

Synthesis and biological evaluation of novel angular fused Pyrrolocoumarins[†]

CHRISTOS KONTOGIORGIS¹, KONSTANTINOS E. LITINAS¹, ARISTEA MAKRI¹,
DEMETRIOS N. NICOLAIDES¹, ANASTASIA VRONTELI¹, DIMITRA
J. HADJIPAVLOU-LITINA², ELENI PONTIKI², & ASIMOULA SIOHOU³

¹Laboratory of Organic Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece, ²Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece, and ³Department of Medical Laboratories, Alexandrion Technological Educational Foundation of Thessaloniki, Thessaloniki 57400, Greece

(Received 12 February 2007; accepted 23 March 2007)

Abstract

Angular pyrrolocoumarins were synthesized from the reaction of 4-hydroxyindole or 5-hydroxyindole with DMAD and PPh₃ and were tested for anti-inflammatory and antioxidant activity. These compounds significantly inhibited the carrageenin-induced paw edema (60.5%–73.4%) and have important scavenging activity. Although their interaction with the free stable radical DPPH is not high, compound **9** is the most potent (73.4%) in the in vivo experiment. Compound **7** seems to be a potent LOX inhibitor. An attempt was made to correlate the biological results with their structural characteristics and physicochemical parameters.

Keywords: Pyrrolocoumarins, Hydroxyindoles, Triphenylphosphine, Dimethylacetylenedicarboxylate, Antioxidant activity, Anti-inflammatory activity

Introduction

Coumarins constitute an important class of compounds with a wide range of biological properties. [1–3] In particular, furocoumarins are important as photochemotherapeutic agents that are used to treat a variety of skin diseases; [4] they have been also found to exhibit antitumor, [5] antioxidant [3] and anti-inflammatory [3] activities. Pyrrolocoumarins are nitrogenated isosteres of furocoumarins, which show photobiological and antitumor [6] activities, cytotoxicity and HIV-integrase inhibition [7] and they have been studied much less extensively.

The synthesis of pyrrolocoumarins is achieved by two main routes. In the first the formation of the pyrrole fragment is achieved from the reaction of aminocoumarins and benzoin, [8] or the Fischer

reaction of the corresponding hydrazones, [9–12] or from amine oxide rearrangement of the corresponding *N*-methyl-*N*-prop-2-ynylcoumarins, [13] or from chloroacetylation of aminocoumarin and subsequent reduction [14]. In the second route the pyranone fragment is introduced by Pechmann cyclization of hydroxyindole [15] or by formylation of hydroxyindole and cyclization with acetic anhydride. [16]

In the course of our continuing interest on the synthesis [17–20] of condensed coumarin derivatives with anti-inflammatory and antioxidant activities [21–23] including furocoumarins, [18,22] we have extended our research to the synthesis and biological evaluation of new fused pyrrolocoumarins as anti-inflammatory, antioxidant and possibly antithrombotic agents. Inflammation, as a complex phenomenon,

Correspondence: K. E. Litinas, Laboratory of Organic Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece. Tel: ++302310997864. Fax: ++302310997679. E-mail: klitinas@chem.auth.gr

[†]Preliminary communication presented at 12th Panhellenic Pharmacology Symposium, January 27–28, 2006, Patras, Greece, Book of Abstrs., p. 28.

is implicated in cardiovascular diseases and coumarin derivatives are used as antithrombotic agents.

Materials and methods

UV-Vis spectra were obtained on a Perkin-Elmer Lambda 20 double beam spectrophotometer and on a Hitachi U-2001 spectrophotometer. IR spectra were obtained with a Perkin-Elmer 1310 spectrophotometer as Nujol mulls. NMR spectra were recorded on a Bruker AM 300 (300 MHz and 75 MHz for ^1H and ^{13}C , respectively) using CDCl_3 as solvent and TMS as an internal standard. Mass spectra were determined on a VG-250 spectrometer at 70 eV under Electron Impact (EI) conditions. Microanalyses were performed on a Perkin-Elmer 2400-II Element analyzer. Dichloromethane (DCM) was used as recrystallization solvent for the new compounds.

