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Anti-inflammatory, Antioxidant and Analgesic Amides*

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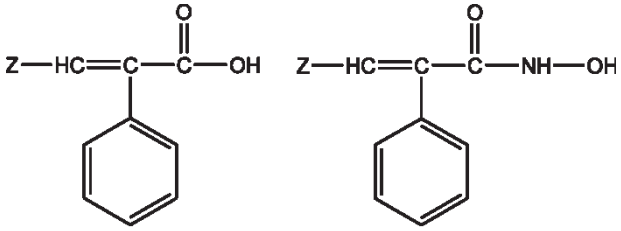
The synthesis of some new aryl acetic acids and amides and a pharmacochemical study and quantitative structure-activity relationships (QSAR) on them are described. The compounds were screened for their biological activity using the carrageenin induced rat paw oedema model and a significant inhibition of oedema occurred (44.1–80.1%) at a concentration of 0.01 mmol/1 kg. The analgesic activity, based on the inhibition of acetic acid-induced writhing in rats was also found to be significant. The compounds were found to interact with the stable free radical 1,1-diphenyl-hydrazyl DPPH and with DMSO (for hydroxyl radicals). The compounds were screened for radical scavenging activity with the xanthine/xanthine oxidase system for $O_2^{\cdot-}$ and for inhibition of soybean lipoxygenase (LOX). The results are discussed in terms of the structural and physicochemical characteristics of the compounds.

Keywords: Cinnamides; Anti-inflammatories; Antioxidants; Soybean lipoxygenase; QSAR

INTRODUCTION

In our previous studies we investigated the antioxidant and lipoxygenase inhibitory activity of some novel aryl-acetic and aryl-hydroxamic acids[1] with the general structures (**1**) and (**2**).



(1) (2)

From the literature[2] it is known that substituted N-cyclo-alkyl benzamides, cinnamides and indole-3-carboxamides possess analgesic and anti-inflammatory activity as well as gastrointestinal irritation liability.

The biosynthetic cascade of arachidonic acid liberated from phospholipids by various stimuli can be metabolized by the cyclooxygenase (COX) pathway to prostaglandins and thromboxane A_2 or by lipoxygenase (LOX) pathways to hydroperoxy-eicosatetranoic acids (HPETE's), hydroxyeicosa-tetranoic acids (HETE's) and leukotrienes (LT's). Inhibition of LOX represents a promising therapeutic target[3,4] for various disease states since the major products of 5-LOX, LT's are important biologically active mediators in a variety of conditions including asthma, psoriasis, ulcerative colitis and rheumatoid arthritis.

Hydroxamic acids[5] are well known to form strong complexes with a variety of transitions metals. This property has been exploited in the use of hydroxa-mates as inhibitors of several metalloenzymes. Since it is generally believed that 5-lipoxygenase contains a catalytically important iron atom, this enzyme is a logical candidate for inhibition by hydroxamic acid-containing molecules. The hydroxamic acid functionality[5] has been incorporated into a wide variety of molecules to produce potent inhibitors of LOX.[5] Pharmacological activities were influenced by the nature of the aryl moiety of the acids and the hydroxamates and by their hydrophobicity.

As a further contribution to the understanding of the structure-activity relationships of this class of compounds, we describe here a new series of amides combining the $\mathbf{Z-CH=C(C_6H_5)-C=O-}$ group with a tertiary or a secondary amidic nitrogen.

*A part of this research has been presented in the 3rd Pan-Hellenic Congress of Free Radicals and Oxidative Stress in Athens, Greece, October 2002.

