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A class of pyrrole derivatives endowed with analgesic/anti-inflammatory activity

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ABSTRACT

We report the synthesis and bio-pharmacological evaluation of a class of pyrrole derivatives featuring a small appendage fragment (carbaldehyde, oxime, nitrile) on the central core. Compound **1c** proved to be extremely effective in vivo, showing an interesting anti-nociceptic profile that is comparable to reference compounds already marketed, hence representing a great stimulus for a further improvement of this class of molecules.

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent a chemically heterogeneous class of drugs which effectively reduce inflammation and relieve pain.[1] NSAIDs efficacy is related to their inhibitory effects towards cyclooxygenase (COX)-2-dependent prostanoids in inflamed peripheral tissues and in central nervous systems, such as spinal cord.[2]

However, variabilities in the analgesic and anti-inflammatory responses to NSAIDs have been detected, and it has been suggested that these variabilities are linked to both pharmacodynamic and pharmacokinetic features (i.e., the extent and duration of COX-2 inhibition at therapeutic plasma concentrations).[3] Approximately one third of the COX-2 selective inhibition attained by selective NSAIDs (named coxibs: such as rofecoxib and celecoxib), has been shown to be dependent on genetic sources of variance, for example, polymorphisms COX-1 and CYP2C9.[4] Both traditional NSAIDs (tNSAIDs) and coxibs are associated with a small, but consistent, increased risk of serious adverse events in the cardiovascular system while tNSAIDs are endowed with enhanced risk of upper gastrointestinal bleeding.[5,6] The degree of COX-2 selectivity assessed in vitro may predict enhanced cardiovascular hazard while that of COX-1 may predict the gastrointestinal toxicity.[5–7] However, other determinants may influence the hazards associated with the use of NSAIDs in patients, such as pharmacokinetic features (i.e., $t_{1/2}$), administered doses and genetic background.[5,8,9] In our continuing research program aimed at developing novel anti-inflammatory agents, we reported several studies on the design, synthesis and activity of pyrrole-based COX-2 inhibitors.[10–22] Structure–activity relationships of these compounds underlined the side chain as a crucial parameter for activity.

In order to further analyse the relationship between side-chain dimension and activity/selectivity, we became interested in the work by Khanna et al. describing the synthesis and biological evaluation of several pyrrole-based compounds, characterised by the

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; (t)NSAIDs, traditional NSAIDs; COX, cyclooxygenase; HWB, human whole blood; AA, arachidonic acid; LPS, lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; FBS, fetal bovine serum; RIA, radioimmunoassay; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2.

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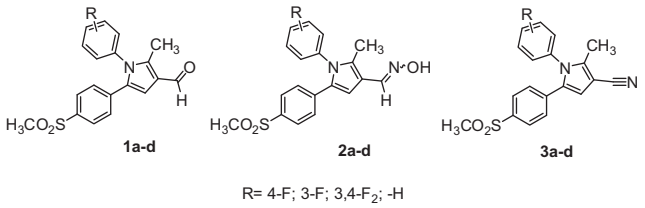


Figure 1. Structures of diphenyl pyrroles **1a–d**, **2a–d** and **3a–d**.

presence of small side chains.[23] Prompted by the stimulus of achieving a deeper knowledge on this class of small-side-chained compounds, we synthesised aldehydes **1a–d**, oximes **2a–d** and nitriles **3a–d** as potential tools for further developing the anti-inflammatory agents armamentarium (Fig. 1).

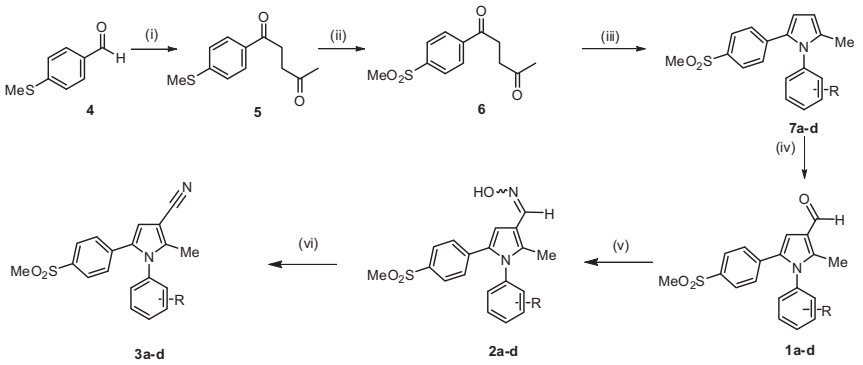
2. Results and discussion

2.1. Synthesis

The synthesis commenced with a Stetter umpolung between 4-methythiobenzaldehyde and methyl vinyl ketone, followed by oxidation of the sulfur by means of Oxone®; the pyrrole core was obtained through a Paal–Knorr condensation (Scheme 1).[10] Compounds **1a–d** were obtained in good yields and good regioselectivity (formation of the C4 regioisomer ranging from 2% to 5% determined on the crude material by ^{1}H NMR) via selective C3-formylation using the Vilsmeier's reagent (the regiochemistry of substitution was established by NOE studies). The material obtained was of sufficient puriprty to progress the synthesis through the next stage. Synthesis of oximes **2a–d** was accomplished by reacting carboxaldehydes **1a–d** with hydroxylamine chloride in the presence of sodium acetate in ethanol/water. After refluxing the mixture for one and a half hour, cooling down the reaction mixture caused precipitation of the oximes **2a–d** as white fine crystalline materials that could be isolated in very good yields. Dehydration of oximes to nitriles **3a–d** was achieved by reacting oximes **2a–d** with a solution of 2,4,6-trichloro[1,3,5]triazine in dimethylformamide for 8 h.[24] Chromatographic purification, followed by recrystallization gave target compounds **3a–d**.

