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INHIBITORY EFFECT OF ARYL THIENYL-KETONES AND -THIOKETONES ON ARACHIDONIC ACID-INDUCED MALONDIALDEHYDE FORMATION IN HUMAN PLATELETS: BIOLOGICAL DATA AND MOLECULAR MODELLING

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A series of anti-thrombotic aryl thienyl-ketones and -thioketones was assayed *in vitro* for their inhibitory effect on malondialdehyde (MDA) production induced by arachidonic acid in human platelets. For several compounds MDA formation was strongly inhibited indicating that the anti-platelet target was situated on the *cyclooxygenase* pathway. A comparison between the inhibition constant K_i and the IC_{50} values revealed *competitive inhibition* kinetics. The molecular structure of one active compound was analysed by X-ray diffraction and theoretical calculations to provide information on its electronic and lipophilic properties.

KEY WORDS: Arachidonic acid, Malondialdehyde, Aryl thienyl ketones, Aryl thienyl thioketones, cyclooxygenase, prostaglandin synthase.

INTRODUCTION

In previous communications[1,2] we described the synthesis and the pharmacological activities of a series of aryl thienyl-ketones and -thioketones (Figure 1). Some of these compounds have been reported to possess a very potent anti-aggregating activity, *in vitro*, on human platelets. However the mode of action of these derivatives has not yet been established. Various reports indicate that multiple mechanisms may exist to explain the anti-thrombotic effects of drugs; some seem to act on platelet cyclic AMP level (methylxanthines, pyrimido-pyrimidine compounds)[3–5] whereas imidazole derivatives are thromboxane synthetase inhibitors,[6] but most anti-platelet drugs (acetylsalicylic acid, sulfinpyrazone and non-steroidal anti-inflammatory compounds) inhibit prostaglandin biosynthesis. The target for the latter inhibitors is the *cyclooxygenase* through different types of inhibition, i.e., *reversible competitive* (most of the common non steroidal anti-inflammatory derivatives),[7] or *irreversible competitive* (acetylsalicylic acid, which acetylates a serine residue in the active site).[8]

Inhibition of prostaglandin synthesis by phenolic compounds has been reported.[9] However, the mechanism for inhibition of cyclooxygenase by these substances is still

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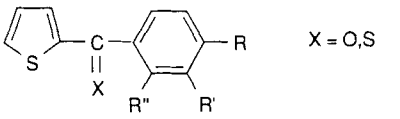


FIGURE 1.

unclear. Phenolic compounds may act as anti-oxidants in a non specific way.[10] It has also been suggested that these inhibitors may compete with arachidonic acid for the enzymatic active site.[11]

In order to establish the thrombocyte target for aryl thienyl-ketones and -thioketones, these compounds were examined for their inhibitory effect on prostaglandin biosynthesis, with regard to the platelet cyclooxygenase. The three dimensional structure of the most active compound (**6**) was analyzed by X-ray diffraction with the aim of providing an understanding of the structure-activity relationships.

MATERIALS AND METHODS

Chemicals

Sodium arachidonate (grade 99% purity) was from SIGMA (USA) and was stored at −20°C in aqueous solution; acetylsalicylic acid and tris (hydroxymethyl) aminomethane (Tris buffer) were from FLUKA (Switzerland). All other reagents and solvents were from PROLABO (France).

The test compounds were obtained according to the methods reported previously[1]. Dimethylsulfoxide was used as a solvent for both test compounds and reference substance (acetylsalicylic acid): the amount (20 μl) of dimethylsulfoxide added to the medium showed no effect on cyclooxygenase activity.

Platelet Cyclooxygenase Activity

Platelets in their own plasma were used as the source of cyclooxygenase. The measurement of malondialdehyde (MDA) production in platelet rich plasma after addition of sodium arachidonate has been proposed by various workers[12,13] as a good marker for the cyclooxygenase activity.

