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Short communication

Synthesis of pyridazine acetic acid derivatives possessing aldose reductase inhibitory activity and antioxidant properties

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Summary — *N*-Acetic acid derivatives of 4-carboxy-6-arylpyridazin-3-ones were synthesized for the dual purpose of inhibiting aldose reductase and exhibiting antioxidant properties. All the prepared compounds showed a significant *in vitro* aldose reductase inhibitory effect (10^{-5} M ≤ IC_{50} ≤ 10^{-4} M). The spatial configuration of the most active derivative **4f** (4-*i*-PrC_6H_4 at C_6, IC_{50} = 0 95 x 10^{-5} M) was compared with pharmacophore requirements of the aldose reductase inhibitor site using a molecular modeling system. The antioxidant action of **4a–f** was also studied *in vitro*. Compound **4c** (4-ClC_6H_4 at C_6, IC_{50} = 1.56 x 10^{-3} M) was the most effective at scavenging the superoxide anion whereas compound **4a** (C_6H_5 at C_6, IC_{50} = 1.28 x 10^{-3} M) was the most active at inhibiting lipid peroxidation. In addition, biological activities (log $1/IC_{50}$) for most of the data sets could be correlated directly to lipophilic, electronic and steric parameters.

pyridazinone /aldose reductase inhibitor / antioxidant

Introduction

Chronic diabetes, a disease that affects over 200 million people worldwide, leads to long-term complications which include neuropathy, nephropathy, retinopathy and cataracts. The progression of these disorders can result in loss of sensory perception and limb function, and in blindness [1–3].

Experimental results generated during the last decade suggest that diabetic pathology may be partly controlled through inhibition of the enzyme aldose reductase [4]. In addition, oxidants such as hydrogen peroxide and oxygen-derived free radicals may play an important role in the physiopathology of tissue damage. Studies have shown that diabetes causes a thrombotic tendency in patients with increased oxidative stress [5]. Recently, it appeared that new therapeutic approaches for the prevention of diabetic complications may be found in free radical scavengers in order to limit the damage caused by oxidant stress [6, 7]. Thus, with the object of delaying the advent of long-term complications of diabetes, we attempted to synthesize a new series of pyridazine derivatives possessing simultaneously aldose reductase inhibitory activity and antioxidant properties.

Specific structural and electronic similarities of apparently diverse aldose reductase inhibitors have been observed through basic studies, and the presence of an acidic proton appears to be of prime importance to inhibiting the enzyme [3]. This result was substantiated by the structures of several recently described inhibitors [8–10]. As regards antioxidant compounds, they also often possess an acidic proton in their chemical structures. This is the case for the natural product gossypol which includes a phenolic function in its skeleton and also presents aldose reductase inhibitory effect [11, 12]. Likewise, the structures of the synthetic antioxidant derivatives oxypurinol and nifedipine contain an acidic or related function [13, 14].

Taking into account the compatibility between the dual requested pharmacological activities and the structural features of new potential active compounds, we decided to synthesize pyridazine derivatives substituted in the 2- and 4-positions by a carboxy group.

