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Original article

Improving the solubility of a new class of antiinflammatory pharmacodynamic hybrids, that release nitric oxide and inhibit cycloxygenase-2 isoenzyme

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ABSTRACT

The development of a novel class of pharmacodynamic hybrids that inhibits COX-2 isoform is reported. These molecules display enhanced nitric oxide releasing properties due to the presence of an ionisable moiety. The *in vivo* analgesic/anti-inflammatory activity was maintained in relation to the parent compounds.

1. Introduction

Side effects related to Non-Steroidal Anti-inflammatory Drugs (NSAIDs) (Fig. 1) have been extensively documented by numerous clinical trials. Long-term therapy with these medicines is often associated with gastrointestinal (GI), renal and haematological effects which reduce patient compliance [1,2].

The development of COX-2 selective NSAIDs (coxibs) (Fig. 2) was meant to circumvent these side effects, by selectively inhibiting the isoenzyme involved in the production of pro-inflammatory mediators. Though clinical trials have shown a reduction of gastrointestinal and renal side effects, an increase in cardiovascular (CV) events was observed suggesting that an exclusive inhibition of COX-2 enzyme could be associated with heart failure and stroke [3,4].

In the last few years many research groups have focused their efforts on the discovery and development of "molecular hybrids"

Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; GI, gastrointestinal; COXIB, cyclooxygenase-2 inhibitor; CV, cardiovascular; COX, cyclooxygenase; NO, nitric oxide; NOS, nitric oxide synthase; CINOD, cyclooxygenase-inhibiting nitric oxide donor; SAR, structure activity relationship; Boc, tert-butyl-oxy carbonyl; Cbz, benzyl-oxy carbonyl; SGF, simulated gastric fluid; PBS, phosphate buffered saline; c-GMP, cyclic guanosine monophosphate; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; E_{max}, maximal vasorelaxing response; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; FBS, fetal bovine serum; PGE$_2$, prostaglandin E_2; RIA, radioimmunoassay; LPS, lipopolysaccharide; Ach, acetylcholine; ipl, intraplantar; ip, intraperitoneal; po, per os.

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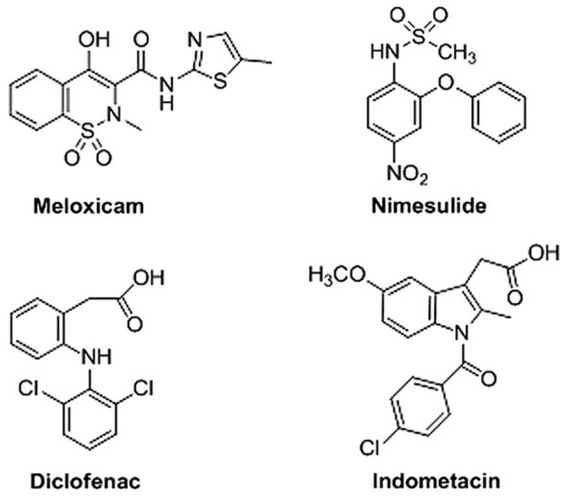


Fig. 1. Chemical structures of Meloxicam, Nimesulide, Diclofenac and Indometacin.

characterised by a nitric oxide (NO) releasing moiety, in order to enhance the pharmacological profile of the parent drug. NO is an important endogenous mediator generated by Nitric Oxide Synthase (NOS) enzymes. While at high concentrations NO is cytotoxic and forms a part of the defence and immunological system, at low concentrations it has important functions in the overall homeostasis of the organism. NO, biosynthesised by endothelial cells, is mainly considered as a fundamental modulator of the cardiovascular function, where it acts as a powerful vasodilator, inhibits platelets activation/aggregation and is involved in ischemic preconditioning, thus ensuring important cardioprotective effects. Increasing evidence suggests a considerable “modulating” effect of nitric oxide as pleiotropic agent, acting in several districts of the body [5–7]. In the GI tract, NO modulates the blood flow contributing to mucosal defence against luminal irritants and it also modulates mucus and bicarbonate secretion. It has a striking role in mucosal healing processes and modulates vascular permeability. The prevention of GI toxicity, by means of nitric oxide, was firstly explored by NicOx combining a nitric oxide releasing moiety with existing NSAIDs. Naproxcinod (AZD3582) and NO-flurbiprofen (HTC-1026) were the first molecules developed as COX-Inhibiting Nitric Oxide Donors namely CINODs (Fig. 3) [8–12].

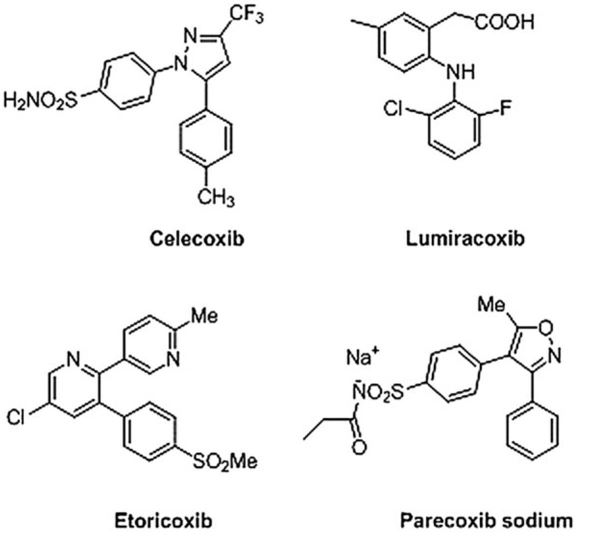


Fig. 2. Chemical structures of Celecoxib, Lumiracoxib, Etoricoxib and Parecoxib sodium.

As part of our ongoing development of analgesic/antiinflammatory candidates, we focused our attention on the design of “Pharmacodynamic Hybrids” endowed with both NO-releasing and COX-2 selective inhibiting properties, with the aim of reducing the side-effects due to a selective COX-2 inhibition [13–21]. In this class of drugs the NO-mediated effect is not directed to the reduction of GI side effects because COX-2 selectivity is a factor of GI safety itself. The NO-releasing properties are addressed to limit the CV toxicity due to COX-2 selective inhibition, which leads to reduced levels of prostacyclin, a vasorelaxing and antiplatelets agents [22]. Indeed the strategy of introducing a NO-releasing moiety to obtain a pharmacodynamics hybrid has been widely used to improve the overall CV profile of these drugs [23]. Several examples involving the “hybridisation” of NSAIDs have been reported; nevertheless very few of these, concern the modification of a coxib-like structure [24–26]. We identified a first class of molecules (Fig. 4) with significant analgesic and antiinflammatory properties, characterised by an ideal NO release.

This class of molecules is characterised by a central core responsible for COX-2 inhibitory activity and a nitric oxide donating moiety which should be able to suppress the side-effects. These molecules showed selective inhibition towards COX-2 isoenzyme with activities in the nanomolar range, lower than that shown by already marketed coxibs (i.e. Celecoxib). Nonetheless their *in vivo* profile, even though describing a very good efficacy, was somehow reduced (in relation to the *in vitro* one), indicating that the pharmacokinetic behaviour plays a crucial role in the fate of this class of molecules [21]. We then moved forward to analyse the solubility of those compounds and as predicted, these molecules were characterised by low water solubility and this feature could heavily affect their absorption [27]. There are two main factors responsible for poor solubility: high crystallinity (zwitterion formation, insoluble salts, strong H-bonding networks, etc) and hydrophobicity related to high log *P* (absence of ionisable moiety, high molecular weight). In our case the problem revealed to be the latter. Formulation strategies represent a fashionable and successful way to get improvement of solubility and bioavailability of active compounds; nevertheless, the study of these systems adds further parameters which results in complexity and expensiveness of the development at early stages [28]. We decided therefore to proceed through chemical modification of the previously identified core (Fig. 5).

An amino group (ionisable moiety) was introduced at the alpha position of the acetic side chain which structure activity relationship (SAR) studies has shown to be one of the few positions where change could be tolerated (compounds **1a**–**d**). The meta-fluorine and para-fluorine substituents at the N1 phenyl ring were chosen for a higher degree of interaction with the target. Since we expected that the NO release could give rise to the nitro-free intermediates as metabolites, we decided to further synthesise (**2a**–**d**) and analyse the activity of these molecules to better understand the biological profile of this class of derivatives.

2. Chemistry

Compounds **1a**–**d** and **2a**–**d** were prepared, using a common route to the synthesis of the main core, namely derivative **9** (Scheme 1) and then following two different strategies for the generation of nitro-oxy and nitro-free compounds (Scheme 2). The synthesis commenced with a Stetter reaction between the 4-methythiobenzaldehyde **3** and methyl vinyl ketone in the presence of a thiazolium salt [13–21]; the obtained compound **4** was oxidised by means of Oxone and subsequently a Paal–Knorr reaction afforded the 1,5-diarylpyrroles **6a**–**b** [13–21]. Acylation of

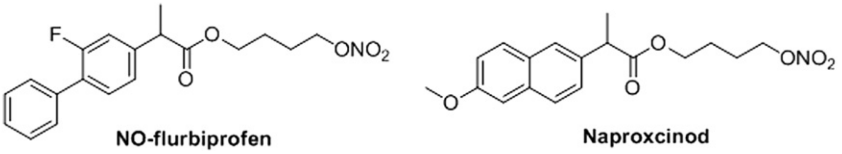


Fig. 3. Chemical structures of NO-flurbiprofen and Naproxcinod.

pyrroles was achieved by a titanium-catalysed regioselective Friedel–Craft on the pyrrole core to obtain compounds **7a–b** [13–21]. The installation of the protected amino functionality was achieved by the formation of the oxime, its subsequent reduction to the amine and protection, giving **10a–b** and **13a–b** in very good yields. Hydrolysis of the ethyl ester was achieved employing really mild conditions and through a subsequent coupling we obtained compounds **12a–b** and **15a–b**. A final deprotection step yielded nitro-free and nitro-oxy compounds. The installation of the α-functionality was afforded using the same strategy (oxime formation, reduction and protection) but employing two different protecting strategies. Indeed, we envisaged these two different routes could be easily converted to the final products, namely the nitro-free and the nitro-oxy derivatives reducing the problem that could have risen using a common protection/deprotection approach. We chose the tert-butyl-oxy carbonyl (Boc) protecting group for the synthesis of the nitro-oxy compounds **1a–d**, whose deprotection in mild acid conditions did not affect the stability of the nitro-oxy side chain. The benzyl-oxy carbonyl (Cbz) protecting group was selected for the route to the nitro-free derivatives since a late stage double deprotection was envisaged to yield compounds **2a–d**. Indeed compounds **15a–d** represent a double protected scaffold where catalytic hydrogenation can get rid of both the nitro and Cbz functionalities.

