Antioxidant and Anti-Inflammatory Activity of Aryl-Acetic and Hydroxamic Acids as Novel Lipoxygenase Inhibitors

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Abstract: Lipoxygenase plays an essential role in the biosynthesis of the leukotrienes. Leukotrienes, as LO metabolites of arachidonic acid (AA), have been implicated as mediators in the pathophysiology of inflammatory diseases, host defense reactions and to play important role in the propagation of the diseases states, exacerbating the local events and ultimately leading to tissue damage.

Simple stable molecules containing the hydroxamic acid functionality have been shown to inhibit 5-lipoxygenase. In fact, several hydroxamates are orally active inhibitors of the enzyme as determined by their ability to block the biosynthesis of leukotriene *in vivo*.

In order to establish the inhibitory utility of simple hydroxamates several ω-phenylalkyl and ω-naphthylalkyl hydroxamic acids were synthesized. In an attempt to expand and delineate these results we tried to synthesize some more for a further pharmacochemical study. Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolic activity and elimination, we tried to determine experimentally their lipophilicity from RPTLC method. The compounds are tested *in vitro* on: a) soybean lipoxygenase inhibition, b) interaction with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical, c) the HO˙ radical mediated oxidation of DMSO, d) inhibition of lipid peroxidation, e) scavenging of superoxide anion radicals f) interaction with glutathione and g) *in vivo* for the inhibition of carrageenin induced rat paw edema.

The compounds have shown important antioxidant activity, medium anti-inflammatory activity and potent inhibition of soybean lipoxygenase as a result of their physichochemical features.

Key Words: Antioxidant agents, anti-inflammatory agents, Lipoxygenase inhibitors, aryl-acetic, aryl-hydroxamic acids.

INTRODUCTION

Eicosanoids are a family of lipid mediators derived from the metabolism of arachidonic acid. The cascade involves two major pathways: a) the lipooxygenase is the first enzyme in a cascade which produces leukotrienes (LTs) while cycloxygenase (COX) initiates the cyclic pathway leading to prostanoids. These eicosanoids have a wide range of biological actions including potent effects on inflammation and immunity.

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 hydroperoxide products [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. The most widely accepted reaction mechanism for the hydroperoxidation is radical- based, where the fatty acid is oxidized by the ferric iron to form a fatty acid radical and a ferrous iron. This ferrous/substrate radical intermediate is then attacked regio- and stereo-specifically by dioxygen to form only the *S*-configured product [7-10].

Lipoxygenase plays an essential role in the biosynthesis of the leukotrienes. Leukotrienes, as LO metabolites of arachidonic acid (AA), have been implicated as mediators in the pathophysiology of inflammatory diseases and host defense reactions [11]. Although leukotrienes may not be involved in the initiating events, they appear to play important role in the propagation of the diseases states, exacerbating the local events and ultimately leading to tissue damage.

Since the elucidation of LO pathway there is an ongoing debate in drug development as to whether it would be better to inhibit the LO enzyme or to antagonize peptide or non-peptido leukotrienes receptors. Pharmacological evidences suggest that LO inhibitors may be superior to LT-receptor antagonists since LO inhibitors block the action of the whole spectrum of LO products [12-13] where as LT-antagonists would produce narrower effects. In addition LT-antagonists appear to prolong the half-lives of LTs by hindering their metabolism [14]. Numerous patents and publications on 5-LO inhibitors have been appeared.

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In regard to its characteristics and mechanism of action, different strategies have been developed to inhibit the LO pathway. Direct approaches, on the one hand, involve [15-17] antioxidants and free radical scavengers, since lipoxygenation occurs *via* a carbon centred radical. These compounds inhibit the formation of this radical or trap it once formed. Moreover many LO inhibitors also inhibit lipid peroxidation acting by scavenging chain-propagating peroxyl free radicals.

a. Iron ligand inhibitors, containing hydroxamic acid or N-hydroxyurea groups that chelate the active site iron, represent potent LO inhibitors. Hydroxamic acids have been widely described as iron-chelators inhibitors.

b. Non-redox competitive inhibitors, which compete with arachidonic acid to bind the enzyme active site and products analogues.

c. A number of LO inhibitory compounds combine the properties of being both chelators and reductants. Many companies over the last decades have reported on compounds that inhibit both lipoxygenase and other enzymes involved in inflammation as approaches to treat a variety of inflammatory diseases.

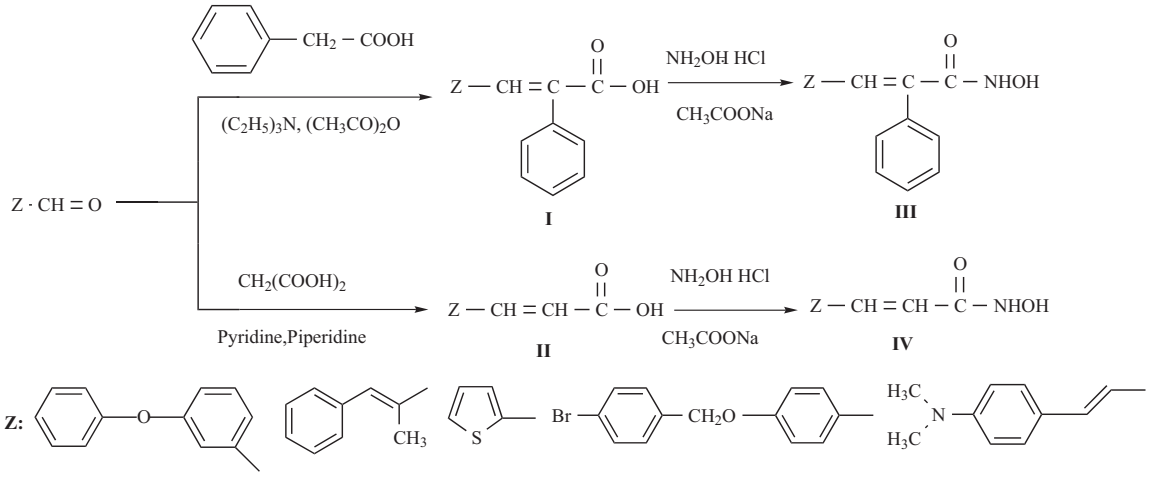
Hydroxamic acids are well known to form strong complexes with a variety of transition metals. This property has been exploited in the use of hydroxamates as inhibitors of several metalloenzymes. Since it is generally believed that lipoxygenase contains a catalytically important iron atom, this enzyme is a logical candidate for inhibition by hydroxamic acid containing molecules [18]. To assist in the selection of more potent hydroxamic inhibitors, a simple hypothesis about the nature of the enzyme - inhibitor binding was devised. According to this, the structures of compounds were matched to a proposed geometry of arachidonic acid when bound to the enzyme. Kerdesky *et al*. [19] showed that when a hydroxamic acid group is positioned at C_5 of arachidonic acid, an inhibitor with a 10-fold enhancement is obtained. This demonstrated that not only the presence but also the position of the hydroxamic acid moiety is important for the inhibition [19]. Jackson *et al*. [20] have developed hydroxamic acids as potent and selective inhibitors of human leukocyte LO, which demonstrate significant oral bioavailability in animals. In order to establish the inhibitory utility of simple hydroxamates several ω-phenylalkyl and ω-naphthylalkyl hydroxamic acids were synthesized by Summers *et al*. [21]. In an attempt to expand and delineate their results we tried to synthesize some more for a further pharmacochemical study. The suggested structural variations could affect both efficacy and their tolerability partly due to differences in their physicochemical properties, which determine their distribution in the body and their ability to pass through and to enter in the interior membranes [22-23]. The hydroxamic acid functionality was incorporated into a wide variety of molecules to produce potent inhibitors of LO. We tried to find out the features that influence the enzyme inhibitory potency, among them were hydrophobicity, aryl substitution and several modifications of the molecule. Recently we have reported a series of aryl-acetic and aryl-hydroxamic acids, having potent inhibitory activity against soybean lipoxygenase and possessing anti-oxidant and antiiflammatory activities [24].