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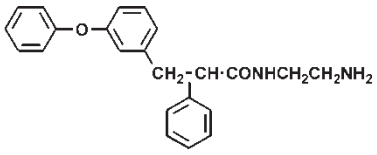
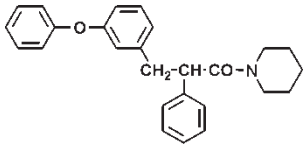
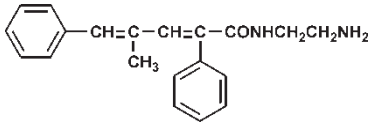
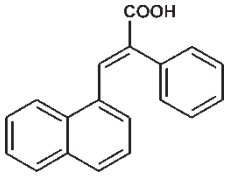
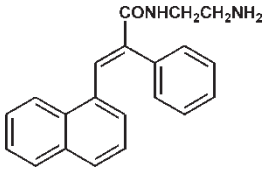
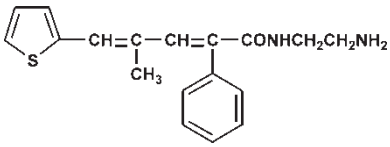
TABLE I Physical data for the newly synthesized compounds

Compound	Structure	R_f	m.p. °C	Yield	M.F.[a]
3i		0.890[b]	118 − 120°C	57.6%	$C_{23}H_{22}N_2O_2$
3ii		0.972[c]	114–116°C	35%	$C_{26}H_{25}NO_2$
3iii		0.849[d]	96–102°C	15%	$C_{20}H_{22}N_2O$
3iv		0.807[d]	Liquid at room temperature	c. 100%	$C_{19}H_{14}O_2$
3v		0.722[e]	197–200°C	35%	$C_{21}H_{20}N_2O$
3vi		0.673[f]	257–260°C	37%	$C_{15}H_{16}N_2OS$

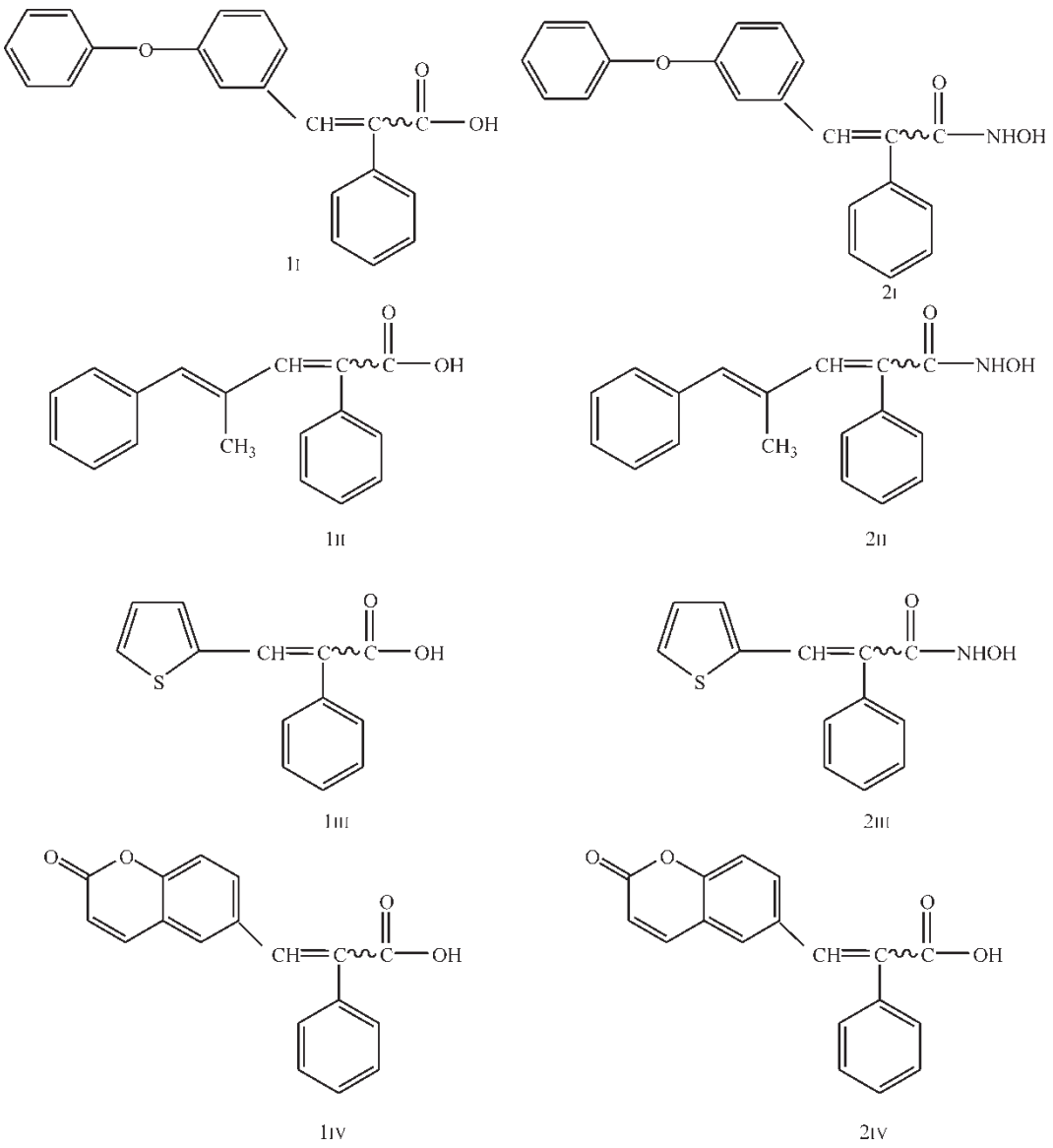
[a] Elemental analysis for molecular formula. [b] Petroleum ether. [c] Ethanol–trichloro methane 1:1. [d] Acetic acid ethyl ester–dichloro methane 2:1. [e] Benzene–acetic acid ethyl ester 1:1. [f] Ethanol–trichloro methane (saturated with ammonium hydroxide solution) 4:1.

