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Original article

Synthesis and evaluation of the antioxidant and anti-inflammatory activity of novel coumarin-3-aminoamides and their alpha-lipoic acid adducts

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ABSTRACT

In the present work a series of novel coumarin-3-carboxamides and their hybrids with the alpha-lipoic acid were designed, synthesized and tested as potent antioxidant and anti-inflammatory agents. The new compounds were evaluated for their antioxidant activity, their activity to inhibit in vitro lipoxygenase and their in vivo anti-inflammatory activity. In general, the derivatives were generally found to present antioxidant and anti-inflammatory activities. Discussion is made based on the results for the structure–activity relationships in order to define the structural features required for activity.

1. Introduction

The association of antioxidants with inflammation stems from the recognition that free radicals are produced during the inflammatory process by macrophages. It has been reported that Reactive Oxygen Species (ROS) are involved in the cyclooxygenase- and lipoxygenase-mediated conversion of arachidonic acid into proinflammatory intermediates. On this basis, several natural and synthetic antioxidants have been tested and shown to possess anti-inflammatory properties [1,2].

Coumarins form an elite class of compounds, which occupy a special role in nature. Pharmacologically, coumarins are flavonoids along with a range of other compounds. Coumarins and their derivatives have been found to exhibit a variety of biological and pharmacological activities and have raised considerable interest because of their potential beneficial effects on human health [3,4]. They have been reported to possess among others: anti-HIV [5–8], anticoagulant [9], antibacterial [10,11], anticancer [12], anthelminthic [13], anti-inflammatory [14–16] and antioxidant activities [17–19]. As a result, coumarins and their derivatives have been the subject of extensive investigations.

The coumarin nucleus incorporates the styryl carbonyl moiety into a rigid framework. It has been reported that styryl carbonyl derivatives possess appreciable anti-inflammatory activity [20–21]. Moreover, coumarin and related derivatives have been used as inhibitors of lipoxygenase (LOX) and cyclooxygenase (COX) pathways of arachidonic acid metabolism [22]. One of the most well-studied coumarin-based anti-inflammatory drugs is cloricromene (8-monochloro-3-b-diethylaminoethyl-4-methyl-7-ethoxy-carbonyl-methoxy coumarin), and several synthetic analogues of this compound have been recently reported and tested for their anti-inflammatory and antioxidant activities [23].

In addition, many coumarin derivatives have special ability to scavenge ROS and to influence processes involving free radical-injury [24,25].

The 4-hydroxycoumarin moiety, widely spread among coumarin natural products, has been the molecular template for the synthesis of a variety of analogues exhibiting important biological activity. Characteristic examples include warfarin,

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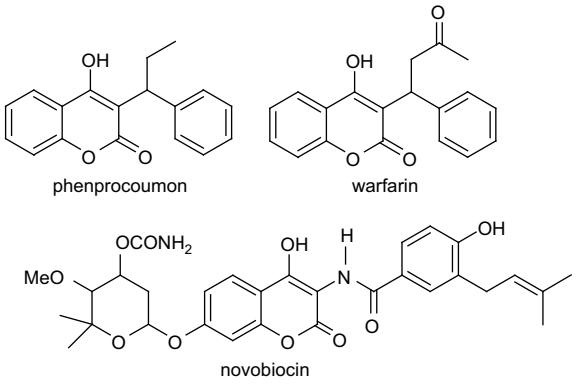


Fig. 1.

a synthetic compound used as a rodenticide and anticoagulant and phenprocoumon with antiviral and anti-HIV activities (Fig. 1) [3]. In addition, aminocoumarin analogues such as novobiocin, chlorobiocin, coumermycin and simocyclinone, which contain an amide bond at position 3 of the heterocyclic ring, are potent antibiotics [26,27]. Moreover, novel 4-hydroxycoumarin-3-carboxamide derivatives have been synthesized as potential drugs for the treatment of Insulin Dependent Diabetes Mellitus (IDDM) [28].

The potential of 4-hydroxycoumarin derivatives as anti-inflammatory and antioxidant agents, prompted us to design and synthesize a series of novel coumarin analogues bearing the 3-carboxamide functionality. Analogous carboxamides based on the structure of 4-hydroxy-quinolinone have been recently synthesized by our group and led to compounds with combined antioxidant and anti-inflammatory activities [29].

Lipoic acid (1,2-dithiolane-3-valeric acid, LA) is a naturally occurring compound present in all kinds of prokaryotic and eukaryotic cells and synthesized by animals and humans. LA exists as both the reduced dithiol form (dihydrolipoic acid – DHLA) and the oxidized disulfide form [30,31]. A great deal of attention has been paid to the antioxidant activity of the LA/DHLA redox couple and their roles as biological thiol antioxidants since they have been shown to possess remarkable antioxidant activity in vitro and in vivo and have been described as universal antioxidants effectively reacting with $RO_2^{\bullet}$, ascorbyl radicals, $HO^{\bullet}$, $NO^{\bullet}$, tocopheryl radicals, $O_2^{\bullet-}$ and hypochlorous acid [32–34].

The combination of the distinct pharmacophores of two different biologically active compounds in the same structure, has been previously reported in literature and is highly likely to lead to hybrid compounds with significant activity [35–38]. Recently we have published some novel quinolinone-3-aminoamides bearing a methyl or phenyl group on the nitrogen heteroatom incorporating the alpha-lipoic acid (LA) moiety and we have examined the influence of this modification on the anti-inflammatory/antioxidant activity of the aminoamides [29]. Therefore, we decided to additionally incorporate alpha-lipoic acid to the coumarin scaffold. Our work reveals that some of the novel hybrid compounds are potent leads and may be useful in the prevention of human diseases attributed to free radical damage.