3. Results and discussion

3.1. Solubility assessment in SGF and PBS solutions

Simulated gastric fluid (SGF-without pepsin) and phosphate buffered saline (PBS) were chosen as ideal media to evaluate the solubility of compounds in acid (pH 1.5 for SGF) and neutral conditions (7.4 pH for PBS) to simulate the gastric and the body fluid environment respectively (Table 1) [29].

Solubility of compounds was assessed using a multi-pulse vortexer (Glass-Col), essential for shaking the samples prepared using stock solutions of compounds in DMSO (20 mM), and then analysing the samples obtained through HPLC. Previously, we determined the solubilities of compounds **18** and **19** (Fig. 6); we found that in SGF these molecules were scantly soluble with concentration ranging below the micromolar values. Similarly, solubility of compounds in PBS ranged the same values of concentration (1.6 μM for **18** and 1.1 μM for **19**). These data could therefore explain the reduced activity found when compounds are tested *in vivo* since it is well known that solubility is a crucial parameter in determining the biological effect, due to the relation between dissolution rate (hence solubility) and absorption. The introduction of an ionisable moiety (amino group) within that scaffold determined a huge increase of solubility as expected; however, we were aware that, even though changes on that position were tolerated, alteration of activity could occur. Analysis of the solubility profiles for **1a** and **1c** underlined a 200-fold improvement of solubility in SGF and around 100-fold in PBS.

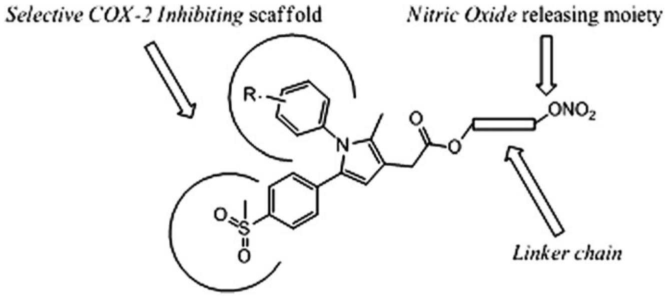


Fig. 4. General structure for selective COX-2 inhibiting nitric oxide donors, previously developed in our research group.

3.2. Cell-based cyclooxygenases inhibition assay

The cell-based assay showed a drop in activity for compounds **1a–d** and **2a–d** in relation to **18** and **19** (Table 2) [21].

We speculate this drop of activity to be related to the presence of the amino group. However, an average micromolar activity associated to all compounds is still present and selectivity towards COX-2 isoenzyme is maintained. Amongst the nitro-oxy derivatives, **1a** proved to exert the best inhibition towards COX-2 (IC_{50} 0.82 μM) while the nitro-free compound **2c** inhibited COX-2 isoform at the lowest IC_{50} value (0.22 μM).

3.3. Reduction of acetic acid-induced abdominal constrictions in mice

The issues related to the acetic acid-induced inflammation indicate that compounds **1a** and **1c** are endowed with activities comparable to compounds **18** and **19** (Table 3). In particular, the presence of an ionisable moiety enhancing solubility seems to overcome the relatively lower activity underlined in the cell-based assay shown by compounds **1a** and **1c**. Compound **1a** bears a three-atom side chain and its activity is higher than the parent compound **19** both at 20 and 40 mg kg^{-1} and comparable to **18** (which has a smaller side chain of two atoms). Compound **1c** shows an activity which is higher than **19** even though the latter presents a smaller side chain. We can assess that though the molecular interaction characterising the amino derivatives are less favourable (cell-based assay) the solubility enhancement gained with the introduction of an ionisable group may be responsible for the slight increase of activity when comparing the side chain length.

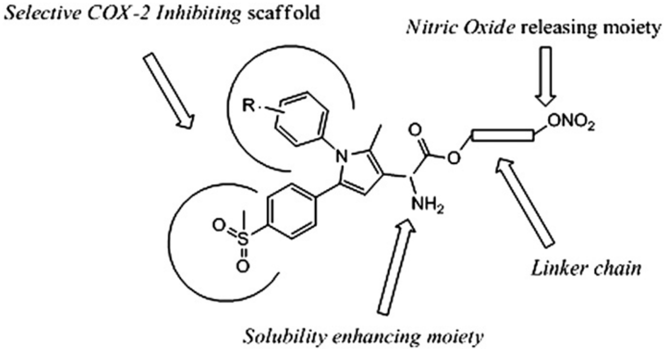
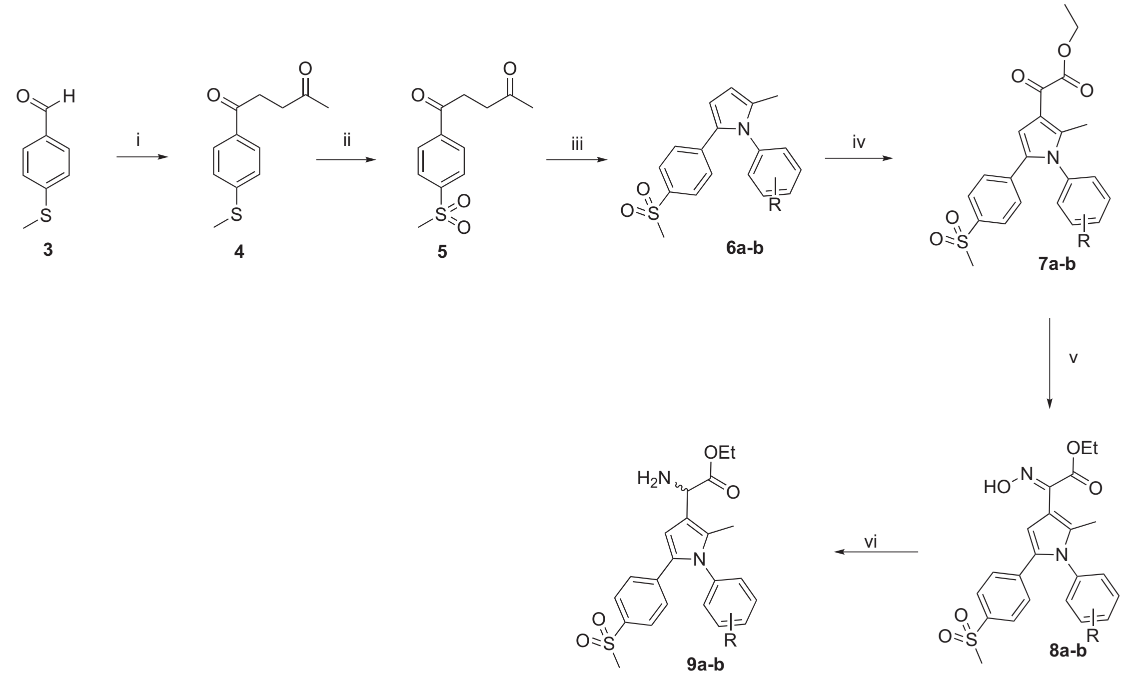


Fig. 5. Novel selective COX-2 inhibiting nitric oxide donors bearing an amino group on the side chain.



Compounds. **6a**: R = 4-F; **6b:** R = 3-F; **7a:** R = 4-F; **7b**: R = 3-F; **8a**: R = 4-F; **8b**: R= 3-F; **9a**: R = 4-F; **9b**: R = 3-F; Reagents and conditions: (i) CH_2=CHCOMe, TEA, 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide, MW; (ii) Oxone, MeOH/H_2O; (iii) $RPhNH_2$, *p*-toluensulfonic acid, EtOH, MW; (iv) ClCOCOOEt, TiCl4, DCM; (v) NH_2OH HCl, NaAcO, dioxane; vi) Zn dust, EtOH, formic acid.

Scheme 1. Synthesis of compounds **9a**–**b**.

If we compare the activities of compounds **1a** and **1c** we can speculate that side chain dimension inversely relates to the analgesic/anti-inflammatory potency of these molecules when performing the writhing test as observed in the previous series of compounds.