In continuation to this effort a series of novel aryl-acetic and hydroxamic acids have been synthesized and biologically evaluated.

CHEMISTRY

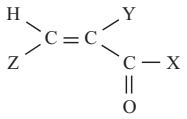
Synthetic Methodology

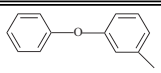
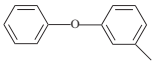
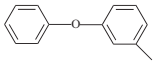
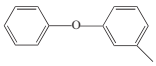
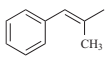
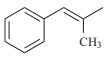
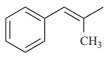
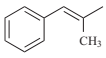
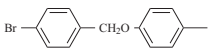
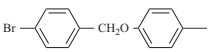
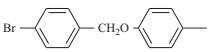
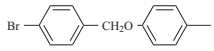
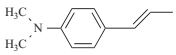
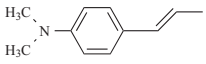
The synthesis of the aryl-acetic acids was accomplished according to the Knoevenangel condensation as indicated to Scheme (**1**). The aryl-acetic acids of series I [21, 24, 25] are obtained by the condensation of the suitable aldehyde with phenylacetic acid and acetic acid anhydride in the presence of triethylamine, while the aryl-acetic acids of series II are obtained by the condensation of the suitable aldehyde with malonic acid in the presence of pyridine and piperidine. Both series I and II are converted to the corresponding hydroxamates with hydroxylamine hydrochloride in the presence of CH_3COONa. Reactions were monitored by thin layer chromatography.



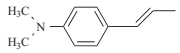
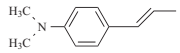
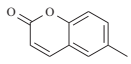
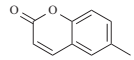
Scheme (1). Synthesis of aryl-acetic and hydroxamic acids.

Table 1. Synthesized Aryl-Acetic Acids and Hydroxamates. Lipophilicity Values: Experimentally Determined R_M and Theoretically Calculated Clog P Values



no	Z	Y	X	Formula*	Rf	clog *P***	R_M# (±SD)	mp ^{0}C	yield%
1i			-OH	$C_{21}H_{16}O_3$ [24]	0.89[a]	5.69	-0.27 (±0.01)[g]	142-144	56.0
1ii		H	-OH	$C_{15}H_{12}O_3$	0.34[b]	4.34	-0.41 (±0.03)[g]	107-109	56.0
1iii			-NHOH	$C_{21}H_{17}NO_3$ [24]	0.84[c]	4.18	-0.65 (±0.01)[h]	174-178	43.7
1iv		H	-NHOH	$C_{15}H_{13}NO_3$	0.46[b]	2.84	-0.56 (±0.02)[h]	62-64	55.9
2i			-OH	$C_{18}H_{16}O_2$ [24]	0.92[d]	4.44	-0.28 (±0.03)[g]	liquid b.p. 86	90.5
2ii		H	-OH	$C_{12}H_{12}O_2$	0.92[c]	3.09	0.81 (±0.02)[g]	liquid b.p. 198-200	81.7
2iii			-NHOH	$C_{18}H_{17}NO_2$ [24]	0.84[c]	2.94	-0.60 (±0.06)[h]	104-106	70.5
2iv		H	-NHOH	$C_{12}H_{13}NO_2$	0.93[c]	1.59	-0.57 (±0.03)[h]	115-117	38.8
3i			-OH	$C_{13}H_{10}O_2S$ [24]	0.77[d]	3.23	-0.52 (±0.02)[g]	165-171	46.6
3ii		H	-OH	$C_7H_6O_2S$ [27]	0.82[d]	1.89	-0.56 (±0.04)[g]	103-105	84.6
3iii			-NHOH	$C_{13}H_{11}NO_2S$ [24]	0.56[d]	1.73	-0.61 (±0.05)[h]	175-177	45.7
3iv		H	-NHOH	$C_7H_7NO_2S$	0.93[d]	0.39	-0.61 (±0.03)[h]	134-136	18
4i			-OH	$C_{22}H_{17}BrO_3$	0.78[e]	6.14	0.15 (±0.01)[g]	196-198	52.7
4ii		H	-OH	$C_{16}H_{13}BrO_3$	0.83[f]	4.79	-0.17 (±0.02)[g]	223-225	52.7
4iii			-NHOH	$C_{22}H_{18}BrNO_3$	0.74[e]	4.64	-0.70 (±0.06)[h]	164-166	41.0
4iv		H	-NHOH	$C_{16}H_{14}BrNO_3$	0.87[f]	3.29	-0.67 (±0.02)[h]	230-231	82.2
5i			-OH	$C_{19}H_{19}NO_2$	0.94[e]	4.74	0.86 (±0.02)[g]	119-121	36.5
5ii		H	-OH	$C_{13}H_{15}NO_2$	0.93[f]	3.22	-0.41 (±0.03)[g]	179-182	36.5

(Table 1. Contd....)

no	Z	Y	X	Formula*	Rf	clog *P***	R_M# (±SD)	mp ^{0}C	yield%
5iii			-NHOH	$C_{19}H_{20}N_2O_2$	0.90[c]	3.24	0.26 (±0.02) [h]	132-134	49.0
5iv		H	-NHOH	$C_{13}H_{16}N_2O_2$ [26]	0.85[f]	1.89	0.51 (±0.02) [h]	74-76	10
6i			-OH	$C_{18}H_{12}O_4$ [24]	0.22[d]	2.86	-0.62 (±0.06) [g]	188-190	36.0
6iii			-NHOH	$C_{18}H_{13}NO_4$ [24]	0.54[c]	1.36	-0.64 (±0.05) [h]	165-168	13.9

[a] CH_2Cl_2, [b] $CHCl_3$, [c] CH_3OH: $CH_3COOC_2H_5$ (1:2), [d] CH_2Cl_2: $CH_3COOC_2H_5$ (1:2), [e] $CHCl_3$: $CH_3COOC_2H_5$ (1:1), [f] $CHCl_3$:CH_3OH (9:1), [g] aryl-acetic acids: CH_3OH: H_2O: CH_3COOH, (77: 23: 0.1) ; [h] aryl-hydroxamic acids: CH_3OH: H_2O, (95: 5).