2. Chemistry

The synthesis of the target compounds includes three steps briefly described as follows: i) Synthesis of functionalized 3-ethoxycarbonyl 4-hydroxycoumarins, ii) synthesis of aliphatic and aromatic coumarin-3-carboxamides, using aliphatic and aromatic diamines and iii) reaction of the resulting aminoamides with activated lipoic acid. The above steps are shown in Schemes 1–3.

Based on our previous work, an efficient short-step synthesis was used to produce functionalized 3-ethoxycarbonyl 4-hydroxycoumarins [39]. These compounds were synthesized via a *C*-acylation–cyclization reaction of diethyl malonate with the *N*-hydroxybenzotriazolyl esters of functionalized acetyl salicylic acids. Our strategy involves activation of the functionalized acetyl salicylic acids **1**, **2** using *N*-hydroxybenzotriazole (HOBt) and *N*,*N*-dicyclohexylcarbodiimide (DCC) and condensation of the resulting *N*-hydroxybenzotriazolyl esters with the anion of diethyl malonate. The intermediates **3**, **4** were converted to the corresponding 3-ethoxycarbonyl 4-hydroxycoumarins **5**, **6** via an intramolecular condensation reaction, using methanolic 10% hydrochloric acid solution (Scheme 1).

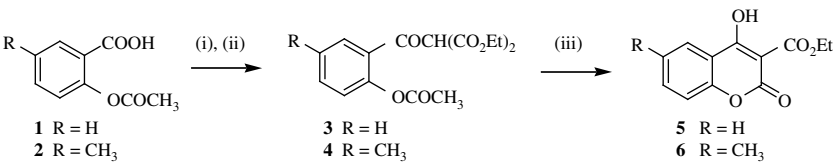
Reaction of coumarins **5**, **6** with the appropriate aliphatic diamines (1,2-ethylenediamine, 1,6-hexamethylenediamine and 1,8-octamethylenediamine) and 1,2-phenylenediamine in refluxing toluene yielded the desired coumarin-3-aminocarboxamides **7**–**13** (Scheme 2), as solids which precipitated in the reaction solution. The compounds were isolated by filtration and washing with diethylether, providing analytically pure compounds without the need of further purification.

Activation of lipoic acid was accomplished by treatment of racemic alpha-lipoic acid with *N*-hydroxysuccinimide and DCC in dichloromethane. The resulting ester **14** is easily prepared in high yields, can be stored for a long time and the *N*-hydroxysuccinimide formed subsequently as a byproduct is a water-soluble compound and can be efficiently removed from the reaction mixture.

The synthesis of hybrid compounds **15**–**19** was achieved by dissolving equimolar amounts of compounds **7**–**13** with the active ester **14** in dichloromethane containing a few drops of dimethylformamide in order to facilitate dissolution. The desired products were obtained after stirring for 48 h, at room temperature, in the absence of light, followed by aqueous work-up and flash column chromatography (Scheme 3).

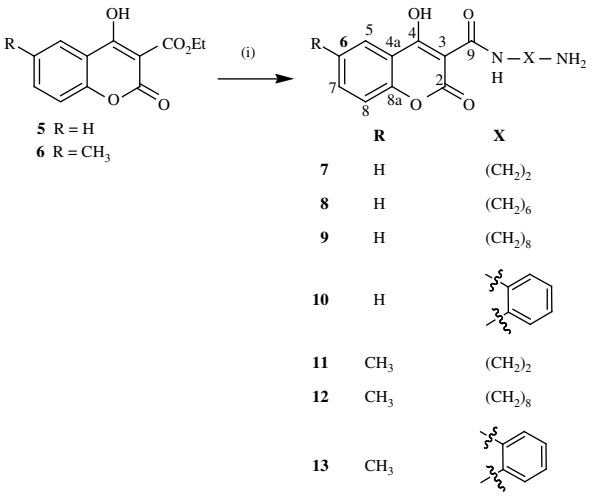
3. Biology

In acute toxicity experiments, the in vivo examined compounds did not present toxic effects in doses up to 0.5 mmol/kg body weight. In almost all cases the animals were survived (80–90%) and looked normal both macroscopically and by autopsy. These results indicate that the synthesized compound demonstrates low general toxicity.



1 R = H
2 R = CH_3
3 R = H
4 R = CH_3
5 R = H
6 R = CH_3

Scheme 1. Synthesis of 3-ethoxycarbonyl 4-hydroxycoumarins. Reagents and conditions: (i) HOBt, DCC, THF; (ii) NaH, diethyl malonate, THF; (iii) HCl/MeOH 10%.

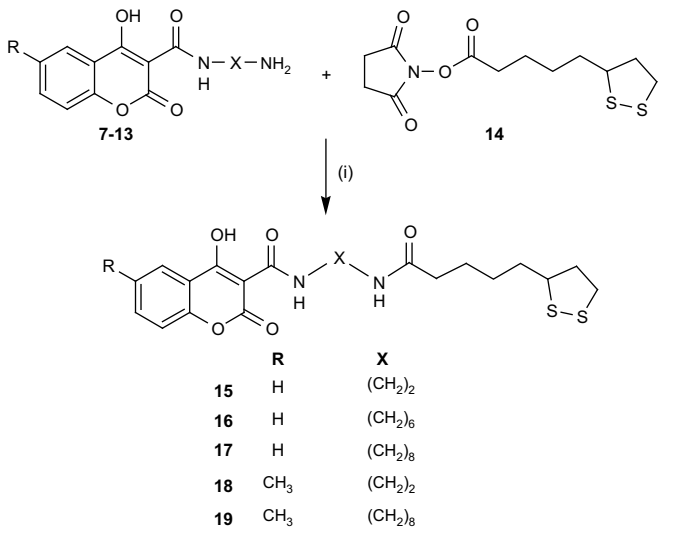


Scheme 2. Synthesis of coumarin-3-carboxamides. Reagents and conditions: (i) diamine, toluene, 110 °C.