2.2. Biological and pharmacological studies

All the compounds were tested in vitro at different concentrations up to 10 μM, to assess their inhibitory activities towards both cyclooxygenases (COX-1 and COX-2) expressed in the murine monocyte/macrophage J774 cell line. Compound selectivity toward COX-2, referred to as selectivity index (SI), is defined as the ratio of



Reagents and Conditions: (i) CH_2=CHCOMe, TEA, 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide, MW, 15min; (ii) Oxone, MeOH/H_2O, rt, 2 hrs; (iii) $RPhNH_2$, p-toluensulfonic acid, EtOH, MW, 45 min; (iv) Vilsmeier reagent, CH_2Cl_2, 0 °C then reflux 1h; (v) hydroxylamine, EtOH, CH_3COONa, H_2O, rt, 1.5 hrs; (vi) TCT, DMF, 8hrs.

Scheme 1. Synthesis of title compounds.

Table 1
In vitro COX-1 and COX-2 inhibitory activities of **1a–d**, **2a–d** and **3a–d** and celecoxib

Compound	*R*	IC_{50} (COX-1)[c] (μM)	IC_{50} (COX-2)[c] (μM)	COX-1/COX-2[d] (SI)
1a[a]	4-F	>10	0.0340	>294.1
1b	3-F	>10	0.0095	>1052.6
1c	3,4-F_2	>10	0.0700	>142.8
1d	–H	>10	0.0340	>294.1
2a	4-F	>10	0.3600	>27.8
2b	3-F	>10	0.4400	>22.7
2c	3,4-F_2	>10	0.1600	>62.5
2d	–H	>10	0.1900	>52.6
3a[b]	4-F	>10	0.0290	>344.8
3b	3-F	>10	0.0130	>769.2
3c	3,4-F_2	>10	0.0022	>454.5
3d	–H	>10	0.3600	>27.8
Celecoxib	–	3.84	0.0610	62.9

[a] Khanna et al. reported 3.23 μM (IC_{50}) towards COX-2 isoform.
[b] Khanna et al. reported 0.75 μM (IC_{50}) towards COX-2 isoform. In both cases Khanna performed the experiment by testing compounds **1a** and **3a** against purified human COX-1 and COX-2.
[c] Results are expressed as the mean (n = 3 experiments) of the percentage inhibition of PGE_2 production by test compounds with respect to control samples and the IC_{50} values were calculated by GraphPad Instat program; data fit was obtained using the sigmoidal dose–response equation (variable slope) (GraphPad software).
[d] In vitro COX-2 selectivity index [IC_{50} (COX-1)/IC_{50}(COX-2)].

the concentration required to inhibit the activity of both isoenzymes by 50% (COX-1/COX-2 IC_{50} ratio).

All compounds did not affect J774 COX-1 activity and this may be related to the fact that the assay was performed in the presence of high concentrations of free arachidonic acid (AA), which may induce a cooperative activation of COX-1, thereby possibly reducing the potency of the compounds.[25] In contrast, all the compounds inhibited J774 COX-2 activity with varying potencies. In particular, these results indicate that the introduction of a 'small' lateral chain installed on the pyrrole core is indeed suitable for fine-tuning the interactions with the biophase (Table 1). Though aldehydes **1a–d**, as well as nitriles **3a–c,** showed a higher degree of inhibition, there is not a unique trend related to the substitution pattern as such. In particular, aldehyde **1b** and nitrile **3c** proved to be the most potent derivatives towards J774 COX-2, showing IC_{50} values of 9.5 nM, and 2.2 nM, respectively and being 8- and 36-fold more potent than celecoxib used as control. On the contrary, oximes **2a–d,** as well as nitrile **3d**, proved to be less active at inhibiting J774 COX-2.

The analgesic activities of the compounds were evaluated in vivo, measuring the reduction of writhes induced by intra-peritoneal injections of acetic acid solution in mice, a model of inflammatory pain.[26] Each compound was orally administered (po) (1–40 mg kg^{-1} dose range) 30 min before the induction of writhes. Results are reported as number of writhes and as percentage of writhes reduction with respect to vehicle-treated mice in Table 2. Aldehyde **1c** showed both the best efficacy and potency inducing a percentage of writhes reduction comparable to that of celecoxib. Interestingly, both **1c** and celecoxib also showed similar potencies at inhibiting COX-2 in vitro. In particular, compound **1c** was significantly active even when dosed at 1 mg kg^{-1} (36% reduction) and it was able to reduce writhes by 60% after administration at 40 mg kg^{-1}; moreover, animals treated with compound **1c** showed a constant response of writhe reduction (about 50%) with absence of dose dependency within the range of 5–20 mg kg^{-1}.

It is worth mentioning that oximes **2a**, **2b** and **2d** showed a similar constant dose response (ranging from 41% to 57%) when administered at higher dose range (20–40 mg kg^{-1}).

Finally, at 40 mg kg^{-1} doses, nitriles **3c** and **3d**, proved to be able to reduce the writhes by 57% and 59%, respectively, while **3a** and **3b** were only moderately active (about 45% and 38% of writhe reduction, respectively).

