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Synthesis and anti-inflammatory/antioxidant activities of some new ring substituted 3-phenyl-1-(1,4-di-*N*-oxide quinoxalin-2-yl)-2-propen-1-one derivatives and of their 4,5-dihydro-(1*H*)-pyrazole analogues

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Abstract—We report the synthesis, anti-inflammatory and antioxidant activities of novel ring substituted 3-phenyl-1-(1,4-di-*N*-oxide quinoxalin-2-yl)-2-propen-1-one derivatives and of their 4,5-dihydro-(1*H*)-pyrazole analogues. The tested compounds inhibit the carrageenin-induced rat paw edema (4.5–56.1%) and present important scavenging activities. Compound **2a** is the most potent (56.1%) in the in vivo experiment and exhibits promising in vitro inhibition of soybean lipoxygenase ($IC_{50} < 1$ μM).

Quinoxalines, including their fused-ring derivatives, display diverse pharmacological activities (antiviral, anticancer, and antibacterial).[1,2] Scientists in Belgium and the United Kingdom have found that quinoxaline is a potential treatment for HIV infection, and works well with lamivudine, abacavir, and efavirenz.[3] Oxidation of both nitrogens of the quinoxaline ring dramatically increased the diversity of certain biological properties, such as antibacterial activity[4a,b,5,6] and hypoxia-selective anticancer activity.[7] Monge et al.[8–12] are involved in the synthesis and biological evaluation of new agents derived from quinoxaline 1,4-di-*N*-oxide and related compounds that have proved to be efficient cytotoxic agents for hypoxic cells in solid tumors. The poor tumor vascular structure, the inefficient blood supply along with a high interstitial pressure generate a variable proportion of viable hypoxic cells in solid tumors which is one of the causes of cell resistance to anticancer treatments. Systemic hypoxia increases reactive oxygen species generation and promotes leukocyte-endothelial adherence via reactive oxidant generation. Thus, antioxidants prevent the increase in leukocyte-endothelial adhesive interactions observed in hypoxia.[13] Bioreductive drugs have been designed to take advantage of the particular metabolic characteristics of hypoxic cells.

The formation of reactive oxygen species (ROS) is characteristic of aerobic organisms that normally defend themselves against these highly reactive species using enzymes, like superoxide dismutase and glutathione peroxidase and naturally occurring antioxidants.[14] ROS, like superoxide radical anion, hydrogen peroxide, and hydroxyl radical, are produced during the inflammation process by phagocytic leukocytes (e.g., neutrophils, monocytes, macrophages, eosinophils) that invade the tissue. Moreover, these reactive species are involved in the biosynthesis of prostaglandins and in the cycloxygenase- and lipoxygenase-mediated conversion of arachidonic acid into proinflammatory intermediates.[15,16]

Persistently high levels of ROS may involve pathological conditions, as the active species can modify essentially

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biological molecules, such as lipids, proteins, and DNA. It is therefore evident that the treatment of the above-mentioned pathophysiological conditions could benefit from the use of drugs that combine antioxidant and anti-inflammatory activity, as has already been proven for a number of commercially available non-steroidal anti-inflammatory drugs (NSAIDs), for example, tolfenamic acid which simultaneously possess radical scavenging properties.[17]

There is increasing evidence from animal models and clinical observations indicating that lipoxygenase (LOX) and their products may play a role in tumor formation and cancer metastasis.[18] Recently the concept has been put forward that LOX activation may be involved in both pro- and anti-tumorigenic effects.[19] Arachidonate lipoxygenase pathway appears to play a role in brain tumor growth as well as inhibition of apoptosis in in vitro studies. Emerging reports now indicate alterations of arachidonic acid metabolism related to carcinogenesis and many anti-inflammatory drugs are being investigated as potential anticancer drugs.

So, on the basis of these results, it seemed interesting to synthesize some novel ring substituted 3-phenyl-1-(1,4-di-*N*-oxide quinoxalin-2-yl)-2-propen-1-one derivatives and their 4,5-dihydro-(1*H*)-pyrazole analogues. Representative compounds have been tested, in order to study their scavenging activities, their role in inflammation, and their inhibition of LOX since LOX inhibitors are able to induce the anti-carcinogenic and/or to inhibit the pro-carcinogenic enzymes responsible for polyunsaturated fatty acid metabolism.