Preparation of Platelet Rich Plasma (PRP) Platelets were obtained from healthy donors who were selected on the basis of abstention from any medication for 14 days prior to the test. Human blood was diluted with 3.8% aqueous sodium citrate solution (9 ml of blood/1 ml of sodium citrate). Blood was then centrifuged at 150 g for 15 min at room temperature and PRP was pipetted from the packed erythrocytes. Platelet number was determined using a Coulter Counter and adjusted to 4.8×10^5 ($\pm 5 \times 10^4$)/μl.

MDA Assay: Measurement of K_i and IC_{50} Different concentrations of the test compounds in dimethylsulfoxide (DMSO) (20 μl) were incubated for 10 min with 2.92 ml PRP at 37°C with constant stirring (1000 rpm). In another experiment, incubation was performed with 20 μl pure DMSO as reference sample. Samples were

then challenged with 60 μl of sodium arachidonate (30 or 120 mM; final concentrations, 0.6–2.4 mM) for 2 min. The reaction was stopped by addition of 1 ml of trichloroacetic acid solution (40% w/v). After vigorous shaking, the mixture was centrifuged at 800 g for 15 min; 2 ml of the supernatant were added to 0.45 ml of a solution of thiobarbituric acid (0.12 M) in tris–HCl (0.26 M; pH = 7) and the mixture was heated on a boiling water-bath for 15 min. After cooling to room temperature, the absorption was read at 532 nm.

The % changes in MDA production (nmol/1.4 $\times$ 10^9 platelets) were calculated with respect to appropriate controls (i.e., dilution liquids without drug). For each compound, the inhibitor concentration which caused 50% inhibition (IC_{50}) of MDA formation was calculated by plotting the percentage inhibition values versus the concentration of inhibitor. The inhibitory constant (K_i) was determined by the method of Dixon[14] by plotting MDA (nmol/1.4 $\times$ 10^9 platelets/min.)$^{-1}$ production against inhibitor concentration.

Crystal Structure Analysis

Suitable crystals of compound **6** for X-ray analysis were obtained by slow evaporation of a solution in ethanol.

$$C_{13}H_{12}O_4S, \quad M = 264.30, \text{ monoclinic, space group } P2_1/c,$$
$$a = 11.223(2), \quad b = 8.442(1), \quad c = 13.472(3)\,\text{Å}, \quad \beta = 105.20(1)°,$$
$$V = 1231.68(2)\,\text{Å}^3, \quad Z = 4, \quad D_c = 1.43\,\text{g cm}^{-3}.$$

A crystal of dimensions 0.30 $\times$ 0.20 $\times$ 0.13 mm was selected from the recrystallized material. Intensity data were collected on a fully automated Enraf-Nonius CAD-4 diffractometer using graphite monochromated Cu K_α radiation ($\bar{\lambda}$ = 1.54178 Å). 1821 symmetry-independent reflections up to $\theta = 60°$ were measured of which 1163 significant diffraction maxima [$I \geqslant 3\,\sigma\,(I)$] were used in the refinements. Data were corrected for Lorentz and polarization effects but not for absorption.

The structure was solved by a combination of direct methods using MULTAN 80[15] and Fourier techniques. It was refined by block-diagonal least squares, initially with isotropic then with anisotropic thermal parameters. Hydrogen atoms were introduced in theoretical positions or in positions derived from difference Fourier synthesis, and were refined with isotropic thermal parameters. The final R factor was 0.034.

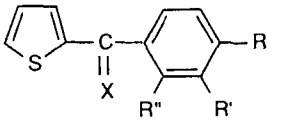
Conformational Analysis

The calculations of conformational energies and energy-minimized geometries were performed with the MM2-(85) molecular mechanics program developed by Allinger,[16] slightly modified by Still in the Macromodel Molecular Modeling System.[17] The construction of input structure for the molecular mechanics calculations was performed with Macromodel.

Electronic Properties Analysis

Atomic charges were calculated with the semi-empirical quantum mechanics method CNDO/2[18] available in CHEM-X.[19] The molecular electrostatic potential (MEP) pattern was calculated with the VSS[20] program available in CHEM-X.

