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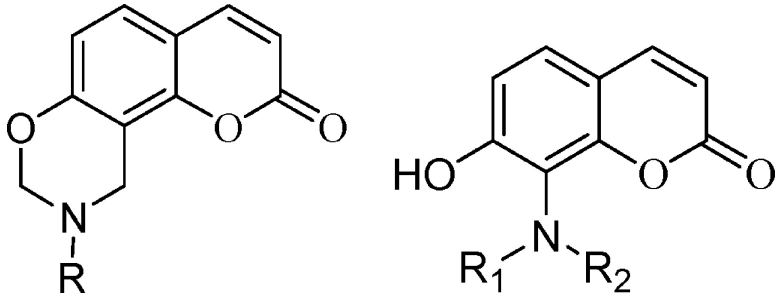

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Synthesis and Antiinflammatory Activity of Coumarin Derivatives†,‡

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The synthesis of several coumarin Mannich bases is described. The structures of the synthesized compounds were confirmed by spectral and elemental analysis. Their lipophilicity was determined experimentally by RPTLC method. All compounds were evaluated for their antiinflammatory and antioxidant activity and for their ability to inhibit in vitro lipoxygenase. The derivatives were found to present antioxidant and antiinflammatory activities. The tested derivatives inhibited carraggeenin-induced hind paw edema. They also significantly suppressed the arthritis induced by Freund's adjuvant. Compound **10**, the most active in vivo, was found to possess protective properties against adjuvant-induced arthritis in rats. The biological in vitro activities were concentration dependent. Hydrophilicity, the presence of a free 7-OH, and steric requirements for the substituent at position 8 are the most important factors in terms of SAR. An attempt was made to correlate several physicochemical properties of the molecules with their in vivo/in vitro activity.

Introduction

Inflammation is a complex phenomenon involving interrelationships humoral and cellular reactions through a number of inflammatory mediators. It is a usual symptom covering different pathologies, and there are still many questions to be answered in order to understand the inflammatory process as well as a need for better-tolerated and more efficient nonsteroidal antiinflammatory drugs. In the pathways of the inflammatory process, the implication of free radicals is particularly important. It has also been reported that antiinflammatory drugs may be effective in the prevention of free radical-mediated damage.[1]

Coumarins have been reported to have multiple biological activities.[2] They have been used to treat such diverse ailments as cancer, burns, brucellosis, and cardiovascular and rheumatic diseases.[3] The coumarin molecule has been shown to possess unique antiedema and antiinflammatory activities. Thus, coumarin derivatives could be particularly effective in the treatment of all high protein edemas.[4−6] Various coumarin-related derivatives are recognized as inhibitors of the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism[7−9] but also of neutrophile-dependent superoxide anion generation.[10] Several natural or synthetic coumarins with various hydroxyl and other substituents were found to inhibit lipid peroxidation and to scavenge hydroxyl radicals and superoxide anion[11] and to influence processes involving free radical-mediated injury, as can some plant phenolics and flavonoids.[12]

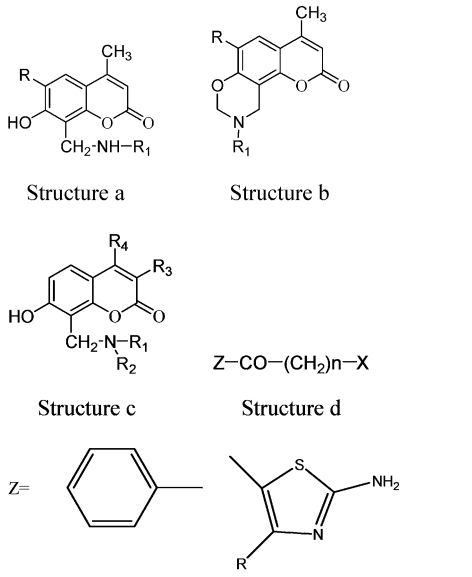

Figure 1.

It has been presented earlier that coumarin Mannich bases play a significant role as biologically active compounds in various diseases (Figure 1, structures a−c].[13−15] Mannich bases were also found to act as antiinflammatory agents (Figure 1, structure d).[16−18]

Thus, we found it interesting to synthesize some coumarin Mannich bases as possible antiinflammatory agents to examine (a) their ability to inhibit various enzymes involved in the arachidonic acid cascade and (b) their antioxidant behavior. The structure of our derivatives combines the biological active group of 7-OH-coumarin and the amine moiety as a new template for antiinflammatory activity. The suggested structural variations could affect both efficacy and their tolerability partly due to differences in their physicochemical prop-

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† This article is dedicated to Professor D. Nicolaides, Laboratory of Organic Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Greece.

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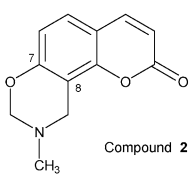
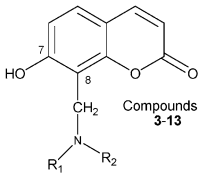
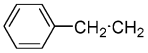
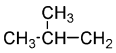
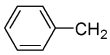
Table 1. Chemical Data of Coumarin Derivatives





Compd	R_1	R_2	Molecular Formula
2	-	-	$C_{12}H_{12}ClNO_3$
3			$C_{15}H_{17}NO_3$
4		H	$C_{18}H_{17}NO_3$
5	$CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2$	H	$C_{15}H_{20}BrNO_3$
6		H	$C_{14}H_{18}BrNO_3$
7	$CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2$	$CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2$	$C_{20}H_{30}BrNO_3$
8		H	$C_{17}H_{16}BrNO_3$
9	$CH_3{\cdot}CH_2$	H	$C_{12}H_{14}BrNO_3$
10			$C_{14}H_{16}N_2O_3$
11			$C_{14}H_{15}NO_4$
12	$NH_2{\cdot}CH_2{\cdot}CH_2$	H	$C_{12}H_{14}N_2O_3$
13	$H_2N{-}CH_2{\cdot}CH_2{\cdot}CH_2$	H	$C_{13}H_{16}N_2O_3$

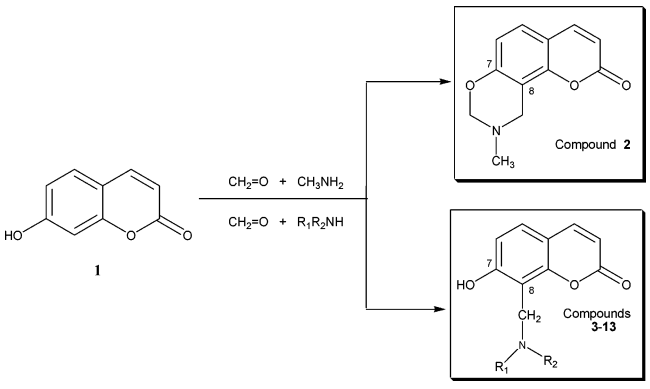
erties, which determine their distribution in the body and their ability to pass through and to enter the interior membranes.[19,20]

Today there is an increased interest in the development of effective nonacidic antiinflammatory agents, since the currently used acidic NSAIDs cause development of untoward side effects in a significant fraction of people.[21,22]

Chemistry. The synthesis of the coumarin derivatives **2**−**13** (Table 1) was accomplished according to the Mannich reaction as indicated in Scheme 1. Examples of 7-hydroxycoumarin condensed with formaldehyde and amines have been reported previously, for example, compounds **3** and **11**,[23,24] which were found to possess potent stimulating activity on the nervous and respiratory system. The synthesis of compound **8** was given in a previous publication.[15] We have included them in our study in order to delineate structure−activity relationships, within these Mannich bases.

