Heterocyclic Aryl(Phenyl)Acetic Acid and Aryl Acetohydroxamic Acids as Antiinflammatory –Antioxidant Agents and Inhibitors of Lipoxygenase and Serine Proteases

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Abstract: Taking into account that compounds bearing a thiazolyl, pyridyl and indolyl, moieties possess a wide spectrum of biological activities which is related to their capacity to transfer electrons and to scavenge reactive oxygen species (ROS), we synthesized some new heterocyclic aryl acetic acids and the corresponding acetohydroxamic acids and we explored their ability to inhibit soybean lipoxygenase, to present antioxidant and anti-inflammatory activities as well as to present serine proteases inhibition. The compounds were found to strongly inhibit lipid peroxidation. and to decrease *in vivo* the carrageenin induced rat paw edema (28-56%). Compound **1i** possesses the highest decrease 56%, *in vivo*. The tested derivatives presented interesting inhibitory activity on soybean lipoxygenase. Compound **5ii** showed significant *in vitro* inhibition on trypsin (IC_{50} 87μM). The results are discussed in terms of structural and physicochemical characteristics of the compounds.

The structures of the synthesized compounds were confirmed by spectral and elemental analysis. Their lipophilicity is experimentally determined by RPTLC method.

Key Words: Antioxidant agents, anti-inflammatory agents, lipoxygenase inhibitors, serine protease inhibitors, heterocyclic aryl -acetic, hydroxamic acids.

INTRODUCTION

Lipoxygenases (LOs) and serine proteases are implicated in the pathophysiology of inflammatory diseases, host defense reactions and play an important role in the propagation of the disease states, exacerbating the local events and ultimately leading to tissue damage.

Lipoxygenases are monomeric proteins that contain a "non–heme" iron per molecule in the active site as high–spin Fe(II) in the native state, and high–spin Fe(III) in the activated state [1-3]. They catalyze the incorporation of dioxygen into 1,4-*cis*,*cis*-pentadiene containing fatty acids (e.g., linoleic and arachidonic acids) to form hydroperoxides [4-6]. The essential iron atom is in the inactive ferrous oxidation state, as isolated, and is activated by 1 equiv of hydroperoxide product ((9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (HPOD)), which oxidizes the iron to the ferric state. Lipoxygenase plays an essential role in the biosynthesis of the leukotrienes. Leukotrienes, as lipoxygenase metabolites of arachidonic acid (AA), have been implicated as mediators in the pathophysiology of inflammatory diseases and host defense reactions.

Hydroxamic acids are well known to form strong complexes with a variety of transition metals. This property has been exploited in the use of hydroxamates as inhibitors of several metalloenzymes. Recently we have reported [7] a series of arylacetic and arylhydroxamic acids, having potent inhibitory activity against soybean lipoxygenase and possessing anti-oxidant and antiiflammatory activities.

In an attempt to expand our research we synthesized some heterocyclic derivatives carrying the same acetic functionalities for a further pharmacochemical study. Indomethacin [8] and tenidap [9] are well known NSAIDs, and have been shown to exert strong antiinflammatory effects. Indole-, containing molecules have been reported to possess a wide variety of biological properties viz., antiinflammatory [10-12], anti-convulsant [13], cardiovascular [14] and antibacterial [15]. Furthermore, substitution of the indole- moiety at 3-position markedly influenced the anti-inflammatory activity [16].

Thiazolyl derivatives are known to possess anti inflammatory as well as antipyretic activities [17]. Meloxicam, for example is a new NSAID with a thiazolyl group.

Coumarins have been shown to possess unique antiedema and anti-inflammatory activities [18, 19] and have been recognized as inhibitors of the lipoxygenase and cyclooxygenase pathways of arachidonate metabolism, [20-22] as well as of the neutrophile dependent superoxide anion generation [23]. 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 [24] and to influence processes involving free radical-mediated injury, as can some plant phenolics and flavonoids [25]. Taking into account all the above observations we designed and synthesised newer heterocyclic derivatives in the hope of obtaining agents with improved biological activities.