*Elemental analyses for molecular formula (± 0.4%), **Theoretically calculated clog *P* values; #R_M values are the average of at least 10 measurements.

Compounds **1i, 1iii, 2i, 2iii, 3i, 3iii, 4i, 4iii, 5i, 5iii, 6i** and **6iii** were available from our previous work [24] and they are included here for further biological evaluation in order to delineate structure activity relationships. The synthesis of compound **5iv** has been reported [26] *via* a different synthetic route. According to this method the corresponding aldehyde reacts with acetic acid ethyl ester in toluene at 40ºC for 6 days to give an ester which was converted into hydroxamic acid using 50% aqueous hydroxylamine and KOH [26]. For the same derivatives we followed our above described synthetic methodology. Compound **3ii** was synthesized by the reaction of the corresponding aldehyde with malonic acid in pyridine, piperidine and now a days is commercially available [27]. Compound **5ii** has been synthesized from the reaction of triethyl 4-phosphonocrotonate with the appropriate aldehyde. An intermediate ethyl ester was produced which was hydrolyzed to give the corresponding acid in a *trans, cis* form [28]. Following our synthetic procedure we have isolate the *trans, trans* form.

The corresponding reactions proceeded smoothly and in good yields (36-90.5 %), compound **5iv** and **6i** were the only exception (10 and 14 %). The structures of the synthesized compounds are given in Table **1** and they are confirmed by UV, IR, ^{1}H-NMR, ^{13}C-NMR, MS and elemental analysis. All the acids present the characteristic absorption in the IR (nujol) as well as the corresponding hydroxamates (cm^{-1} 3200 (N-H), 2970 (O-H), 2950, 1720 (C=O), 1625 (C=C), 1470, 1375). ^{1}H-NMR spectroscopy revealed that the olefinic bond in these compounds adopted the *E*-configuration (trans-derivatives).

Physicochemical Studies

Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolic activity and elimination, we tried to determine experimentally their lipophilicity from RPTLC method as R_M values and to compare them with the corresponding theoretically calculated clog *P* values in n-octanol-buffer [29]. This is considered to be a reliable, fast and convenient method for expressing lipophilicity [30]. Apart from the important role of lipophilicity for the kinetics of biologically active compounds, 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.

From our results (Tables **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 the hydroxamic/carboxylic group, which could disturb the absorption/desorption process.

RESULTS AND DISCUSSION

DISCUSSION

In this paper, we describe the synthesis of some novel aryl-acetic and aryl-hydroxamic acids that were expected to inhibit the enzyme lipoxygenase and offer protection against inflammation and radical attack, by application of standard synthetic methods summarized in Scheme **(1)**.

In acute toxicity experiments, the *in vivo* tested compounds do not present toxic effects in doses up to 0.5 mmoles/kg body weight. The *in vivo* anti-inflammatory effects of the tested compounds were assessed by using the functional model of carrageenin-induced rat paw edema and are presented in Table **2**, as percentage of weight increase at the right hind paw in comparison to the uninjected left hind paw. Carrageenin- induced edema is a non-specific inflammation resulting from a complex of diverse mediators [31]. 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 [32] 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 [33]. As shown in Table **2**, the majority of the investigated compounds induced protection against carrageenin-induced paw edema. The protection ranged up to 70.9 % while the reference drug, indomethacin, induced 47 % protection at an equivalent concentration. The aryl-acetic

Table 2. Inhibition % of Induced Carrageenin Rat Paw Edema (CPE %); In Vitro Inhibition of Soybean Lipoxygenase (LOX) (IC_{50})

compd.	CPE (%)[a] 0.01mM	LOX IC_{50} (μM)
1i	70.9*	no
1ii	41.6*	66
1iii	44.1**	68
1iv	40.3**	78
2i	44.1**	no
2ii	49.0**	95
2iii	34.4*	no
2iv	47.0**	94
3i	65.5*	242
3ii	42.7**	98
3iii	30.5*	375
3iv	36.4*	95
4i	62.3*	81
4ii	44.4*	89
4iii	40.8**	80
4iv	44.6**	100
5i	57.2**	96
5ii	61.8*	79
5iii	45.8*	92
5iv	no	62
6i	37.9*	75
6iii	53.4*	78
Caffeic acid		600
Indomethacin	47*	

[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, * $p<0.01$, ** $p<0.05$; no: no action under the experimental conditions

acids proved to be more potent compared to the corresponding hydroxamic-acids. Aryl-acetic acids **1i**, **3i** and **4i** are the most potent (62.3-70.9 %) whereas aryl-hydroxamic acid **3iii** presents the lowest effect (30.5%) and compound 5iv no effect. The nature of Z substituent as well as the presence of the phenyl-ring and the carboxyl-group seem to be significant for higher inhibition values eg. **1i** > **1ii**, **3i** > **3ii**, **4i** > **4ii**. The ionization constants of these compounds might play a significant role. The three compounds possess high lipophilicity. No conclusion can be extracted for the hydroxamic acids with a phenyl-ring attached at the double bond in comparison to the corresponding without phenyl, with the exception of compounds **5iii** > **5iv**.

Regression analysis was performed to discover whether any correlation existed between anti-inflammatory activity and several physicochemical parameters (lipophilicity, steric and electronic variables). Unfortunately the confidence limits were poor.

Compounds were further evaluated for the inhibition of soybean lipoxygenase LO by the UV absorbance based enzyme assay [34]. While one may not extrapolate the quantitative results of this assay to the inhibition of mammalian 5-LO, it has been shown that inhibition of plant LO activity by NSAIDs is qualitatively similar to their inhibition of the rat mast cell LO and may be used as a simple qualitative screen for such activity.

Perusal of IC_{50} values shows that compound **5iv** is the most active, within the set followed by compounds **1ii** and **1iii** (Table **2**). In this investigation all compounds were studied in order to gain insight their LO-inhibition. The most of the LO inhibitors are antioxidants or free radical scavengers [35], 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+} [36, 37]. 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 ligants for Fe^{3+}. It has been demonstrated that their mechanism of action is presumably related to its coordination with a catalytically crucial Fe^{3+}. We tried to synthesize an iron chelate using the examined compounds as a ligand (data not shown). Thus, we concluded that our compounds could act as iron chelators and have the appropriate characteristics for the fitting at the binding site of the enzyme [21, 25].