In our case 7-hydroxycoumarin, formaldehyde, and the corresponding amine, e.g. primary or secondary, or alicyclic, benzyl-, or phenethylamines in 1:1:1 molar ratio, respectively,[13] were refluxed and stirred in absolute ethanol for 8−12 h. The reactions were monitored by thin-layer chromatography. In the case of compound **2** (R = CH_3) an oxazine ring is formed including 7-O

Scheme 1. Synthesis of Compounds **2**−**13**



(from 7-OH) and 8-C (from the phenyl ring). Compounds **3**−**13** have been identified as 7-hydroxy-8-substituted-aminomethyl coumarin derivatives.

In all cases it was assumed that, as in formylation, the Claisen rearrangement and the Fries shift[13] with 7-hydroxycoumarin, the new substituent would appear at position 8. This indicates that the 8- substituent next to the 7-hydroxy plays a detrimental role in determining the course of the reaction.

In some cases the purification of the new compounds was very difficult. Some derivatives were crystallized

Table 2. Lipophilicity Values: Experimentally Determined R_M and Theoretically Calculated Clog *P* Values, Inhibition % of Induced Carrageenin Rat Paw Edema (CPE %), and in Vitro Inhibition of Soybean Lipoxygenase (LOX) (IC_{50})

compd	Clog *P*[a]	R_M[b] (±SD)	CPE (%)[c,d] 0.01 mmol/kg body weight	LOX[e] IC_{50} μM (% 0.1 mM)
1	1.62	−0.46 (± 0.02)	no[f]	43 ± 6 (99.6)
2	1.37	−0.20 (± 0.02)	53.6** ± 2. 4	42 ± 0.6 (100)
3	2.60	−0.50 (± 0.01)	47.4* ± 1.4	37 ± 0.2 (98.8)
4	3.04	−0.43 (± 0.02)	31.2* ±1.9	41 ± 0.03 (99.6)
5[g]	3.48	−0.38 (± 0.02)	52.7* ± 1.8	59 ± 4 (83.3)
6[h]	2.82	−0.39 (± 0.02)	55.4* ± 2.6	47 ± 2 (85.7)
7[i]	5.64	−0.16 (± 0.01)	46.2** ± 2.2	13 ± 0.5 (99.0)
8[j]	3.22	−0.42 (± 0.017)	47.5** ± 3.1	44 ± 1 (100)
9	1.47	−0.33 (± 0.02)	53.6** ± 1.7	52 ± 0.7 (100)
10	1.11	−0.44 (± 0.01)	77.7* ± 1.4	100 ± 5 (51.6)
11	1.32	−0.44 (± 0.01)	75.7* ± 1.5	≫1000 (26.4)
12	0.76	0.04 (± 0.001)	56.0* ± 2.1	≫1000 (40.7)
13	0.42	ND[k]	53.4* ± 2.9	≫1000 (13.8)
coumarin	nt	nt	30.2** ± 1.8	− (15.1)
warfarin	nt	nt	41.0* ± 2.5	no
NDGA[e]	nt	nt	nt	*e*

[a] Theoretically calculated clog *P* values. [b] R_M values are the average of at least 10 measurements. [c] Each value represents the mean of two independent experiments with five animals in each group; statistical studies were done with student's *T*-test, * $p < 0.01$, ** $p < 0.05$. [d] Indomethacin as a standard 47% (0.01 mM). [e] Nordihydroguaiaretic acid (NDGA) 83.7% (0.1 mM), 94.7% (1 mM). [f] no: no action under the experimental conditions. [g] % CPE at 0.1 mmol/kg 54.7% (±1.3). [h] % CPE at 0.1 mmol/kg 58.6% (±2.9). [i] % CPE at 0.1 mmol/kg 47.5%. [j] % CPE at 0.1 mmol/kg 52.7%; (ED_{50} 0.055 mmoles/kg bw). [k] ND: not determined unter the reported experimental conditions; nt: not tested.

as salts (hydrobromic acid was used for **5**, **6**, **7**, **8**, and **9** and hydrochloric acid for **2**), recrystallized by absolute ethanol, and decomposed upon melting. Most of the derived bases are very soluble in absolute ethanol (**2**, **3**, **5**, **6**, **7**, **8**, **9**). The structures of the compounds are confirmed by UV, IR, ^{1}H NMR, ^{13}C NMR, MS and elemental analysis.

Physicochemical Studies. Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolic activity, and elimination, we tried to determine experimentally from RPTLC[25] the lipophilicity of the synthesized derivatives as R_M values and to compare them with the corresponding to theoretically calculated Clog *P* values[26,27] in *n*-octanol−buffer (Table 1).

From our results (Table 1) it can be concluded that R_M values could not be used as a successful relative measure of the overall lipophilic/hydrophilic balance of these molecules. We could attribute this to the different nature of the hydrophilic and lipophilic phases in the two systems and to the presence of basic nitrogen atoms in the examined compounds, which could disturb the absorption/desorption process.

Discussion

In this investigation, we synthesized some novel coumarin Mannich bases that were expected to offer protection against inflammation and radical attack, by application of standard synthetic methods summarized in Scheme 1.

In acute toxicity experiments, the in vivo examined compounds did not present toxic effects in doses up to 0.5 mmol/kg body weight.