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CHEMISTRY

Synthetic Methodology

The synthesis of the arylacetic acids was accomplished according to the Knoevenangel condensation as indicated in Scheme (**1**). Since an E2 reaction was followed, the outcome of this appears to be controlled by the relative thermodynamic stability of the products, the most stable trans double bond being formed preferentially [26]. The arylacetic acids of series I [**1i**, **2**, **3**, **4** and **5i**] were obtained by condensation of a suitable aldehyde with phenylacetic acid and acetic anhydride in the presence of triethylamine. The arylacetic acids are converted to the corresponding hydroxamates [**1ii**, **5ii**] with hydroxylamine hydrochloride in the presence of CH_3COONa. Reactions were monitored by thin layer chromatography.

All the reactions proceeded smoothly and in general, in good yields. The structures of the synthesized compounds given in Table **1** were confirmed by IR, ^{1}H-NMR, ^{13}C-NMR, MS and elemental analysis. All the carboxylic acids present the characteristic absorption bands in the IR (nujol) and the corresponding hydroxamates [cm^{-1} 3200 (N-H, O-H), 1720 (C=O), 1625 (C=C)]. Coordination of the aromatic and CH= protons was observed in the ^{1}H-NMR spectra and the exact number of protons was given by intergration. The results are consistent with the proposed structures and are in agreement with previous findings [7].

Characteristic fragments, relative intensities (%) from mass spectra are given. In the MS spectra the existence of a daughter ion is assigned by the suggested fragmentanional pattern which is in agreement with the findings from pertinent studies [7].

Physicochemical Studies

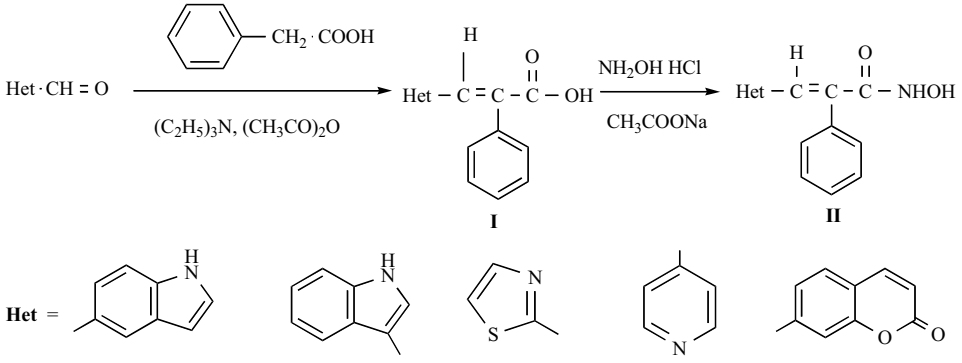
Since lipophilicity is a significant physicochemical property determining the binding and the ADME –Toxicological properties of a molecule, we determined it experimentally by the RPTLC method as R_M values and to compare them with the corresponding theoretically calculated clog *P* values in n-octanol-buffer [26]. RPTLC is considered to be a reliable, fast and convenient method for expressing lipophilicity [27].

However the results shown in Table **1** indicated no relationship among R_M values and clog P. We could attribute this to the different nature of the hydrophilic and lipophilic phases used in the two systems and to the presence of the hydroxamic/carboxylic group, which could strongly influence the absorption/desorption process in RPTLC.

DISCUSSION

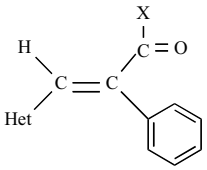
In this paper, we describe the synthesis of some novel arylacetic and arylacetohydroxamic acids and their biological evaluation as inhibitors of the enzyme lipoxygenase and protectants against inflammation and radical attack. Furthermore we evaluated also their acute toxicity.