3.4. Reduction of carrageenan-induced inflammatory condition in rats

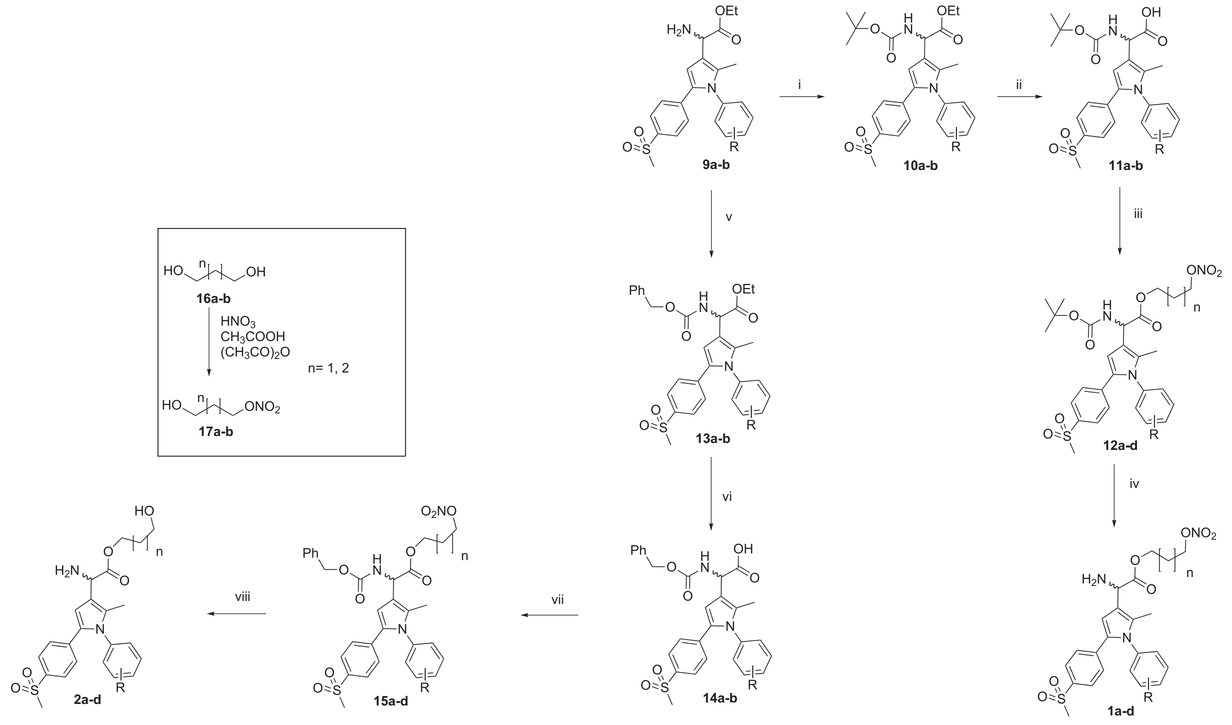
Compound **1a** was selected amongst all derivatives for the carrageenan test. The results were collected every 15 min until activity of compounds could be still detected. The results display an activity which is present already after 15 min (Table 4). The data show the max activity being at 30 min (70% pressure reduction at 40 mg kg^{-1}) and then it decreases after 45 min (53% reduction). The same trend is observed for **18** and **19**, indicating that pharmacokinetics plays a similar role whether or not we introduce an amino group at the alpha position of the acetic side chain. As observed in the acetic acid induced contractions test, the solubility enhancement for compound **1a** seems to be favourable being this compound more active than the corresponding parent compound **19**. Moreover, **1a** is endowed with an activity which is very close to the one displayed by **18**.

3.5. Nitric oxide releasing properties

In order to evaluate the NO-releasing properties, compounds **1a**, **1c**, **18** and **19** were submitted to a functional assay on isolated preparations of vascular smooth muscle; in particular, their vasorelaxing effects were tested on endothelium-denuded rat aortic rings, pre-contracted with 30 mM KCl. As shown in Table 5, all the tested compounds exhibited vasodilator effects. Since the effects of NO on the vascular smooth muscle are mediated by the NO-induced activation of guanylate cyclase and the consequent increase of cytosolic cyclic guanosine monophosphate (cGMP), 1 μM ODQ (inhibitor of guanylate cyclase) was used to identify the mechanism of action. ODQ significantly antagonized the vasorelaxing activity of these derivatives, indicating that these effects can be attributed to the release of NO. In particular, the different levels of potency exhibited by the four derivatives (**18** > **1c** > **1a** > **19**) are likely to reflect different rates of the NO-releasing process. In order to have a further confirmation of the NO-releasing properties, **18** (the compound showing the highest level of potency) was incubated in rat liver homogenate in the presence of opportune cofactors such as glutathione, NADH, and NADPH (all at the concentration 1 mM), i.e. in a biological sample able to metabolize organic nitrates, leading to the production of NO and of the corresponding alcohol derivative. In turn, NO is quickly oxidized to inorganic nitrites and nitrates, the latter being the principal species. As shown in Fig. 7, the incubation of 1 mM **18** was followed by a time-dependent slow formation of the inorganic stable metabolites of NO. The concentrations of nitrite and nitrate reached after 2 h after the incubation of the above derivative were 6 ± 1 mM and 281 ± 80, respectively. This experimental result further demonstrates that these derivatives are endowed with the pharmacological feature of slow NO-releasing agents.

4. Conclusions

In this paper, we report a further structural development of a novel class of pharmacodynamic hybrids, able to produce significant analgesic/anti-inflammatory effects, due to the preferential inhibition of COX-2, and to act as moderate donor of NO. The presence of the latter ancillary mechanism is likely to be a useful pharmacological strategy for attenuating the cardiovascular risk



Compounds. **9a:** R = 4-F; **9b**: R = 3-F; **10a:** R = 4-F; **10b**: R = 3-F; 11a: R = 4-F; **11b**: R = 3-F; **12a**: R = 4-F, n = 1; **12b**: R = 3-F, n = 1; 12c: R = 4-F, n = 2; 12d: R = 3-F, n = 2; 13a: R = 4-F; 13b: R = 3-F **14a:** R = 4-F; **14b**: R = 3-F; **15a**: R = 4-F, n = 1; **15b**: R = 3-F, n = 1; **15c**: R = 4-F, n = 2; **15d**: R = 3-F, n = 2; **2a**: R = 4-F, n = 1; 2b: R = 3-F, n = 1; 2c: R = 4-F, n = 2; 2d: R = 3-F, n = 2; 1a: R = 4-F, n = 1; **1b**: R = 3-F, n = 1; **1c**: R = 4-F, n = 2; **1d**: R = 3-F, n = 2.
Reagents and conditions: (i) $(Boc)_2O$, MeOH; ii) NaOH, MeOH; iii) a) $HO(CH_2)nONO_2$, EDCI, DMAP, DCM; iv) TFA, DCM; v) CbzCl,Na_2CO_3, DCM; vi) NaOH, MeOH; vii) $HO(CH_2)nONO_2$, EDCI, DMAP, DCM; viii) Pd/C, NH_4COO, iprOH, MW.

Scheme 2. Synthesis of compounds **1a**–**d** and **2a**–**d**.

potentially associated with the selective inhibition of COX-2, and mainly due to the reduced biosynthesis of prostacyclin. The experimental results demonstrated that the synthetised compounds were endowed with significant inhibiting activity on the COX-2 isoform, and lower activity on COX-1. Moreover, their nitrooxy function is effectively converted to NO, leading to additional cardiovascular effects, such as the vasorelaxing one, which has been clearly observed in this study. Noteworthy, the related alcoholic derivatives deriving from the biotransformation of the nitrooxy function still behave as COX-2 inhibitors. Moreover, in the new series of derivatives developed in this study, a further structural change has been introduced. Indeed, the analogous compounds previously reported [21] showed some limiting physicochemical aspects, able to negatively influence their pharmacokinetics, and in particular, a very poor levels of water solubility. In order to improve such a potentially negative feature, in these compounds an amino group was introduced. In line with the aims of the work, this structural modification actually led to a clear improvement of the water solubility of the target compounds, without affecting significantly the two cardinal pharmacodynamic properties (i.e., the COX-2 inhibition and the NO-release). Therefore, these novel compounds seem to exhibit promising feature as COX-2-inhibiting antiinflammatory/analgesic agents, endowed with an improved cardiovascular safety.

Table 1
Solubility assessment in SGF and PBS for compounds **1a**, **1c**, **18** and **19**.

Compound	SGF (uM)	PBS (uM)
1a	>200	80.0
1c	>200	70.8
18	<1	1.6
19	<1	1.1

5. Experimental protocols

5.1. Chemistry

Discovery Microwave System apparatus (CEM Srl, Cologno al Serio, Italy) was used to perform the Stetter and Paal–Knorr reactions. All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Microanalyses were carried out by means of a Perkin–Elmer 240C or a Perkin–Elmer Series II CHNS/O Analyzer 2400. Fluka silica gel 60 (230–400 mesh) was used for column chromatography. Fluka TLC plates were used for TLC. Fluka aluminium oxide (activity II–III, according to Brockmann) was used for chromatographic purifications. Fluka Stratocrom aluminium oxide

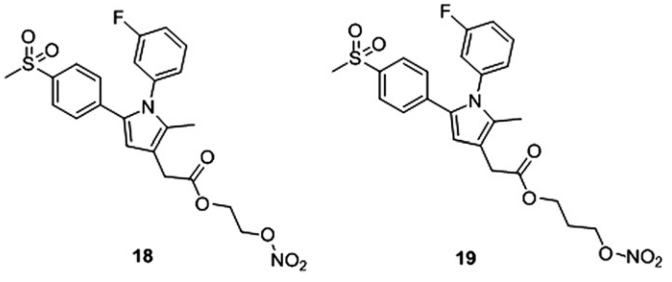


Fig. 6. Chemical structures of compounds **18** and **19**.

Table 2
In vitro assessment of COX-1 and -2 activity for compounds **1a**–**d**, **2a**–**d**.

Compound	% Inhib COX-1 (10 uM)	% Inhib COX-2 (10 uM)	IC_{50} (COX-1) (uM)[a]	IC_{50} (COX-2) (uM)[a]	COX-1/COX-2 (S.I.)[b]
1a	0	95	>10	0.82	>12.20
1b	5	100	>10	1.51	>6.62
1c	1	82	>10	1.00	>10.00
1d	15	59	>10	1.41	>1.35
2a	23	100	>10	0.76	>13.16
2b	30	79	>10	1.19	>8.40
2c	10	100	>10	0.22	>45.45
2d	23	100	>10	1.50	>6.67

[a] Results are expressed as the mean ($n = 3$ experiments) of the % 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).