The in vivo antiinflammatory effects of the tested coumarins were assessed by using the functional model of carrageenin-induced rat paw edema and are presented in Table 2, as the percentage of weight increase at the right hind paw in comparison to the uninjected left hind paw. Carrageenin- induced edema is a nonspecific inflammation resulting from a complex of diverse mediators.[28] Since edemas of this type are highly sensitive to nonsteroidal antiinflammatory drugs (NSAIDs), carrageenin has been accepted as a useful agent for studying new antiinflammatory drugs.[29] This model reliably predicts the antiinflammatory efficacy of the NSAIDs, and during the second phase it detects compounds that are antiinflammatory agents as a result of inhibition of prostaglandin amplification.[30] As shown in Table 2, all the investigated compounds induced protection against carrageenin-induced paw edema. The protection ranged from 31.2 to 77.7% while the reference drug, indomethacin, induced 47% protection at an equivalent concentration. Compounds **10** and **11** were the most potent (75.7 and 77.7%) whereas compound **4** had the lowest effect (31.2%). A slight increase in inhibition values was observed (compounds **5**, **6**, **7**, and **8**, Table 2) with the dose increase from 0.01 to 0.1 mmol. The nature and the presence of the alicyclic amines (piperazinyl, morpholinyl ring) in compounds **10** and **11** (Table 2) seem to be correlated with the higher inhibition values. The ionization constants of these compounds might play a significant role. Both compounds possess low lipophilicity, 1.11 and 1.32 (Table 2). It seems that hydrophilicity (low lipophilicity values) partly affect the antiinflammatory activity, as it can be concluded from the following equation.

$$\%\ \mathrm{CPE} = -0.101\ (\pm 0.079)\ \mathrm{Clog}\ P + 1.881\ (\pm 0.142)$$

$$n = 10,\ r = 0.723,\ r^2 = 0.523,\ q^2 = 0.170,\ s = 0.085,\ F_{1,7} = 8.6,\ a = 0.01$$

Higher lipophilicity does not seem to affect the inhibitory activity, since compound **7** with Clog *P* 5.64 inhibits the carrageenin-induced edema at a range of 46.2%.

Compounds **10** and **11** have been chosen to be examined on adjuvant-induced disease (AID) since both highly inhibited the carrageenin paw edema. Adjuvant-induced disease (AID) is a good experimental model of rheumatoid arthritis and is often used for testing agents for antiinflammatory activity. Rats treated with compounds **10** and **11** did not develop severe arthritis, indicating that these coumarin derivatives exhibited possible immunomodulating activity, an activity to be further confirmed. The time course of adjuvant arthritis development, expressed as arthritic scores, is shown in Table 3. Arthritic score, body weight loss, cahexia, and in vivo metabolism impairment (expressed as the duration of the induced paralysis) were significantly reduced in the experiment (Table 4) (Figure 2). Compounds were found to influence the body weight. The percent change of the body weight for compound **10** was 18.4% and for compound **11** was 7%. In the same experimental protocol phenylbutazone (80 mg/ kg po) gave an arthritic score of 1.5 (for controls, the arthritic score was 9.0) on the 24th day after the administration of FA (Table 3).[25] The duration of zoxazolamine-induced paralysis was reduced to normal levels. Although, *N*-alkyl-7-aminocoumarins are substrates of cytochrome P450-isoenzymes,[31] the present data require further investigation regarding the mechanism of action, to delineate that the

Table 3. Effect of Compounds **1**, **10**, **11** on the Onset (day of appearance after Freund's Adjuvant Injection, FA) and Severity of Arthritis (arthritic scores) in Adjuvant-Induced Disease (AID) Rats

days after FA's injection	AID rats treated with compound **1**[a]	AID rats treated with compound **10**[a]	AID rats treated with compound **11**[a]	AID rat controls[a]
0	0	0	0	0
2	0	0	0	0
4	0	0	0	0
6	0	0	0	0
8	0	0	0	0
10	0	0	0	0
12	0	0	0	0
14	0	0	0	6.6
16	1.2	0	1.25	8.1
18	1.8	3.2	2.5	11.8
20	2.6	3	3.25	14.0
22	5.2	4.8	4.25	10.8
24	4	5.8	3.5	8.8

[a] Number of arthritic scores (SD less than 10%); phenylbutazone 80 mg/kg po gave arthritic score of 0.3 (for controls the arthritic score was 9.0) on the 24th day.

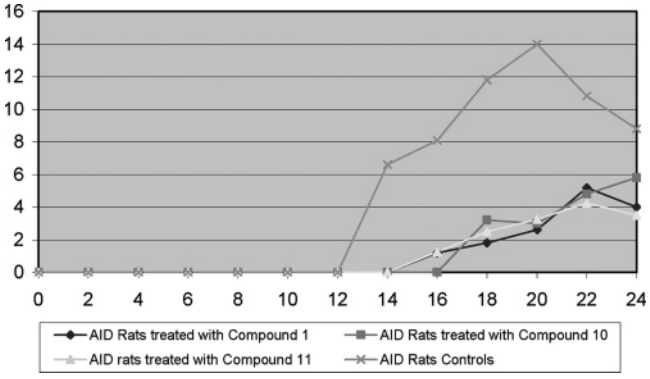


Figure 2. The number of arthritic scores of rats treated with compounds **1**, **10**, **11** compared to rat controls.

hepatoprotective effect of compounds **10** and **11** during inflammation could be mediated via the ability of compound to induce drug-metabolizing enzymes.

Compound **1** (7-hydroxycoumarin) was also tested in the same experiment in order to delineate the role of the 7-hydroxycoumarin in this biological response. From our results it seems that rats treated with 7-hydroxycoumarin did not develop arthritis; the arthritic score, body weight loss, cahexia, and the in vitro metabolic impairment is significantly reduced (Tables 3 and 4, Figure 2). Unfortunately the rats treated with compound **1** presented side effects from the gastrointestinal route (ulcers and hyperemia). On the contrary these effects were not observed after the treatment with compounds **10** and **11**, since both are not acidic.

Concerning the structural features of the active coumarin Mannich bases, our data indicate that the minimal structural requirements for antiinflammatory activity are both a free 7-OH group on the phenyl ring and an alicyclic amine (piperazinyl or morpholinyl ring) or a primary aliphatic amine. This is in accordance with previous findings, where Mannich bases were found to be potent antiinflammatory agents.[15,16]

Compounds were further evaluated for inhibition of soybean lipoxygenase LOX by the UV absorbance-based enzyme assay.[32] 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 2). Perusal of IC_{50} values shows that compound **7** is the most active, within the set, followed by compounds **3** and **4**. Most of the LOX inhibitors are antioxidants or free radical scavengers,[33] since lipoxygenation occurs via a carbon-centered radical. Some studies suggest a relationship between LOX inhibition and the ability of the 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. Several LOX inhibitors are excellent ligands for Fe^{3+}. Many flavonoids and other phenolics such as hydroxycoumarin derivatives inhibit soybean lipoxygenase. 7-Hydroxycoumarin also inhibit the activity of LOX.[6] This inhibition is related to their ability to reduce the iron species in the active site to the catalytically inactive ferrous form.[32,33] Thus the presence of a free 7-OH group could explain the inhibition results of the examined compounds. Although lipophilicity is referred[36] as an important physicochemical property for LOX inhibitors, all the above tested derivatives do not follow this concept with one exception. Compound **7** shows the highest log *P* value 5,64 and it is highly potent.