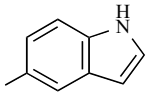
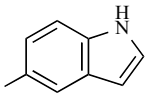
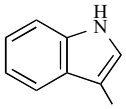
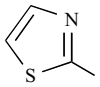
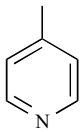
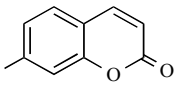
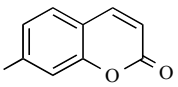
In acute toxicity experiments, *in vivo* the tested compounds do not present toxic effects in doses up to 0.3 mmoles/kg body weight of Fisher 344 rats. The *in vivo* anti-inflammatory effects of the tested compounds were assessed by using the functional model of carrageenin-induced rat paw edema and presented in Table **2**, as percentage of weight increase at the injected right hind paw in comparison to the untreated left hind paw (carrageenin paw edema, CPE % values). The differences observed between the CPE % values of the arylacetic acids and the corresponding hydroxamic acids are not significant (**1i ≈ 1ii**, **5i ≈ 5ii**). Carrageenin- induced edema is a non-specific inflammation resulting from a complex of diverse mediators [28]. The development of the edema induced by carrageenin in the hind paw of rats has been described as a biphasic event. Since 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 [29]. The first phase of the inflammatory response is mediated by histamine and serotonin and the second is mediated by prostaglandins. 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 [30]. As shown in Table **2**, the majority of the investigated compounds induced protection against carrageenin–induced paw edema. The protection ranged up to 56 % while the reference drug, indomethacin, induced 47 % protection at an equivalent molar concentration. Arylacetic acid **1i** is the most potent (56 %) whereas aryl (phenyl)acetic acid **4** presents the lowest activity (28%). The nature of the Het substituent seems to be significant for higher inhibition values eg. **1i > 2>** 5**i.** Indolyl derivatives especially present higher protection values. The



Scheme (**1**): Synthesis of arylacetic and acetohydroxamic acids.

Table 1. Physicochemical Data and Reaction Yields of Aryl-(phenyl) Acetic and Aryl-acetohydroxamic Acids 1-5



No.	Het	X	Formula*	clog *P***	R_M# (±SD)	mp ^{0}C	yield%
1i		-OH	$C_{17}H_{13}N\,O_2$	3.58	-0.610 (±0.022)	195-7	27
1ii		-NHOH	$C_{17}H_{14}\,N_2O_2$	2.08	nd	232-5	59
2		-OH	$C_{17}H_{13}NO_2$	3.58	-0.534 (±0.019)	117-9	71
3		OH	$C_{12}H_9NO_2S$	1.93	-0.146 (±0.00)	277-8	36
4		-OH	$C_{14}H_{11}NO_2$	2.09	-0.615 (±0.023)	193-6	28
5i		-OH	$C_{18}H_{12}O_4$	2.86	-0.532 (±0.028)	81-3	37
5ii		-NHOH	$C_{18}H_{13}NO_4$	1.36	nd	54-6	45

[a] aryl (phenyl)acetic acids**:** CH_3OH: H_2O: CH_3COOH, (77: 23: 0.1) ; *Elemental analyses for molecular formula (± 0.4%), **Theoretically calculated clog *P* values; #R_M values are the average of at least 10 measurements; nd, not determined.

5-indolyl derivatives (**1i**) seems to be more potent than the corresponding 3-substituted (**2**). No role for lipophilicity was found.

Perusal of LO IC_{50} values shows that compound **4** is the most active, within the examined set followed by compound **5i** (Table **2**). For compounds **1i** and **1ii** their % inhibition values at 0.1mM, are indicated in Table **2**. Compound **1ii** presents low activity in several concentrations whereas compound **1i** shows inhibition values in the region of 60% even in lower concentrations. Thus, we were unable to determine their IC_{50} values under our experimental conditions.

In this investigation all compounds were studied in order to gain insight their LO-inhibition. Most of the recognised LO inhibitors are antioxidants or free radical scavengers [31], since lipoxygenation occurs *via* a carbon centered radical. Some studies suggest a relationship between LO inhibition and the ability of the inhibitors to reduce the Fe^{3+} at the active site to the catalytically inactive Fe^{2+} [32, 33]. LOs 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 in the activated state Fe^{3+}. Several LO inhibitors are excellent ligands of the catalytically crucial Fe^{3+}. From our previous data we may hypothesize concluded, that compounds regarded in this paper could act as iron chelators and have the appropriate structural features for an sufficient binding at the catalytic site of the enzyme [34a,b].

Table 2. Inhibition % of Induced Carrageenin Rat Paw Edema (CPE %); *In Vitro* Inhibition of Soybean Lipoxygenase (LO) (IC_{50}) or % Inhibition of LO at 0.1mM; *In Vitro* Inhibition of Trypsin (IC_{50}) ; *In Vitro* Inhibition of Thrombin (TH %)

Compound	CPE %	LO IC_{50} (μM) % (0.1 mM)	IC_{50} μM	TH% 0.1mM
1i	56*	63 %		
1ii	54*	22 %		
2	42*	850		
4	28**	300	500	
5i	32**	350		
5ii	30**	530	87	
indomethacin	47*			
CA		600		
NDGA		83.7% (0.1mM), 94.7% (1 mM)		
Salicylic SODIUM			100	
inogatran				100

[a]Each value represents the mean of two independent experiments with 5 animals in each group, statistical studies were done with student's T-test, * $p<0.05$, ** $p<0.01$; nd not determined; Nordihydroguaiaretic acid -NDGA.