TABLE I
Inhibition of arachidonic acid-induced malondialdehyde formation in human platelets



Compounds	*X*	*R"*	*R'*	*R*	IC_{50}[a] μM
1	O	H	H	OCH_3	28
2	O	H	OCH_3	OCH_3	49
3	O	H	Cl	OCH_3	73
4	O	Cl	Cl	OCH_3	53
5	O	OCH_3	OCH_3	OCH_3	58
6	O	OH	OCH_3	OCH_3	17
7	O	OH	H	OCH_3	23
8	O	OH	H	OH	102
9	S	H	H	OCH_3	36
10	S	H	OCH_3	OCH_3	b
11	S	H	Cl	OCH_3	200
12	S	Cl	Cl	OCH_3	c
Acetyl salicylic acid					30

[a] IC_{50} values were determined from the mean curve of percent inhibition against concentration. The mean curve was obtained from three determinations per concentration.
[b] No activity observed in assay conditions.
[c] At 200 mM concentration, this compound showed 26% inhibition.

Lipophilic Properties Analysis

The molecular lipophilicity potential (MLP) pattern was calculated using the in-house MLP program.[21]

RESULTS

Biological Data

Twelve compounds were evaluated *in vitro* for their effect on platelet malondialdehyde production. Acetylsalicylic acid was included in the tests as reference substance. The results are reported in Table I. The strong inhibitory effect of acetylsalicylic acid on platelet MDA production is in agreement with that previously reported.[22,23]

Among the compounds tested the most active derivatives belonged to the *ketone* series: compounds **1**, **6** and **7** were slightly stronger inhibitors than acetylsalicylic acid itself. With the exception of compound **9**, the *thioketone* derivatives were inactive (**10**) or gave only mild inhibition of MDA production (**11**, **12**).

Two structural requirements on the phenyl ring seem to be involved in determining the potency of the inhibitors on MDA production: the presence of an hydroxy group in the *ortho* position *and* the presence of a methoxy group in the *para* position. Chemical modifications with reference to these substituents decrease biological activity: thus, methylation of the 2-OH group (compare **6** and **5**) or its replacement by a

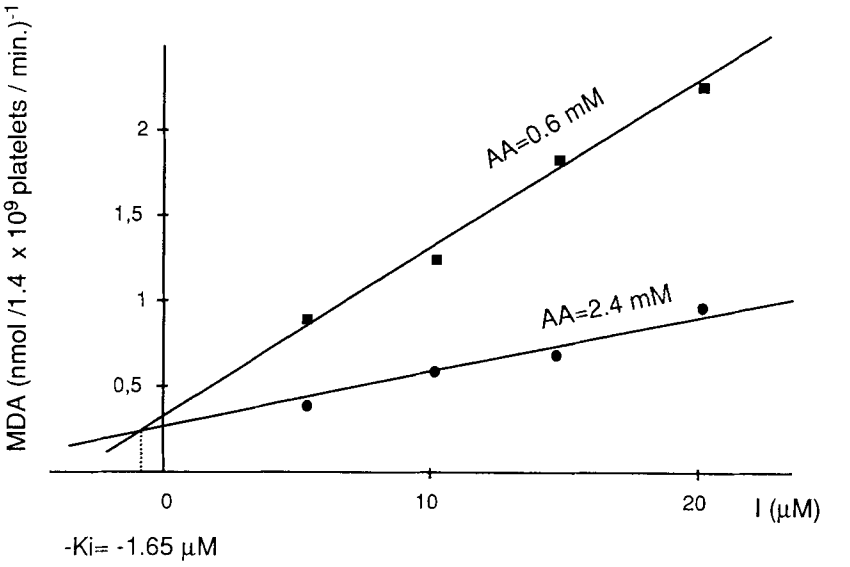


FIGURE 2 Dixon analysis of inhibition by compound **6** of platelet MDA formation under sodium arachidonate stimulation (each point represents the mean of three measurements). A similar plot was obtained with acetylsalicylic acid.

chlorine atom (compare **6** and **4**) or demethylation of the 4-OCH_3 group (compare **7** and **8**) result in a decrease in activity.

