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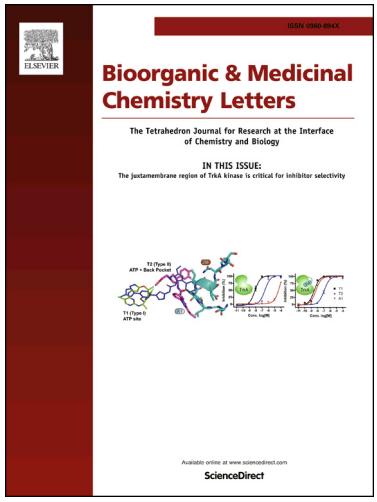

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Easy and rapid preparation of benzoylhydrazides and their diazene derivatives as inhibitors of 15-lipoxygenase

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ABSTRACT

Two series of diaza derivatives were prepared by solvent-free condensation of benzoic acid and 4-substituted phenylhydrazines in order to obtain phenylhydrazides (HYD series) and, by oxidation of these compounds, the corresponding benzoyldiazenes (DIA series). Both sets were evaluated as inhibitors of soybean 15-lipoxygenase activity and antioxidant capability in the FRAP and CUPRAC assays. The most potent inhibitors of both series exhibited IC_{50} values in the low micromolar range. Kinetic studies showed that at least the more active compounds were competitive inhibitors. Docking results indicated that the most potent inhibitor interacts strongly with Ile-839 and iron in the active site.

Lipoxygenases (LOXs) catalyze the production of eicosanoid leukotrienes and lipoxins, both biosynthesized from arachidonic acid (AA)[1] derived from the cell membrane, and important biological mediators of inflammatory processes.[2] This family of enzymes is well conserved among mammalian species[3] and catalyzes stereo- and regiospecific introduction of dioxygen into the polyunsaturated chain of AA as hydroperoxide, which finally is converted to the hydroxyl group.[4] Due to this activity, the name of this enzyme is often preceded by the number of the carbon atom in the AA chain which undergoes oxidation. Thus, using the usual acronym, 5-LOX, 12-LOX and 15-LOX isoforms introduce an oxygen atom at positions C-5, C-12 and C-15 respectively.[5]

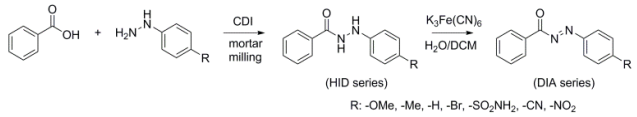
Some benzothiophene derivatives and esters of caffeic acid have been reported to be potent inhibitors of 5-LOX. Examples of these are the commercial Zileuton (IC_{50} = 0.4 μM)[6] and CAPE (IC_{50} = 0.13 μM).[7] Likewise, some phenylhydrazones inhibit the dual cyclooxygenase/peroxidase activity of prostaglandin synthase,[8] phenylhydrazide derivatives selectively inhibit cycloxygenase-2,[9] and pyrazole carboxamides inhibit 15-lipoxygenase-1[10,11] but until now we are not aware of any systematic studies of these compounds as 15-LOX inhibitors. The same is true for the related diazene derivatives.

During the last decades a growing interest has arisen to develop new, greener synthetic strategies whereby the amount of residual waste becomes progressively smaller, and a many such reactions can be found in the literature. An interesting example of a green oxidation of arylhydrazides was proposed by Hashimoto,[12] where a phtalocyanine was used as the oxidant. Another example of a green strategy applicable to our work was reported by Metro,[13] who prepared different amide derivatives by mechanochemical means. Specifically, the latter procedure was used to obtain the first set of compounds in this research.

The aim of the present work was to study the effects of two different structural features on the inhibitory behavior of two families of inhibitors: the influence of the oxidation of the central nitrogen-nitrogen single bond of phenylhydrazides, and the systematic substitution of the *para* position of the *N*-phenyl moiety. Both series proposed here were prepared using short and clean procedures, and some of the resulting compounds proved to be fairly potent inhibitors of 15-LOX.

Scheme I shows the synthesis of both series. The first step is the preparation of phenylhydrazide derivatives (HYD series) where both moieties, hydrazine and benzoic acid, where connected by a solvent-free reaction carried out by grinding the reactants in a simple mortar. The oily material prepared by this method was rinsed with pure water affording a white or yellowish white solid that was crystallized in ethanol to obtain fine needles in almost every case. This procedure is an environmentally friendly coupling reaction, with easy recovery of the desired compounds and negligible formation of side products. The second step in Scheme I is the oxidation of the former substances with potassium ferricyanide[14]. This reaction was carried out by shaking two immiscible solutions, one containing the substituted hydrazide dissolved in dichloromethane, and the other, the ferricyanide in a strongly basic aqueous solution. Using

this simple experimental setup, the formation of diazene is easily detectable by the change of color in the organic phase from colorless to red, keeping the inorganic iron in aqueous solution. Further purification of the oily red products was not needed. Both reactions represent simple and clean methods for the rapid preparation of phenylhydrazides and their phenylcarbonylazo analogs in high yields.