Although lipophilicity is referred to [35, 36a,b, 37] as an important physicochemical property for LO inhibitors, all our tested derivatives do not follow this rule with the exception of compound **1i**. Compounds **1i** and **2** show the highest clog *P* value 3.58 but they show adequate different potency.

Nordihydroguaiaretic acid, a potent lipoxygenase inhibitor, albeit not an antiinflammatory drug, presents 83.7% (0.1mM) and 94.7% (1 mM) inhibition of LO under the same experimental conditions. Thus, lipoxygenase inhibition may, at least partially, contribute to the antiinflammatory activity of this compound. Since there are not strict structural requirements for lipoxygenase inhibition and a large number of chemicals inhibit lipoxygenase activity, several mechanisms of action have been proposed. However, there is no universally accepted approach to evaluate the relative potency of different substances to cause lipoxygenase inhibition. We could only suggest that the lipophilic character of compounds may be involved in lipoxygenase inhibition, possibly influencing their interaction with the active site.

The reducing abilities of the examined compounds were determined by the use of the stable radical DPPH at 0.1mM and 1 mM after 20 and 60 min (Table **3**). This interaction indicates their radical scavenging ability in an iron-free system and expresses the reducing activity (RA) of compounds. Compounds **4**, **5i** and **5ii** present very low reducing activity (RA) at 0.1mM. Thus, we tried to test them in higher concentration (1mM). Under the new experimental conditions the reducing activity was increased. The RA are ranged from 9-82.3%. Both 5-indolyl derivatives interact satisfactorily. For compounds **1ii**, **2**, **4** and **5i** the interaction was found to be increased with the time (from 20 min to 60 min).

Finally we examined the inhibitory activity of our compounds on lipid peroxidation. It is well known that mixing heme proteins with H_2O_2 generates powerfully oxidizing activated heme species and radicals on amino-acids side chains that can cause lipid peroxidation. As a model of such reactions we then used the peroxidation of arachidonic acid by a mixture of heme and H_2O_2. The tested compounds highly inhibit the lipid peroxidation (Table **3**). The inhibition was concentration dependent for compounds **1i** and **1ii**. It seems that the 3-indolyl-acetic acid is more potent than the corresponding 5-indolyl. Lipophilicity does not affect inhibition.

As the last assay considering the role played by proteases in the early stage of inflammatory process we evaluated the ability of a limited number of compounds to inhibit these enzymes. Compound **4** the most potent LO- inhibitor and **5ii** the coumarinyl hydroxamic acid were tested for their inhibition. The combination of a coumarin ring with an hydroxamic group characterizing compound **5ii**, led to high inhibitory activity (Table **2**). Thus we tested compound **5ii** also against thrombin, another enzyme of the serine proteases family which is implicated in the thrombosis cascade. The inhibition seems to be quite low at 0.1mM (25%).

Since most of the biological assays are carried out at pH 7.40, all the tested carboxylic acids should be fully ionised whereas a partial ionisation may be expected for the hydroxamic acids. The non –protonated congeners will pass easily through membranes and will reach the site of action. Furthermore, at the inflamed tissue the pH is about 6-6.5, and from this point of view, derivatives with low pKa values will be more active. Thus, the ionization constants of these compounds might play a significant role. R_M values and ClogP values (both determined for unionised species) do not represent the lipophilicity of the biological form of carboxylic acid at pH 7.40.

Table 3. Interaction with DPPH (RA %)[a]; % Inhibition of Heme Dependent Lipid Peroxidation (LP %)

Compound	RA % 0.1mM		RA % 1mM		LP %	
	20min	60min	20min	60min	0.1mM	1mM
1i	82	82.3	nt	nt	no	29
1ii	63	77	nt	nt	no	43
2	34	31	nt	nt	92	97
4	12	21	39	45	92	95.5
5i	14	18	46	58	81	97
5ii	9	9	65	65	58	98
NDGA	81	82.6	93	95.6	no	26.1
CA					5.5	21.9

NDGA nordihydroguaeretic acid, CA caffeic acid; no: no results under the reported experimental conditions; nt: not tested.