[b] *In vitro* COX-2 Selectivity Index [IC_{50} (COX-1)/IC_{50}(COX-2)].

plates with fluorescent indicator were used for TLC to check the purity of the compounds. ^1H NMR and ^{13}C spectra were recorded with a Bruker AC400 spectrometer in the indicated solvent (TMS as internal standard): the values of the chemical shifts are expressed in ppm. Infrared spectra were recorded neat on a PerkinElmer Spectrum One FT-IR spectrometer using Universal ATR sampling accessories. Purity of compounds was assessed with elemental analysis; analyses which are indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values.

5.1.1. *Procedure for the synthesis of 1-[4-(methylthio)phenyl] pentane-1,4-dione (**4**)*

A solution of 4-methylthiobenzaldehyde **3** (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) was microwave irradiated for 15 min at 70 °C (power 150 W, pressure 60 psi) [13–21]. The reaction mixture was treated with 2 M HCl (10 mL). After extraction with ethyl acetate, the organic layer was washed with aqueous sodium bicarbonate and brine. The organic fractions were dried over sodium sulphate, filtered, and concentrated to give a crude orange liquid. After crystallization from cyclohexane, intermediate **4** was isolated as white needles (78% yield). Analytical data, the melting point, and the ^1H NMR spectrum were consistent with those reported in the literature [13–21].

5.1.2. *Procedure for the synthesis of 1-[4-(methylsulfonyl)phenyl] pentane-1,4-dione (**5**)*

To a solution of **4** (7.8 g, 35 mmol) in methanol (150 mL) Oxone was added (37.7 g, 61.4 mmol dissolved in 150 mL of water) over 5 min. After 2 h the reaction mixture was diluted with water (400 mL) and extracted with dichloromethane (3 × 100 mL). The organic layer was washed with brine (200 mL) and dried over sodium sulphate. After filtration and concentration, the crude material was chromatographed (silica gel, 3:1 hexane/ethyl acetate) to give 5 (90% yield) as a white solid. Analytical data, melting point, and the ^1H NMR spectrum were consistent with those reported in the literature [13–21].

Table 3
Activity of compounds **1a**, **1c**, **18** and **19** assessed with the acetic acid-induced writhing test on the mice.

Compound	Dose per p.o.		N. writhes	% Of writhes reduction
	$mg\ kg^{-1}$	$nmol\ kg^{-1}$		
CMC			33.4 ± 2.5	–
1a	3	5.5	26.9 ± 2.6	19.0
	10	18.4	21.3 ± 2.5*	36.2
	20	36.8	18.1 ± 3.0*	46.1
	40	73.6	13.5 ± 2.8*	59.6
1c	20	35.9	24.5 ± 3.2	26.9
	40	71.8	19.8 ± 2.4*	40.7
18	10	21.0	23.1 ± 2.6	30.8
	20	42.0	17.1 ± 2.3*	48.8
19	40	84.0	9.4 ± 2.5*	71.2
	20	40.8	25.7 ± 2.8	23.3
	40	81.6	24.9 ± 2.2	26.4

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

5.1.3. *General procedure for the preparation of 1,5-diarylpyrroles (**6a**–**b**)*

A solution of **5** (0.58 g, 2.28 mmol) in ethanol (2 mL), the suitable amine (2.28 mmol) and *p*-toluenesulfonic acid (30 mg, 0.17 mmol) was microwave irradiated at 160 °C for 45 min (power 150 W, pressure 150 psi). The crude reaction mixture was concentrated and purified by chromatography on aluminium oxide with a 3:1 cyclohexane/ethyl acetate mixture to give the expected 1,5-diarylpyrroles **6a**–**d** as solids in satisfactory yield. Analytical data, melting points, and the ^1H NMR spectra were consistent with those reported in the literature [13–21].

5.1.4. *General procedure for the preparation of ethyl (1,5diaryl-pyrrol) glyoxylate (**7a**–**b**)*

To a solution of pyrrole **6a**–**b** (4.82 mmol) in anhydrous dichloromethane, under nitrogen flow, $TiCl_4$ (0.53 mL, 4.82 mmol) and ethyl oxalyl chloride (0.54 mL, 4.82 mmol) were added at 0 °C. The solution was stirred for 4 h at rt. The mixture was then poured into cold water and extracted with $CHCl_3$. The organic solution was washed with a saturated aqueous NaCl solution, dried and evaporated *in vacuo*. Purification of the residue by flash chromatography with $CHCl_3$ as the eluent gave a solid which, after re-crystallization from hexane, afforded the expected products **7a**–**b**. Analytical data, melting points, and the ^1H NMR spectra were consistent with those reported in the literature [21].

5.1.5. *General procedure for the preparation of ethyl-2-(hydroxyimino)-(1,5-diarylpyrrol) acetate (**8a**–**b**)*

To a suspension of glyoxylate derivative **7a**–**b** (0.0582 mol) in a mixture of ethanol and 1,4-dioxan (100 mL), hydroxylamine hydrochloride (6.07 g, 0.0874 mol) and sodium acetate (11.9 g, 0.1456 mol) were added. The resulting solution was heated at 90 °C under stirring for 40 h. The solvents were removed *in vacuo* and the mixture was extracted with ethyl acetate (500 mL) and washed with water (100 mL). The organic phase was dried over sodium sulphate, filtered and concentrated to afford the oximes **8a**–**b** as yellowish solid.

*5.1.5.1. Ethyl 2-(hydroxyimino)-2-[2-methyl-5-[4-(methylsulfonyl) phenyl]-1-(4-fluorophenyl)-1H-pyrrol-3-yl]acetate (**8a**).* Yellowish solid, m.p. 103.°C (80% yield); ^1H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.73 (d, 2H, $J = 8.5$ Hz), 7.33 (m, 4H), 7.18 (d, 2H, $J = 8.5$ Hz), 6.55 (s, 1H), 5.67 (s, 1H), 4.18 (q, 2H, $J = 7.4$ Hz), 3.14 (s, 3H), 2.05 (s, 3H), 1.18 (t, 3H, $J = 7.4$ Hz); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 170.02, 159.70, 149.49, 144.50, 141.33, 135.20, 130.25, 129.20, 128.22, 126.11, 123.45, 122.50, 118.03, 115.34, 65.84, 43.15, 27.60, 10.96; ESI-Mass: *m*/*z* 467.105 $[M + Na]^+$; Anal. $C_{22}H_{21}FN_2O_5S$ (C, H, N).

*5.1.5.2. Ethyl 2-(hydroxyimino)-2-[2-methyl-5-[4-(methylsulfonyl) phenyl]-1-(3-fluorophenyl)-1H-pyrrol-3-yl]acetate (**8b**).* Yellowish solid, m.p. 104 °C (79% yield); ^1H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.73 (d, 2H, $J = 8.5$ Hz), 7.33 (m, 4H), 7.18 (d, 2H, $J = 8.5$ Hz), 6.55 (s, 1H), 5.67 (s, 1H), 4.18 (q, 2H), 3.14 (s, 3H), 2.05 (s, 3H), 1.18 (t, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 170.10, 159.66, 149.51, 144.67, 141.33, 138.78, 135.00, 130.75, 129.20, 128.22, 126.06, 123.40, 122.50, 120.66, 118.34, 115.37, 65.99, 43.10, 27.62, 11.06; ESI-Mass: *m*/*z* 467.105 $[M + Na]^+$; Anal. $C_{22}H_{21}FN_2O_5S$ (C, H, N).

Table 4
Activity of compounds **1a**, **18**, **19** and Celecoxib in the carrageenan-induced inflammation.

Pre-treatment i.pl.	Treatment p.o.	Dose	Before treatment	Paw-pressure				Oedema volume (mL)
				After treatment				
				15 min	30 min	45 min	60 min	
Saline	CMC	–	61.2 ± 2.5	65.6 ± 3.4	58.9 ± 3.8	61.4 ± 4.1	63.6 ± 4.0	1.44 ± 0.05
Carrageenan	CMC	–	36.1 ± 3.3	32.8 ± 2.9	30.6 ± 3.2	31.8 ± 2.2	34.8 ± 3.1	2.71 ± 0.08
Carrageenan	**1a**	20	33.8 ± 2.8	40.2 ± 3.6^ (25%)	43.7 ± 3.7^ (44%)	38.3 ± 3.1 (27%)	37.1 ± 3.0 (15%)	2.58 ± 0.05 (15%)
Carrageenan	**1a**	40	32.9 ± 3.0	45.4 ± 3.2* (40%)	50.2 ± 4.4* (70%)	45.6 ± 4.3* (53%)	37.5 ± 2.8 (16%)	2.11 ± 0.09* (45%)
Carrageenan	**18**	20	35.7 ± 3.1	38.7 ± 3.3 (18%)	40.1 ± 3.4^ (35%)	38.7 ± 3.4^ (29%)	35.2 ± 2.8 (10%)	2.52 ± 0.06 (17%)
Carrageenan	**18**	40	29.8 ± 3.1	48.3 ± 3.3* (50%)	54.1 ± 3.7* (80%)	49.7 ± 3.0* (60%)	38.7 ± 3.1 (23%)	2.17 ± 0.08* (40%)
Carrageenan	**19**	40	33.5 ± 2.6	41.2 ± 2.2^ (40%)	42.3 ± 3.8^ (41%)	40.7 ± 2.6^ (40%)	37.9 ± 3.0 (17%)	2.35 ± 0.07* (35%)
Carrageenan	Celecoxib	10	31.7 ± 2.7	45.7 ± 4.2^ (45%)	52.9 ± 3.1* (75%)	48.3 ± 3.4* (56%)	42.5 ± 2.9^ (39%)	2.53 ± 0.05* (17%)

^$P < 0.05$; *$P < 0.01$ in comparison with carrageenan-treated group.