All the chemicals used were of analytical grade and commercially available. 5-Hydroxyindole, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nordihydroguaiaretic acid (NDGA) were from the Aldrich Chemical Co. Milwaukee, WI, (USA). 4-Hydroxyindole was from Alfa Aesar GmbH & Co. Arachidonic Acid (AA), nicotinamido-adenine-dinucleotide (NADH), Nitrotriazolium Blue (NBT), porcine heme, butylated hydroxytoluene (BHT), soybean lipoxygenase, linoleic acid sodium salt and indomethacin were from Sigma Chemical, Co. (St. Louis, MO, USA). *N*-methylphenazonium-methyl sulfate was from Fluka. Carrageenin, type K, is commercially available. Silica gel N^o 60, Merck A.G. was used for column chromatography.

SimplastinR Excel (Bio Merieux) (includes, thromboplastin reagent, aPTT reagent: cephaline – caoline) was used.

Synthesis

General procedure for the reactions of hydroxyindoles with DMAD and PPh₃. Hydroxyindole [0.665 g (5 mmole)] was added to a solution of 1.31 g (5 mmole) triphenylphosphine (PPh₃) in 20 mL dichloromethane (DCM) at 0°C. To the mixture, at that temperature, a solution of dimethylacetylenedicarboxylate (DMAD) [0.6 mL (5 mmole)] in DCM (20 mL) was added dropwise during 45 min. The orange mixture was then refluxed for 6 h. After cooling the mixture was evaporated and separated by column chromatography [ethyl acetate/hexane (1:1)] to give, after the elution of PPh₃, the coumarin derivative (7 or 9).

*Methyl 7-oxo-3,7-dihydropyrano[3,2-*e*]indole-9-carboxylate (7)*. Yellow crystals [0.652 g (54% yield)]; m.p. 224–226°C; IR (Nujol, cm^{-1}): 3370, 1726, 1700; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ 4.09 (s, 3H), 6.55 (s, 2H), 7.24 (d, \mathcal{J} = 8.8 Hz, 1H), 7.37 (t, \mathcal{J} = 2.9 Hz, 1H), 7.62 (d, \mathcal{J} = 8.8 Hz, 1H), 8.59 (brs, 1H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3) δ 53.6, 102.7, 108.9,

112.4, 114.6, 116.8, 120.5, 126.8, 146.1, 148.1, 156.6, 160.4, 165.4; MS m/z 243 (M^+ , 100), 212 (45), 211 (60), 184 (50), 156 (20), 155 (25), 128 (32), 101 (5) Anal. Calcd. for $\text{C}_{13}\text{H}_9\text{NO}_4$ C, 64.18; H, 3.73; N, 5.76. Found: C, 64.33; H, 3.95; N, 5.65%.

*Methyl 2-oxo-2,7-dihydropyrano[2,3-*e*]indole-4-carboxylate (9)*. Yellow crystals [0.466 g (38% yield)]; m.p. 211–212°C; IR (Nujol, cm^{-1}): 3330, 1725, 1710; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ 4.01 (s, 3H), 6.79 (s, 1H), 6.97 (t, \mathcal{J} = 2.9 Hz, 1H), 7.27 (d, \mathcal{J} = 2.9 Hz, 1H), 7.34 (d, \mathcal{J} = 8.8 Hz, 1H), 7.96 (d, \mathcal{J} = 8.8 Hz, 1H), 8.53 (brs, 1H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3) δ 53.0, 101.3, 108.8, 111.1, 114.7, 119.9, 124.9, 128.1, 141.5, 149.8, 154.6, 161.0, 165.2; MS m/z 243 (M^+ , 100%), 215 (42), 184 (30), 156 (10), 128 (25), 101 (5). Anal. Calcd. for $\text{C}_{13}\text{H}_9\text{NO}_4$ C, 64.18; H, 3.73; N, 5.76. Found: C, 64.05; H, 3.83; N, 5.62%.

General procedure for the reactions of 5-hydroxyindole with DMAD and P(OR)₃

Trialkylphosphite (5 mmole) was added to a stirred solution of 5-hydroxyindole [0.665 g (5 mmole)] and DMAD [0.6 mL (5 mmole)] in DCM (50 mL) at room temperature and the yellow solution stirred at room temperature for 4 days. After evaporation of the solvent the resulting mixture was separated by column chromatography [ethyl acetate/hexane (1:1)] to give the indole derivative 14 (after the elution of phosphite).