COX-1 plays a role as a "housekeeping enzyme", for example maintaining the lining of the stomach's endothelial cells and contributing to the normal function of the cardiovascular system via the release of prostacyclin. Thus, inhibition of COX-1 is involved in the appearance of the unwanted side effects. Compounds **10** and **11**, which possess the highest in vivo activity against the CPE test and no side effects from the gastrointestinal route, were also examined for their inhibitory activity against COX-1 at 1 mM (Table 6). Both compounds did not highly inhibit COX-1 (11 and 32%).

In this investigation all compounds were studied in order to gain insight in the mechanism of their antiphlogistic action. It is well-known that free radicals play an important role in inflammatory process.[37]

Table 4. Assessment of the Preventive Action of **10** on the Adjuvant-Induced Disease (AID) Manifestations (body weight change, inflammation-arthritic score, zoxazolamine paralysis)

examined parameters (mean ± SD)	AID rats treated with **1**	AID rats treated with **10**	AID rats treated with **11**	AID rat controls treated only with the liquid vehicle	absolute controls, normal animals, treated with the liquid vehicle only
percent change of body weight (g ± SD)	18.6 ± 8.4	18.4 ± 5.9	7.0 ± 4.2	−14.8 ± 4.5	−18 ± 4.4
inflammation (arthritic scores)[b]	4 (8.8)	5.8 (8.8)	3.5 (8.8)	8.8	no arthritic scores are observed
zoxazolamine paralysis (min ± SD)	232.5 ± 1.4	292 ± 18.8	212.5 ± 1.7	347.0 ± 4.9	218 ± 15.5

[a] Counted on the 24th day after Freund's Adjuvant (FA) injection; figures in parentheses denote score for controls.

Table 5. Interaction % with DPPH (RA %).[a] Competition % with DMSO for Hydroxyl Radical (HO• %)[b]

	RA (%) 20 min			RA (%) 60 min			HO• (%)	
compd	0.1 mM	0.2 mM	0.5 mM	0.1 mM	0.2 mM	0.5 mM	0.01 mM	0.1 mM
1	1.5	2.8	8.3	2.4	5.6	8.9	nt	nt
2	9.1	11.3	19.8	14.8	16.2	28.3	11.3	37.2
3	5.3	6.3	22.7	10.7	12.0	33.9	1.9	46.9
4	12.6	24.1	58.5	20.7	33.4	67.8	no[d]	39.0
5	18.0	22.0	26.0	19.0	22.8	24.8	13.3	81.1
6	20.0	25.0	24.0	21.0	26.0	27.0	27.5	78.4
7	18.0	17.0	20.0	22.0	21.0	25.0	22.1	77.8
8	23.0	29.0	21.0	26.4	39.4	37.0	30.2	51.5
9	13.7	18.0	21.5	12.5	14.6	20.8	65.7	88.0
10	16.4	17.0	26.4	17.5	15	30.6	60.9	75.9
11	35.0	51.6	81.3	41.8	58.4	89.0	75.9	81.1
12	26.6	28.3	48.6	31.7	30.2	55.1	27.5	69.8
13	13.9	9.2	6.2	11.2	11.0	8.7	38	90.9
NDGA[a]	81	80	96.5	82.6	80	96.5	nt	nt
trolox	nt[c]	nt	nt	nt	nt	nt	73.4	88.2
BHT[b]	31.3	52.7	75.4	60	78	98.2	nt	nt
coumarin	4.9	nt	5.8	21	nt	6.7	nt	78.0
warfarin	9.2	nt	10.3	24	nt	12.8	nt	95.8

[a] NDGA (nordihydroguaiaretic acid) [b] BHT (butylated hydroxytoluene). [c] nt: not tested. [d] no: no action under the experimental conditions.

Table 6. In Vitro Inhibition of Cyclooxygenase-1 (COX-1%)

compd	COX-1%, 1 mM
10	11 ± 0.3
11	32 ± 1.3
Sc-560	57.8 ± 4.3

Table 7. % In Vitro Lipid Peroxidation (LP %). % Superoxide Radical Scavenging Activity (PMS %)

	LP (%)		
compd	0.1 mM	1 mM	PMS (%), 1 mM
1	14.6	26.1	80.6
2	no[a]	no	86.1
3	no	7.6	no
4	18.2	5.5	22.2
5	8.6	5.7	no
6	no	no	33.3
7	no	13.7	no
8	19.5	35.6	no
9	no	5.5	no
10	19.5	21.9	no
11	19.5	19.9	58.3
12	no	22.1	no
13	no	ni	50.0
coumarin	9.1	19.2	88.9
warfarin	no	no	25
caffeic acid	no	5.5	86.1
NDGA	no	26.1	no

[a] no: no action show under the experimental conditions

Consequently, compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation and to lead to potentially effective drugs. In fact, many nonsteroidal antiinflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers.[38] Thus, we tested these derivatives with regard to their antioxidant ability and in comparison to well-known antioxidant agents, e.g. nordihydriguaiaretic acid, trolox, caffeic acid (Tables 5 and 7).

The interaction of the examined compounds with the stable free radical DPPH was studied (Table 5). This interaction indicates their radical scavenging ability in an iron-free system. Compounds **2**, **3**, **9**, and **13** were found to have very low activity, followed by compounds **5**, **6**, **7**, **8**, **10**, and **12**. Compound **11** showed the highest interaction (81%) at 0.5 mM, followed by compounds **4** (58.5%) and **12** (48.6%). For most of the compounds the interaction was time and concentration dependent. The time course of DPPH interaction, as affected by various concentrations, is given in Table 5. In general, this interaction expresses the reducing activity of the tested compounds and indicates their ability to scavenge free radicals. It was found that the ethylenediamine derivative, as well as the morpholinyl and the phenethylamine were the most potent in interacting with DPPH.

Preliminary QSAR studies on the values of DPPH interaction have shown that the sterimol properties,[39] e.g. L, the length of the first atom of the substituent R_1, R_2 groups plays a significant role. It seems that the smaller L leads to higher % interaction values.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflamed site, and this is connected to other oxidizing species such as HO•. Hydroxyl radicals are produced by reactions that depend on transition metals, particularly iron.[40] Hydroxy radicals are among the most reactive oxygen species and are considered to be responsible for some of the tissue damage 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• 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 5) compounds **3** and **4** did not show any inhibition at 0.01 mM. Compounds **2** and **5** (0.01 mM) slightly inhibited the oxidation of DMSO (33 mM). Compounds **6**, **7**, **8** showed mild inhibition at 0.01 mM. The inhibition was found to increase as the concentration of the tested compounds was increased. Compound **13** (90% at 0.1 mM) was the most active under these experimental conditions, whereas the "mother compound" coumarin presents 78% competition at the high concentration (0.1 mM), showing that the coumarin nucleus by itself had scavenging activity. The order of decreasing HO• scavenging activity

was **13** > **9** > **5**−**11** > **6** > **7** > **10** > **12** > **8** > **3**. The overall low lipophilicity is correlated with higher competition values. Antioxidants of hydrophilic or lipophilic character are both needed to act as radical scavengers in the aqueous phase or as chain-breaking antioxidants in biological membranes. The most potent compound **13** possesses the lowest Clog *P* value within the set.