Surprisingly, some of the most in vivo effective compounds (e.g., **2a**, **2b**, **2d**, and **3d**) did not show the best results in the corresponding in vitro test. The good in vivo efficacy of these compounds may suggest a high in vivo metabolism with respect to **1a**, **1b**, **1d**, **2c** as well as **3b** showing higher potencies towards COX-2. In addition, differences in protein binding properties among compounds **1**–**3**, might contribute to different in vivo effects.

All the compounds proved to inhibit J774 COX-2 activity in vitro, and this inhibitory effect is reasonably involved in their analgesic and anti-inflammatory activities detected in vivo.

As discussed above, assessment of selectivity towards COX-2 using the J774 cell-based assay cannot be considered the best way to assess the extent of COX-2 selectivity achieved in vivo after dosing the compounds, due to the high concentrations of free AA. Therefore, we studied the COX-2 selective inhibition of three representative compounds––aldehyde **1c**, oxime **2c** and nitrile **3b**–– by using the human whole blood (HWB) assay for COX-1 and COX-2.[27,28] This assay can give information about the inhibitory effects of compounds towards human platelet COX-1 activity, a clinically relevant target related to the gastrointestinal side-effects of NSAIDs;[6] it can also give information about human monocyte COX-2 activity induced in response to inflammatory stimulus, such as lipopolysaccharide (LPS), which is a clinically relevant target related to the anti-inflammatory activity. The assay was performed to predict the actual extent of isozyme inhibition achievable in vivo by circulating drug levels to consider the amount of variables

Table 2
Effect of **1a–d**, **2a–d**, **3a–d**, celecoxib, and vehicle (CMC) in the mouse abdominal constriction test (acetic acid 0.6%)

	Number of writhes (% writhe reduction)					
Compound	CMC	1 mg kg^{-1} (%)	5 mg kg^{-1} (%)	10 mg kg^{-1} (%)	20 mg kg^{-1} (%)	40 mg kg^{-1} (%)
CMC	33.4 ± 2.5	––	––	––	––	––
1a	––	nd	31.7 ± 3.6 (5)	33.6 ± 3.7 (–)	23.3 ± 2.9 (30)	22.9 ± 3.0 (31)
1b	––	nd	33.4 ± 3.1 (–)	20.3 ± 2.2* (39)	18.8 ± 3.4* (44)	nd
1c	––	21.3 ± 2.8* (36)	16.7 ± 3.1* (50)	15.8 ± 2.8* (53)	16.1 ± 2.9* (52)	13.3 ± 2.3* (60)
1d	––	nd	33.0 ± 2.8 (–)	25.8 ± 3.0^ (23)	22.8 ± 3.1* (32)	23.2 ± 2.2* (30)
2a	––	nd	32.6 ± 2.7 (2)	21.5 ± 3.2* (36)	19.8 ± 2.5* (41)	17.8 ± 2.9* (47)
2b	––	nd	33.1 ± 4.0 (–)	22.6 ± 3.5* (32)	19.2 ± 1.7* (42)	18.2 ± 1.7* (45)
2c	––	nd	30.2 ± 4.0 (10)	27.1 ± 2.3 (19)	24.3 ± 3.2^ (27)	nd
2d	––	nd	28.9 ± 2.7 (13)	24.5 ± 3.4^ (27)	15.6 ± 2.5* (53)	14.8 ± 3.1* (57)
3a	––	nd	29.8 ± 2.8 (5)	21.3 ± 2.7* (36)	22.5 ± 3.1* (33)	18.2 ± 3.0* (45)
3b	––	nd	26.4 ± 3.1 (21)	23.9 ± 2.8^ (28)	22.5 ± 3.1* (33)	20.7 ± 2.6* (38)
3c	––	nd	30.7 ± 2.5 (8)	26.9 ± 3.0 (19)	20.6 ± 3.0* (38)	14.2 ± 2.5* (57)
3d	––	nd	35.4 ± 3.3 (–)	24.1 ± 2.3* (28)	19.3 ± 2.8* (42)	13.8 ± 2.9* (59)
Celecoxib	––	19.3 ± 2.5* (42)	16.6 ± 2.2* (50)	14.2 ± 2.3* (57)	13.9 ± 2.7* (58)	10.2 ± 2.1* (69)

All compounds were suspended in 1% CMC and per os (po) administered 30 min before the experiment. 0.6% acetic acid was administered ip. Each value represents the mean of 10 mice.

^ $P < 0.05$.

* $P < 0.01$ in comparison with CMC treated group.