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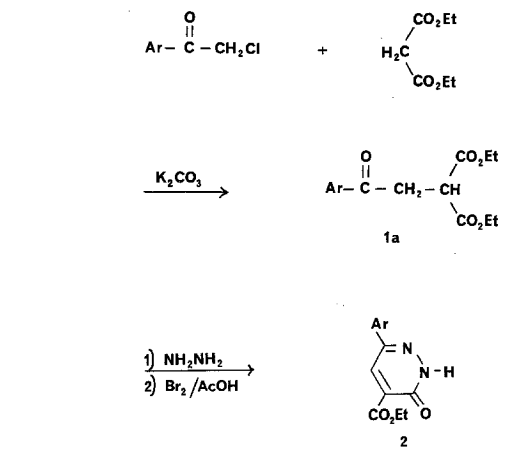
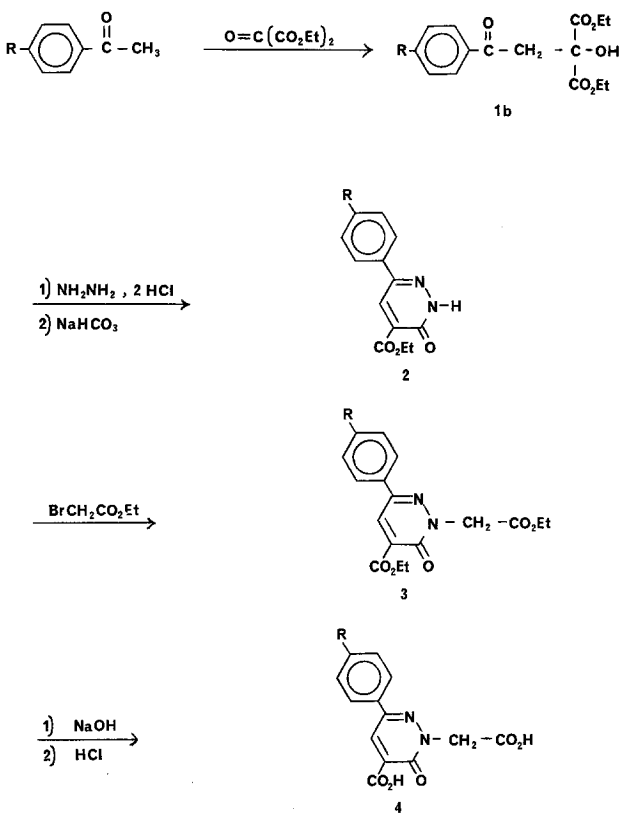


Scheme 1.



Scheme 2.

Chemistry

Two convenient synthetic methods for 4-ethoxycarbonyl pyrldazine derivatives were recently described in the literature [15, 16]. First, introduction of an ethoxycarbonyl group in the 4-position of the pyridazinone ring may be achieved by alkylation of diethyl malonate with an α-chloroketone [15]. Cyclization of the resulting ketodiester **1a** with hydrazine and creation of the 4,5-double bond through bromine/acetic acid oxidation furnished the required pyridazinone **2** (scheme 1). The alternative route we chose for the synthesis of 4-ethoxycarbonyl-6-aryl pyridazinones **2** was developed by Singh and Lesher [16] and involved only 2 steps (scheme 2). Thus, the aldol condensation products **1b** of acetophenones and diethyl mesoxalate were reacted with hydrazine dihydrochloride, and then treated with aqueous sodium bicarbonate to yield **2**. Nucleophilic substitution of the hydrogen atom attached in the 3-position of the pyridazine ring was obtained by reaction of ethyl bromacetate in acetone containing potassium carbonate.

Base-catalyzed hydrolysis of the resulting esters **3** followed by acidification to pH 1 with 5% aqueous sulfuric acid afforded pyridazine acetic acids **4** (table I).

The physical constants of pyridazinones **2**, **3** and **4** were reported in table I. The spectral data of pyridazinones **4a–f** were summarized in table II.

Results and discussion

First, the compounds listed in table I were evaluated *in vitro* for their ability to inhibit aldose reductase

Table I. Physical data for pyridazinones **2**, **3** and **4**.

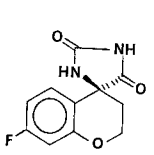
Compound	*R*	*Yield (%)*	*Mp (°C)*	*Formula*
2a	H	53	156[a]	$C_{13}H_{12}N_2O_3$
2b	F	37	265	$C_{13}H_{11}N_2FO_3$
2c	Cl	65	175	$C_{13}H_{11}N_2ClO_3$
2d	Br	66	200	$C_{13}H_{11}N_2BrO_3$
2e	CH_3	65	180	$C_{14}H_{14}N_2O_3$
2f	$CH(CH_3)_2$	37	210	$C_{16}H_{18}N_2O_3$
3a	H	91	oil	$C_{17}H_{18}N_2O_5$
3b	F	92	oil	$C_{17}H_{17}N_2FO_5$
3c	Cl	98	oil	$C_{17}H_{17}N_2ClO_5$
3d	Br	96	oil	$C_{17}H_{17}N_2BrO_5$
3e	CH_3	95	55	$C_{18}H_{20}N_2O_5$
3f	$CH(CH_3)_2$	90	oil	$C_{20}H_{24}N_2O_5$
4a	H	40	240	$C_{13}H_{10}N_2O_5$
4b	F	40	255	$C_{13}H_9N_2FO_5$
4c	Cl	64	271	$C_{13}H_9N_2ClO_5$
4d	Br	59	280	$C_{13}H_9N_2BrO_5$
4e	CH_3	56	268	$C_{14}H_{12}N_2O_5$
4f	$CH(CH_3)_2$	98	152	$C_{16}H_{16}N_2O_5$