Synthesis of the derivatives **2a**–**c**, **3a**–**c**, and **4a**–**c** (Scheme 1) was carried out by a base-catalyzed Claisen–Schmidt condensation,[20,21] establishing a required temperature of −10 °C. The synthesis of compounds **5a**–**c** was carried out by dissolution of derivatives **4a**–**c** in absolute ethanol and subsequent addition of hydrazine hydrate.[22] The starting reagents used (**1a**–**c**) were obtained using the previously described method.[23]

All the synthesized compounds[24] were characterized by infrared, proton nuclear magnetic resonance, elemental analysis of C, H, and N, and melting point.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever, and inflammation. All of the NSAIDs are approximately equivalent in terms of anti-inflammatory efficacy but also cause untoward side effects (such as gastrointestinal ulcers, hemorrhages) in a significant fraction of treated patients and this fact frequently limits therapy. The variations in both efficacy and their tolerability are partly due to differences in their physicochemical properties, which determine their distribution in the body and their ability to pass through and to enter cells.[25,26] Thus partition coefficients such as R_M values are determined experimentally[27] and compared with the corresponding theoretically calculated clog *P* values[28] in *n*-octanol-buffer. From our results (Table 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 basic nitrogen atoms in the examined compounds, which could disturb the absorption/desorption process.

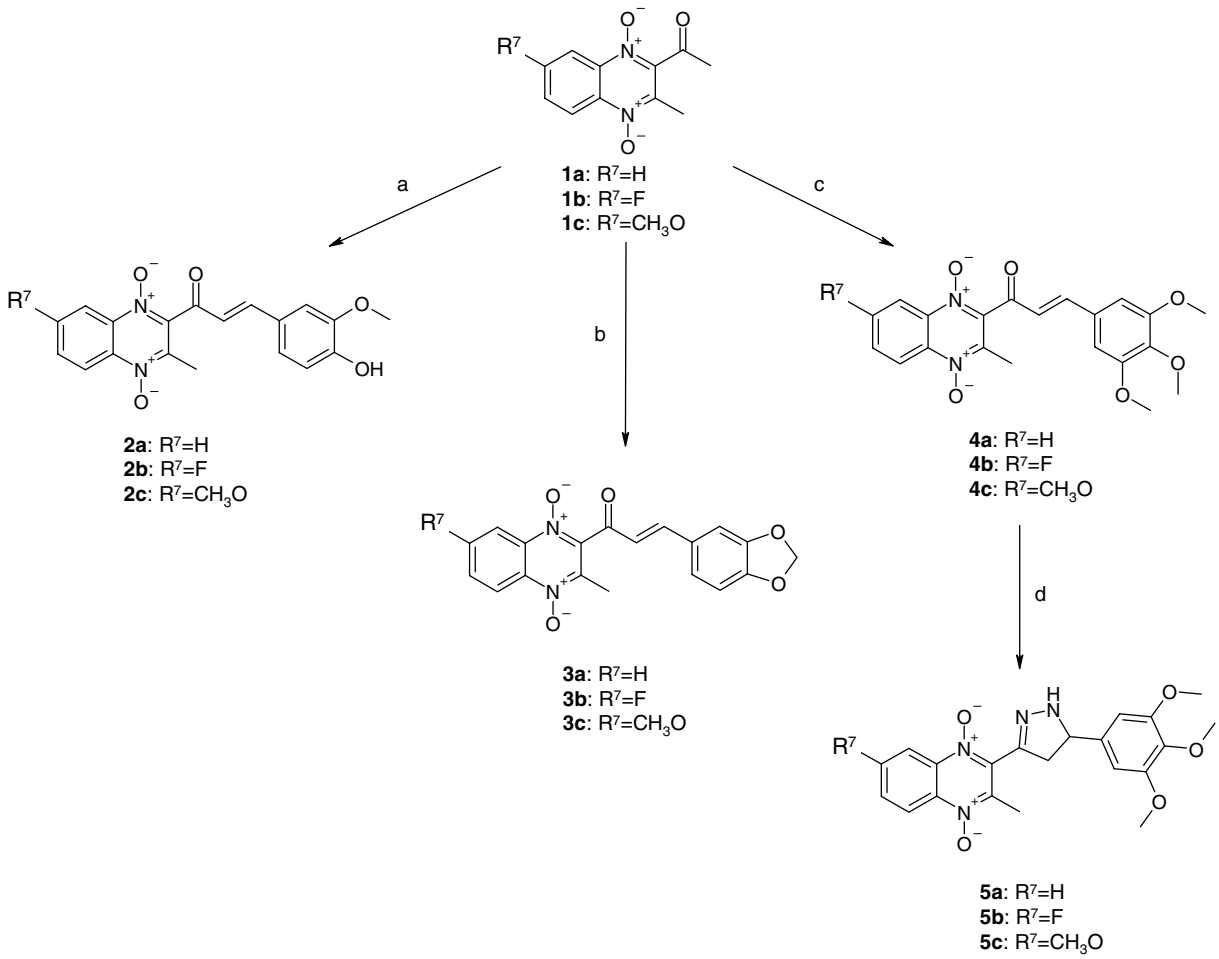
In acute toxicity experiments, the studied compounds did not present in vivo toxic effects at doses up to 0.5 mmol/ml/kg body weight. The in vivo anti-inflammatory effects of the tested compounds were assessed by using the functional model of carrageenin-induced rat paw edema[27] and are presented in Table 1, as percentage inhibition of weight increase at the right hind paw in comparison to the uninjected left hind paw (CPE%).