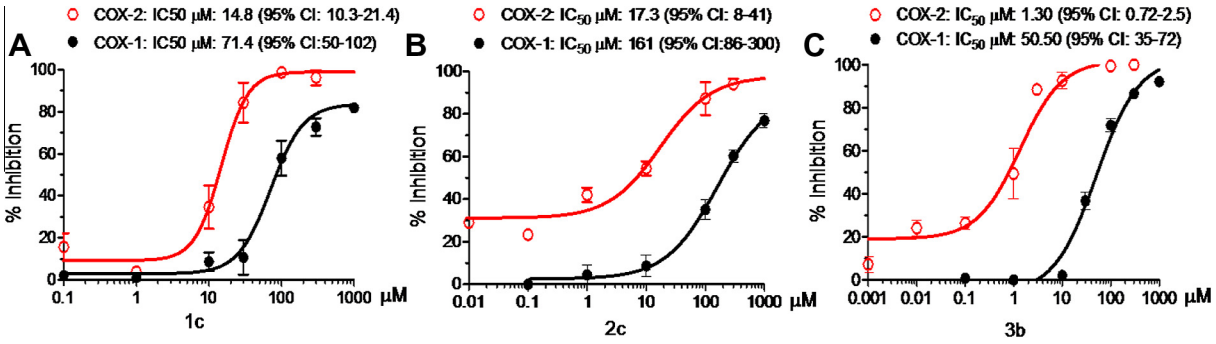


Figure 2. Effects of the compounds **1c**, **2c** and **3b** on COX-1 and COX-2 activity in HWB. Concentration-response curves for inhibition of whole blood COX-1 activity were assessed by measuring serum thromboxane (TX) B_2 levels by the compounds **1c** (A), **2c** (B), and **3b** (C) (0.1–1000 μM). Results were reported as percentage of inhibition (mean ± SEM) from three separate experiments. Concentration-response curves for inhibition of whole blood COX-2 induced by LPS (10 μg/ml) were assessed by measuring PGE_2 levels by the compounds **1c** (0.1–300 μM) (A), **2c** (0.01–300 μM) (B), and **3b** (0.001–300 μM) (C). Results were reported as percentage inhibition (mean ± SEM) from three separate experiments. Open red symbols represent COX-2 inhibition. Closed black symbols represent COX-1 inhibition. Red and black lines represent the concentration-response curves for inhibition of COX-2 and COX-1, respectively, by the compounds **1c**, **2c** and **3b** in vitro.

potentially able to affect drug–enzyme interaction. As shown in Figure 2, compounds **1c**, **2c** and **3b** inhibited LPS-induced whole blood PGE_2 generation (COX-2 assay), in a concentration-dependent fashion with IC_{50} values of 14.8 [95% confidential interval (CI), 10.3–21.4] μM, 17.3 (95% CI, 8–41) μM, and 1.30 (95% CI, 0.72–2.5) μM, respectively. In this assay, celecoxib showed an IC_{50} value of 0.39 (95% CI, 0.29–0.53) mM.[5] All the tested compounds proved to be more potent at inhibiting COX-2 than COX-1. Aldehyde **1c**, oxime **2c** and nitrile **3b** proved to be 4.8-, 9.3- and 38.8-fold more selective towards COX-2, respectively. Nitrile **3b** proved to be the most selective and potent one towards COX-2. Under the same experimental conditions, celecoxib inhibited COX-1 with an IC_{50} value of 12.53 (95% CI, 8.67–17.90) mM, thus showing a COX-1/COX-2 IC_{50} ratio of 30. Compound **3b** hence showed selective activity comparable to celecoxib towards COX-2. These results, even if referred to only one example of each class of synthesized compounds, indicate that nitrile derivatives show a better selectivity towards COX-2 than the other two classes of derivatives. On the contrary, the little reduction of the analgesic efficacy showed in vivo by nitriles with respect to aldehyde **1c**, might be related to reduced bioavailability, metabolism and/or tissue distribution of nitriles. Whether improved COX-2 selectivity of nitrile derivatives might be associated with a better gastrointestinal profile, it requires to be assessed in vivo with appropriate experimental models.

3. Conclusions

Inspired by the work by Khanna et al. we managed to synthesize a class of diarylpyrroles which has been evaluated both biologically and pharmacologically. The results clearly suggest that further modification of the structure is needed to improve the bio-pharmacological profile of these molecules; nonetheless, aldehyde **1c** proved to possess an outstanding anti-nociceptive activity comparable to celecoxib. Oximes **2** showed interesting profiles, but an isosteric replacement of the oxime moiety (in order to generate a geometrical differentiation) is needed to evaluate any steric effect on the interaction with the biological receptor. Further studies are on-going to bring about structural modification in order to undisclose the real potential of this kind of scaffold.

4. Materials and methods

4.1. Chemistry

^{1}H NMR spectra were recorded with a Bruker Avance DPX-400 spectrometer with the residual solvent peak as the internal reference ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.50 ppm). ^{1}H resonances are reported to the nearest 0.01 ppm. ^{13}C NMR spectra were recorded with the same spectrometers with the central resonance of the solvent peak as the internal reference ($CDCl_3$ = 77.16 ppm, DMSO-d_6 = 39.52 ppm). All ^{13}C resonances are reported to the nearest 0.1 ppm. The multiplicity of ^{1}H signals are indicated as: s = singlet, d = doublet, m = multiplet, or combinations of thereof. Infrared spectra were recorded neat on a Perkin–Elmer spectrum one FTIR spectrometer using Universal ATR sampling accessories. Letters in parentheses refer to the relative absorbency of the peak: w = weak, less than 40% of the most intense peak; m = medium, ca. 41–69% of the most intense peak; s = strong, greater than 70% of the most intense peak. Mass spectra were recorded on a API-TOF Mariner by Perspective Biosystem (Stratford, Texas, USA). Purity of compounds was assessed with elemental analysis obtained by a PE 2400 (Perkin–Elmer) analyzer. Purity of target compounds was >95%. Unless stated otherwise, reagents were obtained from commercial sources and used without purification. Unless otherwise stated, heating was conducted using standard laboratory apparatus. TLC analysis was performed on Merck 60 F254 silica gel plates and visualized using both short and long waved ultraviolet light in combination with standard laboratory stains such as acidic potassium permanganate. Melting points were performed on either a Stanford Research Systems MPA100 (OptiMelt) automated melting point system or a Gallenkamp melting point machine and are uncorrected.