*Methyl 7-oxo-3,7,8,9-tetrahydropyrano[3,2-*e*]indole-9-carboxylate (14)*. White crystals [0.413 g (34% yield)] from $\text{P}(\text{OCH}_3)_3$, 0.623 g (51% yield) from $\text{P}(\text{OCH}_2\text{CH}_3)_3$; m.p. 181–183°C; IR (Nujol, cm^{-1}): 3410, 1754, 1726; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ 2.90 (dd, \mathcal{J}_1 = 6.6 Hz, \mathcal{J}_2 = 16.2 Hz, 1H), 3.20 (dd, \mathcal{J}_1 = 1.8 Hz, \mathcal{J}_2 = 16.2 Hz, 1H), 3.67 (s, 3H), 4.30 (dd, \mathcal{J}_1 = 1.8 Hz, \mathcal{J}_2 = 6.6 Hz, 1H), 6.67 (t, \mathcal{J} = 3.0 Hz, 1H), 6.95 (d, \mathcal{J} = 8.3 Hz, 1H), 7.33 (d, \mathcal{J} = 3.0 Hz, 1H), 7.34 (d, \mathcal{J} = 8.3 Hz, 1H), 8.43 (brs, 1H); $^{13}\text{C-NMR}$ (75.5 MHz, CDCl_3) δ 31.4, 39.3, 52.6, 101.1, 110.2, 112.1, 112.4, 126.3, 130.7, 136.1, 151.5, 167.0, 171.5; MS m/z 245 (M^+ , 50%), 186 (49), 185 (100), 158 (32), 157 (36), 142 (6), 130 (51), 102 (7). Anal. Calcd. for $\text{C}_{13}\text{H}_{11}\text{NO}_4$ C, 63.66; H, 4.52; N, 5.71. Found: C, 63.70; H, 4.64; N, 5.63%.

Biological assay

In vivo

Inhibition of the carrageenin-induced oedema [23]. Oedema 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. 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

their maintenance but they were entirely fasted during the experimental period. Our studies were in accordance with recognised guidelines on animal experimentation (Guidelines for the care and use of laboratory animals published by the Greek Government 160/1991, based on EU regulations 86/609).

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 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 carrageenin paw oedema (CPE %) values (Table II). Indomethacin at 0.01 mmol/Kg (47% inhibition of the carrageenin paw oedema CPE) was administered as a standard drug for comparison reasons. Values CPE % are the mean from two different experiments with a standard error of the mean less than 10% ($p < 0.01$ compared with control values).

In vitro

Interaction of the tested compounds with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical [24]. To a solution of DPPH (0.1 mM) in absolute ethanol an equal volume of the compounds (0.1 and 0.2 mM) dissolved in ethanol was added. Ethanol was used as a control solution. After 20 min at room temperature the absorbance was recorded at 517 nm. NDGA was used as an appropriate standard (Table I).

Soybean lipoxygenase inhibition [24]. The tested compounds dissolved in DMSO (final concentration 0.1 mM) were incubated in room temperature with sodium linoleate (0.1 mM) and 0.15 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 (caffeic acid 0.6 mM)

Non-enzymatic assay of superoxide radicals- measurement of superoxide radical scavenging activity [24,25].

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 μ M PMS, 78 μ M NADH, and 25 μ M NBT in 19 μ M 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 pre-incubated for 2 min before adding NADH (Table I). Caffeic acid was used as a standard (0.1 mM)

Competition of the tested compounds with DMSO for hydroxyl radicals [24]. The hydroxyl radicals generated by the Fe^{3+} /ascorbic acid system, were detected by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μ M), 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 I). Trolox was used as a control (Table I).

Measurement of prothrombin time [26]. 50 μ l of the examined compounds (7, 9, 14, warfarin and coumarin at final concentration 0.17 mM) and 50 μ L of plasma from human healthy volunteers were reacted with 100 μ L SimplastinR excel reagent containing rabbit thromboplastin and 100 μ L calcium ions solution dissolved in buffer with stabilizers (CaCl_2). The mixture (final volume 300 μ L) was incubated at 37°C and the time of clot formation was measured (Table III).