5.1.6. *General procedure for the preparation of ethyl 2-amino-(1,5-diarylpyrrol)acetate (**9a–b**)*

To a solution of the crude oxime **8a–b** in ethanol (100 mL) formic acid (100 mL) was added. The solution was then cooled to 0 °C and zinc dust (16.4 g, 0.2522 mol) was added in portions over a period of 1 h. The mixture was allowed to warm up to rt very slowly and then stirred for 19 h. Afterwards, zinc salts were filtered off and the solvents evaporated under reduced pressure. The residue obtained was dissolved in water and the pH adjusted to 10 with 10% NaOH solution and extracted with EtOAc. The combined organic fractions were dried over sodium sulphate, filtered and concentrated *in vacuo* to afford the crude product. The material was then purified by column chromatography (silica gel) eluting with MeOH/$CHCl_3$ (1:20) (*v*/*v*) to afford compounds **9a–b**.

*5.1.6.1. Ethyl 2-amino-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**9a**).* White powder, m.p. 95 °C (50% yield); ^1H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.73 (d, 2H, $J = 8.5$ Hz), 7.30 (m, 4H), 7.18 (d, 2H, $J = 8.5$ Hz), 6.55 (s, 1H), 4.60 (s, 1 H), 4.04 (q, 2H, $J = 7.3$ Hz), 3.14 (s, 3H), 2.05 (s, 3H), 1.74 (s broad, 2H), 1.18 (t, 3H, $J = 7.3$ Hz); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 169.77, 159.42, 144.50, 141.33, 135.20, 130.25, 129.20, 128.22, 126.11, 123.45, 122.50, 115.34, 108.59, 65.84, 58.23, 43.33, 27.60, 10.96; ESI-Mass: *m*/*z* 453.126 $[M + Na]^+$; Anal. $C_{22}H_{23}FN_2O_4S$ (C, H, N).

*5.1.6.2. Ethyl 2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**9b**).* White powder, m.p. 97 °C (47% yield); ^1H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.73 (d, 2H), 7.32 (m, 4H), 7.19 (d, 2H), 6.59 (s, 1H), 4.76 (t, 1H), 4.09 (q, 2H), 3.16 (s, 3H), 2.05 (s, 3H), 1.74 (s, broad 2H), 1.16 (t, 3H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 169.89, 159.27, 143.85, 140.59, 135.72, 130.93, 128.96, 128.13, 125.89, 124.91, 123.92, 122.48, 117.02, 114.84, 108.04, 65.95, 57.99, 42.97, 27.31, 11.02; ESI-Mass: *m*/*z* 453.126 $[M + Na]^+$; Anal. $C_{22}H_{23}FN_2O_4S$ (C, H, N).

5.1.7. *General procedure for the preparation of tert-butyl (ethoxycarbonyl)(1,5-diarylpyrrol)carbamate (**10a–b**)*

To a solution of the amino ester **9a–b** (2.326 mmol) in MeOH (15 mL), Boc anhydride (1.014 g, 4.651 mmol) was added and the reaction mixture stirred for 18 h. The mixture was then diluted with water, adjusted the pH to 2–3 with 1 M HCl and extracted with EtOAc. The combined organic fractions were washed with water, dried over sodium sulphate and evaporated to give the crude product. The residue was purified by column chromatography (silica gel) eluting with EtOAc/hexane (1:20) (v/v) to afford the desired product as white solid.

Table 5
Nitric oxide releasing efficacy and potency for compounds **1a**, **1c**, **18** and **19**.

Compound	E_{max}	pIC_{50}	+ODQ 1 μM
1a	84.0 ± 2.0	5.66 ± 0.03	+
1c	93.0 ± 1.0	5.81 ± 0.03	+
18	69.0 ± 4.0	6.48 ± 0.06	+
19	39.0 ± 1.0	n.c.	+

*5.1.7.1. tert-Butyl (ethoxycarbonyl)-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]methyl carbamate (**10a**).* White solid, m.p. 105 °C (63% yield); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.70 (d, 2H, $J = 8.5$ Hz), 7.20 (m, 4H), 7.05 (d, 2H, $J = 8.5$ Hz), 6.51 (s, 1H), 5.67 (d, 1H, $J = 7.8$ Hz), 5.40 (d, 1H, $J = 7.8$ Hz), 4.15 (q, 2H, $J = 6.8$ Hz), 3.15 (s, 3H), 2.05 (s, 3H), 1.45 (s, 9H), 1.13 (t, 3H, $J = 6.8$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.21, 163.43, 158.20, 140.28, 139.37, 135.81, 132.96, 128.87, 127.93, 126.75, 122.47, 121.34, 118.92, 117.55, 81.68, 65.79, 54.20, 44.25, 28.61, 27.22, 11.20. ESI-Mass: *m*/*z* 553.597 $[M + Na]^+$. Anal. $C_{27}H_{31}FN_2O_6S$ (C, H, N).

*5.1.7.2. 1H-Pyrrol-3-yl]methyl carbamate (**10b**).* White solid, m.p. 109 °C (67% yield); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.69 (d, 2H, $J = 8.4$ Hz), 7.35 (m, 1H), 7.13 (d, 2H, $J = 8.4$ Hz), 7.09 (m, 1H), 6.90 (m, 2H), 6.59 (s, 1H), 5.65 (d, 1H, $J = 7.8$ Hz), 5.39 (d, 1H, $J = 7.8$ Hz), 4.13 (d, 2H, $J = 6.8$ Hz), 3.17 (s, 3H), 2.06 (s, 3H), 1.44 (s, 9H), 1.14 (t, 3H, $J = 6.8$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.32, 168.35, 154.93, 139.72, 137.21, 135.81, 132.86, 128.98, 127.84, 126.75, 122.47, 121.44, 118.92, 117.76, 117.58, 108.43, 81.46, 63.87, 53.96, 43.82, 29.04, 27.27, 11.10. ESI-Mass: *m*/*z* 553.597 $[M + Na]^+$. Anal. $C_{27}H_{31}FN_2O_6S$ (C, H, N).

5.1.8. *Procedure for the preparation of benzyl (ethoxycarbonyl)(1,5-diarylpyrrol)carbamate (**13a–b**)*

To a biphasic solution of amino ester **9a–b** (2.326 mmol) in DCM (10 mL), sodium carbonate (0.481 g, 4.6 mmol) and benzyl

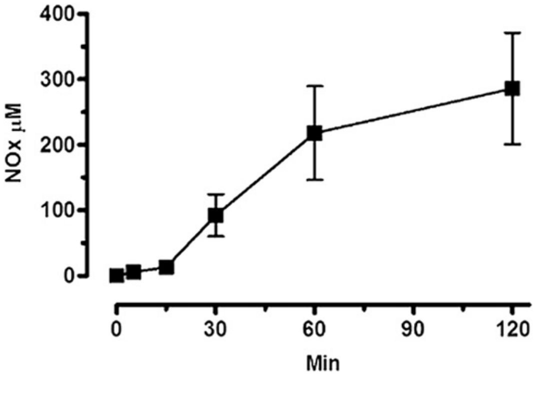


Fig. 7. Time-dependent increase of the concentrations of NOx (nitrites + nitrates) following the incubation of 1 mM **18** in rat hepatic homogenate, containing the opportune cofactors.

chloroformate (0.401 g, 2.352 mmol) were added at 0 °C, the reaction mixture was allowed to warm up to rt and stirred for further 4 h. The mixture was then diluted with water and the two phases separated. The organic fraction was washed with water (50 mL × 2), dried over sodium sulphate and evaporated to give the crude product. The residue was purified by column chromatography (silica gel) eluting with EtOAc/hexane (1:20) (v/v) to afford the desired product as white solid.

*5.1.8.1. N-Benzyl-(ethoxycarbonyl)-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]methylcarbamate (**13a**).* White solid, m.p. 95 °C (65% yield); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.89 (d, 1H, $J = 7.8$ Hz), 7.74 (d, 2H, $J = 8.5$ Hz), 7.51 (m, 5H), 7.19 (m, 6H), 6.50 (s, 1H), 5.16 (d, 1H, $J = 7.8$ Hz), 5.08 (s, 2H), 4.11 (q, 2H, $J = 6.9$ Hz), 3.10 (s, 3H), 2.00 (s, 3H), 1.15 (t, 3H, $J = 6.9$ Hz). ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 168.45, 159.71, 156.04, 142.96, 132.28, 138.86, 137.12, 135.98, 133.27, 130.34, 127.96, 126.51, 126.23, 125.38, 123.91, 122.54, 118.07, 117.44, 67.45, 64.31, 54.11, 43.89, 15.46, 12.01. ESI-Mass: *m*/*z* 587.163 [M + Na]$^+$. Anal. $C_{30}H_{29}FN_2O_6S$ (C, H, N).

*5.1.8.2. tert-Butyl (ethoxycarbonyl)-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-N-benzyl-(ethoxycarbonyl)-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]methylcarbamate (**13b**).* White solid, m.p. 97 °C (61% yield); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 8.01 (d, 1H, $J = 7.8$ Hz), 7.89 (d, 2H, $J = 8.5$ Hz), 7.59 (m, 6H), 7.46 (m, 3H), 7.06 (m, 2H), 6.48 (s, 1H), 5.18 (d, 1H, $J = 7.8$ Hz), 5.15 (s, 2H), 4.16 (q, 2H, $J = 6.8$ Hz), 3.15 (s, 3H), 2.04 (s, 3H), 1.17 (t, 3H, $J = 6.8$ Hz). ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 170.23, 169.27, 155.08, 140.24, 139.23, 137.21, 136.03, 132.78, 130.58, 129.05, 128.04, 127.70, 127.12, 126.65, 124.55, 123.09, 119.25, 118.33, 117.67, 107.28, 67.98, 64.77, 54.05, 44.66, 15.62, 12.03. ESI-Mass: *m*/*z* 587.163 [M + Na]$^+$. Anal. $C_{30}H_{29}FN_2O_6S$ (C, H, N).