Synthesis of 1-[4-(methylthio)phenyl]pentane-1,4-dione (**5**). A solution of 4-methylthiobenzaldehyde **4** (11.97 mL, 0.09 mol), triethylamine (19.5 mL, 0.14 mol), methyl vinyl ketone (5.8 mL, 0.09 mol), and 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide (3.53 g, 0.014 mol), in a 20 mL vial, was microwave irradiated using a CEM apparatus for 15 min at 70 °C (150 W, internal pressure of 150 psi). The reaction mixture was treated with 2 N HCl (10 mL) and extracted with ethyl acetate; the organic layer was washed with aqueous sodium bicarbonate and brine. The organic fractions were dried over sodium sulfate, filtered, and concentrated to give an orange liquid which was crystallized from cyclohexane to give intermediate **5** as white needles (80% yield). ESI-mass: *m/z* 245.063 $[M+Na]^+$, mp, and ^{1}H NMR spectrum were consistent with those reported in the literature.[16,23]

Synthesis of 1-[4-(methylsulfonyl)phenyl]pentane-1,4-dione (**6**). To a solution of **5** (7.8 g, 35 mmol) in methanol (150 mL), a solution of oxone (37.7 g, 61.4 mmol) in water (150 mL) was added over 5 min. After being stirred at 25 °C for 2 h, the reaction mixture was diluted with water (400 mL) and extracted with dichloromethane. The organic layer was washed with brine (200 mL) and dried (Na_2SO_4). After filtration and concentration, the crude material was chromatographed (silica gel, 3:1 hexane/ethyl acetate) to give

6 (90% yield) as a white solid. ESI-mass: *m*/*z* 277.052 $[M+Na]^+$, mp, and ^1H NMR spectrum were consistent with those reported in literature.[16,23]

General procedure for the synthesis of diarylpyrroles (**7a**–**d**). Following the procedure for the Paal–Knorr reaction, a solution of **6** (0.58 g, 2.28 mmol), the opportune aniline (2.28 mmol) and *p*-toluenesulfonic acid (30 mg, 0.17 mmol) in ethanol (2 mL) was microwave irradiated using a CEM apparatus for 45 min at 160 °C (150 W, internal pressure of 150 psi). The reaction mixture was cooled and concentrated. The crude material was purified by chromatography on aluminum oxide with a 3:1 cyclohexane/ethyl acetate mixture, as the eluant, to give the expected 1,5-diarylpyrrole **7** as white needles in satisfactory yield.

1-(4-Fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1*H*-pyrrole (**7a**). ESI-mass: *m*/*z* 352.083 $[M+Na]^+$, mp, and ^1H NMR spectrum were consistent with those reported in literature.[16,23]

1-(3-Fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1*H*-pyrrole (**7b**). ESI-mass: *m*/*z* 352.091 $[M+Na]^+$, mp, and ^1H NMR spectrum were consistent with those reported in literature.[16,23]

1-(3,4-Difluorophenyl)-2-methyl-5-(4-methylsulfonyl)phenyl)-1*H*-pyrrole (**7c**). ESI-mass: *m*/*z* 370.075 $[M+Na]^+$, mp, and ^1H NMR spectrum were consistent with those reported in literature.[16,23]

2-Methyl-5-(4-(methylsulfonyl)phenyl)-1-phenyl-1*H*-pyrrole (**7d**). ESI-mass: *m*/*z* 334.092 $[M+Na]^+$, mp, and ^1H NMR spectrum were consistent with those reported in literature.[16,23]

General procedure for the synthesis of pyrrole-3-carbaldehydes (**1a**–**d**). To a solution of dimethylformamide (8.3 mmol) in dichloromethane (10 mL) at 0 °C, in a round-bottomed flask equipped with a stirring bar, phosphoryl chloride (8.4 mmol) was added dropwise. After 30 min a solution of **7** (3.2 mmol in 10 mL of 100 dichloromethane) was added over 3 min and then refluxed for 1 h. The reaction mixture was cooled down to rt, diluted with saturated carbonate solution (50 mL) and extracted with dichloromethane. The organic layer was washed with brine (200 mL) and water (200 mL) and dried over sodium sulfate. After filtration and concentration, the crude material was crystallized using ethyl acetate to give the aldehyde as an off-white solid.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[4-fluoro-phenyl]-1*H*-pyrrole-3-carboxaldehyde (**1a**). White needles (>95% yield). ESI-mass: *m*/*z* 357,082 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{16}FNO_3S$ calcd C, 63.85; H, 4.51; N, 3.92. Found C, 63.82; H, 4.55; N, 3.98; data, mp, ^1H and ^{13}C NMR spectra were consistent with those reported in the literature.[23]