In order to elucidate the apparent type of inhibition displayed by these drugs a kinetic approach to MDA formation was undertaken according to a Dixon analysis. The results for the best inhibitor of the series (**6**) are shown in Figure 2. A Dixon plot obtained when PRP was incubated with different concentrations of drug before challenge with arachidonic acid afforded the inhibition constant (K_i). In our experimental conditions, the K_i value (1.65 μM) was much lower than the IC_{50} value (17 μM). This finding is in agreement with *competitive inhibition kinetics*.[24]

Crystal Structure Analysis of Compound **6**

A projection of the molecule showing the numbering of atoms is shown in Figure 3. The final coordinates for non-hydrogen atoms are listed in Table II.

Bond lengths and angles within the two rings have the expected values. Our interest focused on the bridge group C_{ar}–C(=O)–C_{ar}. Compared to the average Csp^2–C_{ar} value 1.481 Å,[25] the two C(7)–C_{ar} bonds are not equivalent: C(7)–C(1) = 1.454(4) Å being much shorter than C(7)–C(8) = 1.470(4) Å. The spatial arrangement of the thienyl and the phenyl rings is defined by the torsion angles S(12)–C(8)–C(7)–O(13) = 22(1)° and O(13)–C(7)–C(1)–C(2) = 18(1)°.

Finally the C=O and the hydroxy groups being on the same side of the molecule, there is an intramolecular hydrogen bond O(13). . .H(118)–O(18) with O(13). . . O(18) = 2.573(3) Å, O(13). . .H(118) = 1.79(4) Å and O(13). . .H(118)–O(18) = 148.1(2)°.

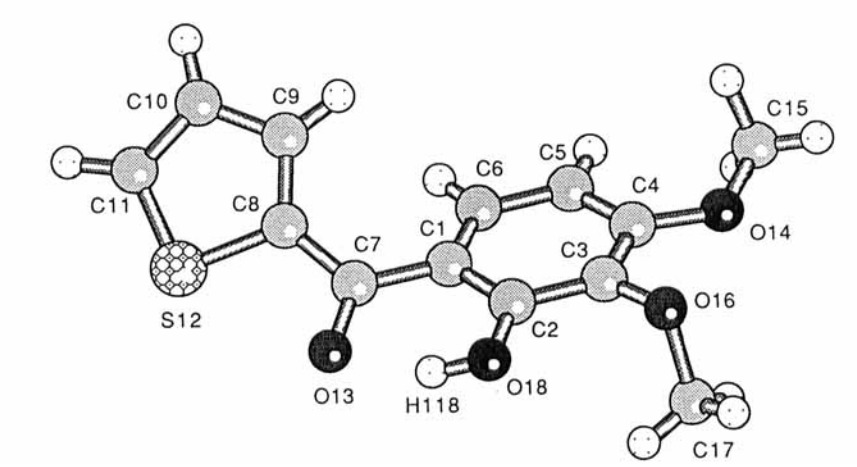


FIGURE 3 Projection of the molecule of **6**.

These results are in favour of a conjugation between the C=O group and the phenyl ring.

Conformational Analysis of Compound 6

The conformational behaviour of the molecule was examined in some detail. An energy-minimized conformation, close to the solid state conformation, was first obtained. Then we studied four degrees of conformational freedom i.e. rotations about the bonds connecting the two rings and rotations of the methoxy groups. Four low-energy conformations within 8 kJ have been obtained (Figure 4). In the first two conformations (**a**, **b**) the C=O and the hydroxy groups are on the same side allowing an intramolecular hydrogen bond as observed in the crystals. They differ by the orientation of the thienyl ring. In the last two conformations (**c**, **d**) the C=O and the hydroxy groups are on opposite sides of the molecule with two different orientations of the thienyl ring and two different orientations for the methoxy groups.

