.

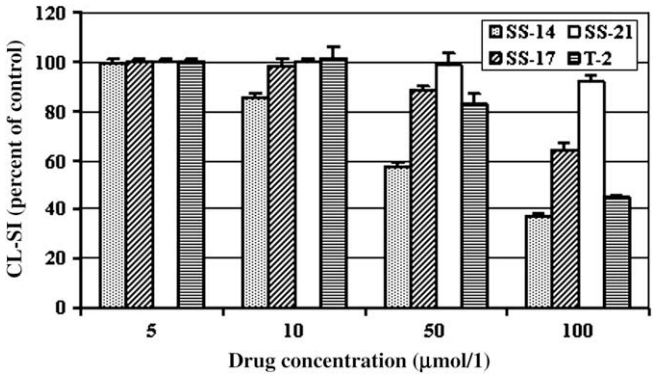

Fig. 8. Relationship between CL-SI and the concentration of the tested compound as bar graph.

(29.6), 63 (9.6), 51 (13), 45 (2.6); TLC: $R_f = 0.5$ (hexane/acetone = 2:1); Anal.: $C_{13}H_{13}NO_5$ (263), (C, H) (calcd/found): %C, 59.31/59.54; %H, 4.98/5.13.

The investigated compounds were 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)-methyl]-3-oxobutanoate (**SS-14**) [6], 4-[1-(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxy-carbonyl)-3-oxobutyl]benzoic acid (**SS-17**) [6], ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**) [6]. Their characteristics and synthetic procedure are presented below.

4.1.2. General procedure for the preparation of condensation products with 4-hydroxycoumarin

Arylmethylene-β-ketoester, obtained in previous reaction, and 4-hydroxycoumarin were mixed in equimolar quantities in 25–30 ml methanol (used as a solvent). Sodium methoxide (0.003 mol) as a basic agent was also added to the reagents. The reaction mixture was boiled and stirred for 60 h under reflux. The reaction was controlled by TLC (hexane/acetone = 2:1 or hexane/acetone/chloroform/methanol = 5:3:2:1). When the quantities of reagents depleted the heating was stopped. The residue from the reaction mixture was filtered off and was washed with hot water, in order to remove the unreacted 4-hydroxycoumarin. After that the residue was dried at room temperature and recrystallized from appropriate solvents (methanol, ethanol or 2-propanol).

*4.1.2.1. Ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(4-hydroxyphenyl)methyl]-3-oxobutanoate (**SS-14**) [6].* White crystals, m.p. 195–197 °C. Purified after recrystallization from ethanol. Yield: 21%; UV–vis: $\lambda_{max} = 214$, 280, 308 nm; FTIR (nujol): 3391.3, 1699.5,

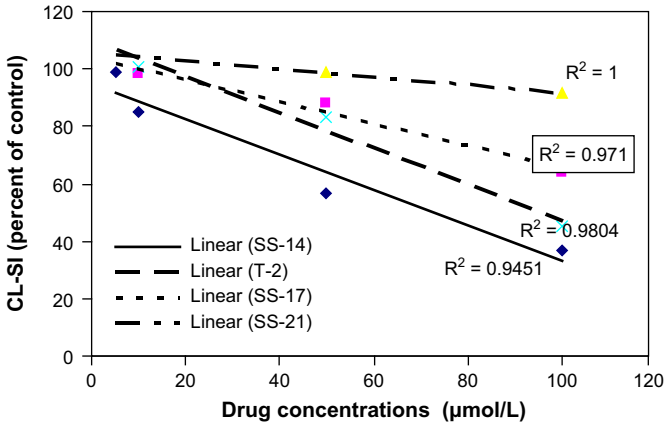

Fig. 9. Linear relationship between concentrations of the tested compounds and CL-SI.

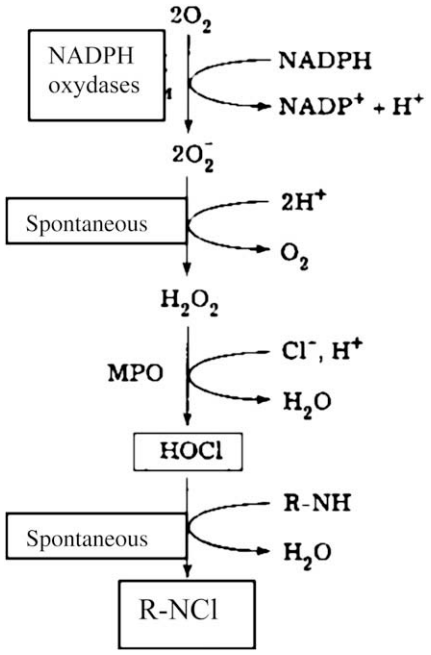

Fig. 10. The metabolic pathway of oxygen explosion [11].