CONCLUSION

Our results pointed out that indolyl derivatives highly inhibit carrageenin –induced paw edema. These compounds may prove useful for treating a variety of inflammatory diseases and may lead to the development of new drugs. In some cases, the tested acids proved to be more potent than the reference drug indomethacin at an equivalent molar dose.

Compound **5ii** will aim the design of compounds that would acquire serine proteases inhibition and lipid peroxidation and to be able to prevent or restore a number of pathological changes implicated within.

For compounds **4**, **5i** and **5ii** the reducing activities RA are in agreement with these on lipid peroxidation which in this case is due to the known reductive properties that aryl (phenyl) acetic acids and acetohydroxamic acids [7] convey.

EXPERIMENTAL SECTION

Materials

All the chemicals used were of analytical grade and commercially available by Merck. 1,1-diphenyl-2-picrylhydrazyl (DPPH), nordihydroguairetic acid (NDGA) are purchased from Aldrich Chemical Co. Milwaukee, WI, (USA). Soybean Lipoxygenase, linoleic acid sodium salt Arachidonic Acid (AA), NADH, porcine heme and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO, USA) and carrageenin, type K, was kindly offered by MEVGAL. For the *in vivo* experiments, male and female Fischer-344 rats (180-240 g) were used. Thrombin 50 NIH-E/mg, Trypsine and albumin come from, Merck KgaA, Substrate: tosyl-Gly-Pro-Arg-pNA from Sigma.

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, USA). UV-Vis spectra were recorded 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 300MHz 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 maintaine-d at 70 eV. Elemental analyses gave acceptable values for C, H and N and were carried out on 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.

1. Synthesis of Phenyl-Substituted Aryl-Acetic Acids I

The reaction was performed according to the literature [7, 34a] upon slight modifications. The compounds reported here were prepared as illustrated in Scheme **1**. The arylacetic acids were synthesized by a Knoevenangel condensation of the suitable aldehyde (0.015 mol) with phenylacetic acid (0.015 mol) and acetic acid anhydride (10 mL) in the presence of triethylamine (5 mL). The mixture was refluxed for 5 hours. The solution was poured into 2 N HCl, then on ice and the precipitate so formed was collected by filtration and recrystallized from 50% aqueous ethanol in the yields indicated in Table **1**.

1i. 3-(1H-indol-5-yl)-2-phenylacrylic acid

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 7.03-7.49 (m,9H), 8.05-8.20 (br, 2H), 10.08 (s, 1H), 11.3(s, 1H) ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 108, 109.2, 110, 112, 117,128.1, 128.8, 129.8, 130.3, 130.2, 131. 3, 132, 134,135, 141.5, 142.7, 173 MS m/e (%): 263 (100), 186 (8.6), 218 (95), 77(26).

2 3-(1H-indol-3-yl)-2-Phenylacrylic Acid

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 6.37(s, 1H), 7.33-7.73 (m, 10H), 8.2(s, 1H), 8.37 (d, 1H) ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 116, 117, 118, 120, 122,, 125.9, 126.9, 127.9, 128.4, 128.6, 129.5, 131, 132, 135, 139, 146, 168 MS m/e (%): 263 (31.7), 218 (100), 186 (7.5), 77 (9.3).

3. 2-phenyl-3-(thiazol-2yl)Acrylic Acid

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 7.14-7.33 (m, 6H), 7.64-8.1 (br, 2H), 10.8 (s, 1H) ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 119, 126, 126.5, 127.8, 128.2, 128.4, 133, 136, 139, 144, 167, 170 MS m/e (%): 263 (25.8), 219 (100), 218 (40), 77(8.1).

4 2-phenyl-3 pyridin-4-yl-Acrylic Acid

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 7.14(br, 1H), 7.21-7.61 (m, 7H), 8.69-8.71 (br, 2H), 11 (s, 1H) ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 120.7, 124, 126, 126.4, 128.3, 128.6, 128.8, 133, 141, 144, 145, 150, 151, 167 MS m/e (%): 225 (36.9), 180 (44), 103(14), 77 (59.4), 45(24).