Scheme I. Synthesis of phenylhydrazide derivatives (HYD series) and azocarbonyl derivatives (DIA series).

To evaluate the ability of both sets of compounds to inhibit the activity of 15-LOX an initial screen was performed, whose results are shown in Table 1.

Table 1. Screening of 15-LOX inhibition by HYD and DIA series at 10 μM inhibitor. *DIA-4SA (sulfamide) not evaluated due to poor solubility.

Inhibitor	Inhibition (%)	Inhibitor	Inhibition (%)
HYD-4OMe	73 ± 3.3	DIA-4OMe	19 ± 4.6
HYD-4Me	93 ± 5.3	DIA-4Me	44 ± 7.8
HYD-4H	33 ± 5.5	DIA-4H	87 ± 7.6
HYD-4Br	28 ± 2.2	DIA-4Br	44 ± 3.8
HYD-4SA	10 ± 2.2	DIA-4SA	*
HIYD-4CN	9 ± 1.6	DIA-4CN	80 ± 4.1
HYD-4NO_2	17 ± 2.1	DIA-4NO_2	34 ± 4.6

The comparison of inhibition values would seem to indicate that, in the HYD series, an electron donor group at the *para* position increases and an electron attractor decreases activity, as is particulary noticeable with methyl and methoxyl substitution. When the central nitrogen-nitrogen bond is oxidized to diazene, this trend seems to be reversed, although the electron-withdrawing *para*-cyano group has no apparent effect on the inhibitory activity. This behavior is more evident when the values of inhibition for each HYD and its DIA counterpart are compared. If the inhibitory activity is mediated by hydrogen bond formation in the enzyme's active site, it may be supposed that there is a correlation between the effect of the electron donor group and the basicity of the secondary amine in the inhibitor's dinitrogen bridge. This tendency indicates that arylhydrazide derivatives with electron donating aryl groups are better candidates for the design of 15-LOX inhibitors. In contrast, although DIA-4H and DIA-4CN are practically as potent as HYD-4Me and HYD-4OMe, the screening data do not suggest a design strategy for additional diazene analogs, although the smaller size of the cyano group (E_s = -0.51) as compared to methyl and bromine (E_s = -1.24 and -1-34 respectively)[18] might explain the strong inhibition by DIA-4CN.

The most potent 15-LOX inhibitors in our series were chosen to obtain their IC_{50} values and kinetic parameters (Table 2).

Table 2. Inhibition and kinetic constants for 15-LOX inhibition by HYD and DIA compounds.

Inhibitor	IC_{50} (μM)	K_i (μM)	K_m (μM)	V_{max} (μM/s)	Type of Inhibition
HYD-4Me	5.35 ± 0.80	0.81	89.6	0.46	competitive
DIA-4H	6.97 ± 0.59	N.D	N.D	N.D	-
DIA-4CN	9.78 ± 0.72	3.89	139.8	0.57	competitive

The most potent inhibitor of the series (HYD-4Me), shows an IC_{50} value five and three times higher than the well-known inhibitors boswellic acid (IC_{50} = 1 μM)[15] and baicalein (IC_{50} = 1.6 μM),[16] respectively, but it is 3.4 times more potent than the commercial 15-LOX inhibitor 4-methyl-2-(4-methylpiperazinyl)-pyrimido[4,5b]benzothiazine.[17] The diazene derivatives DIA-4H and DIA-4CN inhibit 15-LOX to a somewhat lesser extent but in the same range as HYD-4Me. Even though the IC_{50} values of these compounds lie far from the desirable nanomolar range, they incorporate good molecular scaffolds that could be used to introduce a broad variety of structural modifications taking advantage of their simple synthesis and the ready availability of many differently substituted benzoic acids and arylhydrazines.

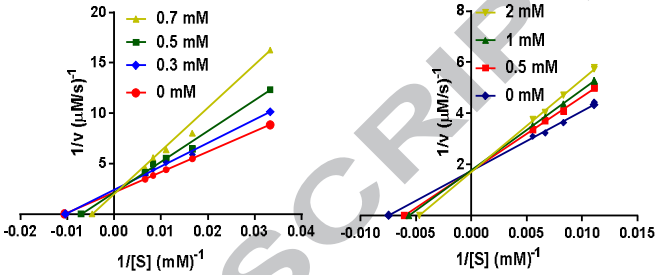


Figure 1. Lineweaver-Burk plots for two representative 15-LOX inhibitors of the HYD and DIA series. Left, HYD-4Me and right, DIA-4CN.