The new derivatives and known compounds are shown in Table I and in Scheme 1.

MATERIALS AND METHODS

Melting points (uncorrected) were determined on a MEL-Temp II (Lab. Devices, Holliston, MA, USA), IR as a neat film or as a Nujol mull and ^{1}H-NMR(300 MHz) and ^{13}C-NMR(75 MHz) in $CDCl_3$ with tetramethylsilane as internal standard. IR spectra were recorded with a Perkin-Elmer 597 or a 554 double beam spectrophotometer (The Perkin-Elmer Corporation Ltd., Lane Beaconsfield, Bucks, England) and a NMR spectra with a Bruker Analytische (Messtechnik GmbH, Rheinstetten, Germany). Chemical shifts were expressed in δ (ppm) values. MS spectra were determined on a VG-250 spectrometer (VG-Labs., Tritech, England) with ionization energy maintained at 70 eV. All the compounds gave spectra consistent with their structures. Elemental analyses were obtained within the range (±0.4%) on a Perkin-Elmer 240B CHN analyzer. Thin layer chromatography (TLC) was performed on silica gel (60 F_{254}, Merck, Darmstadt, Germany).

Carrageenin K- type was commercially available. $CDCl_3$, DMSO-d_6, tetramethylsilane, Nujol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nordihydroguairetic acid (NDGA) were purchased from the Aldrich Chemical Co. (Milwaukee, WI, USA) whereas soybean lipoxygenase [EC. 1.13.11.12], xanthine oxidase, xanthine sodium linoleate and indomethacin were purchased from Sigma Chemical, Co. (St. Louis, MO, USA). All the chemicals used



SCHEME 1 The reported aryl acetic acids[1] and hydroxamates.[1]

were of analytical grade and commercially available from Merck (KgaA 64271 Darmstaadt, Germany).