[a]Literature value: 158°C [15].

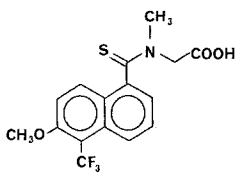
Table II. Spectral data of pyridazinones **4a–f**.

Compound	*IR (KBr) ν (cm^{-1})*	*^{1}H-NMR chemical shift (δ ppm) (in DMSO-d_6)*
4a	3300–2300, 1750, 1720, 1640, 1580, 1510, 1440	5.0 (s, 2H, CH_2), 7.7 (m, 5H, C_6H_5), 8.5 (s, 1H, CH=), 9.7 (m, 2H, 2 OH)
4b	3300–2400, 1750, 1720, 1640, 1600, 1580, 1510, 1440	5.1 (s, 2H, CH_2), 7.8 (m, 4H, C_6H_4), 8.5 (s, 1H, CH=), 12.0 (m, 2H, 2 OH)
4c	3100–2300, 1740, 1720, 1630, 1580, 1490, 1450	5.0 (s, 2H, CH_2), 7.7 (m, 4H, C_6H_4), 8.5 (s, 1H, CH=), 9.5 (m, 2H, 2 OH)
4d	3100–2500, 1740, 1720, 1630, 1580, 1440	5.0 (s, 2H, CH_2), 7.7 (m, 4H, C_6H_4), 8.5 (s, 1H, CH=), 9.5 (m, 2H, 2 OH)
4e	3300–2400, 1760, 1720, 1635, 1570, 1500, 1460	2.4 (s, 3H, CH_3), 5.0 (s, 2H, CH_2),7.5 (m, 4H, C_6H_4), 8.4 (s, 1H, CH=), 9.5 (m, 2H, 2 OH)
4f	3300–2300, 1740, 1710, 1630, 1500, 1450	1.3 (d, 6H, 2 CH_3), 3.0 (m, 1H, CH), 5.0 (s, 2H, CH_2), 7.7 (m, 4H, C_6H_4), 8.4 (s, 1H, CH=), 9.8 (m, 2H, 2 OH)

obtained from pig lens. The inhibitory IC_{50} values are shown in table III and lie between 1.5×10^{-4} and 0.95×10^{-5} M. The isopropyl derivative **4f** exhibited the highest activity of the compounds substituted at the 4-position of the phenyl nucleus (**4b–e**). Nevertheless this activity was moderate in comparison with reference compounds such as sorbinil and tolrestat (10^{-8} M < IC_{50} < 10^{-6} M) [17, 18].



Sorbinil



Tolrestat

Substitution of the aromatic ring with an electron-withdrawing group (**4b–d**) was less effective than substitution with an electron-donating group (**4e–f**).

On the other hand, the influence of both lipophilicity and steric hindrance of the substituents on the phenyl ring did not clearly appear. In order to understand the real significance of the R substituent contribution, we have attempted to correlate the observed aldose reductase inhibitory activity (log $1/IC_{50}$) with log k_w as lipophilic index, Hammett's constant (σ_p) as electronic parameter, and Taft's steric factor (E_s). These 2 values have been taken from the compilation by Hansch and Leo [19]. In a preliminary QSAR study, we found that it was impossible to correlate significantly the biological activity of all compounds with the chosen physical parameters. It appeared that the observed aldose reductase inhibitory activity of derivative **4c** was fundamentally different from the calculated value, whatever equation was used. This may suggest that in **4c**, other factors than those taken into account to establish equation of correlation could influence biological activity. This hypothesis was corroborated by the fact that **4c** and **4d** revealed equipotent activity, while the lipophilicity and the electronic and steric parameters were notably different. Thus, a new regression analysis excluding compound **4c** resulted in the following equation, where the standard error of estimate is given in parentheses for the fitted values:

$$\log (1/IC_{50}) = 0.712\ (\pm 0.017) \log k_w - 3.140\ (\pm 0.057)\ \sigma_p + 0.213\ (\pm 0.018)\ E_S + 2.661\ (\pm 0.031) \quad [1]$$