To study the type of inhibition induced by both series of compounds, HYD-4Me and DIA-4CN were selected (Figure 1). The Lineweaver-Burk plots indicate that the inhibition was competitive for both derivatives, suggesting that these inhibitors compete with arachidonic acid to reach the active site without affecting the theoretical maximum rate of conversion to product. The active site of this oxidoreductase enzyme has a non-heme iron atom which plays a key role in the introduction of a hydroxyl group at the C-15 position of the fatty acid by generating the allyl radical in the fatty acid chain and subsequently stabilizing the hydroperoxide radical formed by addition of molecular oxygen.[19] With these results, it is possible to hypothesize that the inhibitor blocks the access of arachidonic acid to the active site by generating specific interactions with the iron and/or an amino acid residue present in the active site of 15-LOX, lowering the K_m. As an approach to understand the likely molecular interactions in the environment of the metal we built molecular models of possible complexes with the inhibitors (Figure 2). Our results indicate that both inhibitors are able to reach the active site of 15-LOX, which is in agreement with the competitive inhibition found by kinetics experiments. A detailed analysis of the models shows HYD-4Me establishing basically two different interactions. One of these arises between the aromatic amine NH group and the iron atom (3.6 Å). The second interaction found is a hydrogen bond (2.3 Å) between the hydrogen of the amide NH group and the carboxyl of the C-terminal amino acid residue Ile-839. It is interesting to note that if hydrogen bonding contributes to the strength of binding in the active site, and/or if subsequently this capability is enhanced by electron donor substituents at the *para* position of the phenyl ring, it would be possible to determine some grade of correlation between the presence of the substituents and the effective acidity parameter $\Sigma\alpha_2^H$ of Abraham,[20] which indicates precisely the ability to donate a hydrogen atom toward a hydrogen acceptor. In order to evaluate which interaction would be mainly responsible for the inhibition, the series of 4-substituted anilines proposed by Abraham was taken as a model due to their structural analogy with the phenylhydrazide derivatives of this work. In the aniline series, higher values of acidity are observed for 4-chloroaniline (0.30) and lower values for 4-methoxy- and 4-methylaniline

(0.23), which means that electron donor groups weaken the hydrogen donating ability. This tendency is opposite to the behavior observed in the HYD series, which seems to suggest that the higher affinity for the active site of the phenylhydrazides with electron donor groups obeys mainly to complex formation between the NH group and iron rather than hydrogen bond formation between -N´H- and Ile-839.

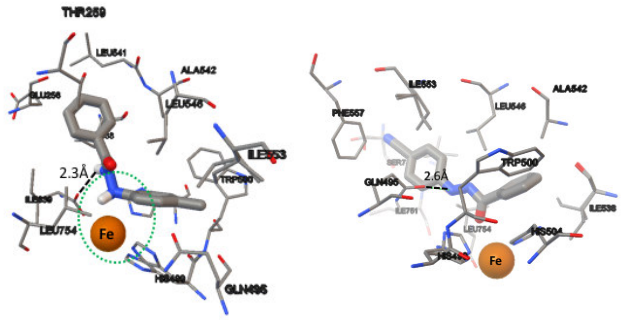


Figure 2. Enzyme-inhibitor complexes and main molecular interactions in the 15-LOX binding site obtained by docking calculations with HYD-4Me (left) and DIA-4CN (right).

In the case of DIA-4CN the docking results do not show any interaction between the iron atom and the inhibitor, presumably due to the reduced ability to form a complex involving the -CON=N- moiety versus the bidentate -CONHNH- central group of HYD-4Me. This results in a considerable gap between the isoleucine-histidine-iron complex and the diazene inhibitor, associated with the benzoyl moiety's approach to the hydrophobic loop in the catalytic domain of LOX.[21] This seems reasonable based on the increased hydrophobic character of the diazene. On this basis we can suggest that iron complexation by HYD-4Me is important for its 15-LOX inhibitory activity, while DIA-4CN acts primarily by blocking the access of the substrate to the α-helical domain of the catalytic site. The docking energies support this notion, as the interaction with HYD-4Me (-4.67 kcal/mol) is stronger than with DIA-4CN (-3.66 kcal/mol).