Chemical Synthesis

General Procedure for the Synthesis of Aryl-acetic Acids[1]

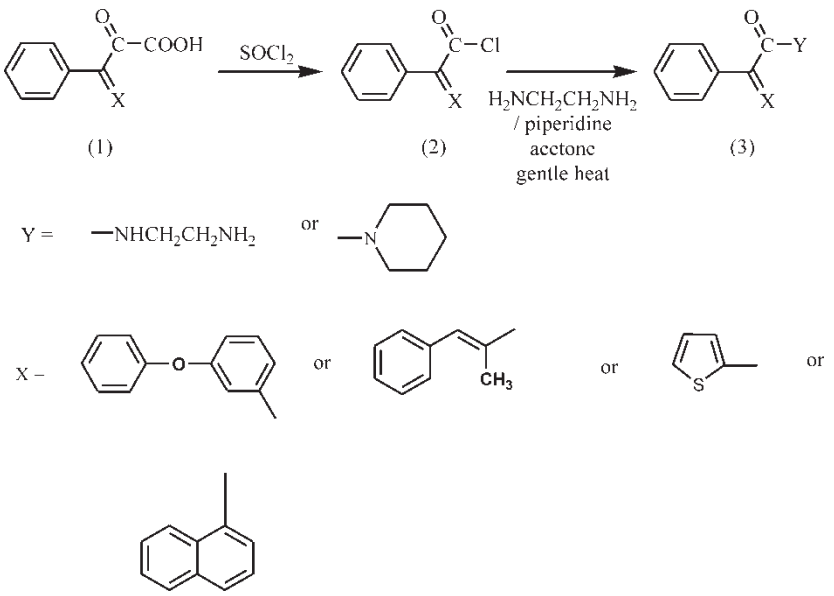
The compounds reported here were generally prepared as illustrated in Scheme 2. The aryl-acetic acids were synthesized by a Knoevenangel condensation between an aldehyde (0.015 mol) and phenylacetic acid (0.015 mol) with acetic acid anhydride (10 mL) in the presence of triethylamine (5 mL) with refluxing for 5 h. The solution was then poured into 2 N HCl, then onto ice. The precipitate formed was collected by filtration and recrystallized from 50% aqueous ethanol, otherwise the residue was extracted with 3×100 mL $CHCl_3$ and the collected organic phase dried over Mg_2SO_4 and evaporated.

General Procedure for the Synthesis of Aryl-acetyl Chlorides[6]

Thionyl chloride (16 mmol) was added dropwise to the corresponding acid (equimolar amount) with stirring and the mixture stirred at room temperature for 45 min (TLC monitoring). The volatile materials were then removed under reduced pressure and the resulting solid used without further purification in the subsequent step. (IR Nujol 1740 cm^{-1} C = O).

General Procedure for the Synthesis of Amides[6]

The appropriate amine dissolved in dry acetone (30 ml) was added dropwise to a solution of



SCHEME 2 Synthetic procedure for compounds (**3**).

the corresponding acid chloride (equimolar amount) in the same solvent (30 ml) and the mixture was stirred at room temperature for 45 min (TLC monitoring) and then refluxed for 1 h. After removal of the acetone under vacuum, the residue was purified by recrystallization from ethanol (see Table I). The infra red, MS and NMR spectral data for compounds (**3**) are collected in Tables II and III.

Pharmacochemical Evaluation

Effect of the Test Compounds on the OH Radical-mediated Oxidation of DMSO[7]

The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system were detected by the determination of formaldehyde produced from the oxidation of DMSO according to Nash.[7] 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 (0.1 mM) and ascorbic acid (10 mM). After 30 min incubation at 37°C the reaction was stopped by the addition of CCl_3COOH (17% w/v) and the absorbance was recorded at λ 412 nm. The results are shown in Table VI.

Interaction of the Test Compounds with 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH), a Stable Free Radical[8]

To a solution of DPPH (0.1 mM) in absolute ethanol an equal volume of the compounds (0.1 mM and 0.2 mM) dissolved in ethanol was added. As control a solution ethanol was used. After periods of 20 min and 90 min at room temperature the absorbance was recorded at λ 517 nm and the percent reduction was estimated. Acetylsalicylic acid (0.1 mM) was used as an appropriate standard. The results are summarized in Table V.