It seems that LOX % inhibition in vitro is correlated with the ·HO• radical scavenging activity as well as with the DPPH interaction values (% LOX is log % inhibitory activity on LOX induced by the tested compounds at 1 mM, RA % the log % interaction with DPPH, and % OH the log % of the competition with DMSO for hydroxyl radicals at the same concentration).

$$\%\ \mathrm{LOX} = 2.070\ (\pm 1.061)\ \%\ \mathrm{RA} + 3.033\ (\pm 1.954)\%\ \mathrm{OH} - 6.753\ (\pm 4.906) \quad (2)$$

$$n = 9,\ r = 0.890,\ r^2 = 0.792,\ q^2 = 0.596,\ s = 0.168,\ F_{2,9} = 11.36,\ \alpha = 0.01$$

The tested compounds did not highly inhibit the lipid peroxidation. In some cases a small inhibition increase is observed by increasing their concentration, e.g. compound **1**, **8**, **10**, **12** (Table 7). Nonenzymatic superoxide anion radicals were generated. Compounds **1**, **2**, **11**, and **13** present high scavenging activity (Table 7). 7-Hydroxycoumarin, the "mother molecule", seems to strongly scavenge superoxide radicals. In general, lipophilicity does not positively influence the scavenging activity.

We tried to linearly correlate the expressions of antiinflammatory, antioxidant, free radical scavenging activity, and LOX inhibition activity for all the tested compounds. None of these correlations were satisfactory enough ($r < 0.6$). Presumably, these activities proceed via at least partially different mechanisms.

Attempts to correlate these expressions of activity with R_M values in a linear or nonlinear regression analysis gave statistically nonsignificant correlations. Unfortunately the number of compounds is not enough to calculate a combination of all the effects.

Conclusion

The present study has shown that certain coumarin Mannich bases possess high antiinflammatory activity. Their synthesis is almost simple with satisfactory yields. Most of them are potent HO• scavengers and inhibit in vitro soybean lipoxygenase. Hydrophilicity, the presence of a free 7-OH, and steric requirements for the substituent at position 8 are the most important factors in terms of SAR. Although the antiinflammatory mechanism explaining the activity on CPE remains unclear, the in vivo antiinflammatory activity of the synthesized compounds seems to be related with their high HO• scavenging and reducing activity, in vitro.

Two compounds, **10** and **11**, were found to present a promising antiinflammatory profile: combining immunomodulating high antiedematous ability with no side effects from the gastrointestinal route and significant inhibitory activity on LOX. These compounds constitute an interesting template for the evaluation of new synthetic lipoxygenase inhibitors and may be helpful for the design of new therapeutic tools against inflammation.

Experimental Section

Materials. All the chemicals used were of analytical grade and commercially available from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and nordihydroguairetic acid (NDGA) were purchased from the Aldrich Chemical Co., Milwaukee, WI. Soybean lipoxygenase, linoleic acid sodium salt arachidonic acid (AA), nicotinamidoadenine dinucleotide (NADH), nitrotetrazolium Blue (NBT), porcine heme, butylated hydroxytoluene (BHT), and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO) and carrageenin, type K, was commercially available. For the in vivo experiments, male and female Fischer-344 rats (180−240 g) were used, and the kit for COX activity assay was purchased from Cayman. *N*-Methylphenazonium-mehtyl sulfate was purchased by Fluka. Freund's Adjuvant (FA) referred to 0.6 mg desiccated *Mycobacterium butyricum* was suspended in 0.1 mL liquid paraffin.

Synthesis All starting materials were obtained from commercial sources and used without further purification. Melting Points (uncorrected) were determined on a MEL-Temp II (Lab. Devices, Holliston, MA). UV−vis spectra were obtained on a Perkin-Elmer 554 double beam spectrophotometer and on a Hitachi U-2001 spectrophotometer. Infrared spectra (film as Nujol mulls) were recorded with Perkin-Elmer 597 spectrophotometer (The Perkin-Elmer Corporation Ltd., Lane Beaconsfield, Bucks, England) and a Shimadzu FTIR-8101M. The ^{1}H nucleic magnetic resonance (NMR) spectra were recorded at 300 MHz on a Bruker AM 300 spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) in $CDCl_3$ or DMSO using tetramethylsilane as an internal standard unless otherwise stated. ^{13}C NMR spectra were obtained at 75.5 MHz on a Bruker AM 300 spectrometer in $CDCl_3$ or DMSO solutions with tetramethylsilane as internal reference unless otherwise stated. Mass spectra were determined on a VG-250 spectrometer (VG-Labs., Tritech, England) with ionization energy maintained at 70 eV. Elemental analyses were obtained on an acceptable range (±0.4%) in a Perkin-Elmer 240B CHN analyzer (The Perkin-Elmer Corporation Ltd.). Reactions were monitored by thin-layer chromatography (TLC) by Fluka, on aluminum cards precoated with 0.2 mm of silica gel and fluorescent indicator.

General Procedure. The reaction was performed according to the literature[9−11,30,31] after modification. To a solution of the appropriate amines (0.005 mol) in 30 mL of absolute ethanol was added aqueous formaldehyde (37%) (0.01 mol). After 0.5 h of gentle refluxing at 60 °C, the 7-hydroxycoumarin (**1**) was added (0.005 mol), dissolved in 30 mL of absolute ethanol. The duration of the reaction was 8−12 h, and the reaction was monitored by TLC. The solvent was removed under reduced pressure.

Gerneral Procedure for Salts Preparation. A. A cold alcohol solution of the product was treated with concentrated hydrochloric acid, and the hydrochloride was obtained. The salt was purified by absolute ethanol/ethyl ether.

B. Following the procedure of Burke et al.,[41] to a solution of the reaction mixture in propanol-1 (85% propanol-1) was added hydrobromic acid in ethanol absolute. After 30 min of gentle refluxing at 60 °C and cooling, ethyl ether was added dropwise. Upon cooling and filtration, the product was obtained.