In the above equation, the number of compounds was $n = 5$, the squared correlation coefficient was $r^2 = 0.999$, and the residual standard deviation was $s = 0.009$ and $F = 2924.431$ ($p = 0.016$).

The relationship in the above equation indicates that an important aldose reductase inhibitory activity is associated with electron-donating substituents on the phenyl nucleus (negative σ_p) whereas the influence of the steric parameter E_S on the activity was secondary. On the other hand, the compounds should be sufficiently lipophilic to exhibit the highest aldose reductase inhibitory effect. The data used in the analysis and log ($1/IC_{50}$) values recalculated from the equation [1] are given in table IV and represented graphically in figure 1.

On the basis of its biological properties, pyridazinone **4f** was chosen for a molecular modeling study

Table III. Biological activities of pyridazinones **4a–f**.

Compound	*Aldose reductase inhibitory activity* IC_{50} *(x 10^{-5} M)*	*Superoxide anion scavenging activity* IC_{50} *(x 10^{-3} M)*	*Lipid peroxidation inhibitory activity (% at 1 mg/ml)*
4a	8.30	2.33	97 IC_{50} = 1.28 x 10^{-3} M
4b	15.00	2.39	11 (NS)
4c	7.00	1.56	7 (NS)
4d	7.00	2.10	34
4e	3.70	2.08	21 (NS)
4f	0.95	1.87	6 (NS)
Sorbinil	0.11[a]	ND	ND
Tolrestat	0.0034[a]	ND	ND
Cyanidanol	ND	0.17	IC_{50} = 1.37 x 10^{-5} M

[a]Reference [17]; ND: not determined; NS: not significant.

using Alchemy III software (Tripos Associates, Saint-Louis, MO, USA). Compound **4f** was built in Alchemy III and minimized (converging to $\Delta E < 0.01$ kcal/mol per iteration) using the Tripos molecular mechanics force field. Thus it appeared that **4f** can adopt a low energy conformation (fig 2) corresponding to the pharmacophore model of Kador *et al* [2, 20]. Derivative **4f** included a pyridazinone ring as a lipophilic region separated by 3.6 Å from a group possessing an acidic proton, as represented by the carboxylic function of the acetic chain.

A secondary lipophilic ring constituted of the phenyl nucleus located 5.9 Å from the acetic chain could enhance hydrophobic interactions with the aldose reductase inhibitor site.

In addition both the carboxylic function in the 4-position and the isopropyl group, 3.7 and 7.7 Å re-

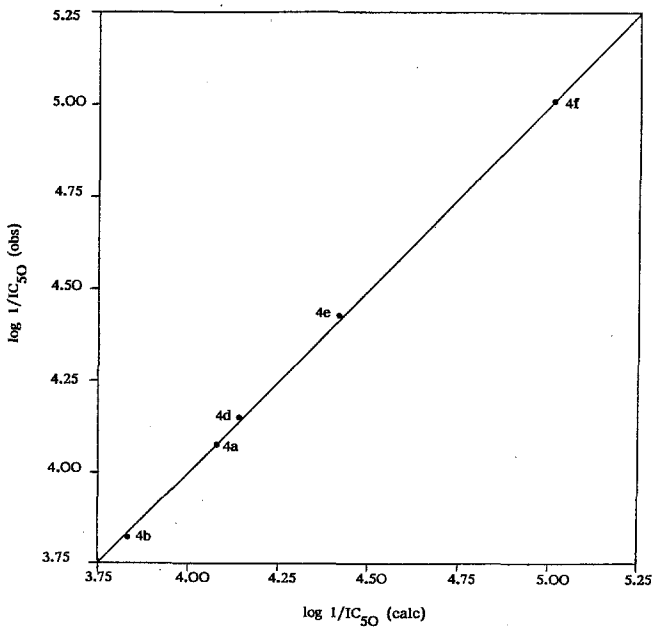
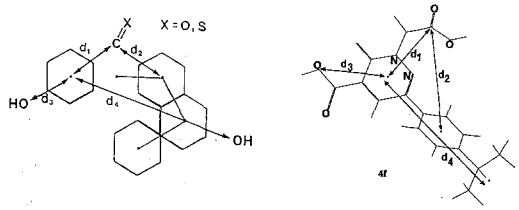