Because the active site of this oxidoreductase contains a redox active iron atom which is the key to the initial radical generation from the at least doubly unsaturated arachidonic (or linolenic) acid, two different antioxidant capability assays were carried out. These are based on the change of oxidation state of iron and copper, namely, the Ferric Reducing Antioxidant Power (FRAP)[22] and Cupric Reducing Antioxidant Capacity (CUPRAC)[23] assays respectively. The results for the HYD and DIA series are shown in Figure 3.

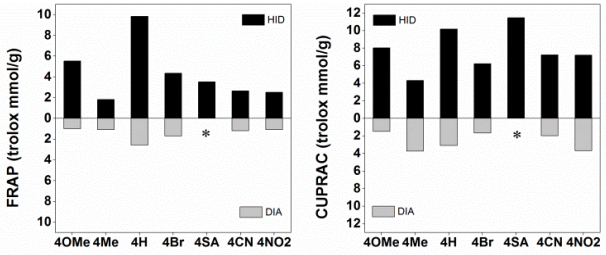


Figure 3. Antioxidant capacity of the HYD and DIA series evaluated by: left, Ferric Reducing Antioxidant Power (FRAP) assay, and right, Cupric Reducing Antioxidant Capacity (CUPRAC) assay. *Not evaluated due to poor solubility.

The same marked difference of antioxidant capacity of the HYD and DIA series shown by different methods indicates beyond doubt that the HYDs are much better able to reduce the active Fe(III) to the inactive Fe(II). Secondly, the differences of antioxidant capacity values for different compounds of both series are not significant when the CUPRAC and FRAP methods are analyzed. In spite of this, it is interesting to point out the greater antioxidant capacity exhibited by the unsubstituted phenylhydrazide (HID-4H) in both assays.

Since metal reductive capability is not related to the enzyme inhibitory potency, it seems that some other property should enhance activity in the DIA series. So far we have considered the electronic nature of the substituent at the *para* position, azo bond formation, the hydrogen bonding ability of the -NH- group, and now the antioxidant capacity of the inhibitors. Due to the hydrophobic nature of the catalytic domain, it would come as no surprise if the hydrophobicity of the inhibitor plays a crucial role in accessing the iron-histidine complex. To evaluate this effect, both series were subjected to a preliminary assessment using the ALOGPS 2.1 software,[24] The results are shown in Figure 4. From these, it is noteworthy that dehydrogenation of the central CONH-NH- moiety of the HYD inhibitors enhances their lipophilicity by about two log units. This remarkable difference might explain the behavior of DIA-4CN found by docking calculations, where this compound is unable to reach the metallic center of catalytic domain due to hydrophobic interactions between its extended conjugated system and the lipophilic helix in the active site. Although the 15-LOX inhibition by both families of compounds is competitive, the molecular interactions involved occur at different loci in the active site of the enzyme. For the sake of comparison, log *P* values were calculated with the same software for competitive inhibitors of similar molecular weight, ebselen,[25] zileuton,[26] baicalein,[16] and ML351.[27] The figures obtained were 1.91, 2.01, 3.19 and 4.57 respectively, in the same range as the lipophilicities of the inhibitors studied here (log *P* ca. 2-4), suggesting that such values are appropriate for the design of new 15-LOX inhibitors.

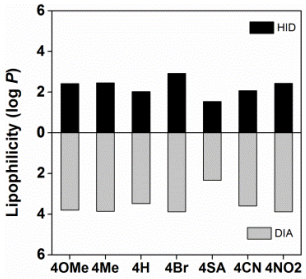


Figure 4. Lipophilicity of the HYD and DIA series calculated using ALOGPS 2.1 software.

In conclusion, two sets of dinitrogen derivatives, the HYD and DIA series, were prepared by "one pot", two-phase reactions by clean and easy procedures. This methodology is environmentally friendly and provides the desired products in high yields. Both series of compounds were shown to be low micromolar competitive 15-LOX inhibitors. The biochemical results show that the more potent HYD derivatives are those that bear an electron donor *para*-substituent, but no such relationship is clear in the DIA series. These differences were interpreted tentatively on the basis of docking calculations for two representative molecules (HID-4Me and DIA-4CN) indicating that compounds belonging to each of the two series may interact with different loci of the same active site. Besides, the combination of moderate lipophilicity with a greater propensity of the HYD series to reduce the active Fe(III), suggests the synthesis of new analogs that could be good leads for the design of useful 15-LOX inhibitors.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Graphical Abstract

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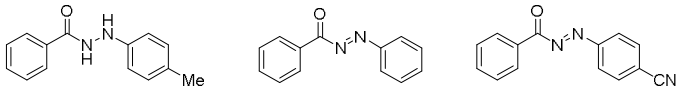

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