3.1. In vivo anti-inflammatory activity

The in vivo anti-inflammatory effects of the tested coumarins were assessed by using the functional model of carrageenin-induced rat paw edema and are presented in Table 1, as percent inhibition of induced rat paw edema. Acute inflammation is due to the release of chemical mediators, which cause edema as a result of extravasations of fluid and proteins from the local microvasculature and accumulation of polymorphonuclear leukocytes at the inflammatory site. Carrageenin-induced inflammation is a non-specific inflammation resulting from a complex of diverse mediators [40]. This model is conventional, sensitive, and accepted for screening of newer anti-inflammatory agents [41]. Further, this model reliably predicts the anti-inflammatory efficacy based on the inhibition of prostaglandin amplification [42]. As shown in Table 1, all the investigated compounds inhibited carrageenin-induced paw edema. The protection ranged from 41 to 73% while the reference drug, indomethacin, induced 47% protection at an equivalent dose. The coumarin–lipoic acid conjugates **16** and **19** were found highly potent. Their potency remains high even in lower doses (Table 1). The nature and the presence of moiety X within the coumarin-3-carboxamides seem to influence the inhibition values in comparison to the starting materials **5** and **6**. Thus, amides **7**–**10** derived from compound **5** present a decrease in the induced carrageenin rat paw edema (CPE) % values. Between compounds **7**–**9** the increase of the number of the CH_2 groups (from 2 to 8) led to a decrease in activity. The decrease in the presence of an *ortho*-phenyl-diamine is lower (compound **10** = 57%, compound **5** = 63%, Table 1). The amides **11**–**13**, derived from compound **6** led to more potent derivatives. However the observed differences in CPE % values among **11**–**13** are not significant.

Lipoic acid–coumarin-3-aminoamides hybrids showed higher anti-inflammatory activity than lipoic acid alone (29.6% inhibition in the carrageenin-induced rat paw edema). The presence of the lipoic group in the adducts **16** and **19** is correlated with higher activity in comparison to compounds **8** and **12**. However the adduct **17** presents lower activity than compound **9** (a coumarin-3-carboxamide derivative). The results show that, in the case of the synthesized coumarin derivatives, overall lipophilicity does not increase in parallel to inhibition (Table 1), with the exception of compounds **12** and **19** (compound **12** = *c* log *P* 4.01, CPE % = 59 and compound **19** = *c* log *P* 6.18. and CPE % = 73).



Scheme 3. Synthesis of coumarin–lipoic acid conjugates. Reagents and conditions: (i) CH_2Cl_2, DMF, r.t.

3.2. In vitro antioxidant activity

It is well known that free radicals play an important role in the inflammatory process [43]. Many non-steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers [44]. Consequently, compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation and to lead to potentially effective drugs. Thus, we tested the novel amides and hybrids with regard to their antioxidant ability and in comparison to well-known antioxidant agents e.g. nordihydroguaiaretic acid and trolox (Tables 1 and 2). The potential of the antioxidant activity is shown in Tables 1 and 2. The antioxidant activity has been evaluated in two in vitro

Table 1
Competition % of compounds with DMSO for hydroxyl radical (HO˙ %); inhibition of soybean lipoxygenase percent LOX %; percent inhibition of induced carrageenin rat paw edema (CPE %) at 0.01 mmol/kg body weight. Calculated lipophilicity *c* log *P*[51].

Compounds	*c* log *P* [51]	˙OH %[a]	LOX %[b]	CPE %[c] 0.01 mmol/kg
5	2.83	91	93	63**
6	3.33	81	93	41*
7	1.21	91	100	59**
8	2.46	100	52	49**
9	3.51	no	no	48**
10	2.26	no	23	57*
11	1.71	100	14	59**
12	4.01	100	30	59**
13	2.76	100	22	60**
16	4.62	96	18	72[d] **
				61[e] *
17	5.68	no	33	43*
19	6.18	100	8.3	73[d] *
				60[e] *
Indomethacin		nt	nt	47**
Lipoic acid	2.39	38.6	29.1	29.6**
NDGA		nt	84	nt
Trolox		88.2	nt	nt

**$P < 0.01$, *$P < 0.05$; no: no action under the reported experimental conditions; nt: not tested; Nordihydroguaiaretic acid (NDGA).
[a] Final concentration for the tested compounds 0.1 mM.
[b] Final concentration for the tested compounds 0.1 mM.
[c] Statistical studies were done with student's *t*-test.
[d] The in vitro results were performed at 0.01 mmol/kg.
[e] The in vitro results were performed at 0.001 mmol/kg.

Table 2
Interaction with DPPH-reducing activity % (RA %).