3-Methyl-3,4,4a,10a-tetrahydro-2H-1,5-dioxa-3-aza-phenanthren-6-one (2). According to the general procedure, 7-hydroxycoumarin was reacted with methylamine in absolute ethanol. Following the general procedure for salt preparation A, the hydrochloride was obtained. (12%, salt). The salt decomposes upon melting. UV (ethanol absolute) λ_{max}: 348, 279, 236, ϵ_{max}: 1795, 1935, 2132; IR (Nujol) (cm^{-1}): 1710−1720 (C═O), 1250 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 7.72 (d, J = 10.2, 1H), 7.47 (d, J = 8.9, 1H), 7.18 (d, J = 8.9, 1H), 6.22 (d, J = 10.2, 1H), 4.65 (br, s, 3H), 4.55 (br, s, 2H), 2.83 (br, 3H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 165.7, 153.7, 145.8, 143.9, 126.6, 113.6, 112.6, 112.1, 107.8, 84.5, 46.9, 40.0; MS: 218 [M+], 217 (35.7), 174 (41.5), 146 (100), 90 (43.8), 89 (39.2); Anal.: ($C_{12}H_{12}ClNO_3$) C, H, N.

7-Hydroxy-8-piperidin-1-ylmethyl-chromen-2-one (3). According to the general procedure, 7-hydroxycoumarin was reacted with piperidine in absolute ethanol. The product was collected and recrystallized from ethanol (52%): mp 173−175 °C; UV (ethanol absolute) λ_{max}: 382, 307, 237, ϵ_{max}: 2175, 1886, 2082; IR (Nujol) (cm^{-1}): 3450−3300 (O−H), 1720−1700 (C═O), 1300(C−N); ^{1}H NMR ($CDCl_3$): δ 9.41 (s, 1H), 7.61 (d, J = 8.9, 1H), 7.26 (d, J = 8.9, 1H), 6.73 (d, J = 8.9, 1H), 6.17 (d, J = 8.9, 1H), 4.0 (s, 2H), 2.9−2.2 (br, 4H), 1.8−1.5 (br, 6H); ^{13}C NMR ($CDCl_3$): 163.4, 161.4, 153.1, 144.3, 143.9, 127.8, 113.9, 111, 107.7, 54.3, 53.8, 25.6, 23.6; MS: 259 [M^+], 175 (36), 146 (24), 98 (26), 84 (100); Anal.: ($C_{15}H_{17}NO_3$) C, H, N.

7-Hydroxy-8-(phenethylamino-methyl)-chromen-2-one (4). According to the general procedure, 7-hydroxycoumarin was reacted with phenethylamine in absolute ethanol. The product was collected and recrystallized from ethanol. The product was collected and recrystallized from ethanol (36%): mp 120−121 °C; UV (ethanol absolute) λ_{max}: 390, 305, 239, ϵ_{max}: 2308, 1890, 2089; IR (Nujol) (cm^{-1}): 3400−3300 (O−H, N−H), 1720−1710 (C═O), 1250 (C−N); ^{1}H NMR ($CDCl_3$): δ 7.61−7.58 (d, J = 8.91, 1H), 7.33−7.22 (m, 7H), 6.78−6.75 (d, J = 8.91,1H), 6.18−6.15 (d, J = 8.91, 1H), 4.30 (br, 2H), 4.17−3.44 (br, 1H), 3.03 (tr, J = 6.4, 2H), 2.91 (tr, J = 6.4, 2H); ^{13}C NMR ($CDCl_3$): 161.5, 161.4, 153.6, 144.2, 143.9, 129.3, 128.7, 128.4, 126.9, 114.1, 111.9, 111.5, 101.1, 49.0, 34.8, 33.5; MS: 295 [M^+], 204 (43), 175 (70), 145 (18), 91 (100); Anal. ($C_{18}H_{17}NO_3$) C, H, N.

3-Pentyl-3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-6-one (5). The reaction followed the general procedure using 1-pentylamine. The hydrobromide was made according to the general procedure for salt preparation B (8% salt): mp 198−201 °C; UV (ethanol absolute) λ_{max}: 347, 278, 240, ϵ_{max}: 1730, 1862, 2056; IR (Nujol) (cm^{-1}): 3450−3300 (O−H), 1730−1720 (C═O),1250 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 11.20 (s, 1H), 8.78 (m, 2H), 7.89 (d, J = 9.4, 1H), 7.55 (d, J = 8.5, 1H), 7.03 (d, J = 8.5, 1H), 6.23 (d, J = 9.4, 1H), 4.28 (s, 2H), 3.08−2.92 (m, 2H), 1.86−1.69 (m, 2H), 1.60−1.05 (m, 4H), 0.92−0.79 (br, s, 3H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 160.2, 159.7, 154.1, 144.3, 130.3, 112.6, 111.7, 111.3, 105.0, 47.3, 38.5, 28.3, 25.0, 21.8, 13.7; MS: 261 [M^+], 204 (38.6), 175 (100), 57 (95.7); Anal.: ($C_{15}H_{20}BrNO_3$) C, H, N.

3-Isobutyl-3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-6-one (6). According to the general procedure, 7-hydroxycoumarin was reacted with isobutylamine absolute ethanol. The hydrobromide was made according to the general procedure for salt preparation B (12% salt): mp 232−234 °C; UV (ethanol absolute) λ_{max}: 321, 209, ϵ_{max}: 1743, 3757; IR (Nujol) (cm^{-1}): 3450−3300 (O−H), 1720−1710 (C═O), 1250 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 11.6 (s, 1H), 8.01 (d, J = 9.09, 1H), 7.65 (d, J = 10.92, 1H), 7.43 (d, J = 9.12, 1H), 6.3 (d, J = 9.09, 1H), 4.46 (s, 2H), 3.37−3.25 (s, 2H), 3.02−3.00 (br-s, 2H), 2.36−2.22 (br, 2H), 0.98 (s, 6H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 160.2, 159.2, 154.3, 144.5, 131.0, 112.3, 111.6, 111.3, 103.6, 48.7, 45.5, 36.1, 23.8; MS: 247 [M^+] (59.3), 204 (79.4), 175 (96.3), 146 (57.9), 100.24 (96.8); Anal. ($C_{14}H_{18}BrNO_3$) C, H, N.

8-Dipentylaminomethyl-7-hydroxy-chromen-2-one (7). The reaction was made according to the general procedure. Dipentylamine was used. The hydrobromide was made according to the general procedure for salt preparation B. The product was recrystallized from acetone (7%, salt): mp 127−128 °C; UV (ethanol absolute) λ_{max}: 348, 274, 240, ϵ_{max}: 1612, 1757,1963; IR (Nujol) (cm^{-1}): 3400−3250 (O−H), 1720−1730 (C═O), 1270 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 11.42 (s, 1H), 7.99 (d, J = 8.7, 1H), 7.65 (d, J = 8.7, 1H), 7.01 (d, J = 8.7, 1H), 6.27 (d, J = 8.7, 1H), 4.39 (s, 2H), 3.35 (s, 2H), 2.92−3.21 (br, 4H), 2.03−1.74 (m, 4H), 1.53−1.23 (m, 8H), 1.08−0.79 (m, 6H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 158.8, 157.8, 152.9, 143.0, 129.4, 110.9, 110.1, 109.8, 102.7, 51.5, 42.8, 26.7, 21.1, 20.1, 12.1; MS: 332 [M+], 274 (93.5), 260 (23.4), 175 (86.4), 158 (32.7), 147 (53.6), 100 (95.8); Anal. ($C_{20}H_{30}BrNO_3$) C, H, N.