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[3-fluoro-phenyl]-1*H*-pyrrole-3-carboxaldehyde (**1b**). Off-white needles (>95% yield), mp 170 °C. FT-IR (neat, cm^{-1}) *v*: 2770 (w), 1673 (s), 1590 (s), 1506 (s), 1300 (s), 1140 (s); ^1H NMR (DMSO-d_6 400 MHz) ppm: 9.93 (s, 1H), 7.85 (m, 1H), 7.52 (m, 1H), 7.40 (m, 2H), 7.31 (m, 1H, *J* = 8.6 Hz), 7.08 (d, 2H, *J* = 8.6 Hz), 6.94 (s, 1H), 3.17 (s, 3H), 2.37 (s, 3H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 187.09, 163.43, 142.70, 138.91, 138.11, 137.00, 131.54, 128.84, 128.55, 117.40, 117.02, 116.03, 112.03, 107.02, 106.82, 44.33, 10.89; ESI-mass: *m*/*z* 380.073 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{16}FNO_3S$ calcd C, 63.85; H, 4.51; N, 3.92. Found C, 63.80; H, 4.57; N, 3.95.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[3,4-difluoro-phenyl]-1*H*-pyrrole-3-carboxaldehyde (**1c**). Off-white needles (>95%yield), mp 178 °C. FT-IR (neat, cm^{-1}) *v*: 2785 (w), 1671 (s), 1592 (s), 10 1500 (s), 1304 (s), 1133(s); ^1H NMR ($CDCl_3$ 400 MHz) ppm: 9.97 (s, 1H), 7.77 (m, 3H), 7.55 (m, 1H), 7.36 (d, 2H, *J* = 8.7 Hz), 7.22 (m, 1H), 6.98 (s, 1H), 3.20 (s, 3H), 2.39 (s, 3H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 187.09, 163.03, 150.31, 140.70, 138.90, 138.10, 137.09, 131.50, 128.93, 128.59, 118.03, 117.33, 15 109.22, 108.8, 107.02, 44.39, 10.99; ESI-mass: *m*/*z* 398.064 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{15}F_2NO_3S$ calcd C, 60.79; H, 4.03; N, 3.73. Found C, 61.00; H, 3.99; N, 3.78.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-phenyl-1*H*-pyrrole-3-carboxaldehyde (**1d**). Off-white needles (>95%yield), mp 160 °C. FT-IR (neat, cm^{-1}) *v*: 2782 (w), 1679 (s), 1588 (s), 1503 (s), 1298 (s), 1106 (s); ^1H NMR ($CDCl_3$ 400 MHz) ppm: 10.00 (s, 1H), 7.73 (d, 2H, *J* = 8.6 Hz), 7.46 (m, 3H), 7.22 (d, 2H, *J* = 8.6 Hz), 7.16 (m, 2H), 6.95 (s, 1H), 3.02 (s, 3H), 2.40 (s, 3H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 186.69, 161.30, 140.30, 139.33, 138.04, 135.89, 133.70, 129.04, 128.55, 128.44, 125.40, 111.10, 109.57, 44.20, 11.44. ESI-mass: *m*/*z* 362.083 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{17}NO_3S$ calcd C, 67.25; H, 5.05; N, 4.13. Found C, 67.33; H, 5.00; N, 4.18.

General procedure for the synthesis of pyrrole-3-carboximes (**2a**–**d**). To a solution of aldehyde **1** (1 mmol) in ethanol (5 mL), hydroxylamine chloride water solution (1 mmol in 4 mL of water) and sodium acetate (1 mmol) were added. The reaction was refluxed for 1 h and a half. At the end the precipitated formed was filtered and collected to give the oxime **2** as white powder.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[4-fluoro-phenyl]-1*H*-pyrrole-3-carboxime (**2a**). White powder (>95%yield). FT-IR (neat, cm^{-1}) *v*: 3285 (w), 1596 (s), 1518 (s), 1307 (s), 1149 (s), 956 (s), 774 (s); ^1H NMR (400 MHz, DMSO-d_6): (ppm) 10.94 (s, 1H), 10.61 (s, 1H), 8.11 (s, 1H), 7.70 (d, 4H, *J* = 40 8.4 Hz), 7.42–7.31 (m, 9H), 7.21 (d, 4H, *J* = 8.4 Hz), 6.74 (s, 2H), 3.15 (s, 6H), 2.13 (s, 6H); ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 159.66, 148.20, 138.92, 138.11, 136.73, 135.19, 133.30, 128.81,128.50, 123.22, 117.13, 116.10, 106.98, 47.88, 10.96; ESI-mass: *m*/*z* 395.084 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{17}FN_2O_3S$ calcd C, 61.29; H, 4.60; N, 7.52. Found C, 61.25; H, 4.64; N, 7.58.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[3-fluoro-phenyl]-1*H*-pyrrole-3-carboxime (**2b**). White powder (>95%yield). FT-IR (neat, cm^{-1}) *v*: 3292 (w), 1590 (s), 1510 (s), 1297 (s), 50 1140 (s), 950 (s), 766 (s); ^1H NMR (400 MHz, DMSO-d_6) ppm: 10.98 (s, 1H), 10.65 (s, 1H), 8.14 (s, 1H), 7.78 (d, 4H, *J* = 8.6 Hz), 7.53–7.43 (m, 2H), 7.36–7.33 (m, 6H), 7.18 (d, 4H, *J* = 8.4 Hz), 6.77 (s, 2H), 3.19 (s, 6H), 2.19 (s, 6H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 163.55, 149.00, 143.00, 138.88, 138.21, 55 135.20, 133.10, 131.00, 128.77, 128.43, 117.21, 117.10, 112.28, 107.28, 106.90, 47.99, 11.06. ESI-mass: *m*/*z* 395.084 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{17}FN_2O_3S$ calcd C, 61.29; H, 4.60; N, 7.52. Found C, 61.27; H, 4.63; N, 7.50.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[3,4-ifluorophenyl]-1*H*-pyrrole-3-carboxime (**2c**). White powder (>95%yield). FT-IR (neat, cm^{-1}) *v*: 3280 (w), 1582 (s), 1522 (s), 1300 (s), 1117(s), 943 (s), 760 (s); ^1H NMR (400 MHz, DMSO-d_6): (ppm) 10.98 (s, 1H), 10.66 (s, 1H), 8.14 (s, 1H), 7.79 (d, 4H, *J* = 8.5 Hz), 7.64–7.69 (m, 2H), 7.66 (m, 2H), 7.43–7.23 (m, 5H), 7.15 (m, 2H), 6.77 (s, 2H), 3.19 (s, 6H), 2.19 (s, 6H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 153.35, 148.33, 146.30, 138.98, 138.56, 138.31, 135.10, 133.13, 128.87, 128.53, 118.61, 117.70, 117.44, 108.88, 106.89, 48.01, 11.12. ESI-mass: *m*/*z* 413.075 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{16}F_2N_2O_3S$ calcd C, 58.45; H, 4.13; N, 7.18. Found C, 58.50; H, 4.10; N, 7.20.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-phenyl-1*H*-pyrrole-3-carboxime (**2d**). White powder (>95%yield). FT-IR (neat, cm^{-1}) *v*: 3280 (w), 1599 (s), 1520 (s), 1289 (s), 1167 (s), 75 955 (s), 770 (s); ^1H NMR 400 MHz, DMSO-d_6): (ppm) 10.92 (s, 1H), 10.59 (s, 1H), 8.10 (s, 1H), 7.68 (d, 4H, *J* = 8.6 Hz), 7.45–7.32 (m, 11H), 7.23 (d, 4H, *J* = 8.6 Hz), 6.76 (s, 2H), 3.10 (s, 6H), 2.15 (s, 6H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 148.23, 141.22, 138.98, 138.16, 135.11, 133.17, 129.43, 128.89, 128.63, 80 125.66, 121.65, 106.91, 117.24, 47.66, 11.02. ESI-mass: *m*/*z* 377.094 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{18}N_2O_3S$ calcd C, 64.39; H, 5.12; N, 7.90. Found C, 64.42; H, 5.15; N, 7.88.