Measurement of activated partial thromboplastin time [27]. 50 μ L of the tested compounds and 50 μ L plasma from human healthy volunteers were incubated with 100 μ L of cephaline – caoline at 37°C. After that 100 μ L of CaCl_2 (0.025 M) was added and the time was measured (Table III).

Table I. Interaction % with DPPH (RA %); Competition % with DMSO for hydroxyl radical ($\text{HO}\cdot$ %); % Superoxide radical scavenging activity (PMS %).

Comp.	RA % 0.1 mM 20 min	RA % 0.1 mM 60 min	RA % 0.2 mM 20 min	RA % 0.2 mM 60 min	$\text{HO}\cdot$ (%) 0.01 mM	$\text{HO}\cdot$ (%) 0.1 mM	PMS% 0.1 mM
7	21.4	20.5	33	44.2	97	94.4	no
9	12.2	3.6	4.3	20.5	96	no	84.4
14	29.5	46.6	61.8	69.6	95	88.5	no
NDGA	81	82.6	80	80	nt	nt	nt
Warfarin	9.2	10	24	23.2	nt	nt	no ^a
Trolox	nt	nt	nt	nt	73.4	88.2	nt
Caffeic Acid	nt	nt	nt	nt	nt	nt	47

^a25% (at 1 mM); nt: not tested; no: not determined under the reported experimental conditions.

In vitro / In vivo assays. Each experiment *in vitro* was performed at least in triplicate and the standard deviation of absorbance was less than 10%. Analysis of variance (ANOVA test) was used to assess significant differences. Differences at $p < 0.05$ were considered to be significant. *In vivo* statistical studies were done with student's T-test.

Results and discussion

Synthesis

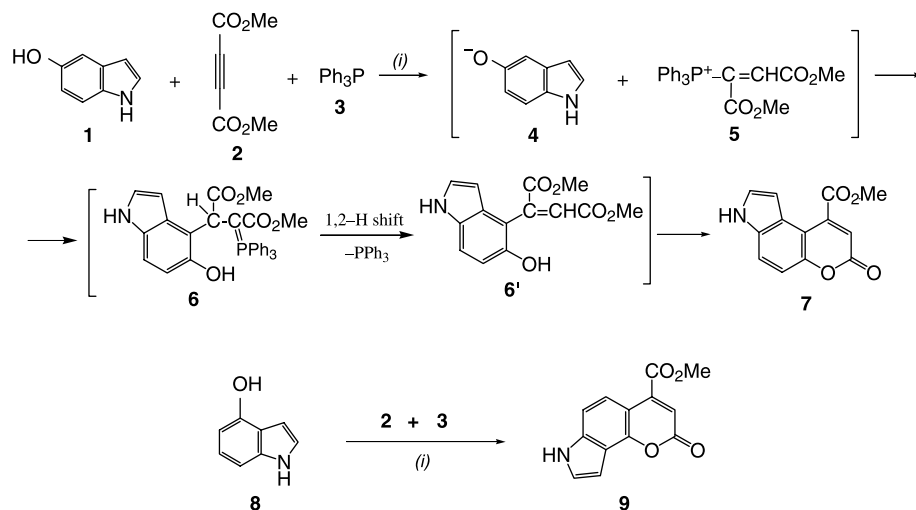
Synthesis was achieved in one step by a new pathway from hydroxyindoles and is based on the recently [28–29] reported formation of 4-methoxycarbonylcoumarin derivatives by the reaction of phenols with PPh_3 and DMAD. The reaction studied and the products obtained are depicted in Schemes 1–2.

In a DCM solution of 5-hydroxyindole (**1**) was added dropwise at 0°C an equimolar amount of DMAD (**2**) in the presence of PPh_3 (**3**) and the resulted orange solution was refluxed for 6 h to give compound **7** (54% yield) after separation of the complex reaction mixture by column chromatography