Although lipophilicity is referred [38a,b, 39] as an important physicochemical property for LO inhibitors, all the above tested derivatives do not follow this concept with the exception of compounds **1ii**, **1iii**, **4ii** and **4iii**. Compound **4i** shows the highest clog *P* value 6.14 but it is not highly potent (81μM). The presence of a thienyl-group is correlated to higher activity as well as a p-substitution eg. compound **5i** > **2i** and **5ii** > **2ii**.

Compounds **1i, 2i** and **2iii** did not show inhibitory activity under the reported experimental conditions. The absence of the phenyl ring seems to improve the inhibitory activity. Preliminary QSAR studies on the IC_{50} values on soybean LO have shown that molar refractivity of Z substituents plays a significant role. I_{NHOH} is an indicator variable which takes the value 1 for the hydroxamic acids. The positive sign indicates that the hydroxamates are more potent than the corresponding aryl-acetic acids.

The negative sign with MR_Z indicates that fitting to a macromolecule of limited steric capacity is important.

$$\log 1/IC_{50}\text{-LO} = 0.075\ (\pm 0.041)\ \text{clog}\ P + 0.126\ (\pm 0.080)\ I_{NHOH} - 0.095\ (\pm 0.039)\ MR_Z + 0.234\ (\pm 0.095) \quad \textbf{(1)}$$

n = 16, r = 0.846, r^2 = 0.716, s = 0.048, $F_{3,12}$ = 10.142, α = 0.01

Three compounds were omitted from the derivation of the above equation. Eq. 1 is not sharp in terms of r^2. However it illustrates in terms of QSAR the most significant structural characteristics.

Taking alone for LO inhibition of the hydroxamates eq. 2 was derived. It seems that MR_{-Z} in a parabolic model better explains the LO inhibitory activity.

$\log 1/IC_{50}$-LO = -0.374 (± 0.107) MR_{-Z} + 0.035 (± 0.011) MR_{-Z}^2 + 1.057 (± 0.240)

n = 9, r = 0.975, r^2 = 0.951, s = 0.025, $F_{2,6}$ = 55.5, α = 0.01 **(2)**

MR_{-Z} optimum = 5.364 (± 0.388) from 5.070 to 5.847

An attempt to derive a correlation for the aryl-acetic acids did not lead to a significant equation.

COX-1 plays the role as a "housekeeping enzyme", for maintaining the lining of the stomach and in endothelial cells 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. Compound **1i**, which posses the highest *in vivo* activity against the CPE test and no LO inhibitory activity and compounds **2i** and **6i** were also examined against COX-1 at 1mM (Table **3**). Compound **1i** is potent (57.8 %), compound **6i** does not highly inhibit COX-1 (15.1 %) whereas compound **2i** is highly potent.

Table 3. In Vitro Inhibition of Cyclooxygenase-1 (COX-1 %)

compd.	COX-1 %, 1 mM
1i	57.8
2i	100
6i	15.1
Sc-560	57.8

The reducing abilities of the examined compounds were determined by the use of the stable radical DPPH at 0.05 and 0.1mmol/lt after 20 and 60 min (Table **4**). This interaction indicates their radical scavenging ability in an iron-free system and expresses the reducing activity of compounds [14]. The reducing abilities are ranged from 50.6-99.7 % for the acids and from 57.9- 98.5 % for the hydroxamates. Most of the compounds were found to interact with DPPH, but the order of this activity did not proceed in parallel with the time and the increase of concentration. 4-Bromo-(benzyloxy)-phenyl- derivatives (**4i, 4ii, 4iii, 4iv**), as well as the 4-Dimethylamino-phenyl- derivatives (**5i, 5ii, 5iii, 5iv**) present the higher interaction with DPPH.

Preliminary QSAR studies on the values of DPPH interaction (0.05mM, 60 min) have shown that the steric effects as molar refractivity of Z-substituents (MR_{-Z}) are significant.

Eq. 3 refers to the subgroup of hydroxamic acids.

DPPH % = 12.992 (± 9.162) I_{-PH} + 4.448 (± 3.601) MR_{-Z} + 65.400 (± 17.599) **(3)**

n = 9, r = 0.858, r^2 = 0.737, s = 5.383, $F_{2,6}$ = 8.40, α = 0.05

Indicator I_{-PH} assigns 1 for the presence of a phenyl ring (Y = $-C_6H_5$) and its positive sign indicates an increase when a phenyl-ring is present.

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, which depend on transition metals, particularly iron [36]. Hydroxy radicals are among the most reactive oxygen species and are considered to be responsible for some of the tissue damage occurring in inflammation. It has been claimed that hydroxyl radical scavengers could serve as protectors, thus increasing prostaglandin synthesis.

The competition of the synthesized compounds 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 only compounds **2i, 3i** and **5i** showed high inhibition at 0.001 mM, whereas all the others did not shown any inhibition (Table **4**). The tested compounds at 0.01 mM inhibited highly (71.1-96.7) the oxidation of DMSO. Compounds **1i, 3ii, 4ii, 5ii** did not show any inhibition. For compounds **2iv, 4i, 5iv** the inhibition was found to be increased as the concentration of the tested compounds was increased. In general the most of the compounds present high inhibition at 0.1mM.

Lipophilicity does not seem to affect competition. Antioxidants at hydrophilic or lipophilic character are both needed to act as radical scavengers in the aqueous phase or as chain-breaking antioxidants in biological membranes.

Preliminary QSAR studies on the values of DMSO competition have shown that steric properties eg. the molar volume (MgVol) and the molar refractivity of Z substituent (MR_{-Z}) play significant role as well as the presence of a phenyl group.