Scavenging Activity of Superoxide Anion Radical[9]

The superoxide anion was generated by the xanthine–xanthine oxidase system and measured by the nitroblue tetrazolium (NBT) method.[9]

TABLE II Spectral characteristics of the synthesized compounds

Compound	IR ν C=O cm^{-1}	MS m/z (%)[a]
3i	1650	299(8.3), 93(12.2), 77(100), 44(67.9)
3ii	1660	[M^+] 10.5, 299(10.2), 271(10.2), 84(68.8), 77(83.7)
3iii	1620	262(10.2), 117(13.1), 77(46.7), 44(100)
3iv	3150 cm^{-1}, 2750 cm^{-1}, 1680 cm^{-1}	[M^+] (64.1), 230 (100), 229 (89.5), 139 (8.7), 198 (18.9), 77 (35.5)
3v	1660	257(9.1), 229(16.1), 44(68.4)
3vi	1650	185(22.3), 96(12.3), 77(20.1), 71(46.8), 70(100), 44(35.5)

[a] Relative intensities.

TABLE III Spectral characteristics of the synthesized compounds

Compound	^{1}H-NMR	^{13}C-NMR
3i	δ, ppm, 0.88(s, 1H), 1.24–1.25 (br, 5H), 6.67–7.56 (br, 14H), 9.17 (s, H)	δ, ppm, 27.1, 36.5, 45.7, 79.6, 118.2, 119.1, 120.3, 120.6, 126.7, 127.1, 127.4, 127.7, 128.1, 128.5, 129.2, 129.7, 130.2, 154.9
3ii	δ, ppm, 1.17–1.64 (br, 10H), 9.10–9.13 (d, 1H), 6.67–7.80 (br, 14H)	δ, ppm, 44.5, 76.6, 77.0, 77.1, 77.4, 118.9, 120.4, 129.8, 152.0, 156.5, 158.8
3iii	δ, ppm, 0.83–2.92 (br, 7H), 3.4 (s, 3H), 6.89–7.57 (br, 10H), 7.9 (s, 1H)	δ, ppm, 40.8, 41.0, 41.1, 43.8, 46.8, 47.7, 77.5, 77.9, 78.3, 90.8, 118.5, 128.3, 128.9, 129.7, 136.7, 166.44
3iv	δ, ppm, 7.14–8.16 (m, 12H), 8.66 (s, 1H), 10.41 (s, 1H)	δ, ppm, 76.5, 77.0, 77.4, 123, 128.9, 131.1, 172
3v	δ, ppm, 6.68 (d, 1H), 7.5–8.2 (m, 8H), 9.6 (br, 1H), 1.66–1.70 (tr, 2H), 1.87–1.95 (m, 3H), 2.91–3.15 (s, 2H)	
3vi	δ, ppm, 1.18–1.23 (m, 3H), 1.18–2.17 (m, 5H), 3.44–3.71 (m, 2H), 7.1–7.26 (m, 5H), 7.50–7.53 (d, 1H), 7.71–7.87 (m, 2H), 8.05 (d, 1H)	δ, ppm, 30.86, 56.19, 95.8, 111.0, 117.6, 125.3, 127.3, 127.9, 128.0, 128.4, 128.6, 128.9, 140.7, 158.0

To the reaction mixture in phosphate buffer pH 7.4 (0.1 M) containing xanthine, NBT and test compound (1 mM final concentration), xanthine oxidase (0.07 U/mL) was added. After incubating for 10 min at room temperature the absorbance was recorded at λ 560 nm.

Soybean Lipoxygenase Inhibition[9]

The tested compounds dissolved in 60% v/v aqueous ethanol (final concentration 0.1 mM), were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of enzyme solution (3×10^3 dilution of lyophilized enzyme w/v in 0.9% NaCI). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with nordihydroguaretic acid (0.1 mM), an appropriate standard inhibitor. The results are summarized in Table VI.