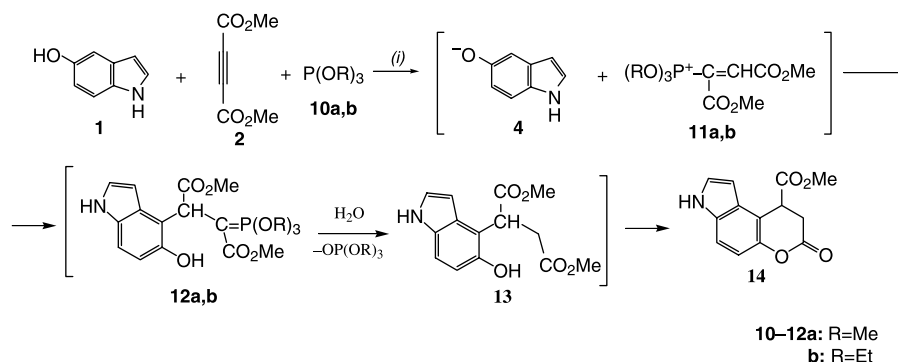
with hexane/ethyl acetate (1:1) as eluent. The reaction of 4-hydroxyindole (**8**) with equimolar amounts **2** and **3** led to compound **9** (38% yield).

From the reaction of **1** with **2** in the presence of trimethylphosphite (**10a**) or triethylphosphite (**10b**) at room temperature over 4 days, compound **14** was isolated, after separation by column chromatography of the complex mixtures (34% or 51% yield, respectively). The $^1\text{H-NMR}$ spectra of the products **7** and **14** showed both two doublets for the phenyl protons, in agreement with their angular structures.

The formation of compound **7** proceeds through the intermediates depicted in Scheme 1. An initial addition of the conjugate base, **4**, to vinyltriphenylphosphonium salt, **5**, leads, after a few transformations involving 1,2-H shift and abstraction of PPh_3 from the intermediate ylide **6**, to the formation of the substituted indolol **6'**, which by lactonization results in compound **7**. A similar mechanism has been suggested for the formation of coumarins from the reactions between **2**, **3** and phenols. [28,29] On the basis of analogous literature data for the reaction of 2-naphthol with phosphites in the presence of DMAD, [30] it is reasonable to assume that the intermediate



Scheme 1. Reagents and conditions: (i) DCM, 0°C (45 min) and the reflux (6 h).



Scheme 2. Reagents and conditions: (i) DCM, r.t. (4 days).

ylides **12a,b** (Scheme 2) are formed by attack of the carbon atom of the anion **4** to the initially formed adducts **11a,b**. Hydrolysis of ylides **12a,b** followed by lactonization of the resulted intermediate succinate **13** leads to the dihydro-compound **14**.

Biological studies

The reported derivatives were tested for their antioxidant, anti-inflammatory and anticoagulant activities. Non-steroidal anti-inflammatory drugs (NSAIDs) have a broad spectrum of effects and it has been suggested that the variations in both efficacy and their tolerability are partly due to differences in their physicochemical properties, which determine their distribution in the body and their ability to pass through and to enter the interior of membranes. [31–32] In acute toxicity experiments, the *in vivo* examined compounds did not present toxic effects in doses up to 0.5 mmol/kg body.

To assess the anti-inflammatory activity of the pyrrolocoumarins the rat carrageenin-induced paw Oedema assay was employed as a model for acute inflammation. It detects compounds that are anti-inflammatory agents, as a result of inhibition of prostaglandin amplification. Edema was induced in the right hind paw of Fischer 344 rats (150–200 g) by the intradermal injection of 0.1 ml 2% carrageenin in water. The tested compounds 0.01 mmol/kg body weight, were dissolved in water and were given intraperitoneally simultaneously. Indomethacin was included as a reference drug. It is of interest that all the tested compounds possessed significant protection which ranged from 60.5 to 73.4%. The small differences among *clogP* values (1.24–1.60) do not support the view that lipophilicity could affect the biological responses (Table II). The compounds concerned were further evaluated for inhibition of soybean lipoxygenase LOX by the UV absorbance based enzyme assay. [24] 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 II). Examination of LOX % inhibition and/or IC₅₀'s values shows that compound **7** is the most active, while compounds **9** and **14** present moderate inhibitory activity on LOX. Most of the LOX inhibitors are antioxidants or free radical scavengers [33] since lipoxygenation occurs via a carbon-centered radical. Several LOX inhibitors are excellent ligands for Fe⁺³. Many coumarinyl and pyrrolyl derivatives inhibit soybean lipoxygenase. This inhibition is related to their ability to reduce the iron species in the active site to the catalytically inactive ferrous form. [34] Nowadays, antioxidants that exhibit DPPH radical scavenging activity are increasingly receiving attention. They have been reported to have interesting anticancer, anti-ageing and anti-inflammatory activities. DPPH is a stable free radical that can accept an electron or hydrogen radical and thus be converted into a stable, diamagnetic molecule. DPPH has an odd electron and so has a strong absorption band at 517 nm. All compounds were tested for their interaction with the stable free radical DPPH and NDGA was used as reference compound. This interaction indicates their radical scavenging activity in an iron free system and expresses their reducing activity. The tested compounds were found to have low activity at 0.1 mM. Not many changes were observed with time of reaction (compound **14** presents an increase). Compounds **7** and **14** showed higher interactions with an increase in concentration. Compound **14** presents the highest activity at 0.1 mM. and 0.2 mM. In general the results did not proceed in parallel with the increase of time and concentration (Table I). The competition of compounds with DMSO for OH· radicals [24], generated by the Fe³⁺/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. Compound **9** could not be tested at 0.1 mM due to solubility problems. Compounds **7**, **9**, **14** significantly inhibited the DMSO oxidation at 0.01 mM concentration. The inhibition was not significantly affected by an increase in concentration (Table I).