Compounds	0.1 mM	0.1 mM	0.5 mM	0.5 mM
	RA % 20 min	RA % 60 min	RA % 20 min	RA % 60 min
5	15	27	20	13
6	18	8	20	33
7	42	25	No	No
8	3	3	5	5
9	17	8	9	19
10	90	93	85	83
11	48	59	70	65
12	58	68	72	78
13	74	65	70	78
16	15	18	23	12
17	21	21	32	25
19	69	65	89	87
Lipoic acid	20	27	5	7
NDGA	81	83	97	98

No: no action under the reported experimental conditions; Nordihydroguaiaretic acid (NDGA); the final concentrations of compounds were 0.1 mM and 0.5 mM.

tests. In view of the differences among the test systems available, the results of a single assay can give only a suggestion on the protective potential of tested compounds. Among the plethora of methods used for the evaluation of the antioxidant activity, the DPPH test is very useful in the micromolar range demanding minutes to hours for both lipophilic and hydrophilic samples. In the cases where the structure of the electron donor is not known this method can afford data on the reduction potential of the sample, and hence can be helpful in comparing the reduction potential of unknown materials. The competition of the compounds with DMSO for hydroxyl radicals has been used, whereas antioxidant capability as well as lipid peroxidation inhibitory activity was assayed by the inhibition of the oxidizing enzyme lipoxygenase [45].

The interaction of the examined compounds with the stable free radical DPPH was studied (Table 2). This interaction indicates their radical scavenging ability in an iron-free system using a stable free radical which can accept an electron or hydrogen atom [45]. In general, compounds **5**, **6**, **8**, **9**, **16** and **17** were found to have very low activity, whereas compound **10** showed the highest interaction (90%) at 0.1 mM, followed by compounds **13**, **19**, **12** and **11**. Not many changes in interaction % values were observed between the amides and the corresponding LA hybrids. The adduct **17** and compound **9** (a coumarin-3-carboxamide derivative) present almost equal interaction values. Compound **12** presents lower activity than the corresponding adduct **19**. Introduction of the lipoic acid moiety improves the DPPH % interaction with compound **19**. For most of the compounds the interaction was not time and concentration dependent. The time course of DPPH interaction, as affected by two different concentrations is given in Table 2. In general, this interaction expresses the reducing activity of the tested compounds and indicates their ability to scavenge free radicals.

Lipophilicity does not seem to play a significant role under the reported experimental conditions.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflamed site. Superoxide anion and H_2O_2 are the Reactive Oxygen Species (ROS) primarily formed by phagocytic cells. At sites of inflammation superoxide anion participates in reaction producing H_2O_2, the highly reactive hydroxyl radical and possibly singlet $^1O_2$. When superoxide dismutase comes in contact with the superoxide, it reacts with it and forms H_2O_2 which is dangerous in the cell because it can easily transform into a hydroxyl radical (via reaction with Fe^{2+} Fenton chemistry) one of the most destructive free radicals [46]. Hydroxy radicals are considered to be responsible for some of the tissue damages occurring in inflammation. Hydroxy radicals formed in the body, can lead to the generation of carbon-centered and peroxyl radicals. It has been claimed that hydroxyl radical scavengers could serve as protectors, thus increasing prostaglandin synthesis.

The competition of coumarins with DMSO for HO$^\bullet$ generated by the Fe^{3+}/ascorbic acid system, expressed as percent inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. In these experiments (Table 1) compound **9** and its adduct compound **17** did not show any inhibition at 0.1 mM. Compound **10** also did not present any activity. Compounds **8**, **11**–**13**, **19** and **16** were found to be very potent followed by compounds **5** and **7** (equipotent) and **6**. It is important that compounds **8** and **12** and their adducts **16** and **19** are highly potent at 0.1 mM. In compound **9** with a $(CH_2)_8$ methylenic chain the competition disappears. The "mother compound" coumarin presents 78% competition at the concentration of 0.1 mM showing that the coumarin nucleus implicated by itself in the scavenging procedure.

The assay for lipoxygenase (LOX) activity was carried out according to the UV absorbance based enzyme assay [47] using soybean lipoxygenase. While one may not extrapolate the quantitative results of this assay to the inhibition of mammalian 5-LOX, it has been shown that inhibition of plant LOX activity by NSAIDs is qualitatively similar to their inhibition of the rat mast cell LOX and may be used as a simple qualitative screen for such activity. (Table 1) Perusal of LOX % inhibition values shows that compound **7** [X = $(CH_2)_2$] is the most active, within the set, followed by compounds **5** and **6**. Compounds **16** and **19** (adducts of LA and compounds **8** and **12**) present lower LOX inhibition value compared to compounds **8** and **12**. On the contrary compound **17** (LA's hybrid of compound **9**) presents 33% LOX inhibition (compound **9** does not present any inhibition under the reported experimental conditions). The majority of the LOX inhibitors referred in literature acts as: a) antioxidants or free radical scavengers [48], since lipoxygenation occurs via a carbon-centered radical, b) inhibitors to reduce Fe^{3+} at the active site to the catalytically inactive Fe^{2+} (LOXs contain a "non-heme" iron per molecule in the enzyme active site as high-spin Fe^{2+} in the native state and the high-spin Fe^{3+} in the activated state) and c) excellent ligands for Fe^{3+}. In case of compounds **5**–**7** their LOX inhibition is correlated with their high hydroxyl radical scavenging activity.

4. Conclusions

In the present study, as a first step, we have successfully developed a synthetic route for the preparation of novel hybrids of coumarins and lipoic acid. Their synthesis is almost simple with satisfactory yields. We have then shown that the synthesized compounds, both the aminoamides and the final hybrids, possess significant anti-inflammatory activity in vivo and also that **16** and **19** are potent $^\bullet$OH scavengers. Moreover, an overview of the results of the in vivo experiment reveals that the tested coumarin–lipoic acid hybrids **16** and **19** exhibit significantly higher activity than lipoic acid. Also adducts **16** and **19**, seem to be more potent in vivo than the corresponding aminoamides **8** and **12**.