In Vivo Assays

Inhibition of Carrageenin-induced Edema[9]

Edema was induced in the right hind paw of Fisher 344 rats (both sexes, 150–200 g) by an intradermal injection of 0.1 mL 2% w/v carrageenin in water. Pregnant females were excluded from the experiments. The animals, bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water *ad libitum*. During the experimental period food but not water was withheld. These studies were in accordance with recognized 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 amides **3i**, **3ii**, **3iii**, **3iv**, **3v**, **3vi** as well as the acids **1ii** and **1iii**, at 0.01 mmol/Kg body weight, were ground in a mortar, suspended in water with few drops of Tween 80 and given intraperitoneally (i.p) at the same time as the carrageenin. The rats were sacrificed by decapitation 3.5 h after the carrageenin injection. The experiment was repeated twice for each compound (two groups of 6 animals). 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 (injected with water) and expressed as a percent inhibition of the edema (CPE % values Table IV). Indomethacin at 0.01 mmol/Kg (47%) was administered as a standard drug for comparison. Values for CPE % are the mean from two different experiments with a standard error of the mean less than 10%. Statistical studies were made with student's T-test ($p < 0.01$ or $p < 0.05$ compared with control values).

Antinociceptive Screen[10]

The writhing reflex was used.[9] Compounds, which showed the best anti-inflammatory activity were

TABLE IV *In vivo* inhibition (%) of carrageenin-induced rat paw edema

Compound	Dose mmol/kg	CPE%	Clog P^b	W %
3i	0.01	52.3**	4.715	nt
3ii	0.01	53.7**	6.340	26.2
1ii[a]	0.01	42.3**	4.440	nt
3iii	0.01	45.5***	3.470	no
3iv	0.01	80.1**	4.761	25.7
3v	0.01	36.0**	3.791	56.5
1iii[a]	0.01	65.5**	3.233	nt
3vi	0.01	29.2***	2.263	nt
indomethacin	0.01	57.0	4.180	nt
Acetylsalicylic acid (sodium salt)	0.1	nt		93.3

[a] Corresponds to the reported structures in Scheme 1; $^{**}p < 0.01$, $^{***}p < 0.05$, compared to the controls (student's test); nt = not tested; no = no results under the reported experimental conditions. [b] Lipophilic parameter clog *P* (theoretically calculated values using Biobyte's program[19]).

TABLE V % Interaction of the examined compounds and reference drugs with the stable free radical DPPH

	0.1 mM		0.2 mM		0.5 mM	
Compound	20 min	60 min	20 min	60 min	20 min	60 min
3i	**no***	2.64	27.91	26.43	40.22	47.58
3ii	**no**	**no**	12.96	10.8	37.03	47.14
3iii	10.33	19.82	60.88	60	67.03	81.5
3iv	**no**	**no**	**no**	**no**	**no**	1.97
3v	**no**	**no**	**no**	**no**	**no**	**no**
3vi	25.05	37	60.88	61.34	81.54	96.7

*no = no results under the experimental conditions; nor-dihydrogouaretic acid (NDGA) = 94.4% at 0.1 mM; acetylsalicylic acid = 80.6% at 0.1 mM.

administered i.p. (the same dose as for previous *in vivo* test) to a group of 7 Fischer rats (120–170 g), 30 min prior to the i.p. administration of 1 ml/100 g body weight of 0.6% acetic acid. Sodium acetylsalicylate at 0.1 mmol/100 g body weight was administered i.p. as a standard drug for comparison. After 5 min, the number of stretches characterized by repeated contractions of the abdominal musculature accompanied by extension of the hind limbs was counted every 5 min for the next 30 min. The total number of writhes exhibited by each animal in the test group was recorded and compared to that of a vehicle treated control group. The % antinociceptive activity (a.a) was calculated as:

$$\%\mathbf{a.a} = (\mathbf{n} - \mathbf{n}'/\mathbf{n}) \times \mathbf{100}$$

(**n** average number of writhes of control group, $\mathbf{n}'$ average number in the test group). For the tested compounds the analgesic activity is shown in Table IV and Figure 1.