DMSO-0.1 = -0.162 (± 0.071) MgVol + 14.441 (± 5.643) I_{-PH} + 7.482 (± 3.160) MR_{-Z} + 96.601 (± 5.669) **(4)**

n = 18, r = 0.833, r^2 = 0.695, s = 2.535, $F_{3,14}$ = 10.613, α = 0.01

For the subgroup of hydroxamic acids:

DMSO-0.1 = 18.591 (± 8.350) MR_{-Z} - 1.997 (± 0.932) MR_{-Z}^2 + 53.473 (± 17.608) **(5)**

n = 10, r = 0.896, r^2 = 0.803, q^2 = 0.323, s = 2.369, $F_{2,7}$ = 14.23, α = 0.01

MR_{-Z} optimum = 4.655 (±0.392) from 4.327 to 5.110

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

Table 4. Interaction % with DPPH (RA %)[a]; Competition % with DMSO for Hydroxyl Radical ($HO^{\cdot}$ %)[b]

compd.	RA % 0.05mM		RA % 0.1mM		$HO^{\cdot}$ (%)		
	20min	60min	20min	60min	0.001mM	0.01mM	0.1mM
1i	50.6	93.9	60.3	60.5	no	no	97.1
1ii	72.4	66.2	72.2	94.8	no	no	90.2
1iii	75.1	98.1	87.2	80.9	no	94.6	96.6
1iv	93	94.9	86.7	92.6	no	no	96.4
2i	98.6	92.1	99.7	71.5	94.3	97.2	97.7
2ii	75.1	86.1	75.6	55.3	94.2	98.7	nd
2iii	82.8	92.1	57.9	79.7	no	91.4	96.3
2iv	91	75.4	74.9	97.9	no	72.6	95.3
3i	72.7	93.4	70.8	68.8	91.3	93.9	94.3
3ii	70.2	94	76.3	62.5	no	87.9	87.1
3iii	98.4	94.9	71	73.5	no	89.2	87.6
3iv	85.4	75.4	72.3	77.5	no	86.8	85.7
4i	77.6	77.1	85.4	97.3	no	71.1	91.6
4ii	74.7	72.9	81.8	93.5	no	91.2	90.5
4iii	91.8	86.8	87.4	98.5	no	90.7	94.1
4iv	94	96.4	75.3	85	no	94.9	85.6
5i	82	74.4	94	81.5	99.1	96.7	93.3
5ii	85	87.6	88.2	89.9	no	no	95.3
5iii	94	84	92.2	96.9	no	57.9	68.7
5iv	89.5	87.3	88.2	91.7	no	92.2	96.7
6i	76.1	81.6	66.4	91.5	no	96.2	96.7
6iii	72	98.5	63.1	96	no	95.1	95.5
BHT			31.3	60			
NDGA			81	82.6			
Caffeic acid			9.3	9.02			
Trolox					nt	73.4	88.2

no: no action under the experimental conditions; nt: not tested; nd: not determined

model of such reactions we used the peroxidation of arachidonic acid by a mixture of heme and H_2O_2.

The tested compounds did not highly inhibit the lipid peroxidation (4.5-59.6%). In some cases a small inhibition increase is observed by increasing their concentration e.g. compounds **1iii**, **2iv**, **4ii**, **4iii** and **4iv** (Table **5**). Lipophilicity does not affect inhibition.

Compounds **1ii, 1iii 1iv, 4i, 4iii, 5ii, 5iv, 6i** and **6iii** which present higher LO inhibition have been tested against the Fe^{3+}-iron stimulated peroxidation of lineloic acid. Compound **6iii** presents the higher inhibition (98.3 at 0.01mM) followed by compounds **4i, 4iii, 6i** (Table **6**).

Non enzymatic superoxide anion radicals were generated. The majority of the compounds present high scavenging activity. Lipophilicity does not seem to increase the scavenging activity. Compound **1iii** did not present biological response. It seems that as concentration increases decreases the inhibitory activity. Compounds **2i, 2iii, 3i, 5iv** and **6iii** present dissolution problems at higher concentrations (1mM) (Table **5**). Coumarin derivatives seem to strongly scavenge superoxide radicals.

Table 5. % Inhibition of Heme Dependent Lipid Peroxidation (LP %); % Superoxide Radical Scavenging Activity ($O_2^{\cdot-}$)

compd.	A % LP-0.1mM	A % LP-1mM	$O_2^{\cdot-}$ (%)0.1mM	$O_2^{\cdot-}$ (%)1mM
1i	no	32.1	no	no
1ii	no	37.8	67.9	72.3
1iii	19.5	44.2	3.2	52.2
1iv	no	30.1	77.7	70
2i	no	11.5	75.4	*
2ii	no	31.4	88.8	66.6
2iii	no	23.7	68.8	*
2iv	19.5	43.6	62.9	100
3i	no	0.60	52.1	*
3ii	no	29.5	82.1	95.7
3iii	no	no	65.6	65.2
3iv	no	30.8	63.5	87
4i	no	4.50	92.1	100
4ii	7.3	59.6	90	100
4iii	19.5	44.2	92.1	100
4iv	22	41.3	98.8	100
5i	no	no	92.5	100
5ii	no	no	69.2	100
5iii	no	no	82.8	100
5iv	no	no	95	*
6i	no	23.1	70	71
6iii	no	28.8	80.4	*
Caffeic acid	5.5	21.9		86.1
NDGA	no	26.1		

no: no action under the experimental conditions, * dissolution problems

A number of α,β-unsaturated ketones have demonstrated preferential reactivity towards thiols in contrast to amino and hydroxyl groups [40] and hence these compounds may be free from the problems of mutagenicity and carcinogenicity which are associated with a number of alkylating agents used in cancer chemotherapy [41]. Alkylation with a cellular thiol such as glutathione GSH may occur with the α,β-unsaturated acids, leading to the adducts A and we have studied this possibility.

Glutathione conjugation is an important pathway by which reactive electrophilic compounds are detoxified. It protects vital cellular constituents against chemical reactive species by virtue of its nucleophilic sulphydryl group and constitutes an *in vivo* antioxidant protective mechanism. The nucleophilic addition of GSH to electron-deficient carbon double bonds occurs mainly in compounds with α,β-unsaturated double bonds. In most instances the double bond is rendered electron deficient by resonance or conjugation with a carbonyl group. It should be emphasized that not all α,β-unsaturated compounds are conjugated with GSH (**1ii, 2i, 2ii, 3iv, 4iii, 4iv, 5i, 5iii** and **5iv**) .

For compounds **1i, 1ii, 1iii, 1iv, 2ii, 2iii, 2iv, 3iii, 4i, 4ii, 4iv, 6i** and **6iii** an alkylation may occur leading to the adducts A:

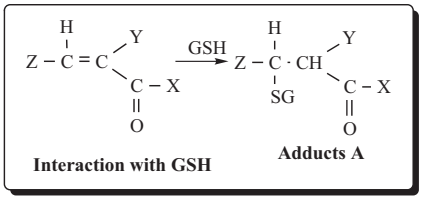
For compounds **2ii, 2iii, 3iii** and **4iv** the alkylation proceed, higher when the concentration of GSH is high (10GSH). Compound 2iii presents the higher alkylation. It seems that the stereochemistry of substituent Z affects the alkylation.

We tried to linearly correlate the expressions of anti-inflammatory, antioxidant, free radical scavenging activity and LO inhibition activity for all 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 non-linear regression analysis gave statistically non significant equations.