General procedure for the synthesis of pyrrole-3-carbonitriles (**3a**–**d**). A solution of 2,4,6-trichloro[1,3,5]triazine (1 mmol) in 0.2 mL of dimethylformamide was stirred for 30 min at room temperature. Carboxime **2** (1 mmol), dissolved in 1.5 mL of

dimethylformamide, was added, and the reaction mixture was stirred at room temperature for 8 h. The reaction mixture was then diluted with water (2 mL), extracted with ethyl acetate (50 × 3 mL), washed with saturated carbonate solution (50 mL), 1 N HCl (50 mL) and brine (50 mL). After filtration and concentration, the crude mixture obtained was purified by chromatography on silica gel with 4:1 cyclohexane/ethylacetate mixture as eluent, to give **3** as a white solid. Recrystallization from ethanol gave **3** as white crystals.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[4-fluoro-phenyl]-1*H*-pyrrole-3-carbonitrile (**3a**). White needles (80% yield). ESI-mass: *m*/*z* 377.073 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{15}FN_2O_2S$ calcd C, 64.39; H, 4.27, N, 7.90. Found C, 64.45; H, 4.30; N, 7.98; mp, ^1H and ^{13}C NMR spectra were consistent with those reported in the literature.[23]

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[3-fluoro-phenyl]-1*H*-pyrrole-3-carbonitrile (**3b**). White needles (76.8% yield), mp 219 °C. FT-IR (neat, cm^{-1}): 2220 (w), 1600 (s), 1519 (s), 1320 (s), 1013 (s), 944 (s); ^1H NMR (400 MHz, $CDCl_3$): (ppm) 7.78 (d, 2H, *J* = 8.4 Hz), 7.50–7.44 (m, 1H), 7.23 (d, 2H, *J* = 8.4 Hz), 105 6.99–6.92 (m, 3H), 6.71 (s, 1H), 3.05 (s, 3H), 2.35 (s, 3H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 159.59, 140.66, 138.94, 138.01, 137.50, 133.41, 129.23, 128.89, 128.56, 117.55, 117.39, 116.00, 111.25, 108.02, 106.77, 45.00, 11.21; ESI-mass: *m*/*z* 377.074 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{15}FN_2O_2S$ calcd C, 64.39; H, 4.27; N, 7.90. Found C, 64.42; H, 4.26; N, 7.95.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-[3,4-difluoro-phenyl]-1*H*-pyrrole-3-carbonitrile (**3c**). White needles (78.0% yield), mp 220 °C. FT-IR (neat, cm^{-1}) *v*: 2225 (w), 1605 (s), 1516 (s), 115 1300 (s), 1023 (s), 958 (s); ^1H NMR (400 MHz, $CDCl_3$): (ppm) 7.79 (d, 2H, *J* = 8.3 Hz), 7.29–7.25 (m, 1H), 7.21 (d, 2H, *J* = 8.3 Hz), 7.06–7.02 (m, 1H), 6.95–6.93 (m, 1H), 6.68 (s,1H), 3.05 (s, 3H), 2.30 (s, 3H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 159.66, 146.76, 138.64, 138.38, 137.53, 132.91, 128.78, 128.77, 117.44, 116.60, 108.14, 107.00, 44.89, 11.11. ESI-mass: *m*/*z* 395.064 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{14}F_2N_2O_2S$ calcd C, 61.29; H, 3.79; N, 7.52. Found C, 61.35; H, 3.82; N, 7.58.