TABLE II
Final positions ($\times 10^4$) with estimated standard deviations in parentheses for compound **6**

Atoms	*x*	*y*	*z*	Atoms	*x*	*y*	*z*
C(1)	−456(2)	7980(3)	353(2)	C(10)	−3199(3)	5112(4)	−2071(3)
C(2)	251(3)	8374(3)	1354(2)	C(11)	−4269(3)	5874(5)	−2085(3)
C(3)	1531(3)	8450(3)	1579(2)	S(12)	−4066(1)	7310(1)	−1170(1)
C(4)	2111(2)	8242(3)	800(2)	O(14)	3358(2)	8401(3)	1088(2)
C(5)	1429(3)	7907(3)	−210(2)	C(15)	4024(3)	8047(5)	338(3)
C(6)	167(2)	7775(3)	−412(2)	O(16)	2230(2)	8655(2)	2577(1)
C(7)	−1790(3)	7837(3)	144(2)	C(17)	2265(3)	10246(4)	2965(2)
O(13)	−2350(2)	8423(3)	741(2)	O(18)	−287(2)	8654(3)	2127(1)
C(8)	−2508(2)	6943(4)	−749(2)	H(118)	−1086(31)	8672(42)	1856(26)
C(9)	−2179(3)	5721(4)	−1304(2)				

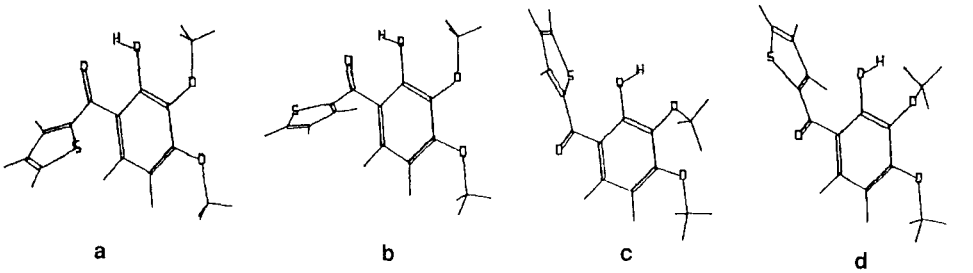


FIGURE 4 Low-energy conformations for compound **6**.

Electronic Properties of Compound ***6***

Figure 5 shows the 3-D electrostatic potential negative areas generated by the oxygenated groups over the molecule in its solid-state conformation and surrounding space. The thienyl ring is considered as a positive area.

Lipophilic Properties Analysis of Compound ***6***

Figure 6 shows the 3-D molecular lipophilicity potential over the molecule in its solid