Table 6. Stability Studies and Incubation with Glutathione (GSH)

a/a	l_{max}	e_{max}
1i	270	1117
1i + 2GSH	270	660
1i + 10GSH	270	1002
1ii	270	1776
1ii + 2GSH	270	1791
1ii + 10GSH	270	1619
1iii	270	1247
1iii + 2GSH	270	922
1iii + 10GSH	270	1220
1iv	280	469
1iv + 2GSH	280	391
1iv + 10GSH	280	429
2i	290	580
2i + 2GSH	290	593
2i + 10GSH	290	547
2ii	280	503
2ii + 2GSH	280	513
2ii + 10GSH	280	full alkylation
2iii	290	128
2iii + 2GSH	290	full alkylation
2iii + 10GSH	290	full alkylation
2iv	270	1627
2iv + 2GSH	270	1568
2iv + 10GSH	270	1514
3i	300	1075
3i + 2GSH	300	1037
3i + 10GSH	300	1034
3ii	300	1134
3ii + 2GSH	300	1078
3ii + 10GSH	300	1235
3iii	300	1200
3iii + 2GSH	300	1053
3iii + 10GSH	300	983
3iv	300	1128
3iv + 2GSH	300	1092
3iv + 10GSH	300	1235
4i	290	830
4i + 2GSH	290	718
4i + 10GSH	290	734
4ii	280	1400
4ii + 2GSH	280	1047
4ii + 10GSH	280	1303
4iii	290	621
4iii + 2GSH	290	600
4iii + 10GSH	290	609
4iv	280	891
4iv + 2GSH	280	908
4iv + 10GSH	280	674
5i	290	64
5i + 2GSH	290	100
5i + 10GSH	290	90
5ii	250	712
5ii + 2GSH	250	647
5ii + 10GSH	250	no result
5iii	290	56
5iii + 2GSH	290	85
5iii + 10GSH	290	88
5iv	250	1009
5iv + 2GSH	250	1180
5iv + 10GSH	250	1213
6i	280	1607
6i + 2GSH	280	1339
6i + 10GSH	280	1313
6iii	280	3767
6iii + 2GSH	280	3379
6iii + 10GSH	280	3229



CONCLUSION

From our results it is obvious that aryl acetic acids highly inhibit carrageenin-induced paw edema compared to the corresponding hydroxamic acids. In many cases, the aryl

acetic acids proved to be more potent than the reference drug indomethacin at an equivalent concentration.

It seems that for compound **1i** (which does not inhibit LOX under our experimental conditions, but highly protects 70.9% from carrageenin paw edema), the possible mechanism of action is correlated with the COX inhibition.. Compound **2i** seems to be a COX-1 inhibitor whereas compound **6i** presents dual inhibitory activity (COX/LOX). The results from their conjugation with glutathione (GSH) indicate the possibility of alkylation. This is important since GSH- conjugation is a pathway by which reactive electrophile compounds are detoxified.

Table 7. Effects of the Tested Compounds on the Fe^{3+}-ion Stimulated Peroxidation of Linoleic Acid

a/a	0.01mM
1ii	no
1iii	no
1iv	no (78.5%, 0.1mM)
4i	84.3
4iii	IC_{50} 0.1mM
5ii	no
5iv	no (93.6%, 0.1mM)
6i	88.9
6iii	98.3

no: no results under the reported experimental conditions

In general, the antioxidant activity of the tested compounds is correlated with the *in vivo* anti-inflammatory activity.

The experimental results as well as the QSAR analysis indicate the features that influence activities and must be taken under consideration in our future research:

- The value of the molar refractivity e.g. the bulk of substituent Z is the most important group for LOX inhibitory activity. The optimum MR_Z value (5.364) taken from eq.2 will be very helpful in our design process.
- The presence of the phenyl group is the second structural feature which will be included in the new compounds

EXPERIMENTAL SECTION

Materials

All the chemicals used were of analytical grade and commercially available by Merck, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), nordihydroguairetic acid (NDGA) are purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). Soybean Lipoxygenase, linoleic acid sodium salt Arachidonic Acid (AA), NADH, Nitrotetrazolium Blue (NBT), porcine heme and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO, USA) 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 by Cayman. N-methylphenazonium-methyl sulfate was purchased by Fluka.

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 obtained on a Perkin-Elmer 554 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 ^1H 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 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.

1a. Synthesis of Phenyl-Substituted Aryl-Acetic Acids I [21, 24, 25]

The reaction was performed according to the literature [21, 24, 25] after modification. The compounds reported here were generally prepared as illustrated in Scheme **1**. The aryl-acetic 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) [5,6]. The mixture was refluxed for 5 hours. The solution was poured into 2 N HCl, then on ice and a precipitate was formed which was collected by filtration and recrystallized from 50 % aqueous ethanol. In case that no precipitate was formed after the ice dropping an extraction with 3x100 mL $CHCl_3$ was made and the organic phase was collected and dried over Mg_2SO_4.

1b. General Procedure for the Synthesis of Aryl-Acetic Acids II

The compounds reported here were generally prepared as illustrated in Scheme **1**. The aryl-acetic acids were synthesized by a Knoevenangel condensation of the suitable aldehyde. The malonic acid was dissolved in pyridine. Aldehyde and piperidine were added. The mixture was refluxed under water until the emission of CO_2 stops. Then the solution was poured into 2 N HCl, on ice and a precipitate was formed which was collected by filtration and recrystallized from water or from 3:1 water/ethanol. For

compounds that no precipitate was formed after the ice dropping, an extraction with 3 x 100 mL $CHCl_3$ or CH_2Cl_2 was made and the organic phase was collected and dried over Mg_2SO_4.

General Procedure for the Synthesis of Aryl-Hydroxamic Acids III and IV [21, 24, 25]

Hydroxamates were synthesized from the corresponding acids dissolved in ethanol 95°. 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 that the mixture was poured into ice. The residue was collected by filtration and recrystallized from C_2H_5OH 95° /H_2O mixture. Compounds were not crystallized they were liquids 2i and 2ii.

3-(3-Phenoxy-phenyl)-2-phenyl-acrylic Acid (1i) [24]

3-(3-Phenoxy-phenyl)-acrylic Acid (1ii):

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 230, 270, $_{max}$:15240, 14450; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): δ 6.3-6.4 (d, 1H), J = 15, 7.01-7.07 (m, 3H), 7.13-7.17 (m, 2H), 7.26-7.39 (m, 4H), 7.69-7.75 (d, 1H), 10.9 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 116.5, 117.8, 117.9, 119.2, 120.9, 123.2, 123.8, 122.1, 129.9, 130.3, 135.8, 146.3, 158.0, MS m/e (%): 239.7 [M+], 194 (14), 165 (59), 104 (7.6), 94 (66), 77 (77), 45 (31).