5i. 3-(2-oxo-2H-chromen-7-yl)-2-Phenylacrylic Acid

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 6.34-6.37 (br, 1H), 7.14-7.37(m, 9H), 7.64-7.67 (br, 1H), 12.3 (s, 1H) ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 115.4, 117.1, 119,122,124, 125.5, 127.4, 128.3, 128.5, 129, 132, 133,137.6, 137, 143, 143.9, 157, 165.8.

2. General Procedure for the Synthesis of Aryl-Hydroxamic Acids II

Hydroxamic acids were synthesized from the corresponding carboxylic acids [7, 34] dissolved in ethanol 95^o. To this solution was added an equimolar solution of hydroxylamine hydrochloride and CH_3COONa. The mixture was refluxed with stirring and monitored by TLC. After cooling in room temperature, the mixture was poured into ice. The residue was collected by filtration and recrystallized from C_2H_5OH 95^o /H_2O mixture.

1ii. 3-(1H-indol-5-yl)-2-phenylacrylamide

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 6.76-7.55 (br, 10H), 7.94 (s, 1H), 8.06-8.26 (m, 3H) ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 106, 112, 117, 118, 120, 125, 128, 130, 131, 132, 134, 136, 138, 142, 143, 144, 171 MS m/e (%): 247 (13), 219 (13), 77(12).

5ii. 2-N-hydroxy-3- (2-oxo-2H-chromen-7-yl)-2-phenylacrylamide

^{1}H-NMR (DMSO-d_6, $CDCl_3$): 6.35 (d, 1H), 7.08-7.37 (m, 10H), 7.66-7.68 (br, 1H), 12.3 (s, 1H).

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. The mobile phase was a methanol/water mixture (77/23, v/v) containing 0.1 acetic acid for the arylacetic acids. The plates were developed in closed chromatography tanks saturated with the mobile phase at 24^{o}C. Spots were detected under UV light or by iodine vapours. R_M values were determined from the corresponding R_f values (from ten individual measurements) using the equation $R_M = \log [(1/R_f) -1]$ [38].

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

In Vivo Experiments

Inhibition of the Carrageenin-Induced Edema [7]

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 carried out in accordance with recognised guidelines on animal experimentation.

The tested compounds 0.01 mmol/kg body weight, were suspended in water, with few drops of Tween 80 and ground in a mortar before use and were given intraperitoneally simultaneously with the carrageenin injection. The rats were euthanized 3.5 h after carrageenin injection. The difference between the 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 in 0.01 mmol/kg (47%). Values CPE % are the mean from two different experiments with a standard error of the mean less than 10 % [7].

In Vitro Experiments

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

Determination of the Reducing Activity of the Stable Radical 1,1-diphenyl-picrylhydrazyl (DPPH) [7]

To a solution of DPPH 0.1 mM in absolute ethanol an equal volume of the compounds dissolved in ethanol was added. As control solution ethanol was used. The concentrations of the solutions of the compounds were 0.1 and 1 mM. After 20 and 60 min at room temperature the absorbance at 517nm was recorded.

Soybean Lipoxygenase Inhibition Study In Vitro [7]

In vitro study was evaluated as reported previously. The tested compounds dissolved in ethanol were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of enzyme solution (1/9 x 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.

Heme Protein-Dependent Lipid Degradation [7]

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

In Vitro Inhibition of Trypsin Induced Proteolysis [39]

0.2 mL of 100μg/mL trypsin was added to 1.77 mL of phosphate buffer (0.1M) pH 7.6 including the tested compounds (30 μL in DMSO) and preincubated at 35° C for 30 min and then 1 ml of 0.1 g/100ml albumin (in phosphate buffer)was added and incubated at 35° C for 20 min. After the incubation 3 ml 5% trichloroacetic acid was added to the incubated solution and allowed to set to room temperature for 1 h. Then the solution was filtered by filter paper and the absorption of the filtered solution was measured at 280 nm.

In vitro Inhibition of Thrombin

[40] As a substrate tosyl-Gly-Pro-Arg-pNA was used at 1mM final concentration. Compounds were dissolved at a final concentration of 0.1mM in a Tris-buffer (0.05 M Tris, 0.154 M NaCl, ethanol 5%, pH=8.0). Three minutes after the addition of bovine thrombin (2.5 unit /mg), the reaction was ended by adding 1ml acetic acid 50%. The absorption of the released p-nitro-aniline was measured at 405 nm.

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