Fig 1. Plot of observed *versus* calculated aldose reductase inhibitory activity (from equation [1]) of pyridazinones **4a–f**.



$2.8 \leq d_1 \leq 3.8$ Å
$2.8 \leq d_2 \leq 6.1$ Å
$2.8 \leq d_3 \leq 3.8$ Å
$8.0 \leq d_4 \leq 9.3$ Å

$d_1 = 3.6$ Å
$d_2 = 5.9$ Å
$d_3 = 3.7$ Å
$d_4 = 7.7$ Å

Fig 2. Comparison between pyradazione **4f** and pharmacophore model of aldose reductase inhibitors proposed by Kador [2, 20].

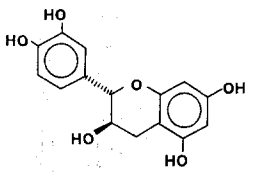
Table IV. Biological activities, Hansch analyses and capacity factors (log k_w) of pyridazinones **4a–f**.

Compound	*Aldose reductase inhibitory activity*		*Superoxide anion scavenging activity*		*log k_w*
	log 1/IC$_{50}$ (obs)	*log 1/IC$_{50}$ (calc)*[a]	*log 1/IC$_{50}$ (obs)*	*log 1/IC$_{50}$ (calc)*[b]	
4a	4.08	4.08	2.63	2.61	1.99
4b	3.82	3.83	2.62	2.64	2.04
4c	–	–	–	–	3.10
4d	4.15	4.14	2.67	2.68	3.45
4e	4.43	4.42	2.68	2.69	2.10
4f	5.02	5.02	2.73	2.71	3.17

[a,b]Calculated from equations [1] and [2], respectively.

spectively from the center of the pyridazinone, could enhance binding to this site.

Furthermore, pyridazinones **4a–f** were evaluated as superoxide anion scavengers and compared with cyanidanol used as reference compound.



Cyanidanol

All the compounds were approximately 10 times less active than cyanidanol and equipotent to one another with IC_{50} values ranging from 1.56 to 2.39 x 10^{-3} M (table III). Since there were no great differences in antioxidant properties of **4a–f**, the structure-dependent radical scavenging behavior for this series of derivatives may be attributed to changes in the steric environment of the phenyl nucleus.

Consistent with this hypothesis, a regression analysis using capacity factor (log k_w), Hammett's constant (σ_p) and Taft's steric factor (E_s) gave significant correlation as follows:

$$\log (1/IC_{50}) = -0.060\ (\pm 0.014)\ E_s + 2.611\ (\pm 0.016) \quad [2]$$

$n = 5$; $r^2 = 0.855$; $s = 0.019$; $F = 17.729$ ($p = 0.022$)

Similarly to aldose reductase inhibitory activity, compound **4c** should be excluded from this last study in order to obtain a significant equation. From equation [2], it appeared that the highest superoxide anion scavenging activity corresponded to a bulky substituent on the phenyl ring. The data used in the analysis and log ($1/IC_{50}$) values recalculated from equation [2] are given in table IV and represented graphically in figure 3.