RESULTS AND DISCUSSION

Synthesis

The compounds in Table I were prepared by the general procedure outlined in Scheme 2. The reactions proceeded smoothly but not always in high yields (35–100%); for compound **3iii** the yield was very low (15%). The structures of intermediates and final products were confirmed by elemental as well as spectroscopic analyses (Tables I, II and III).

TABLE VI *In vitro* effects of the examined compounds and reference drugs on the HO· mediated oxidation of dimethyl sulphoxide % and % inhibition of soybean lipoxygenase (LOX%)

Compound	HO·%	LOX%
3i	38.3	no
3ii	82.7	36.8
3iii	94.2	no
3iv	no	no
3v	76.15	45.5
3vi	94.95	no

no = no result under the reported experimental conditions.

Biological Studies

The *in vivo* anti-inflammatory effects of the tested compounds were assessed by using the functional model of carrageenin-induced rat paw oedema and are presented (Table IV) as percentage of weight increase in the right hind paw in comparison to the uninjected left hind paw. Carrageenin- induced oedema is a non-specific inflammation resulting from a complex of diverse mediators.[11] Since on edemas of this type are highly sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), carrageenin has been accepted as a useful agent for studying new anti-inflammatory drugs.[12] This model reliably predicts the anti-inflammatory efficacy of the NSAIDs and during the second phase it detects compounds that are anti-inflammatory agents as a result of inhibition of prostaglandin amplification.[13] As shown in Table IV, all the investigated compounds induced protection against carrageenin -induced paw on edema. The protection ranged from 29.2–80.1% while the reference drug, indomethacin, induced 47% protection at an equivalent concentration. Compound **3iv** was the most

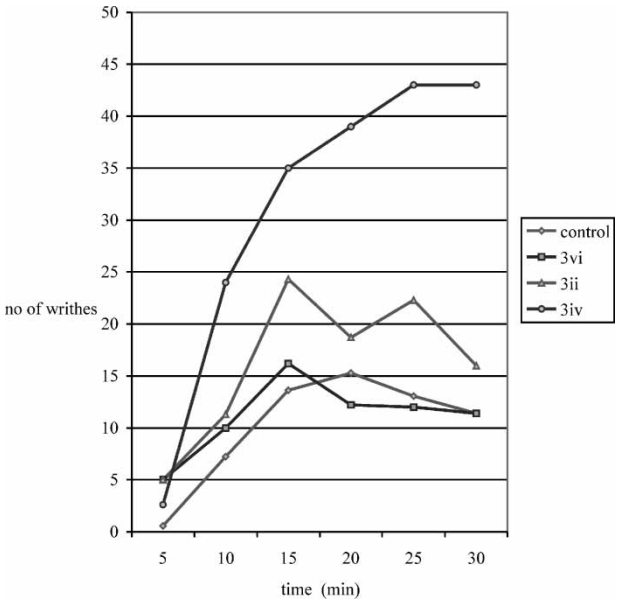


FIGURE 1 Antinociceptive activity of some of the tested activity of some of the tested t.

potent (80.1%) whereas compound **3vi** had the least effect (29.1%). Comparing the results (Table IV), it seems that the tested aryl acetic acids are more potent inhibitors than the corresponding amides, with the exception of the acid **1ii** (42.3%, Table V) and its corresponding amide **3iii** (45.5%, Table V). Within the amides, **3ii** (53.7%) was found to be the most potent.