N-Hydroxy-3-(3-phenoxy-phenyl)-2-phenyl-acrylamide [24]

N-Hydroxy-3-(3-phenoxy-phenyl)-acrylamide (1iv)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 230, ε_{max}: 13770; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): δ 6.34-6.40 (d, 1H) *J = 15*, 7.00-7.32 (b, 9H), 7.64-7.70 (d, 1H) *J = 18*, 8.80 (s, 1H), 10.9 (s, 1H); MS m/e (%): 240, 162 (34.6), 85 (11.5), 77 (100), 60 (11), 54 (23.7).

4-Methyl-2,5-diphenyl-penta-2,4-dienoic Acid (2i) [24]

4-Methyl-5-phenyl-penta-2,4-dienoic Acid (2ii)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 220, 280, ε_{max}:10610, 6890; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): δ 1.66 (s, 3H), 6.07-6.10 (d, 1H) *J = 9.3,* 6.72-6.73 (d, 1H) *J = 3,* 6.76-7.60 (b, 5H), 8.07-8.10 (d, 1H) *J = 9*, 9.59 (s, 1H)); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 15.8, 117.0, 125.5, 126.4, 127.6, 128.3, 128.5, 128.6, 133.3, 133.7, 151.8, 168.0**,** MS m/e (%): 77 (97.81), 51 (100), 50 (83.7).

4-Methyl-2,5-diphenyl-penta-2,4-dienoic Acid Hydroxy-amide (2iii) [24]

4-Methyl-5-phenyl-penta-2,4-dienoic Acid Hydroxyamide (2iv)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 220, 260, 280 ε_{max}:13520, 16390, 14860; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): δ 2.1 (s, 3H), 6.73 (s, 1H), 7.27-7.29 (d, 1H) *J = 6,* 7.31-7.38 (m, 5H), 7.40-7.44 (d, 1H) *J = 12,* 7.9 (s, 1H), 9.6 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 13.1, 118.9, 123.1, 127.9, 128.4, 129.4, 129.5, 131.5, 133.1, 137.7, 155.5, 162.9**,** MS m/e (%): 143 (48.9), 117 (12.5), 77 (24).

2-Phenyl-3-thiophen-2-yl-acrylic Acid (3i) [24]

3-Thiophen-2-yl-acrylic Acid (3ii)

According to our general synthetical procedure.

N-Hydroxy-2-phenyl-3-thiophen-2-yl-acrylamide (3iii) [24]

N-Hydroxy-3-thiophen-2-yl-acrylamide (3iv)

According to the above reported synthetical procedure, UV (ethanol absolute) λ_{max}: 210, 300, ε_{max}: 9470, 13710; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 6.22-6.27 (d, 1H) *J = 15*, 7.2-7.3 (m, 1H), 7.41-7.43 (m, 1H), 7.8-7.9 (m, 3H), 9.4 (s, 1H), ; ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 128.2, 129.2, 131.5, 139.3, 150.7, 157.7, 166.2; MS m/e (%): 154 [M+] (13.3), 69 (52), 60 (11.8), 44 (100).

3-[4-(4-Bromo-benzyloxy)-phenyl]-2-phenyl-acrylic Acid (4i)

According to the reported general procedure 1a. UV (ethanol absolute) λ_{max}: 230, 300 ε_{max}: 18590, 16700; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 5 (s, 2H), 6.7-6.8 (d, 2H), 7-7.1 (d, 2H), 7.2-7.3 (m, 5H), 7.39-7.41 (m, 2H), 7.5-7.6 (d, 2H), 7.78 (s, 1H), 11.01 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 69.2, 114.6, 122, 126.4, 127.4, 127.5, 128, 128.4, 128.8, 128.9, 129, 129.2, 129.8, 131.7, 132.6, 132.7, 135.7, 141.9, 143.2, 157.6, 181.3; MS m/e (%): 409 [M+] (15), 193 (20), 171 (94), 169 (100), 89 (25), 77(11).

3-[4-(4-Bromo-benzyloxy)-phenyl]-acrylic Acid (4ii)

According to our above reported synthetical procedure. UV (ethanol absolute) λ_{max}: 220, 290, ε_{max}: 12570, 11680; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 5.01-5.06 (m, 2H), 6.70-6.98 (m, 3H), 7.2-7.3 (b, 4H), 7.33-7.4 (m, 2H), 7.69-7.74 (d, 1H) *J* = 15, 9.4 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 76.6, 111.2, 114, 127.8, 128, 128.9, 129, 129.7, 131.7, 132.4, 138.6, 151.2, 151.4, 157.7, 157.8, 180.81.

3-[4-(4-Bromo-benzyloxy)-phenyl]-N-hydroxy-2-phenyl-acrylamide (4iii)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 220, 300, ε_{max}: 15030, 12820; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 4.96 (s, 2H), 6.70-6.74 (d, 2H), 6.99-7.02 (d, 3H), 7.04-7.26 (m, 2H), 7.39-7.41 (m, 4H), 7.47-7.50 (d, 2H), 7.8 8 (s, 1H), 11 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 69.2, 114.6, 115, 123, 125.5, 126.3, 127, 128, 128.2, 128.6, 128.9, 129, 130, 130.8, 131.5, 132.5, 132.7, 139.8, 141.9, 160.9, 164.8; MS m/e (%): 409 [M+] (14), 171 (94), 169 (100).

3-[4-(4-Bromo-benzyloxy)-phenyl]-N-hydroxy-acrylamide (4iv)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 230, 290, ε_{max}: 15170, 14770; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 4.70-5.10 (m, 2H), 6.28-6.34 (d, 1H) *J* = 18, 6.69-6.72 (d, 2H), 6.9-7.0 (d, 2H), 7.2-7.3 (m, 2H), 7.5-7.53 (m, 2H), 7.59-7.65 (m, 1H) *J* = 18, 8 (s, 1H), 11.9 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 76.5, 114.5, 115.3, 117.2, 118.1, 126.9, 127.1, 128, 129.1, 129.4, 131.6,

131.8, 139.5, 144.3, 151.6, 161.5; MS m/e (%): 169 (100), 164 (11), 90 (47.9), 89 (53.3), 61 (7.8).

5-(4-Dimethylamino-phenyl)-2-phenyl-penta-2,4-dienoic Acid (5i)

According to the general synthetical procedure. UV (ethanol absolute) $_{max}$: 210, 260, 360, ε_{max}: 13250, 11780, 13340; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 2.6-3.3 (b, 6H), 6.54-6.57 (m, 2H), 6.60-6.63 (m, 1H), 6.69-6.74 (m, 1H), 7.3-7.4 (m, 6H), 7.41-7.43 (d, 2H), 12.2 (s, 1H) ; ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 43.5, 113.8, 114.1, 124, 125.6, 125.9, 126, 126.2, 127.3, 127.6, 128.6, 129.8, 131.8, 132.5, 136.8, 137, 148.8, 159.8, MS m/e (%): 248 (100), 171.8 (42.7), 77 (21.5).