*5.1.9. Procedure for the preparation of protected (Boc and Cbz) (1,5-diarylpyrrol)glycine (**11a**–**b**, **14a**–**b**)*

To a solution of the amino ester **9a**–**b** (2.326 mmol) in MeOH (15 mL), a solution of NaOH (0.2367 g, 5.917 mmol) in water (3 mL) was added and the reaction mixture stirred for 17 h. The mixture was then concentrated *in vacuo*, diluted with water (2 mL) and the acid precipitated through the addition of HCl. The solid was collected to give the product as off white solid.

*5.1.9.1. N-tert-Butyl-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]-2-amino-acetic acid (**11a**).* White solid, m.p. 120 °C (60% yield); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 12.70 (s, 1H), 7.81 (d, 1H, $J = 7.8$ Hz), 7.75 (d, 2H, $J = 8.5$ Hz), 7.35 (m, 4H), 7.15 (d, 2H, $J = 8.5$ Hz), 6.63 (s, 1H), 5.09 (d, 1H, $J = 7.8$ Hz), 3.17 (s, 3H), 2.02 (s, 3H), 1.43 (s, 9H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 170.23, 162.38, 157.75, 140.28, 139.77, 136.23, 133.06, 128.71, 127.86, 126.88, 121.98, 122.01, 119.32, 117.33, 82.01, 53.87, 44.15, 28.53, 11.11. ESI-Mass: *m*/*z* 525.147 [M + Na]$^+$. Anal. $C_{25}H_{27}FN_2O_6S$ (C, H, N).

*5.1.9.2. N-tert-Butyl-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]-2-amino-acetic acid (**11b**).* White solid, m.p. 128 °C (64% yield); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 12.75 (s, 1H), 7.85 (d, 1H, $J = 7.8$ Hz), 7.72 (d, 2H, $J = 8.4$ Hz), 7.37 (m, 1H), 7.16 (d, 2H, $J = 8.4$ Hz), 7.12 (m, 1H), 6.90 (m, 1H), 6.64 (s, 1H), 5.10 (d, 2H, $J = 7.8$ Hz), 3.15 (s, 3H), 2.10 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 170.84, 169.17, 155.62, 140.23, 138.12, 136.04, 133.11, 129.01, 128.13, 126.88, 123.98, 122.21, 117.45, 117.32, 116.82, 108.99, 82.31, 63.74, 55.05, 42.46, 29.00, 18.26, 10.90. ESI-Mass: *m*/*z* 525.147 [M + Na]$^+$. Anal. $C_{25}H_{27}FN_2O_6S$ (C, H, N).

*5.1.9.3. N-(Benzyloxy)carbonyl-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]-2-amino-acetic acid (**14a**).* White solid, m.p. 130 °C (>95% yield); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 12.81 (s, 1H), 7.81 (d, 1H, $J = 7.8$ Hz), 7.79 (d, 2 H, $J = 8.5$ Hz), 7.68–7.37 (m, 9H), 7.16 (d, 2H, $J = 8.5$ Hz), 6.63 (s, 1H), 5.09 (d, 1H, $J = 7.8$ Hz), 5.06 (s, 2H), 3.21 (s, 3H), 2.03 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 171.03, 169.27, 156.98, 143.08, 140.23, 136.91, 134.21, 133.09, 130.88, 127.97, 127.34, 126.32, 124.55, 122.91, 119.00, 118.23, 117.43, 106.99, 63.21, 53.92, 43.71, 11.18. ESI-Mass: *m*/*z* 559.132 [M + Na]$^+$. Anal. $C_{28}H_{25}FN_2O_6S$ (C, H, N).

*5.1.9.4. N-(Benzyloxy)carbonyl-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]-2-amino-acetic acid (**14b**).* White solid, m.p. 127 °C (>95% yield); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 12.81 (s, 1H), 7.89 (d, 1H, $J = 7.8$ Hz), 7.70 (d, 2H, $J = 8.6$ Hz), 7.52 (m, 1H), 7.47 (m, 7H), 7.32 (d, 2H, $J = 8.6$ Hz), 7.21 (m, 1H), 6.64 (m, 1H), 5.11 (d, 1H, $J = 7.8$ Hz), 5.09 (s, 2H), 3.15 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 171.22, 168.97, 155.85, 141.02, 139.18, 138.12, 136.58, 132.93, 128.56, 128.02, 127.90, 126.76, 126.54, 126.00, 124.21, 124.00, 117.11, 116.50, 115.99, 106.97, 63.74, 55.05, 42.46, 11.02. ESI-Mass: *m*/*z* 559.132 [M + Na]$^+$. Anal. $C_{28}H_{25}FN_2O_6S$ (C, H, N).

*5.1.10. General procedure for the preparation of protected (Cbz and Boc) α-(1,5-diarylpyrrole)-glycine nitroxyalkyl esters (**12a**–**d**, **15a**–**d**)*

To a solution of the suitable protected α-1,5-diarylpyrrole-glycine (**11a**–**b** and **14a**–**b**) (0.1 mmol) in dichloromethane (5 mL), under nitrogen atmosphere, an excess of hydroxyalkyl nitrate (**17a**–**b**) (0.3 mmol), DMAP (0.1 mmol), and EDCI (0.2 mmol) were added in sequence. The reaction was quenched with water after 3 h and extracted with chloroform. The organic layer was washed with 1 M HCl, $NaHCO_3$ saturated solution, brine and dried over sodium sulphate. The filtration and concentration of the organic phase gave a crude material, which was purified by chromatography on silica gel using petroleum ether/chloroform/ethyl acetate, 4:4:1 (v/v/v), as the eluent to give the desired product in good yield.

*5.1.10.1. 3-(Nitroxy)propyl N-tert-butyl-(ethoxycarbonyl)-2-(1-(4-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino-acetate (**12a**).* White solid, m.p. 112 °C (73% yield) ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (d, 2H, $J = 8.4$ Hz), 7.35 (m, 4H), 7.15 (d, 2H, $J = 8.4$ Hz), 6.63 (s, 1H), 5.59 (d, 1H, $J = 6.8$ Hz), 5.33 (d, 1H, $J = 6.8$ Hz), 4.64 (t, 2H, $J = 7.0$ Hz), 4.28 (t, 2H, $J = 7.0$ Hz), 3.05 (s, 3H), 2.15 (s, 3H), 1.98 (m, 2H), 1.46 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.24, 168.72, 155.24, 138.72, 135.83, 134.88, 132.50, 129.21, 127.17, 126.74, 123.02, 123.33, 118.86, 117.32, 81.97, 64.05, 61.34, 52.94, 44.03, 29.50, 27.82, 11.24. ESI-Mass: *m*/*z* 628.174 [M + Na]$^+$. Anal. $C_{27}H_{32}FN_3O_9S$ (C, H, N).

*5.1.10.2. 3-(Nitroxy)propyl] N-tert-butyl-(ethoxycarbonyl)-2-(1-(3-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino-acetate (**12b**).* White solid, m.p. 114 °C (71% yield) ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.83 (d, 2H, $J = 8.4$ Hz), 7.40 (m, 1H), 7.12 (m, 3H), 6.90 (m, 2H), 6.65 (s, 1H), 5.55 (d, 1H, $J = 7.8$ Hz), 5.30 (d, 1H, $J = 7.8$ Hz), 4.71 (t, 2H, $J = 7.0$ Hz), 4.30 (t, 2H, $J = 7.0$ Hz), 3.05 (s, 3H), 2.17 (s, 3H), 1.99 (m, 2H), 1.40 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 170.27, 168.88, 154.23, 141.40, 138.07, 136.29, 134.52, 130.01, 128.13, 126.68, 123.12, 121.43, 117.32, 117.06, 118.11, 108.31, 81.93, 62.85, 60.56, 54.82, 43.83, 28.51, 27.31, 10.90. ESI-Mass: *m*/*z* 628.174 [M + Na]$^+$. Anal. $C_{27}H_{32}FN_3O_9S$ (C, H, N).

*5.1.10.3. 4-(Nitroxy)butyl N-tert-butyl-(ethoxycarbonyl)-2-(1-(4-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino-acetate (**12c**).* White solid, m.p. 114 °C (75% yield) ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.75 (d, 2H, $J = 8.5$ Hz), 7.36 (m, 4H), 7.15 (d, 2H, $J = 8.5$ Hz), 6.65 (s, 1H), 5.59 (d, 1H, $J = 6.8$ Hz), 5.33 (d, 1H, $J = 6.8$ Hz), 4.65 (t, 2H, $J = 7.6$ Hz), 4.10 (t, 2H, $J = 7.6$ Hz), 3.05

(s, 3H), 2.15 (s, 3H), 1.73 (m, 2H), 1.58 (m, 2H), 1.46 (s, 9H). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 170.82, 169.04, 156.23, 139.01, 135.82, 134.65, 132.46, 129.02, 126.89, 125.38, 124.21, 123.13, 118.15, 116.85, 109.95, 81.88, 65.82, 63.00, 53.28, 44.03, 25.24, 23.82, 10.98. ESI-Mass: m/z 642.190 [M + Na]$^+$. Anal. C$_{28}$H$_{34}$FN$_3$O$_9$S (C, H, N).

*5.1.10.4. 4-(Nitroxy)butyl N-tert-butyl-(ethoxycarbonyl)-2-[1-(3-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)]-1H-pyrrol-3-yl]-2-amino-acetate (**12d**).* White solid, m.p. 118 °C (68% yield) ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.83 (d, 2H, $J = 8.5$ Hz), 7.40 (m, 1H), 7.12 (m, 3H), 6.90 (m, 2H), 6.65 (s, 1H), 5.55 (d, 1H, $J = 7.8$ Hz), 5.30 (d, 1H, $J = 7.8$ Hz), 4.70 (t, 2H, $J = 7.6$ Hz), 4.11 (t, 2H, $J = 7.6$ Hz), 3.05 (s, 3H), 2.17 (s, 3H), 1.71 (m, 2H), 1.63 (m, 2H), 1.40 (s, 9H). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 169.82, 167.54, 153.90, 142.04, 138.75, 135.81, 133.81, 129.85, 128.00, 127.41, 122.31, 123.56, 117.20, 117.06, 117.33, 107.02, 79.68, 65.79, 62.09, 54.82, 44.28, 27.94, 26.82, 22.54, 12.03. ESI-Mass: m/z 642.190 [M + Na]$^+$. Anal. C$_{28}$H$_{34}$FN$_3$O$_9$S (C, H, N).