On the other hand, most of pyridazinone derivatives displayed a very weak lipid peroxidation inhibitory activity (table III) except for compound **4a** with an

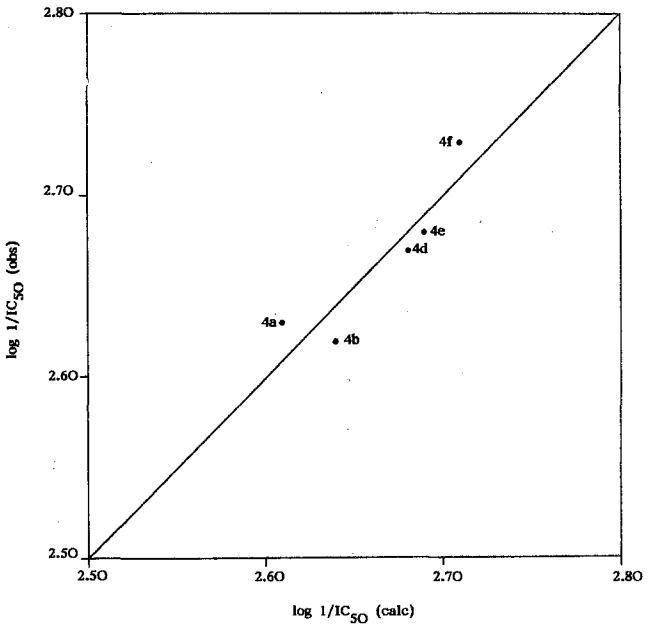


Fig 3. Plot of observed *versus* calculated superoxide anion scavenging activity (from equation [2]) of pyridazinone derivatives.

IC_{50} value of 1.28 x 10^{-3} M. Cyanidanol exhibited an IC_{50} value of 1.37 x 10^{-5} M in this test. Thus, pyridazinone **4a**, seemed to be the most interesting derivative of this series, since it was effective in the 3 biological tests used. Nevertheless compound **4f** could also be useful for the treatment of ocular diabetic complications, taking into account both its potent aldose reductase inhibitory effects and superoxide anion scavenging activity.

In conclusion, 4-carboxypyridazin-2-yl acetic acids represent a new class of derivatives which combines aldose reductase inhibitory properties and antioxidant effects in the same molecule.

Experimental protocols

Chemistry

All compounds were checked for their structures by IR, ^{1}H-NMR and elemental analyses. The IR spectra were obtained with a Beckman 4240 spectrophotometer and ^{1}H-NMR spectra were recorded on a Varian EM 360A spectrometer with tetramethylsilane as an internal standard. Elemental analyses (C, H, N, F, Cl and Br within ± 0.4% of theoretical values) were performed at the Service Central d'Analyses, Centre National de la Recherche Scientifique, 69390 Vernaison, France. Melting points were determined on a Reichert apparatus and were uncorrected. Starting materials, acetophenones and diethyl mesoxalate were purchased from Janssen Chimica, Noisy-le-Grand, France.

*Ethyl 2-ethoxycarbonyl-2-hydroxy-4-aryl-4-oxo butanoates **1b***
Compounds **1b** were prepared according to a literature procedure [16].

*General procedure for 4-ethoxycarbonyl-6-aryl-(2H)-pyridazin-3-ones **2***
A mixture of the appropriate compound **1b** (10 mmol) and hydrazine dihydrochloride (1.05 g, 10 mmol) in ethanol (50 ml) was refluxed with stirring for 18 h and then cooled to room temperature. The resulting precipitate was filtered off and suspended in ethanol (20 ml). The mixture was neutralized to pH 7 with 5% aqueous sodium hydrogenocarbonate. The resulting white solid was collected, washed with water and recrystallized from ethanol.

*General procedure for ethyl 4-ethoxycarbonyl-6-aryl-3-oxo-pyridazin-2-yl acetates **3***
A solution of pyridazinone **2** (5 mmol), potassium carbonate (1.03 g, 7.5 mmol) and ethyl bromacetate (1.25 g, 7.5 mmol) in anhydrous acetone (50 ml) was refluxed with stirring for 24 h. The hot mixture was then filtered off to eliminate mineral products. The filtrate was evaporated to dryness and the solid or the oily residue collected. The crude esters **3** were used without further purification.