All the tested compounds (dose ip 0.01 mmol/ml/kg body weight), after 3.5 h,[29,30] induced protection (ranged from 4.5% to 56.1%) against carrageenin induced paw edema while the reference drug indomethacin (IMA) induced 47% protection at an equivalent dose.[29] Compounds **2a** and **4a** were the most potent (56.1% and 55.4%, Table 1) and presented almost equipotent effect. Among derivatives **2a**, **2b**, and **2c**, compound **2a** was found to be the most potent followed by **2b** and **2c** (**2b** > **2c**). The existence of the pyrazolyl ring decreases the biological response (compounds **5a** and **5b**) whereas the condensed ring –OCH_2O– (compound **3a**) is correlated with a very significant loss in inhibition.

Compounds **2a** and **4a**, the most potent in vivo, as well as compounds **2b**, **2c**, **3a**, **5a**, and **5b** were further evaluated for inhibition of soybean lipoxygenase LOX by the UV absorbance based enzyme assay.[29] Lipoxygenases oxidize certain fatty acids at specific positions to hydroperoxides that are the precursors of leukotrienes, which contain a conjugated triene structure. It is known that soybean lipoxygenase, which converts linoleic to 13-hydroperoxylinoleic acid, is inhibited by NSAIDs in a qualitatively similar way to that of the rat mast cell lipoxygenase and may be used in a reliable screen for such activity. Perusal of % inhibition values or IC_{50} values (Table 1) shows that compound **2a** (IC_{50} < 1 μM) is the most active, within the set, followed by compounds **3a**, **4a**, **5b**, and **2b**.

Most of the LOX inhibitors are antioxidants or free radical scavengers, since lipoxygenation occurs via a carbon-centered radical. Although lipophilicity is referred[31–33] to as an important physicochemical property for LOX inhibitors, all the above tested derivatives do not follow this concept.

Many non-steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers. Compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation and to lead to potentially effective drugs. Thus, we tested the new derivatives with regard to their antioxidant ability and in comparison to well-known antioxidant agents, for example, nordihydriguaiaretic acid (NDGA), trolox,



Scheme 1. Reagents and conditions: (a) 3-methoxy-4-(tetrahydro-pyran-2-yloxy)-benzaldehyde, 3% NaOH/methanol, −10 °C; HCl 35%; (b) benzo[1,3]dioxole-5-carbaldehyde, 3% NaOH/methanol, −10 °C; (c) 3,4,5-trimethoxy-benzaldehyde, 3% NaOH/methanol, −10 °C; (d) NH_2NH_2, ethanol, rt.

Table 1. Experimentally determined R_M[a] values and theoretically calculated clog P[17] values; inhibition% of induced carrageenin rat paw edema (CPE%) at 0.01 mmol/ml/kg; in vitro inhibition of soybean lipoxygenase (LOX) (IC_{50})/% 100 μM

Compound	R_M	clog P[b]	CPE%[c]	LOX IC_{50} (% 100 μM)
2a	−0.582 (±0.02)	0.05	56.1*	<1 μM
2b	−0.645 (±0.02)	0.34	36.3*	85 μM
2c	−0.654 (±0.04)	0.31	21.3*	55.7% (100 μM)
3a	−0.579 (±0.02)	0.83	4.5*	31 μM
4a	−0.622 (±0.01)	0.17	55.4**	42 μM
5a	−0.355 (±0.03)	0.58	24.3**	67.3% (100 μM)
5b	−0.397 (±0.03)	0.87	23.4*	70 μM
IMA			47*	
Caffeic acid				600 μM

IMA, indomethacin.

[a] R_M values are the average of at least 10 measurements; each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

[b] Theoretically calculated values of lipophilicity.