3-Benzyl-3,4-dihydro-2*H*-1,5-dioxa-3-aza-phenanthren-6-one (8). According to the general procedure, 7-hydroxycoumarin was reacted with benzylamine absolute ethanol. The hydrobromide was made according to the general procedure for salt preparation B. The product was recrystallized with methanol and ether (16%, salt): mp 222−225 °C; UV (ethanol absolute) λ_{max}: 348, 302, 238, ϵ_{max}: 1713, 1837, 2053; IR (Nujol) (cm^{-1}): 3400−3300 (O−H), 1720−1730 (C═O), 1280 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 9.28 (s, 1H, disappears in addition of D_2O), 7.96 (d, J = 8.9, 1H), 7.67−7.31 (br, 7H), 6.80 (d, J = 8.9, 1H), 6.28 (d, J = 8.9, 1H), 4.26 (br-s, 2H), 4.23 (br-s, 2H), 3.35 (br-s, 2H, disappears in addition of D_2O); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 160.2, 159.6, 154, 144.6, 131.6, 130.3, 128.9, 128.5, 112.4, 111.4, 111.2, 105.4, 50.3, 37.9; MS: 281 M+], 190 (67.7), 175 (60.2), 146 (70.0), 106 (90.8), 91 (100); Anal: ($C_{17}H_{16}NO_3Br$) C, H, N.

8-Ethylaminomethyl-7-hydroxy-chromen-2-one (9). According to the general procedure, 7-hydroxycoumarin was reacted with ethylamine in absolute ethanol. Following the general procedure for salt preparation B, the hydrobromide was obtained. (22% salt); mp 236−237 °C; UV (ethanol absolute) λ_{max}: 338, 259, ϵ_{max}: 3771, 3766; IR (Nujol) (cm^{-1}): 3400−3300 (O−H), 1730−1720 (C═O), 1250 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 11.36 (br-s, 1H), 7.72 (d, J = 10.17, 1H), 7.47 (d, J = 5.1, 1H), 7.19 (d, J = 8.91, 1H), 6.23 (d, J = 5.1, 1H), 4.22 (br-s, 2H), 3.36 (br-s, 2H), 3.04 (br-s, 2H), 1.26 (m, 3H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 160.0, 159.7, 154.0, 144.7, 130.5, 113.1, 112.3, 111.3, 105.4, 53.7, 37.8, 22.4; MS 219 [M+] (35), 176 (100), 146 (74), 118 (55), 91 (53); Anal.: ($C_{12}H_{14}NO_3Br$) C, H, N.

7-Hydroxy-8-piperazine-1-ylmethyl-chromen-2-one (10). According to the general procedure, 7-hydroxycoumarin was reacted with piperazine in absolute ethanol. The product was collected and was recrystallized with dichloromethane and ether (35%): mp 231−233 °C; UV (ethanol absolute) λ_{max}: 322, 219, ϵ_{max}: 2079, 2034; IR (Nujol) (cm^{-1}): 3450 (O−H), 3050 (N−H), 1730−1720 (C═O); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 9.42 (s, 1H), 7.62 (d, J = 9.5, 2H), 7.30 (d, J = 8.3, 1H), 6.78 (d, J = 8.3, 1H), 6.20 (d, J = 9.5, 1H), 4.09 (s, 2H), 2.72−2.96 (br, 9H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 163.6, 162.4, 154.5, 145.6, 129.7, 115.2, 112.9, 109.0, 108.7, 54.7, 53.8, 52.3; MS: 260 [M^+], 175 (64.9), 105 (37.7), 85 (63); Anal.: ($C_{14}H_{16}N_2O_3$) C, H, N.

7-Hydroxy-8-morpholin-4-ylmethyl-chromen-2-one (11). According to the general procedure, 7-hydroxycoumarin was reacted with morpholine in absolute ethanol. The product was collected and was recrystallized with ethanol absolute and dried (13%): mp 231−233 °C; UV (ethanol absolute) λ_{max}: 348, 316, 231, ϵ_{max}: 2040, 2199, 2411; IR (Nujol) (cm^{-1}): 3450−3300 (O−H), 3200−3150 (N−H), 1720−1710 (C═O), 1250 (C−N); ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ 9.42 (s, 1H), 7.86 (d, J = 9.84, 1H), 7.47 (d, J = 8.85, 1H), 6.93 (d, J = 8.85, 1H), 6.17 (d, J = 9.84, 1H), 3.7−4.1 (br, 6H), 2.50−2.60 (m, 4H); ^{13}C NMR (DMSO-d_6, $CDCl_3$): 160.2, 159.4, 153.6, 144.5, 127.6, 112.7, 112.4, 111.4, 106.6, 61.9, 53.1, 50.3; MS 261 [M+], 175 (9.4), 87 (93); Anal.: ($C_{14}H_{15}NO_4$) C, H, N.

8-[(2-Aminoethylamino)-methyl]-7-hydroxy-chromen-2-one (12). According to the general procedure, 7-hydroxycoumarin was reacted with 1,2-diamine-ethane in absolute ethanol. The product was collected and was recrystallized with ethanol absolute and dried (43%): mp: 215−217 °C; UV (ethanol absolute) λ_{max}: 211, 294, ϵ_{max}: 2934, 721; IR (Nujol) (cm^{-1}): 3350−3250 (O−H), 3150−3050 (N−H), 1700−1680 (C═O), 1250 (C−N); ^{1}H− NMR (DMSO-d_6, $CDCl_3$): δ 8.02−8.00 (d, J = 9.09, 2H), 7.66−7.62 (d, J = 10.92, 1H), 7.44−7.43 (d, J = 9.12, 1H), 6.31−6.28 (d, J = 9.09, 1H), 3.92−2.88 (br, 4H), 4.30 (s, 2H), 2.18−1.98 (br, 2H), 1.56−1.32 (br, 2H),; ^{13}C NMR (DMSO-d_6, $CDCl_3$): 160.1, 159.2, 153.9, 144.7, 133.2, 112.4, 111.5, 111.3, 106.3, 54.6, 44.0, 36.4; MS: 218 (9.6), 144 (14), 130 (20), 55 (100); Anal.: ($C_{12}H_{14}N_2O_3$) C, H, N; ($C_{12}H_{15}BrN_2O_3$) C, H, N.