The in vivo anti-inflammatory activity of **12** and **19** seems to be related with their high HO$^\bullet$ scavenging and reducing activities, whereas the high competition of compound **16** with DMSO for hydroxyl radicals is correlated with its in vivo potency. More specifically, it is evident that, coumarin aminoamides **11**–**13** exhibit satisfactory combined antioxidant–anti-inflammatory activity therefore the design of this type of dual acting molecules should be further explored based on the structural features of these compounds. Among the final hybrids, the hybrid adducts **16** and **19**

present the highest in vivo activity from all the tested compounds, they are more effective than the corresponding aminoamides **8** and **12** therefore worth further study.

5. Experimental section

Melting points were determined on a Galenkamp MFB-595 melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian Gemini 2000, 300 MHz spectrometer. Elemental analyses were recorded on a Euro EA3000 Series Euro Vector CHNS Elemental Analyser.

5.1. General procedure for the synthesis of coumarin-3-carboxamides

3-Ethoxycarbonyl 4-hydroxycoumarins **5**, **6** (1 mmol) were mixed with the appropriate diamine (2 mmol) in dry toluene (5 mL). The mixture was stirred at 110 °C for 4 h. The precipitate formed was filtered off, washed with diethylether and dried in vacuo.

5.1.1. N-(2-aminoethyl)-4-hydroxy-2-oxo-2H-chromene-3-carboxamide (**7**) [49]

White solid, mp 245–247 °C. Obtained from the reaction of 3-ethoxycarbonyl-4-hydroxycoumarin (**5**) with 1,2-ethylenediamine. Yield: 79%; ^{1}H NMR ($CDCl_3/CF_3COOD$) $\delta = 3.47$ (br s, 2H, CH_2NH_2), 3.79–3.86 (t, $J = 4.8$ Hz, 2H, $NHCH_2$), 7.41 (d, $J_{7,8} = 8.4$ Hz, 1H, H-8), 7.48 (pseudotriplet, $J = 7.6$ Hz, 1H, H-6,), 7.81 (pseudotriplet, $J = 8.1$ Hz, 1H, H-7), 8.09 (dd, $J_{5,6} = 7.8$ Hz, $J_{5,7} = 1.4$ Hz, 1H, H-5), 9.64 (br s, 1H, CONH); Anal. Calcd for $C_{12}H_{12}N_2O_4$: C, 58.06, H, 4.87, N, 11.28. Found C, 58.32, H, 4.73, N, 11.31.

5.1.2. N-(6-aminohexyl)-4-hydroxy-2-oxo-2H-chromene-3-carboxamide (**8**) [50]

White solid, mp 212–214 °C. Obtained from the reaction of 3-ethoxycarbonyl-4-hydroxycoumarin (**5**) with 1,6-hexamethylenediamine. Yield: 65%; ^{1}H NMR ($CDCl_3/CF_3COOD$) $\delta = 1.15$–1.49 (m, 8H, $CH_2(CH_2)_4CH_2$), 2.75 (br s, 2H, CH_2NH_2), 3.31 (br s, 2H, $NHCH_2$), 7.06–7.45 (m, 2H, H-6, H-8), 7.62 (pseudotriplet, $J = 7.9$ Hz, 1H, H-7), 7.78 (d, $J = 8.0$ Hz, 1H, H-5), 10.28 (br, 1H, CONH); Anal. Calcd for $C_{16}H_{20}N_2O_4$: C, 63.14, H, 6.62, N, 9.20. Found C, 63.26, H, 6.67, N, 9.34.

5.1.3. N-(8-octylamino)-4-hydroxy-2-oxo-2H-chromene-3-carboxamide (**9**)

White solid, mp 219–222 °C. Obtained from the reaction of 3-ethoxycarbonyl-4-hydroxycoumarin (**5**) with 1,8-octamethylenediamine. Yield: 71%; ^{1}H NMR ($CDCl_3/CF_3COOD$): $\delta = 1.24$–1.45 (m, 8H, $(CH_2)_2(CH_2)_4(CH_2)_2$), 1.67–1.72 (m, 4H, $CH_2(CH_2)_4CH_2$), 3.14 (t, $J = 6.7$ Hz, 2H, CH_2NH_2), 3.46 (t, $J = 6.9$ Hz, 2H, $NHCH_2$), 7.37 (dd, $J_{7,8} = 8.1$ Hz, $J_{6,8} = 1.2$ Hz, 1H, H-8), 7.46 (dtd, $J_{6,8} = 1.2$ Hz, $J_{6,7} = 7.7$ Hz, $J_{5,6} = 7.8$ Hz, 1H, H-6), 7.77 (dtd, $J_{5,7} = 1.4$ Hz, $J_{6,7} = 7.7$ Hz, $J_{7,8} = 8.1$ Hz, 1H, H-7), 8.09 (dd, $J_{5,7} = 1.4$ Hz, $J_{5,6} = 7.8$ Hz, 1H, H-5), 9.42 (br s, 1H, CONH); Anal. Calcd for $C_{18}H_{24}N_2O_4$: C, 65.04, H, 7.28, N, 8.43. Found C, 65.27, H, 7.15, N, 8.21.