*General procedure for 4-carboxy-6-aryl-3-oxopyridazin-2-yl acetic acids **4a–f***
The appropriate ester **3** (5 mmol) was suspended in ethanol (50 ml) and sodium hydroxide (1.2 g, 30 mmol) was added. The reaction mixture was refluxed for 4 h and then ethanol was removed.

The residue was dissolved in water (40 ml) and acidified to pH 1 with an aqueous solution of 5% sulfuric acid. The resulting precipitate was filtered off, washed with water and dried. Acids **4a–f** were recrystallized from ethanol.

Biology

Aldose reductase inhibiting activity
This activity was evaluated *in vitro* according to a technique adapted from Varma and Kinoshita [21]. The experiments were carried out at 25°C over 5 min with the enzyme extracted from pig lens. Aldose reductase activity was assayed spectrophotometrically following the 340 nm oxidation of NADPH to NADP, using DL-glyceraldehyde as a substrate. The reaction mixture contained 0.1 M phosphate buffer (pH 6.2), 0.25 mM NADPH, 1.5 mM DL-glyceraldehyde and the enzyme in a total volume of 1 ml. Each assay, repeated 5 times, was performed with the supernatant of lens homogenates at suitable dilution and after 15 min preincubation with several effector concentrations. The percentage inhibition for each compound was calculated by comparing the reaction rate of the solution containing the inhibitor with that of the control.

Superoxide anion scavenging
The technique of Slater and Eakins [22] utilizing the interactions of NADPH, phenazine methosulfate (PMS), molecular oxygen and nitro blue tetrazolium (NBT) was used for evaluating superoxide anion scavenging.

The NADP/PMS/O_2/NBT system involves the intermediate formation of the superoxide anion radical ($O_2^{-\bullet}$) from the interaction of reduced PMS with O_2; the superoxide anion radical then reducing NBT to the highly colored formazan.

The reaction can be followed by measuring the absorbance of the formazan at 578 nm. The incubation mixture contained phosphate buffer (200 µl, 76 mM, pH 7.4), PMS (200 µl, 10.8 µM), NBT (200 µl, 172 µM) and NADH + H^+ (200 µl, 360 µM). Each assay repeated 5 times was performed after 5 min incubation with several effector concentration.

Lipid peroxidation
Iron-dependent peroxidation of male rat liver microsomes was assayed as previously described [23]. Liver microsomes were prepared according to the technique of May and McCay [24]. Lipid peroxidation was initiated by the system ADP-Fe^{2+}/ascorbate [25], which produced hydroxyl radicals ($OH^\bullet$). Incubations of microsomal fractions (1 mg protein/ml) were carried out at 37°C in 7 ml 0.1 M potassium phosphate buffer (pH 7.4) containing 2 mM ADP/16 µM $FeSO_4$. Lipid peroxidation was started upon addition of 0.5 mM ascorbate to the above incubation mixture. The extent of polyunsaturated fatty acids peroxidation was measured spectrophotometrically at 535 nm by the rate of malondialdehyde formed after 90-min microsomal incubation.

Lipophilicity measurements
Lipophilicity was determined by reversed-phase high-performance liquid chromatography using literature procedures [26, 27]. A Varian 5000 liquid chromatograph equipped with a detector operating at 254 nm was used. A Varian CDS 111L integrator was used for peak registration and calculation of retention times. An ODP column (15 cm x 6 mm ID) prepacked with octadecyl copolymer gel, particle size 5 µm, was used as a non-polar stationary phase. Mobile phases were prepared volumetrically from 50:50 to 90:10 combinations of methanol/acetic acid (0.085 N, pH = 3). The flow rate was 1 ml/min. Isocratic capacity factors k_i were defined as k_i =

$(t_r-t_o)/t_o$ where t_r is solute retention time and t_o is column dead time determined using methanol as the non-retained compound. Log k_w was used as the lipophilic index, obtained by linear extrapolation of log k_i to 100% water.

Determination of IC_{50}

IC_{50} values were obtained by graphic estimation from log concentration–response curves.

The Student's *t*-test was used to assess the statistical significance between means of unpaired data compared to the control group. $P < 0.05$ was statistically significant. Thus all percentage inhibition values given in the tables for each compound are statistically significant unless otherwise indicated.

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