*5.1.10.5. 3-(Nitroxy)propyl N-benzyl-(ethoxycarbonyl)-2-(1-(4-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino-acetate (**15a**).* White solid, 118 °C (62% yield); ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.69 (d, 2 H, $J = 8.4$ Hz), 7.55 (m, 9H), 7.16 (d, 2 H, $J = 8.4$ Hz), 6.64 (s, 1H), 5.54 (d, 1H, $J = 7.7$ Hz), 5.32 (d, 1H, $J = 7.7$ Hz), 5.10 (s, 2H), 4.66 (t, 2H, $J = 6.8$ Hz), 4.39 (t, 2H, $J = 6.8$ Hz), 3.00 (s, 3H), 2.20 (s, 3H), 1.95 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 170.83, 168.72, 154.98, 143.23, 139.01, 135.82, 134.65, 132.43, 131.80, 127.98, 127.49, 127.29, 127.17, 126.89, 123.45, 123.22, 119.05, 116.92, 115.88, 66.82, 66.24, 60.45, 44.03, 27.8, 11.24. ESI-Mass: m/z 662.158 [M + Na]$^+$; Anal. C$_{31}$H$_{30}$FN$_3$O$_9$S (C, H, N).

*5.1.10.6. 3-(Nitroxy)propyl N-benzyl-(ethoxycarbonyl)-2-(1-(3-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino acetate (**15b**).* White solid, 115 °C (66% yield); ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.70 (d, 2H, $J = 8.5$ Hz), 7.52 (m, 1H), 7.47 (m, 7H), 7.32 (d, 2H, $J = 8.5$ Hz), 7.21 (m, 1H), 6.64 (m, 1H), 5.50 (d, 1H, $J = 7.8$ Hz), 5.29 (d, 1H, $J = 7.8$ Hz), 5.10 (s, 2H), 4.67 (t, 2H, $J = 6.9$ Hz), 4.29 (t, 2H, $J = 6.9$ Hz), 3.00 (s, 3H), 2.20 (s, 3H), 1.95 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 170.83, 169.04, 155.28, 142.47, 142.06, 138.07, 136.29, 134.31, 130.83, 128.79, 127.64, 127.32, 127.13, 126.40, 124.08, 121.31, 117.51, 117.08, 116.92, 108.09, 67.20, 63.04, 60.93, 54.82, 43.83, 28.51, 10.90. ESI-Mass: m/z 662.158 [M + Na]$^+$; Anal. C$_{31}$H$_{30}$FN$_3$O$_9$S (C, H, N).

*5.1.10.7. 4-(Nitroxy)butyl N-benzyl-(ethoxycarbonyl)-2-(1-(4-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino acetate (**15c**).* White solid, 121 °C (61% yield); ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.65 (d, 2 H, $J = 8.5$ Hz), 7.53 (m, 9H), 7.17 (d, 2 H, $J = 8.5$ Hz), 6.64 (s, 1H), 5.55 (d, 1H, $J = 7.6$ Hz), 5.34 (d, 1H, $J = 7.6$ Hz), 5.12 (s, 2H), 4.65 (t, 2H, $J = 7.0$ Hz), 4.11 (t, 2H, $J = 7.0$ Hz), 3.05 (s, 3H), 2.15 (s, 3H), 1.73 (m, 2H), 1.58 (m, 2H). ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 171.00, 168.66, 154.99, 143.21, 139.34, 135.04, 134.09, 132.40, 131.81, 128.22, 127.39, 127.21, 127.07, 126.09, 123.31, 123.20, 118.95, 117.55, 116.09, 66.67, 66.04, 60.05, 44.03, 27.81, 27.55, 11.21. ESI-Mass: m/z 676.184 [M + Na]$^+$; Anal. C$_{32}$H$_{32}$FN$_3$O$_9$S (C, H, N).

*5.1.10.8. 4-(Nitroxy)butyl N-benzyl-(ethoxycarbonyl)-2-(1-(3-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1H-pyrrol-3-yl)-2-amino acetate (**15d**).* White solid, 119 °C (64% yield); ^{1}H NMR (400 MHz, CDCl$_3$): δ (ppm) 7.70 (d, 2H, $J = 8.4$ Hz), 7.55 (m, 1H), 7.47 (m, 7H), 7.29 (d, 2H, $J = 8.4$ Hz), 7.21 (m, 1H), 6.64 (s, 1H), 5.48 (d, 1H, $J = 7.7$ Hz), 5.31 (d, 1H, $J = 7.7$ Hz), 5.12 (s, 2H), 4.60 (t, 2H, $J = 7.0$ Hz), 4.07 (t, 2H, $J = 7.0$ Hz), 3.05 (s, 3H), 2.10 (s, 3H), 1.75 (m, 2H), 1.60 (m, 2H); ^{13}C NMR (100 MHz, CDCl$_3$): δ (ppm) 170.92, 169.00, 155.20, 142.45, 142.21, 138.07, 136.29, 134.31, 130.83, 128.79, 127.64, 127.32, 127.03, 126.41, 124.08, 121.89, 117.51, 117.08, 116.97, 108.09, 67.20, 63.44, 61.45, 54.82, 43.83, 27.33, 28.51, 10.91. ESI-Mass: m/z 676.184 [M + Na]$^+$; Anal. C$_{32}$H$_{32}$FN$_3$O$_9$S (C, H, N).

*5.1.11. General procedure for the preparation of α-(1,5-diarylpyrrole)-glycine hydroxyalkyl esters (**2a–d**)*

To a solution of the opportune Cbz-protected α-1,5-diarylpyrrole-glycine (**15a–d**) (0.32 mmol) in isopropanol (2 mL), ammonium formate (0.081 g, 1.3 mmol) and Pd/C (0.07 g) were added. The solution was microwave irradiated at 80 °C for 5 min (power 150 W, pressure 170 psi). The reaction mixture was passed through Celite® and then poured into water (20 mL). The pH was adjusted to 10–12 (using NaOH 10% solution) and the mixture extracted with chloroform. The organic layer was then dried over sodium sulphate. The filtration and concentration of the organic phase gave a material, which was identified to be the product without the need of any further purification.

*5.1.11.1. 3-Hydroxypropyl 2-amino-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**2a**).* Yellowish powder, m.p. 107 °C (>95% yield); FT-IR (neat, cm^{-1}) ν: 3340 (m), 3320 (m), 2852 (w), 1736 (s), 1593 (s), 1302 (s), 1140 (s), 1098 (s); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.90 (d, 2H, $J = 8.4$ Hz), 7.70 (d, 2H, $J = 8.4$ Hz), 7.29 (d, 2H, $J = 8.4$ Hz), 7.08 (m, 2H), 6.56 (s, 1H), 4.78 (t app., 1H), 4.45 (s, 1H), 4.15 (m, 2H), 3.57 (m, 2H), 3.06 (s, 3H), 2.24 (s br., 2H), 2.18 (s, 3H), 1.90 (m, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 171.30, 159.87, 147.30, 146.78, 141.51, 135.00, 130.32, 129.25, 128.78, 126.20, 123.48, 122.53, 117.12, 64.10, 61.00, 58.88, 43.07, 27.62, 11.06; ESI-Mass: m/z 483.137 [M + Na]$^+$; Anal. C$_{23}$H$_{25}$FN$_2$O$_5$S (C, H, N).

*5.1.11.2. 3-Hydroxypropyl 2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**2b**).* Yellowish powder, m.p. 110 °C (>95% yield); FT-IR (neat, cm^{-1}) ν: 3345 (m), 3322 (m), 2858 (w), 1741 (s), 1590 (s), 1300 (s), 1145 (s), 1090 (s); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.71 (d, 2H, $J = 8.4$ Hz), 7.41 (m, 1H), 7.15 (d, 2H, $J = 8.4$ Hz), 7.05 (m, 1H), 6.97 (m, 2H), 6.59 (s, 1H), 4.79 (t app., 1H), 4.40 (s, 1H), 4.13 (m, 2H), 3.56 (m, 2H), 3.11 (s, 3H), 2.20 (s br., 2H), 2.19 (s, 3H), 1.92 (m, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 171.44, 158.57, 148.78, 144.00, 139.50, 138.56, 137.66, 135.54, 131.92, 128.90, 128.66, 126.20, 123.44, 122.59, 117.11, 64.09, 60.88, 58.80, 43.10, 27.60, 11.01; ESI-Mass: m/z 483.137 [M + Na]$^+$; Anal. C$_{23}$H$_{25}$FN$_2$O$_5$S (C, H, N).

*5.1.11.3. 4-Hydroxybutyl 2-amino-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**2c**).* Yellowish powder, m.p. 101 °C (>95% yield); FT-IR (neat, cm^{-1}) ν: 3349 (m), 3324 (m), 2861 (w), 1731 (s), 1588 (s), 1298 (s), 1132 (s), 1079 (s); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.72 (d, 2H, $J = 8.5$ Hz), 7.37 (d, 2H, $J = 8.5$ Hz), 7.15 (d, 2H, $J = 8.5$ Hz), 7.09 (m, 2H), 6.60 (s, 1H), 4.78 (t app., 1H), 4.41 (s, 1H), 4.17 (m, 2H), 3.55 (m, 2H), 3.10 (s, 3H), 2.20 (s br., 2H), 2.19 (s, 3H), 1.89 (m, 4H); ^{13}C NMR (100 MHz, DMSO-*d6*): δ (ppm) 171.31, 159.90, 147.40, 146.71, 141.50, 135.02, 130.32, 129.67, 128.80, 126.21, 123.43, 122.51, 117.03, 74.34, 64.10, 60.90, 43.11, 24.62, 22.88, 11.16; ESI-Mass: m/z 497.152 [M + Na]$^+$; Anal. C$_{24}$H$_{27}$FN$_2$O$_5$S (C, H, N).