5.1.4. N-(2-aminophenyl)-4-hydroxy-2-oxo-2H-chromene-3-carboxamide (**10**)

Green solid, mp >280 °C. Obtained from the reaction of 3-ethoxycarbonyl-4-hydroxycoumarin (**5**) with 1,2-phenylenediamine. Yield: 44%; ^{1}H NMR ($CDCl_3/CF_3COOD$): $\delta = 7.46$–7.49 (d, $J_{7,8} = 7.9$ Hz, 1H, H-8), 7.54 (br s, 1H, aromatic), 7.56–7.6 (pseudotriplet, $J = 7.9$ Hz, 1H, H-6), 7.58–7.63 (br s, 1H, aromatic), 7.70–7.78 (m, 2H, aromatics), 7.84–7.89 (dtd, $J_{5,7} = 1.5$ Hz, $J_{6,7} = 7.7$ Hz, $J_{7,8} = 7.9$ Hz, 1H, H-7), 8.15–8.18 (dd, $J_{5,7} = 1.5$ Hz, $J_{5,6} = 8.1$ Hz, 1H, H-5), 11.4 (br s, 1H, CONH); Anal. Calcd for $C_{16}H_{12}N_2O_4$: C, 64.86, H, 4.08, N, 9.45. Found C, 64.68, H, 4.21, N, 9.63.

5.1.5. N-(2-aminoethyl)-4-hydroxy-6-methyl-2-oxo-2H-chromene-3-carboxamide (**11**)

White solid, mp 252–256 °C (dec). Obtained from the reaction of 3-ethoxycarbonyl-4-hydroxy-6-methyl coumarin (**6**) with 1,2-ethylenediamine. Yield: 65%; ^{1}H NMR ($CDCl_3/CF_3COOD$): $\delta = 2.48$ (s, 3H, CH_3), 3.46 (br s, 2H, CH_2NH_2), 3.83 (t, $J = 6.9$ Hz, 2H, $NHCH_2$), 7.29 (d, $J_{7,8} = 8.2$ Hz, 1H, H-8), 7.62 (dd, $J_{5,7} = 1.2$ Hz, $J_{7,8} = 8.2$ Hz 1H, H-7), 7.88 (d, $J_{5,7} = 1.2$ Hz, 1H, H-5), 9.78 (br s, 1H, CONH); Anal. Calcd for $C_{13}H_{14}N_2O_4$: C, 59.54, H, 5.38, N, 10.68. Found C, 59.77, H, 5.41, N, 10.35.

5.1.6. N-(8-aminooctyl)-4-hydroxy-6-methyl-2-oxo-2H-chromene-3-carboxamide (**12**)

White solid, mp 221–224 °C (dec). Obtained from the reaction 3-ethoxycarbonyl-4-hydroxy-6-methyl coumarin (**6**) with 1,8-octamethylenediamine. Yield: 98%; ^{1}H NMR ($CDCl_3/CF_3COOD$): $\delta = 1.34$–1.39 (m, 8H, $(CH_2)_2(CH_2)_4(CH_2)_2$), 1.6–1.69 (m, 4H, $CH_2(CH_2)_4CH_2$), 2.47 (s, 3H, CH_3), 3.15 (t, $J = 6.7$ Hz, 2H, CH_2NH_2), 3.45 (t, $J = 6.9$ Hz, 2H, $NHCH_2$), 7.3 (d, $J_{7,8} = 8.0$ Hz, 1H, H-8), 7.59 (d, $J_{7,8} = 7.9$ Hz, 1H, H-7), 7.87 (s, 1H, H-5), 9.81 (br s, 1H, CONH); Anal. Calcd for $C_{19}H_{26}N_2O_4$: C, 65.88, H, 7.57, N, 8.09. Found C, 65.79, H, 7.64, N, 8.21.

5.1.7. N-(2-aminophenyl)-4-hydroxy-6-methyl-2-oxo-2H-chromene-3-carboxamide (**13**)

Green solid, mp 196–199 °C (dec). Obtained from the reaction of 3-ethoxycarbonyl-4-hydroxy-6-methyl coumarin (**6**) with 1,2-phenylenediamine. Yield: 48%; ^{1}H NMR ($CDCl_3/CF_3COOD$): $\delta = 2.46$ (s, 3H, CH_3), 6.8 (m, 2H, $CONHCCCCHCHCCNH_2$), 7.12 (m, 1H, $CONHCCCCCCHCNH_2$), 7.29 (m, 1H, $CONHCCHCCCCCNH_2$), 7.43–7.53 (m, 2H, H-7, H-8), 7.85 (s, 1H, H-5), 10.91 (s, 1H, CONH); Anal. Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80, H, 4.55, N, 9.03. Found C, 66.04, H, 4.38, N, 9.18.

5.2. Synthesis of the final compounds: general procedure

Coumarin-3-aminocarboxamides **7**–**13** (0.46 mmol) were mixed with *N*-(lipoyloxy)succinimide (**14**) (0.14 g, 0.46 mmol) in dichloromethane (3 mL) and drops of DMF were added to facilitate dissolution of the reactants. The mixture was stirred and light-protected overnight. Drops of H_2O were added to the mixture and afterwards it was extracted with dichloromethane (3×10 mL) and washed with H_2O. The organic extracts were dried (Na_2SO_4) and concentrated in vacuo to afford the final compounds which were triturated with diethylether and filtered off.