1622.3, 1599.2, 1464.1, 821, 760 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H) (aliphatic), 2.0 (s, 3H) (aliphatic), 4.1 (q, 2H) (aliphatic), 4.4 (m, 1H) (aliphatic), 4.6 (m, 1H) (aliphatic), 6.9 (m, 2H) (aromatic), 7.3 (m, 1H) (aromatic), 7.4 (m, 1H) (aromatic), 7.6 (m, 2H) (aromatic), 7.9 (m, 1H) (aromatic), 8.1 (m, 1H) (hydroxyl), 8.6 (s, 1H) (hydroxyl); EIMS: m/z (%) = 396 (0.09, M^+), 364 (0.09), 350 (0.09), 321 (0.09), 307 (0.9), 279 (0.4), 266 (56.1), 265 (100), 249 (31.6), 237 (10.5), 221 (2.6), 210 (2.6), 181 (1.8), 165 (1.8), 153 (2.6), 146 (7), 130 (7), 121 (19.3), 118 (17.5), 102 (4.4), 92 (15.8), 85 (12.3), 76 (2.6), 63 (10.5), 53 (2.6), 46 (0.09); TLC: $R_f = 0.48$ (hexane/chloroform/acetone/methanol= 5:3:2:1); Anal.: C_{22} H_{20} O_7 (396), (C, H) (calcd/found): %C, 66.66/66.36; %H, 5.09/5.13.

*4.1.2.2. 4-[1-(4-Hydroxy-2-oxo-2*H*-chromen-3-yl)-2-(ethoxycarbonyl)-3-oxobutyl]benzoic acid (**SS-17**) [6].* White crystals, m.p. 150–155 °C. Purified after recrystallization from methanol. Yield: 28%; UV–vis: $\lambda_{max} = 208$, 282, 308 nm; FTIR (nujol): 3442, band 3300–2400, 1732.3, 1693.7, 1612.7, 1462.4, 1109, 846.3, 756.2 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H) (aliphatic), 2.1 (s, 3H) (aliphatic), 3.9 (q, 2H) (aliphatic), 4.2 (m, 1H) (aliphatic), 4.6 (m, 1H) (aliphatic), 7.2 (m, 1H) (aromatic), 7.4 (m, 1H) (aromatic), 7.5 (m, 2H) (aromatic), 7.6 (m, 2H) (aromatic), 7.8 (m, 1H) (aromatic), 8.0 (m, 1H) (hydroxyl), 12.83 (s, 1H) (carboxyl); EIMS: m/z (%) = 424 (1.3, M^+), 392 (0.4), 378 (17.5), 360 (1.8), 335 (48.2), 317 (19.3), 307 (9.6), 294 (44.7), 293 (34.2), 265 (6.1), 257 (50.9), 250 (22.8), 249 (100), 239 (5.3), 229 (1.8), 215 (12.3), 205 (1.8), 187 (2.6), 173 (2.6), 165 (4.4), 146 (2.6), 130 (6.1), 120 (19.3), 102 (6.1), 92 (30.7), 75 (6.1), 64 (8.8), 51 (2.6); TLC: $R_f = 0.62$ (hexane/chloroform/glacial acetic acid = 10:10:4); Anal.: $C_{23}H_{20}O_8$ (424) (C, H) (calcd/found): %C, 65.09/65.07; %H, 4.75/4.90.

*4.1.2.3. Ethyl 2-[(4-hydroxy-2-oxo-2*H*-chromen-3-yl)(3-nitrophenyl)methyl]-3-oxobutanoate (**SS-21**) [6].* White crystals, m.p. 135–140 °C. Purified after recrystallization from ethanol. Yield: 37%; UV–vis: $\lambda_{max} = 210$ nm; FTIR (nujol): 3335, 1732.3, 1674.4, 1620.4, 1529.7, 1068.7, 763, 736 cm^{-1}; ^1H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H) (aliphatic), 1.9 (s, 3H) (aliphatic), 3.9 (q, 2H) (aliphatic), 4.4 (m, 1H) (aliphatic), 4.6 (m, 1H) (aliphatic), 7.2 (m, 1H) (aromatic), 7.4 (m, 1H) (aromatic), 7.5 (m, 1H) (aromatic), 7.7 (m, 3H) (aromatic), 7.9 (m, 2H) (aromatic), 9.8 (s, 1H) (hydroxyl); EIMS:

m/*z* (%) = 425 (4.4, M^+), 382 (4.4), 361 (12.3), 336 (38.6), 320 (1.8), 308 (2.6), 294 (58.8), 278 (100), 266 (8.8), 257 (14.9), 249 (48.2), 248 (91.2), 239 (1.8), 220 (8.8), 205 (1.8), 176 (3.5), 165 (10.5), 139 (5.3), 130 (15.8), 120 (71.9), 101 (13.2), 92 (68.4), 85 (18.4), 75 (14.9), 64 (17.5), 51 (6); TLC: $R_f = 0.34$ (hexane/acetone = 2:1).