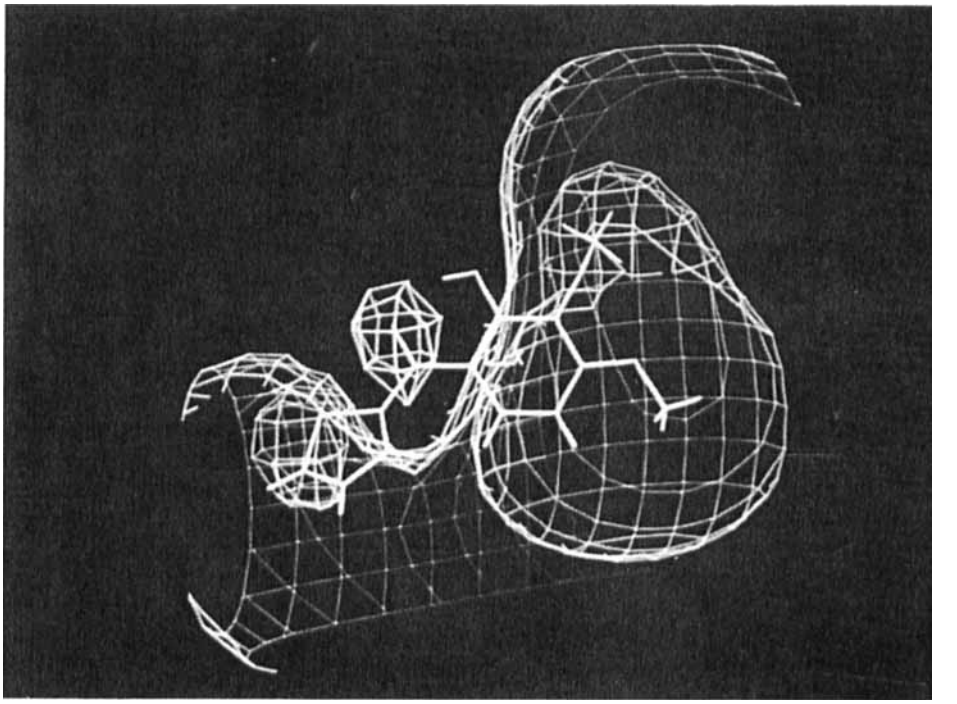


FIGURE 5 3-D electrostatic map generated with use of VSS from charges calculated by CNDO/2 with the QM interface within CHEM-X. Only negative areas ($-20\,\text{kcal mol}^{-1}$) have been depicted.

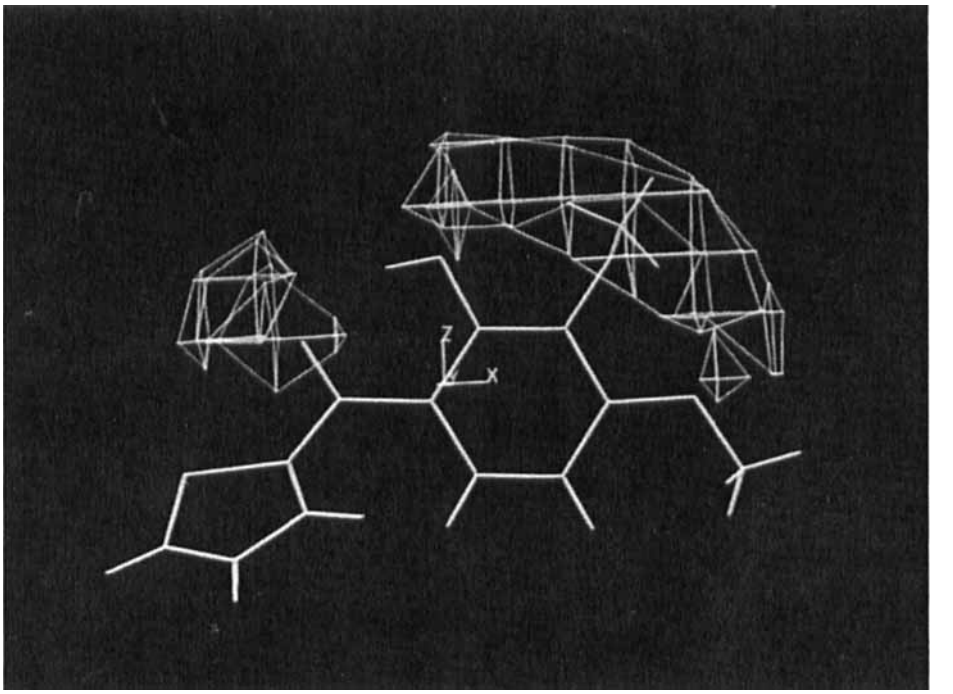


FIGURE 6 3-D molecular lipophilicity potential with use of MLP in-house program. There is a single hydrophilic region around the carbonyl group.

state conformation and surrounding space. A small hydrophilic region is concentrated on the carbonyl group and corresponds to a negative electrostatic potential area. A wide hydrophobic region presents two maxima, one corresponding to the thienyl ring and the other to the dimethoxyphenyl moiety. It should be noted that the methoxy groups induce a negative electrostatic potential but an hydrophobic area.