5.2.1. N-(2-(4-(1,2-dithiolan-3-yl)butanamido)ethyl)-4-hydroxy-2-oxo-2H-chromene-3-carboxamide (**15**)

Yellowish solid, mp 151–153 °C. Obtained from the reaction of *N*-(2-aminoethyl)-4-hydroxy-2-oxo-2*H*-chromene-3-carboxamide (**7**) with *N*-(lipoyloxy)succinimide (**14**). Yield: 75%; ^{1}H NMR ($CDCl_3$): $\delta = 1.41$–1.5 (m, 2H, $CH_2CH_2CH_2CHS$), 1.61–1.72 (m, 4H, $CH_2CH_2CH_2CHS$), 1.89 (sextet, $J = 6.6$ Hz, 1H, SCH_2CHH), 2.17–2.23 (m, 2H, $NHCOCH_2$), 2.44 (sextet, $J = 5.9$ Hz, 1H, SCH_2CHH), 3.06–3.17 (m, 2H, CH_2S), 3.48–3.65 (m, 5H, $NH(CH_2)_2NH$, CHS), 6.00 (br, 1H, NHCO), 7.33–7.4 (m, 2H, H-6, H-8), 7.69 (dtd, $J_{7,8} = 7.5$ Hz, $J_{7,6} = 7.6$ Hz, $J_{7,5} = 1.5$ Hz, 1H, H-7), 8.04 (dd, $J_{5,6} = 7.8$ Hz, $J_{5,7} = 1.5$ Hz, 1H, H-5), 9.41 (br s, 1H, CONH), 17.67 (s, 1H, OH); Anal. Calcd for $C_{20}H_{24}N_2O_5S_2$: C, 55.03, H, 5.54, N, 6.42. Found C, 54.83, H, 5.42, N, 6.56.

5.2.2. N-*(6-(5-(1,2-dithiolan-3-yl)pentanamido)hexyl)-4-hydroxy-2-oxo-2*H*-chromene-3-carboxamide (**16**)*

Yellowish solid, mp 128–134 °C. Obtained from the reaction of *N*-(6-aminohexyl)-4-hydroxy-2-oxo-2*H*-chromene-3-carboxamide (**8**) with *N*-(lipoyloxy)succinimide (**14**). Yield: 50%; ^{1}H NMR ($CDCl_3$) $\delta = 1.33–1.71$ (m, 14H, $NHCH_2(CH_2)_4CH_2NH$, $(CH_2)_3CHS$), 1.9 (sextet, $J = 6.5$ Hz, 1H, SCH_2CHH), 2.14–2.19 (m, 2H, $NHCOCH_2$), 2.45 (sextet, $J = 6.5$ Hz, 1H, SCH_2CHH), 3.06–3.28 (m, 4H, $(CH_2)_4CH_2NH$, CH_2S), 3.45 (q, $J = 6.6$ Hz, 2H, $NHCH_2(CH_2)_4$), 3.56 (q, $J = 7.2$ Hz, 1H, CHS), 5.47 (br, 1H, NHCO), 7.26–7.39 (m, 2H, H-6, H-8), 7.69 (dd, $J_{7,5} = 1.8$ Hz, $J_{7,6} = 8.2$ Hz, 1H, H-7), 8.05 (dd, 1H, H-5, $J_{5,6} = 8.2$ Hz, $J_{5,7} = 1.8$ Hz), 9.26 (br, 1H, CONH), 18.18 (s, 1H, OH); Anal. Calcd for $C_{24}H_{32}N_2O_5S_2$: C, 58.51, H, 6.55, N, 5.69. Found C, 58.62, H, 6.59, N, 5.51.

5.2.3. N-*(8-(5-(1,2-dithiolan-3-yl)pentanamido)octyl)-4-hydroxy-2-oxo-2*H*-chromene-3-carboxamide (**17**)*

Yellow solid, mp 112–117 °C. Obtained from the reaction of *N*-(8-aminooctyl)-4-hydroxy-2-oxo-2*H*-chromene-3-carboxamide (**9**) with *N*-(lipoyloxy)succinimide (**14**). Yield: 59%; ^{1}H NMR ($CDCl_3$): $\delta = 1.29–1.73$ (m, 18H, $NHCH_2(CH_2)_6CH_2NH$, $(CH_2)_3CHS$), 1.9 (sextet, $J = 6.6$ Hz, 1H, SCH_2CHH), 2.17 (t, $J = 7.1$ Hz, 2H, $NHCOCH_2$), 2.46 (sextet, $J = 6.6$ Hz, 1H, SCH_2CHH), 3.06–3.19 (m, 2H, CH_2S), 3.23 (q, $J = 6.7$ Hz, 2H, $(CH_2)_6CH_2NH$), 3.43 (q, $J = 6.3$ Hz, 2H, $NHCH_2(CH_2)_6$), 3.56 (quintet, $J = 7.2$ Hz, 1H, CHS), 5.47 (br, 1H, NHCO), 7.32–7.38 (m, 2H, H-6, H-8), 7.66 (dtd, $J_{7,8} = 7.2$ Hz, $J_{7,6} = 7.2$ Hz, $J_{7,5} = 1.6$ Hz, 1H, H-7), 8.04 (dd, $J_{5,6} = 8.0$ Hz, $J_{5,7} = 1.6$ Hz, 1H, H-5,), 9.26 (br, 1H, CONH), 18.23 (s, 1H, OH); Anal. Calcd for $C_{26}H_{36}N_2O_5S_2$: C, 59.97, H, 6.97, N, 5.38. Found C, 60.21, H, 6.77, N, 5.46.