2-Methyl-5-[4-(methylsulfonyl)phenyl]-1-phenyl-1*H*-pyrrole-3-carbonitrile (**3d**). White needles (79.3% yield), mp 197 °C. FT-IR (neat, cm^{-1}) *v*: 2215 (w), 1607 (s), 1510 (s), 1309 (s), 1019 (s), 950 (s); ^1H NMR (400 MHz, $CDCl_3$): (ppm) 7.45 (d, 2H, *J* = 8.4 Hz), 7.30 (m, 3H), 7.20 (m, 2H), 7.18 (d, 2H, *J* = 8.4 Hz), 6.70 (s, 1H), 3.05 (s, 3H), 2.19 (s, 3H). ^{13}C NMR ($CDCl_3$ 100 MHz) ppm: 138.90, 138.51, 133.57, 133.09, 129.42, 129.68, 128.40, 128.44, 125.55, 121.13, 117.38, 112.05, 110.65, 44.80, 11.55. ESI-mass: *m*/*z* 359.083 $[M+Na]^+$; Elem. Anal. for $C_{19}H_{16}N_2O_2S$ calcd C, 67.84; H, 4.79; N, 8.33. Found C, 67.80; H, 4.81; N, 8.35.

4.2. Biology

In vitro study. The murine monocyte/macrophage J774 cell line was grown in DMEM supplemented with 2 mM glutamine, 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum (FBS) and 1.2% Na pyruvate. Cells were plated in 24-well culture plates at a density of 2.5 × 105 cells/mL or in 60 mm-diameter culture dishes (3 × 106 cells/3 mL/dish) and allowed to adhere at 37 °C in 5% CO_2 for 2 h. Immediately before the experiments, culture medium was replaced by fresh medium without FBS and cells were stimulated as described.[29] To evaluate COX-1 activity, cells were pre-treated with test compounds (0.01–10 μM) for 15 min and further incubated at 37 °C for 30 min with 15 μM AA in order to activate the constitutive COX-1. At the end of the incubation, the supernatants were collected for the measurement of prostaglandin E_2 levels by radioimmunoassay (RIA). On the other hand, to evaluate COX-2 activity, cells were stimulated for 24 h with *Escherichia coli* lipopolysaccharide (10 μg/mL) to induce COX-2, in the absence or presence of test compounds, at the concentrations previously reported. Celecoxib was utilized as reference compound for selectivity index. The supernatants were collected for the measurement of PGE_2 by RIA. Throughout the time the experiments lasted, triplicate wells were used for the various conditions of treatment. Results are expressed as the mean, for three experiments, of the percent inhibition of PGE_2 production by test compounds with respect to control samples. The IC_{50} values were calculated by GraphPad Instat program, data fit was obtained using the sigmoidal dose–response equation (variable slope) (GraphPad software).

In vitro HWB assay. Compounds **1c**, **2c** and **3b** were evaluated for COX-1 and COX-2 selectivity in HWB assays. Whole blood was withdrawn from three healthy volunteers (age range 30 ± 3 years). Informed consent was obtained from each subject. Compounds **1c**, **2b** and **3b** were dissolved in DMSO, and 2 μL of vehicle, or stock solution of the compounds were added to 1 mL of whole blood to give final concentrations of 0.001–1000 μM. To evaluate COX-2 activity, 1 mL aliquots of peripheral venous blood samples containing 10 IU of sodium heparin were incubated in the absence or in the presence of LPS (10 μg/mL) (Sigma–Aldrich, St. Louis, MO) with or without increasing concentrations (0.001–300 mM) of the compounds (**1c**, **2b** or **3b**) for 24 h at 37 °C.[28] The contribution of platelet COX-1 was suppressed by pretreating the subjects with 300 mg aspirin 48 h before sampling. PGE_2, as an index of monocyte COX-2 activity, was measured in plasma by previously described and validated RIAs.[25] Moreover, peripheral venous blood samples were drawn from the same donors when they had not taken any NSAIDs during the 2 weeks preceding the study. Aliquots (1 mL) of whole blood were immediately transferred into glass tubes in the presence of vehicle (DMSO), or different concentrations (0.1–1000 mM) of the compounds, and allowed to clot at 37 °C for 1h. Whole blood TXB_2, as a reflection of maximally stimulated platelet COX-1 activity in response to endogenously formed thrombin, was measured in serum by previously described and validated RIA.[27] For each experiment, the results were reported as percentage of inhibition of PGE_2 or TXB_2 production by test compounds with respect to control samples (DMSO vehicle). The IC_{50} values were calculated by using GraphPad Prism (version 5.00 for Windows, GraphPad).

4.3. Pharmacology

In vivo analgesic/anti-inflammatory study. Animal handling was carried out according to the European community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). The ethical policy of the University of Florence conforms with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described herein was obtained from the animal subjects review board of the University of Florence. For the experiment described male Swiss albino mice (23–25 g) were used. The animals were fed with a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. The analgesic/anti-inflammatory activity of the new compounds was investigated in the mouse abdominal constriction test.[30] Mice, randomly distributed in different groups, were injected intraperitoneally (ip) with 0.6% acetic acid and, 5 min later, the number of writhes due to abdominal constriction was counted for further 10 100 min. All compounds at the doses of 1, 5, 10, 20 and 40 mg kg^{-1}, or the vehicle (1% carboxymethylcellulose) were administered per os (po) 30 min before acetic acid injection.

4.4. Statistical analysis

Results were expressed as the means ± SEM and an analysis of variance was performed by ANOVA. A Fisher's protected least significant difference procedure was used as post-hoc comparison. *P* values of less than 0.05 or 0.01 were considered significant. Data were analyzed using the 'Origin 7.5' software.

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