Ethyl 2-[(3,4,5-trimethoxyphenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**T-2**) is a brand new compound. Its synthetic procedure can be presented as follows.

4.1.2.4. Synthetic procedure for ***T-2***

4.1.2.4.1. Knoevenagel reaction between 3,4,5-trimethoxybenzaldehyde and ethylacetoacetate. About 1.96 g (0.01 mol) of 3,4,5-trimethoxybenzaldehyde, 5.2 g (0.04 mol) of ethylacetoacetate, 1.7 g (0.02 mol) of piperidine, and 1.2 g (0.02 mol) of glacial acetic acid were consequently poured into a round-bottomed flask. The reaction mixture was stirred with electromagnetic stirrer at room temperature for 2 h. 20 ml of ether was added at the end of the reaction time. The reaction mixture was left to stay for 2 days at room temperature and white-yellow crystals were formed. After that new quantities of 5–10 ml of ether was added and the crystal residue was filtered off in vacuo. These crystals were washed, dried at room temperature and recrystallized from appropriate solvents.

4.1.2.4.1.1. Ethyl-2-(3,4,5-trimethoxy)-phenylmethyleneacetoacetate

White crystals. The product was recrystallized from ethanol. M.p. 159–160.5 °C.; UV–vis $\lambda_{max} = 206$, 236, 320 nm; Anal. $C_{16}H_{20}O_6$ (308) (C, H) = (calcd/found): %C, 62.33/61.8; %H, 6.54/6.64.

4.1.2.4.2. Michael condensation between ethyl-2-(3,4,5-trimethoxy)-phenylmethyleneacetoacetate and 4-hydroxycoumarin. About 1.99g (0.0064 mol) of ethyl-2-(3,4,5-trimethoxy)phenylmethy leneacetoacetate, 2.09 g (0.0257 mol) of 4-hydroxycoumarin and 20 ml dioxane were poured and mixed in a round-bottomed flask. After that 1–2 ml of piperidine was added. The reaction mixture was stirred under reflux for 30 h. Then 20 ml of water was added to the reaction mixture and the stirring under reflux was continued for 2 h. After that the heating was stopped for 48 h. The reaction mixture was stirred under reflux for additional 30 h, according to TLC control. At the end of the reaction time the mixture was left at room temperature and white crystal residue was formed.

4.1.2.4.2.1. Ethyl 2-[(3,4,5-trimethoxyphenyl)(4-hydroxy-2-oxo-2*H*-chromen-3-yl)methyl]-3-oxobutanoate (**T-2**)

White crystals. The substance was recrystallized from ethanol. Yield: 32%, m.p. 155–157 °C, $R_f = 0.38$ (hexane/acetone = 2:1); UV–vis: $\lambda_{max} = 206$, 234 nm; ^{1}H NMR (DMSO, 250 MHz): $\delta = 1.0$ (t, 3H), 2.0 (s, 3H), 3.5 (m, 3H), 3.8 (m, 6H), 4.2 (q, 2H), 7.2 (m, 4H), 7.6 (m, 2H), 11.2 (s, 1H); EIMS: *m*/*z* (%) = 470 (0.4), 450 (0.4), 420 (44.7), 406 (0.4), 392 (0.4), 374 (17.4), 359 (0.8), 347 (28), 331 (4.5), 318 (1.5), 301 (100), 286 (6.1), 260 (26.5), 245 (9.8), 232 (8.3), 210 (1.5), 199 (4.5), 181 (5.3), 168 (8.3), 153 (5.3), 135 (5.3), 115 (6.8), 98 (9.8), 77 (6.1), 65 (2.3), 53 (4.5).

4.2. Investigation of the antioxidant properties

The luminol-dependent chemiluminescence (CL) was used for registration of ROS (reactive oxygen species) with LKB 1251 luminometer (Bioorbit, Turku, Finland) set at 310 K and connected with an AT-type computer. Data collection was performed by MultiUse program, version 1.08 (Bioorbit, Turku, Finland).

Luminol-dependent CL in a system of NaOCl generated ClO^-. The sample contained the following substances in 1 ml PBS: 0.1 mM luminol, 0.06 mM NaOCl and the tested drug at concentrations between 1 and 100 μM, or a buffer for the controls. The chemiluminescence was registered after the addition of NaOCl using the "flash assay" option of the MultiUse program, every 50 ms. The ratio of CL in the presence and in the absence of the drug was termed CL scavenging index (CL-SI).

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