5.2.4. N-*(2-(4-(1,2-dithiolan-3-yl)butanamido)ethyl)-4-hydroxy-6-methyl-2-oxo-2*H*-chromene-3-carboxamide (**18**)*

Yellow solid, mp 106–109 °C. Obtained from the reaction of *N*-(2-aminoethyl)-4-hydroxy-6-methyl-2-oxo-2*H*-chromene-3-carboxamide (**11**) with *N*-(lipoyloxy)succinimide (**14**). Yield: 52%; ^{1}H NMR ($CDCl_3$): $\delta = 1.4–1.8$ (m, 6H, $(CH_2)_3CHS$), 1.89 (sextet, $J = 6.5$ Hz, 1H, SCH_2CHH), 2.17–2.23 (m, 2H, $NHCOCH_2$), 2.37–2.49 (m, 4H, CH_3, SCH_2CHH), 3.06–3.18 (m, 2H, CH_2S), 3.47–3.62 (m, 5H, $NH(CH_2)_2NH$, CHS), 6.12 (br, 1H, NHCO), 7.2–7.26 (m, 1H, H-8), 7.48 (d, $J = 8.1$ Hz, 1H, H-7), 7.79 (s, 1H, H-5), 9.41 (br, 1H, CONH), 17.61 (s, 1H, OH); Anal. Calcd for $C_{21}H_{26}N_2O_5S_2$: C, 55.98, H, 5.82, N, 6.22. Found C, 55.71, H, 5.93, N, 6.31.

5.2.5. N-*(8-(5-(1,2-dithiolan-3-yl)pentanamido)octyl)-4-hydroxy-6-methyl-2-oxo-2*H*-chromene-3-carboxamide (**19**)*

Yellowish solid, mp 139–141 °C. Obtained from the reaction of *N*-(8-aminooctyl)-4-hydroxy-6-methyl-2-oxo-2*H*-chromene-3-carboxamide (**12**) with *N*-(lipoyloxy)succinimide (**14**). Yield: 33%; ^{1}H NMR ($CDCl_3$): $\delta = 1.3–1.7$ (m, 18H, $NHCH_2(CH_2)_6CH_2NH$, $(CH_2)_3CHS$), 1.9 (sextet, $J = 6.7$ Hz, 1H, SCH_2CHH), 2.17 (t, $J = 7.5$ Hz, 2H, $NHCOCH_2$), 2.39–2.48 (m, 4H, CH_3, SCH_2CHH), 3.06–3.17 (m, 2H, CH_2S), 3.23 (q, $J = 6.6$ Hz, 2H, $(CH_2)_6CH_2NH$), 3.43 (q, $J = 6.6$ Hz, 2H, $NHCH_2(CH_2)_6$), 3.56 (quintet, $J = 7.1$ Hz, 1H, CHS), 5.5 (br, 1H, NHCO), 7.2-7.26 (m, 1H, H-8), 7.46 (d, $J = 8.1$ Hz, 1H, H-7), 7.81 (s, 1H, H-5), 9.27 (br, 1H, CONH), 18.16 (s, 1H, OH); Anal. Calcd for $C_{27}H_{38}N_2O_5S_2$: C, 60.65, H, 7.16, N, 5.24. Found C, 60.82, H, 7.28, N, 5.09.

5.3. Biological experiments

5.3.1. Experiments in vivo

5.3.1.1. Acute toxicity tests. Rats were divided into 5 groups (6–8 animals each), in which different doses (0.1–0.5 mmol/kg body weight) of the test compounds at the lethal range were injected. Mortality was recorded 24 h post-injection.

5.3.1.2. Inhibition of the carrageenin-induced edema [29]. Edema was induced in the right hind paw of Fisher 344 rats (150–200 g) by the intradermal injection of 0.1 mL 2% carrageenin in water. Both sexes were used. Females pregnant animals were excluded. Each group was composed of 6–15 animals and the experiment was repeated twice. The animals, which have been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum during the maintenance but they were entirely fasted during the experiment period. Our studies were in accordance with recognised guidelines on animal experimentation.

The tested compounds 0.01 mmol/kg body weight, were suspended in water, with few drops of Tween 80 and ground in a mortar before use and were given intraperitoneally simultaneously with the carrageenin injection. The rats were euthanized 3.5 h after carrageenin injection. The difference between the weights of the injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (treated with water) and expressed as a percent inhibition of the edema CPE % values (Table 1). Indomethacin in 0.01 mmol/kg (47%), was used as a reference compound. Each value represents the mean obtained from 6 to 15 animals in two independent experiments with a standard error of the mean less than 10% (Table 1).

5.3.2. Experiments in vitro

In the in vitro assays each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

5.3.2.1. Determination of the reducing activity of the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH) [29]. To a solution of DPPH in absolute ethanol (final concentration 0.05 mM), the tested compounds in final concentration 0.1 and 0.5 mM, dissolved in ethanol, were added. As control solution ethanol was used. The concentrations of the solutions of the compounds were 0.1 and 0.5 mM. After 20 and 60 min at room temperature the absorbance was recorded at 517 nm (Table 2). Nordihydroguaiaretic acid (NDGA) was used as a reference compound.

5.3.2.2. Competition of the tested compounds with DMSO for hydroxyl radicals [29,52,53]. The hydroxyl radicals were generated by mixing the Fe^{3+}/ascorbic acid system. The methyl radical is produced by trapping the hydroxyl radical with DMSO. One part of this methyl radical is then changed to formaldehyde. Using this principal, we determined the production of hydroxyl radical according to Nash [52,53]. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (concentration 0.1 mM) and ascorbic acid (10 mM). After 30 min of incubation (37 °C) the reaction was stopped with CCl_3COOH (17% w/v) (Table 1) and the formaldehyde formed was detected spectrophotometrically at 412.

5.3.2.3. Soybean lipoxygenase inhibition study in vitro [29]. In vitro study was evaluated as reported previously. The tested compounds dissolved in ethanol were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor (Table 1).

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