Since most of the non-steroidal anti-inflammatory agents possess analgesic properties (non-opioid),[14] antinociceptive activity was examined in an attempt to elucidate the underlying mechanism of action. The writhing test was used for this assay because it is considered to be more specific for this type of antinociceptive activity[15,16] (Table IV, Figure 1). From the tested compounds, it was found that only compound **3vi** inhibited the acetic acid-induced writhing significantly (56.5% Table IV). Compound **3iii** was found to be inactive whereas compounds **3ii** and **3iv** showed low inhibition (26.2% and 25.7% Table IV). It seems that amide **3v** is significantly more potent than the corresponding acid **3iv**.

Perusal of Table V shows that anti-inflammatory activity and antinociception do not proceed in parallel. This however is not unexpected, since the case of aspirin and paracetamol is similar.[11,17]

All compounds were tested for their interaction with the stable free radical DPPH (Table V). This interaction indicates their radical scavenging ability in an iron-free system. Compounds **3ii** and **3vi** showed the highest interactions (81.5–96.7%). This interaction was time- and concentration-dependent. Compounds **3i** and **3ii** at 0.1 mM did not show any interaction during the experimental period (20–60 min) but, for these compounds the interaction seemed to be increased by an increase in concentration. Compounds **3iv** and **3v** were found to be inactive. In general, this interaction expresses the reducing activity of the tested compounds and indicates their ability to scavenge free radicals.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflammed site, and this is connected to other oxidizing species such as the hydroxyl radical (HO·) Hydroxyl radicals are produced by reactions that depend on transition metals, particularly iron.[18] Thus, the superoxide anion radical and HO· scavenging abilities of these coumarins were tested.[§] The superoxide anion radical was measured by the reduction of NBT to formazan. The assay was also adapted to assess the ability of antioxidants to react with $O_2^{\cdot-}$. However, none of the amides tested at a final concentration of 1 mM, showed any significant ability to scavenge $O_2^{\cdot-}$ (data not shown).

The competition of compounds with DMSO for HO· generated by the Fe^{3+}/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. In these experiments (Table VI) compound **3iv** did not show any inhibition, whereas compounds **3ii**, **3iii**, **3v**, **3vi** (0.1 mM) markedly inhibited (76.1–94.9%) the oxidation of DMSO (33 mM). Compound **3i** was found to have low potency (38.3%). The order of decreasing HO· scavenging activity was **3vi** > **3iii** > **3ii** > **3v** > **3i**.

Inhibitory activities were measured against soybean lipoxygenase, *in vitro*. Except for compounds **3ii** and **3v** (36.8 and 45.5%, Table VI) no inhibition was observed on soybean lipoxygenase (LOX) under our experimental conditions. Since iron is present in the active site of LOXs, several LOX-inhibitors are excellent ligands for Fe^{3+}. In our case we were unable to synthesize an iron chelate using the examined compounds as a ligand. Thus it was concluded that they might not produce LOX inhibition as iron chelators.

Concerning the structural feature of the active amides our data indicates that the -$NHCH_2CH_2NH_2$ group is correlated with higher antioxidant activity. In terms of anti-inflammatory activity, the acids seemed to be more active compared to the corresponding amides. The 1-napthalinyl- acid was found to be the most potent compound followed by the 2-thienyl derivative. The role of the napthalinyl group in the design and synthesis of potent and selective anti-inflammatory agents has been already shown e.g. naproxen.

Lipophilicity is an important physicochemical parameter for the absorption of biologically active compounds. Antioxidants of hydrophilic or hydrophobic character are both needed to act as radical scavengers in the aqueous phase or as chain—breaking antioxidants in biological membranes. In our case lipophilicity does not seem to affect the biological activities of the tested compounds.

Regression analysis[19] was performed to discover whether any correlation existed between anti-inflammatory activity and several physicochemical parameters (lipophilicity, steric and electronic variables). Unfortunately the number of derivatives was unsufficient to calculate a combination of all the effects. Using an Indicator variable e.g. I_{amide} (assigning 1 for the presence of an amidic group) gave $r = 0.766$.

A poor relationship exists ($r = 0.711$) between anti-inflammatory activity CPE% and % interaction with DPPH.

Acknowledgements

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