8-[(3-Aminopropylamino)-methyl]-7-hydroxy-chromen-2-one (13). According to the general procedure, 7-hydroxycoumarin was reacted with 1,3-diaminopropane in absolute ethanol. The product was collected and was recrystallized with ethanol absolute and dried (17%): mp 217−219 °C; UV (ethanol absolute) λ_{max}: 326, 207, ϵ_{max}: 652, 887; IR (cm^{-1}):

3400−3300 (O−H), 3200−3150 (N−H), 1720−1700 (C=O), 1250 (C−N),; MS: 175 (25.1), 174 (46), 133 (28), 103 (42), 84 (100); Anal.: ($C_{13}H_{16}N_2O_3$) C, H, N.

Physicochemical Studies. (a) Determination of lipophilicity as R_M values. Reversed phase TLC (RPTLC) was performed on silica gel plates impregnated with 55% (v/v) liquid paraffin in light petroleum ether.[25] The mobile phase was a methanol/water mixture (75/25, v/v) containing 4% aqueous ammonia (27%). The plates were developed in closed chromatography tanks saturated with the mobile phase at 24 °C. Spots were detected under UV light or by iodine vapors. R_M values were determined from the corresponding R_f values (from 10 individual measurements) using the equation $R_M = \log [(1/R_f) - 1]$ (Table 2).

(b) Determination of lipophilicity as Clog *P*. Lipophilicity was theoretically calculated as Clog *P* values in *n*-octanol−buffer by CLOGP Program of Biobyte Corp.[27]

Biological Experiments. Experiments in Vivo. Toxicity of the Examined Compounds. Toxicity experiments were carried out using both male and female Fischer 344 rats. Pregnant females were excluded. The tested compounds were dissolved in water (salts) or suspended with few drops of Tween 80 and ground in water and administered by intraperitoneal injection at various concentrations. Mortality was recorded after 24 h.

Inhibition of the Carrageenin-Induced Edema.[25] 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. Pregnant females were excluded. Each group was composed of 6−15 animals. 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 recognized guidelines on animal experimentation.

The tested compounds, 0.01 mmol/kg body weight, were suspended in water with a few drops of Tween 80 and ground in a mortar before use (bases) or dissolved in water (salts) and were given intraperitoneally simultaneously. The rats were euthanized 3.5 h after carrageenin injection. The difference between the weight 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 2. Indomethacin at 0.01 mmol/kg gave 47% inhibition. Values CPE % are the mean from two different experiments with a standard error of the mean less than 10%.

Induction of Adjuvant-Induced Disease (AID).[25] Groups of five animals were used. Rats were divided into three groups: groups 1 and 2 were injected[19] with Freund's Adjuvant (FA) intradermally, into the base of the tails of the animals, and were treated with compound **10**, which was found to be the most active in the carrageenin paw edema test. Compound **10** was injected ip in a dose of 0.001 mmol/kg from day zero once every other day for the following 24 days. Group 3, used as an absolute control, was injected with liquid vehicle only. Adjuvant arthritis was developed ca. 14 days post FA administration. Arthritic score was measured every 2 days from the commencement (14th day). For quantification of arthritis (arthritic score), a single point was assigned for each inflammaed wrist or ankle area and an additional point was given for each involved phalangeal joint, up to a maximum of five points per extremity (Table 7). On the 24th day and at least 12 h after the last injection, animals were administered zoxazolamine ip 10 mg/1 mL/g, as an aqueous suspension with a few drops of Tween 80, and the duration of the paralysis and the inflammation were assessed on the 24th day (Table 4). The experiment was conducted in duplicated.

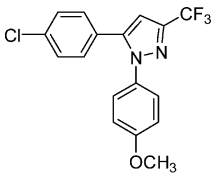
Experiments in Vitro. In the in vitro assays each experiment was performed at least in triplicate, and the standard deviation of absorbance was less than 10% pf the mean.

Determination of the Reducing Activity of the Stable Radical 1,1-Diphenyl-picrylhydrazyl (DPPH).[25] To a solution of DPPH (0.1 mM) in absolute ethanol was added an equal volume of the compounds dissolved in ethanol. As control solution ethanol was used. The concentrations of the solutions of the compounds were 0.01, 0.02, and 0.05 mM. After 20 and 60 min at room temperature, the absorbance was recorded at 517 nm (Table 5).

Competition of the Tested Compounds with DMSO for Hydroxyl Radicals.[25] The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system were detected according to Nash by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 ìM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (concentration 0.01 mM and 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 3).

Soybean Lipoxygenase Inhibition Study in Vitro.[25] The 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 ($1/3 \times 10^4$ 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 (nordihydroguaiaretic acid 0.1 mM 83.7% inhibition; 1 mM 94.7% inhibition) (Table 5).

In Vitro Cyclooxygenase-1 (COX-1) Inhibition Study.[42] Cyclooxygenase (COX) activity was determined by using arachidonic acid (AA) as substrate and *N*,*N*,*N*,*N*-tetramethylphenylenediamine (TMPD) as cosubstrate, as described by Kulmacz and Lancs.[41] The reaction mixture (1 mL) contained 0.75 μM heme, 128 ìM TMPD, 80 μM AA, and 1.5 μg of enzyme, in 0.1 M Tris/HCl (pH 8.5). The oxidation of substrate, starter of the reaction, was measured at 37 °C by monitoring the increase of absorbance at 611 nm. The absorption due to the spontaneous oxidation of TMPD was subtracted from the initial rate of oxidation observed in the presence of AA. The inhibition of the studied compounds was determined after preincubation for 6 min with the enzyme in the presence of heme and TMPD, and the reaction was started by adding AA. For coumarins, 10 μL of scalar dilutions of the inhibitors in ethanol/water 1/10 (v/V) was added. Sc-560 has been used as a comparison COX-1 inhibitor (Table 6).



Sc-560

Heme Protein-Dependent Lipid Degradation.[43] Heme (50 μM), arachidonic acid (0.4 mM), the compounds at the various concentrations tested, and H_2O_2 (0.5 mM) were incubated together for 10 min at 37 °C in KH_2PO_4−KOH buffer (50 mM, pH 7.4). The product of peroxidation was detected using the TBA test.[44] The compounds were added in DMSO solution, which has no effect on the assay (Table 7).

Nonenzymatic Assay of Superoxide Radicals. Measurement of Superoxide Radical Scavenging Activity.[45] The superoxide producing system was set up by mixing phenazine methosulfate (PMS), NADH, and air (oxygen). The production of superoxide was estimated by the nitroblue tetrazolium method. The reaction mixture containing 3 μM PMS, 78 μM NADH, and 25 μM NBT in 19 μM phosphate buffer pH 7.4 was incubated for 2 min at room temperature and the absorption measured at 560 nm against a blank containing PMS. The tested compounds were preincubated for 2 min before adding NADH (Table 7).

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