DISCUSSION

Prostaglandin H synthase catalyses two reactions: the bis-dioxygenation of arachidonic acid to form prostaglandin G_2 (*cyclooxygenase* activity), and the reduction of hydroperoxides to the corresponding alcohols (*peroxidase* activity). Although these two enzymatic activities seem to be localized on distinct functional areas on the surface of the synthase,[26,27] the topography of the cyclooxygenase site remains unclear. The models proposed by Scherrer[28] and by Gund and Shen[29] have a cationic binding region normally occupied by the carboxyl group of arachidonic acid which accommodates the arylacetic acids inhibitors. In the interpretation of Appleton and Brown,[30] based on the structural similarity between the peroxy radical precursor of prostaglandin G_2 and the non steroidal antiinflammatory drugs, the carboxyl group of the drug is assumed to bind to an oxygenation site of the enzyme. However, phenolic inhibitors are not accommodated well by all these models.

Our results for cyclooxygenase inhibition by aryl thienyl ketones indicate that the

potency of these drugs depends on the following characteristics:

(1) The hydroxyl group on the aromatic ring seems essential to generate a strong anti-cyclooxygenase activity, since the methoxy derivative **5** is less active than the corresponding phenol **6**. However, the non-hydroxyl compound **2** maintains part of the activity, as well as the chloro derivative **4**. This observation indicates that the hydroxyl group alone does not account for the inhibitory effect against cyclooxygenase. This strongly suggests that the phenolic group could act as an anchor by interaction with a binding site on the enzyme different from the catalytic site. Humes and coworkers,[31] like Rotilio and coworkers,[32] have proposed the existence of a supplementary binding site in addition to the catalytic site on the cyclooxygenase. This multiple enzymatic sites hypothesis could offer an explanation for the results of the present study.

(2) In compound **6**, the distance between the mass-centres of the thienyl and the phenyl rings is close to 5 Å. In fact, most of cyclooxygenase inhibitors are characterized by two ring systems interconnected directly or through a short "bridge".[33] According to Salvetti and coworkers,[34] these areas must be hydrophobic and non coplanar to fit the cyclooxygenase site; this feature constitutes a necessary condition for enzyme inhibition. The distances between these areas, estimated from the CPK models for cyclooxygenase inhibitors or calculated from X-ray data are in the range 4.5–5.6 Å.[35] Our results agree with the mean value and our calculations of MLP confirm the hydrophobic character of the two ring systems.

(3) In the crystals of compound **6** the methyl C atoms of the two methoxy groups are turned away from each other and are not coplanar with the benzene ring. However, this spatial arrangement does not seem responsible for the biological activity since it is observed both for the fairly active compound **2**, and for the inactive compound **10**.[38] In the most active compound **6**, the methoxy group in the 3-position is almost perpendicular to the phenyl ring, but MM calculations show no energy barrier and both methoxy groups can easily accommodate a planar arrangement with the phenyl ring (Figure 4). Comparison of biological results for compounds **7** and **8** shows that the 4-methoxy group is an important structural feature for inhibitory activity towards cyclooxygenase.

In summary, our work favours the hypothesis that aryl thienyl ketones react with at least two sites on cyclooxygenase: (a) *The 2-hydroxyl group* could allow the drug to fit on the enzyme. This interaction is considered to occur on a supplementary site distinct from the catalytic one and could enhance the stability of the drug-enzyme complex. (b) *The methyl of the 4-methoxy group* could display a steric effect by masking a residue close to the enzyme active site and contributes to the hydrophobic character of the molecule. The activity in the cyclooxygenase inhibition should be conditioned by previous anchoring of the drug through the phenolic hydroxyl and by suitable positioning of the 4-methoxy group.

CONCLUSION

The data obtained in these *in vitro* enzyme inhibition tests show that ortho-hydroxyaryl thienyl ketones constitute an interesting new series of anti-cyclooxygenase drugs which are distinct from the classical acidic inhibitors. The physico-chemical studies could partly explain the binding mechanism for the investigated compounds. Recent papers[36,37] studying the possible modes of interaction of benzoic and salicylic acids

with cyclooxygenase have shown that an electrostatic orientation effect seems to make an important contribution to the approach of inhibitor molecules toward their receptor site. Our results agree well with these findings.

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