5-(4-Dimethylamino-phenyl)-penta-2,4-dienoic Acid (5ii)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 210, 260, 380, 400, ε_{max}: 7500, 8570, 14580, 18410, ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 2.9-3.1 (d, 6H), 6 (s, 1H), 6.6-6.7 (m, 2H), 6.82-6.87 (m, 2H), 6.94 (s, 1H), 7.3-7.5 (m, 2H), 8.4 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 39.0, 39.8, 114.1, 117.0, 118.2, 122.0, 123.0, 125.0, 133.0, 148.1, 150.2, 171.2.; MS m/e (%): 217 [M+] (46.7), 157 (45.5), 128 (100), 116 (21.9), 77 (21.9).

5-(4-Dimethylamino-phenyl)-2-phenyl-penta-2,4-dienoic Acid Hydroxyamide (5iii)

According to the general synthetical procedure. UV (ethanol absolute) λ_{max}: 210, 270, 370, ε_{max}: 14330, 11090, 15960; ^{1}H-NMR (DMSO-d_6, $CDCl_3$): 2.83-3.20 (b, 6H), 6.6-6.7 (m, 2H), 6.7-6.8 (m, 1H), 6.9-7 (m, 1H), 7.2-7.21 (m, 3H), 7.22-7.24 (m, 2H), 7.30-7.35 (m, 2H), 7.40-7.46 (d, 1H) J = 18, 8.4 (s, 1H), 10.9 (s, 1H); ^{13}C-NMR (DMSO-d_6, $CDCl_3$): 59.3, 111.3, 112.4, 122.7, 125.1, 126.4, 126.5, 127.2, 127.5, 129.1, 129.8, 130.3, 132.4, 134, 134.3, 138.9, 146.3, 162.6; MS m/e (%): 248 (100), 205 (56), 190 (14), 172 (99), 128 (70), 118 (19), 44 (13.8).

5-(4-Dimethylamino-phenyl)-penta-2,4-dienoic Acid Hydroxyamide (5iv)

According to our general procedure.

3-(2-Oxo-2H-chromen-6-yl)-2-phenyl-acrylic Acid (6i) [24]

N-Hydroxy-3-(2-oxo-2H-chromen-6-yl)-2-phenyl-acrylamide (6iii) [24]

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 aryl-acetic acids and methanol/water mixture (95/5, v/v) for the aryl-hydroxamic 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 $R_M = \log [(1/R_f) -1]$ [42].

***b) Determination of Lipophilicity as Clog* P**

Lipophilicity was theoretically calculated as Clog *P* values in n-octanol-buffer by CLOGP Programme of Biobyte Corp. [30].

Biological Experiments

Experiments In Vivo

Inhibition of the Carrageenin-Induced Edema [24, 43, 44]

Edema was induced in the right hind paw of Fisher 344 rats (150-200 g) by the intradermal injection of 0.1 ml 2% carrageenin in water. Both sexes were used. Females pregnant 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 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 % [43, 44].

Experiments In Vitro

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

Determination of the Reducing Activity of the Stable Radical 1,1-diphenyl-picrylhydrazyl (DPPH) [24, 45]

To a solution of DPPH 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 0.05mM. After 20 and 60 min at room temperature the absorbance was recorded at 517nm .

Competition of the Tested Compounds with DMSO for Hydroxyl Radicals [24, 46, 47]

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 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds (concentration 0.01 mM and 0.1mM) and ascorbic acid (10 mM). After 30 min of incubation (37^o C) the reaction was stopped with CCl_3COOH (17 % w/v).

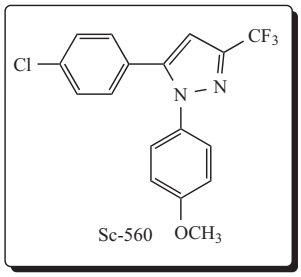
Soybean Lipoxygenase Inhibition Study In Vitro [24, 48]

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.

In Vitro Cyclooxygenase-1 (COX-1) Inhibition Study [49]

Cyclooxygenase (COX) activity was determined by using arachidonic acid (AA) as substrate and N,N,N,N-tetramethylphenylenediamine (TMPD) as co-substrate, as described by Kulmacz and Lancs. The reaction mixture (1 ml) contained 0.75 µM heme, 128 µM TMPD, 80 µM arachidonic acid (AA) and 1.5 µg 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 substrated from the initial rate of oxidation observed in presence of AA. The inhibition of the compounds 1i, 2i and 6i 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. Sc-560 has been used as a comparison COX-1 inhibitor.



Heme Protein-Dependent Lipid Degradation [50, 51]

50µM heme, arachidonic acid (0.4mM) the compounds at the various concentrations tested, 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 [50, 51]. The compounds were added in DMSO solution, which has no effect on the assay.

Non Enzymatic Assay of Superoxide Radicals-Measurement of Superoxide Radical Scavenging Activity [52]

The superoxide producing system was set up by mixing phenazine methylsulfate (PMS), NADH and air -oxygen. The production of superoxide was estimated by the nitroblue tetrazolium method. The reaction mixture containing compounds, 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.

Effects of the Test Compounds on the Fe^{+3} -Ion-Stimulated Peroxidation of Linoleic Acid [53]

Linoleic acid sodium salt (90mg) was dissolved and diluted to 50mL with 0.2M phosphate buffer pH 7.4 and oxygen (100%) bubbled through the solution. 2mL aliquots of the linoleic acid sodium solution were incubated for 2h at 37°C with 2.5mM Fe^{+3} ($FeSO_4$) and 1mM of the test compounds. The amount of peroxidation which occurred during this time was measured by the 2-thiobarbituric acid (TBA) method. The amount of TBA-reactive material in each sample was determined by measuring the absorbance of the aqueous layer at 535 nm. The inhibition of Fe^{2+}-stimulated oxidation of linoleic acid caused by each compound is the mean value of three or five experiments.

Stability Studies and Incubation with Glutathione (GSH) [40]

Solutions of the compounds were prepared in water using phosphates buffer solution (PBS) pH 7.4 and in order to achieve dissolution the solvent contained approximately 10% v/v alcohol. The concentrations of the solutions were chosen so that the absorption maxima were between 0.5 and 1. The test compounds are incubated for 24h at 37–C and their UV spectra were recorded. All determinations were carried out in duplicate. The error limits of the ε values were approximately 2%.

The experiment was repeated in the presence of GSH using thiol/test compound, 2/1 and 10/1 and incubation at 37–C for 24h. and their UV spectra were recorded. The results are given in Table **6**.

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