Non-enzymatic superoxide anion radicals were generated [25] by mixing phenazine methosulfate

Table II. Inhibition % LOX / IC₅₀ values of soybean lipoxygenase; Inhibition % of induced carrageenin rat paw edema (CPE %) at 0.01 mmol/Kg body weight; Theoretically calculated lipophilicity values. [35]

Compounds	LOX % 0.1 mM	LOX % IC ₅₀ (mM)	CPE % ^a	Clog P ^b
7	62.4(0.01 mM) 71 (0.1 mM)		67.8*	1.60
9		0.325 mM	73.4 **	1.60
14		0.35 mM	60.5 *	1.24
Warfarin	no	no	41 **	
Caffeic acid		0.6 mM	nt	
Indomethacin	nt		47 *	

^astatistical studies were done with student's T-test, * p < 0.01, ** p < 0.05; ^b Biobyte Corp [35]; no: no action under the reported experimental conditions; nt: not tested.

Table III. PT and aPTT values (sec) induced by the tested compounds

Compounds	PT (s)	aPT (s)
7	24	105
9	21.5	86
14	24	102
Warfarin	>50	
Coumarin	19	35
Control	19	60

(PMS), NADH and air –oxygen and the production of superoxide was estimated by the nitroblue tetrazolium method. Compounds 7 and 14 did not show any activity under the experimental conditions but 9 showed high scavenging activity.

Quick et al [26] introduced a prothrombin time (PT) assay as a screening test for factors VII, X, V and II which allows an accurate evaluation of the capacity of the extrinsic pathway; whereas the aPTT (partial activated thromboplastin time assay) is used to evaluate the efficacy to inhibit the intrinsic pathway of coagulation. The method consists in measuring the time up to clot formation after adding the “PR reagent” (a combination of tissue thromboplastin and Ca^{+2}). [27] All the tested compounds showed medium PT values whereas the aPTT values compared to the control were found to be significant. Compounds 7 and 14 presented comparable activities in both tests. Coumarin was also tested under the same experimental conditions and the results are given in Table III. Since all these derivatives contain this functional structure, the biological results should be correlated with this.

The above compounds constitute an interesting template for the evaluation of new synthetic lipoxigenase inhibitors and may be helpful in the design of new therapeutic tools against inflammation. From our results, it can be concluded, that lipophilicity is not the main property responsible for the anti-inflammatory/antioxidant activity of the investigated coumarins. The *in vivo* antiinflammatory activity of the synthesized compounds seems to be related with their high HO-scavenging ability and their good LOX inhibitory activity

For the C5-C6 fused derivatives, the absence of the double bond between C3-C4 is correlated with a decrease in the *in vivo* biological responses (compound 7, 67.8% and 14, 60.5%). The angular derivative 9 (fused at C7-C8) is more potent *in vivo* compared to compound 7. In general, stereochemistry of the molecules seems to affect their biological activities.

Acknowledgements

The authors gratefully acknowledge support of this work through the program PYTHAGORAS II of EPEAEK II (MIS: 97436/073). We would like also to thank Dr C. Hansch and Biobyte Corp. 201 West 4th

Str., Suite 204, Claremont CA California, 91711, USA for free access to C-QSAR programme.

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