[c] Statistical studies were done with Student's t-test, $^*p < 0.01$, $^{**}p < 0.05$.

and caffeic acid. The interaction of the examined compounds with the stable free radical DPPH was studied by the use of the stable 1,1-diphenyl-2-picrylhydrazyl radical DPPH at 0.05 and 0.1 mM after 20 and 60 min (Table 2). This interaction indicates their radical scavenging ability in an iron-free system. Compounds **2b** and **2c** interact with DPPH in a concentration and time dependent manner, whereas compounds **2a**, **3a**, and **4a** do not present any interaction at 0.05 mM. Slight differences are observed between the compounds **2c** and **5b** with the time and the concentration whereas compound **2a** presents reducing ability at 0.1 mM. The presence of the condensed ring –OCH_2O– (compound **3a**) diminishes the reducing activity. Preliminary QSAR[28] studies on the values of DPPH interaction have shown that the molar refractivity (MR) of substituents R^7 plays a significant role. Molar refractivity (MR) is related not only to the volume of the substituents but also to the London dispersive forces. Thus, high molar refractivity values of substituent R^7– (MR for R^7–OCH_3 = 0.78, MR for R^7–H = 0.103, and MR for R^7–F = 0.092) affect the reducing ability. No role for lipophilicity is defined.

The insertion of a pyrazolyl ring (compounds **5a** and **5b**) increases the reducing ability. The R^7 = –F substitution

Table 2. Interaction % with DPPH at 0.05 mM and at 0.1 mM

Compound	DPPH % 20 min, 0.05 mM	DPPH % 60 min, 0.05 mM	DPPH % 20 min, 0.1 mM	DPPH % 60 min, 0.1 mM	HO· % 0.1 mM	PMS % 0.1 mM
2a	No	No	39.4	56	100	12.5
2b	36.4	38.4	45.3	52	99.7	No
2c	45	59	51	70.4	97.3	No
3a	No	No	1	3	96	No
4a	No	No	No	No	99	100
5a	42	46	31	38	93.4	No
5b	58	67	51.6	68	99.3	No
NDGA	68	72	81	82.6		
CA acid						45
Trolox					73.4	

Competition % with DMSO for hydroxyl radical (HO· %); superoxide radical scavenging activity (PMS %).
No, no result under the experimental conditions; NDGA, nordihydroguaiaretic acid; CA acid, caffeic acid; each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

in compound **5b** is correlated with an increase compared to compound **5a**. Further investigations are in progress in order to have a detailed structure–activity relationship study on their interaction with DPPH.

It is consistent that rates of reactive oxygen species (ROS) production are increased in most diseases.[14] Cytotoxicity of $O_2^{-}\cdot$ and H_2O_2 in living organisms is mainly due to their transformation into ·OH, reactive radical metal complexes, and $^1O_2$. During the inflammatory process, phagocytes generate the superoxide anion radical at the inflamed site and this is connected to other oxidizing species as ·OH. Hydroxyl 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 compounds with dimethylsulfoxide (DMSO) for OH radicals,[27,29] generated by the Fe^{3+}/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. All the tested derivatives show high inhibition of DMSO (33 mM) oxidation at 0.1 mM (Table 2). Lipophilicity is not well correlated with the results. 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.

Non-enzymatic superoxide anion radicals were generated.[29] The superoxide producing system was set up by mixing phenazine methosulfate (PMS), nicotinamide adenine dinucleotide NADH and air–oxygen. The production of superoxide was estimated by the nitroblue tetrazolium method. The majority of the compounds does not present scavenging activity at 0.1 mM (Table 2), with the exception of compound **4a** (100%) which is the most potent.

The antiradical activity of the tested compounds supports, at least in part, the in vivo anti-inflammatory activity.

Further investigation is in progress to delineate the physicochemical properties implicated in the in vivo response. 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 found to be poor.

For the in vivo results the following equation was derived:

$$\log\%(\mathrm{CPE}) = -1.312(\pm 0.797)\mathrm{clog}P + 1.902(\pm 0.367)$$
$$n = 6, \quad r = 0.916, \quad r^2 = 0.839, \quad q^2 = 0.485,$$
$$s = 0.183, \quad F_{1,4} = 20.932, \quad \alpha = 0.05$$

Hydrophilicity (lipophilicity with negative sign) is the most significant parameter. Compound **5b**, the more lipophilic, is not included in the regression. This fact proceeds in parallel to the observation that low lipophilicity is highly involved to the biological response. Attempts to correlate the in vivo/in vitro expressions of activity with R_M values in a linear or non-linear regression analysis gave statistically non-significant correlations. Unfortunately the number of compounds is not enough to calculate a combination of all the effects.

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