*5.1.11.4. 3-Hydroxybutyl 2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**2d**).* Yellowish powder, m.p. 98 °C (>95% yield); FT-IR (neat, cm^{-1}) ν: 3353 (m), 3311 (m), 2861 (w), 1744 (s), 1590 (s), 1300 (s), 1147 (s), 1092 (s); ^{1}H NMR (400 MHz, DMSO-*d6*): δ (ppm) 7.74 (d, 2H, $J = 8.5$ Hz), 7.43 (m, 1H), 7.15 (d, 2H, $J = 8.5$ Hz), 7.11 (m, 1H), 6.97 (m, 2H), 6.52 (s, 1H), 4.80 (t app., 1H), 4.39 (s, 1H), 4.18 (m, 2H), 3.53 (m, 2H), 3.13 (s, 3H), 2.21 (s br., 2H), 2.18 (s, 3H), 1.86 (m, 4H); ^{13}C NMR (100 MHz, DMSO-*d6*):

δ (ppm) 171.39, 158.60, 148.90, 144.05, 139.54, 138.56, 137.66, 135.50, 131.91, 128.88, 128.60, 126.20, 123.44, 122.59, 117.18, 74.70, 64.10, 61.20, 43.34, 25.69, 23.44, 11.09; ESI-Mass: m/z 497.152 $[M + Na]^+$; Anal. $C_{24}H_{27}FN_2O_5S$ (C, H, N).

5.1.12. General procedure for the preparation of α-(1,5-diarylpyrrole)-glycine nitroxyalkyl esters (1a–d)

To a solution of the opportune Boc-protected α-1,5-diarylpyrrole-glycine (**12a**–**d**) (0.40 mmol) in dioxane (2 mL), an excess of trifluoroacetic acid (4 mL) was added dropwise at 0 °C. The solution was microwave irradiated at 60 °C for 40 min (power 150 W, pressure 170 psi). The reaction mixture was poured into ice/water (20 mL) and the pH adjusted to 12–13 (using ammonia solution) and the mixture extracted with chloroform. The organic layer was then dried over sodium sulphate. The filtration and concentration of the organic phase gave a crude material, which was purified through column chromatography (alumina) using MeOH/DCM 1:20 (v/v) to obtain the product as a yellowish powder.

*5.1.12.1. 3-(Nitroxy)propyl 2-amino-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**1a**).* Yellowish powder, m.p. 121 °C (75% yield); FT-IR (neat, cm^{-1}) ν: 3344 (m), 2890 (w), 1736 (s), 1685 (s), 1590 (s), 1302 (s), 1147 (s), 1090 (s), 880 (m); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.70 (d, 2H, $J = 8.4$ Hz), 7.29 (d, 2H, $J = 8.4$ Hz), 7.11 (d, 2H, $J = 8.4$ Hz), 7.08 (m, 2H), 6.61 (s, 1H), 4.65 (t, 2H, $J = 7.2$ Hz), 4.50 (s, 1H), 4.39 (t, 2H, $J = 7.2$ Hz), 3.01 (s, 3H), 2.20 (s, 3H), 1.95 (m, 2H), 1.80 (s br., 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.33, 159.91, 147.33, 146.80, 141.53, 135.00, 130.32, 129.22, 128.76, 126.19, 123.45, 122.50, 117.09, 74.34, 64.08, 60.98, 53.07, 27.62, 11.06; ESI-Mass: m/z 528.122 $[M + Na]^+$; Anal. $C_{23}H_{24}FN_3O_7S$ (C, H, N).

*5.1.12.2. 3-(Nitroxy)propyl 2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**1b**).* Yellowish powder, m.p. 117 °C (73% yield); FT-IR (neat, cm^{-1}) ν: 3340 (m), 2887 (w), 1738 (s), 1681 (s), 1592 (s), 1300 (s), 1149 (s), 1091 (s), 891 (m); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.70 (d, 2H, $J = 8.5$ Hz), 7.39 (m, 1H), 7.18 (d, 2H, $J = 8.5$ Hz), 7.10 (m, 1H), 7.03 (m, 2H), 6.60 (s, 1H), 4.67 (t, 2H, $J = 7.2$ Hz), 4.48 (s, 1H), 4.38 (t, 2H, $J = 7.2$ Hz), 3.07 (s, 3H), 2.23 (s, 3H), 1.98 (m, 2H), 1.90 (s br., 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.44, 158.57, 148.78, 144.00, 139.50, 138.56, 137.66, 135.54, 131.92, 128.90, 128.66, 126.20, 123.44, 122.59, 117.11, 74.58, 64.11, 61.03, 43.00, 27.69, 11.11; ESI-Mass: m/z 528.122 $[M + Na]^+$; Anal. $C_{23}H_{24}FN_3O_7S$ (C, H, N).

*5.1.12.3. 4-(Nitroxy)butyl 2-amino-2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**1c**).* Yellowish powder, m.p. 102 °C (58% yield); FT-IR (neat, cm^{-1}) ν: 3347 (m), 2894 (w), 1742 (s), 1689 (s), 1587 (s), 1298 (s), 1157 (s), 1099 (s), 877 (m); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.69 (d, 2H, $J = 8.5$ Hz), 7.25 (d, 2H, $J = 8.5$ Hz), 7.15 (d, 2H, $J = 8.5$ Hz), 7.07 (m, 2H), 6.60 (s, 1H), 4.68 (t, 2H, $J = 7.2$ Hz), 4.48 (s, 1H), 4.39 (t, 2H, $J = 7.2$ Hz), 3.07 (s, 3H), 2.23 (s, 3H), 2.01 (m, 4H), 1.90 (s br., 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.31, 159.90, 147.40, 146.71, 141.50, 135.02, 130.32, 129.67, 128.80, 126.21, 123.43, 122.51, 117.03, 74.34, 64.10, 60.90, 43.11, 24.62, 22.88, 11.16; ESI-Mass: m/z 542.137 $[M + Na]^+$; Anal. $C_{24}H_{26}FN_3O_7S$ (C, H, N).

*5.1.12.4. 4-(Nitroxy)butyl 2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1H-pyrrol-3-yl]acetate (**1d**).* Yellowish powder, m.p. 113 °C (57% yield); FT-IR (neat, cm^{-1}) ν: 3350 (m), 2892 (w), 1740 (s), 1693 (s), 1580 (s), 1292 (s), 1150 (s), 1088 (s), 870 (m); ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.68 (d, 2H, $J = 8.4$ Hz), 7.42 (m, 1H), 7.18 (d, 2H, $J = 8.4$ Hz), 7.10 (m, 1H), 7.05 (m, 2H), 6.61 (s, 1H), 4.70 (t, 2H, $J = 7.2$ Hz), 4.50 (s, 1H), 4.37 (t, 2H, $J = 7.2$ Hz), 3.12 (s, 3H), 2.20 (s, 3H), 2.03 (m, 4H), 1.90 (s br., 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 171.39, 158.60, 148.90, 144.05, 139.54, 138.56, 137.66, 135.50, 131.91, 128.88, 128.60, 126.20, 123.44, 122.59, 117.18, 74.70, 64.10, 61.20, 43.88, 25.69, 23.44, 11.09; ESI-Mass: m/z 542.137 $[M + Na]^+$; Anal. $C_{24}H_{26}FN_3O_7S$ (C, H, N).

5.1.13. General procedure for the preparation of hydroxylalkyl nitrates (17a–b)

To a solution of diol (20 mmol) in ethyl acetate (100 mL) nitric acid (65% w/v, 16 mmol), glacial acetic acid (97 mmol), and acetic anhydride (97% w/v, 59 mmol) were added. The mixture was allowed to react overnight. The reaction was quenched with 20% KOH solution. The pH was adjusted to 12–13, and the aqueous phase was extracted with ethyl acetate (3 × 100 mL), washed with NaCl saturated solution, and dried over Na_2SO_4. After filtration, the organic layers were concentrated *in vacuo*, and the crude product was purified by chromatography on silica gel, using petroleum ether/chloroform/ethyl acetate, 3:1:1 (v/v/v), as eluent, to obtain the expected nitroester in good yield. Analytical data, melting points, and the ^1H NMR spectra were consistent with those reported in the literature [21].

5.2. Bio-pharmacological testing

5.2.1. Pharmacology

In Vitro Anti-Inflammatory Study. The *in vitro* profiles of compounds **1a**–**d** and **2a**–**d**, related to their inhibitory activity towards both COX-1 and COX-2 isoenzymes, were evaluated through cell-based assay employing murine monocyte/macrophage J774 cell lines. The cell line was grown in DMEM supplemented with 2 mM glutamine, 25 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum (FBS), and 1.2% sodium pyruvate. Cells were plated in 24-well culture plates at a density of 2.5×10^5 cells/mL or in 60 mm diameter culture dishes (3×10^6 cells per 3 mL per dish) and allowed to adhere at 37 °C in 5% CO_2 for 2 h. Immediately before the experiments, the culture medium was replaced with fresh medium and cells were stimulated as described previously [15].

The evaluation of COX-1 inhibitory activity was achieved by pretreating cells with test compounds (10 μM) for 15 min and then incubating them at 37 °C for 30 min with 15 μM arachidonic acid to activate the constitutive COX. For the compounds with COX-1 inhibition higher than 50% (at 10 μM), the cells were also treated with lower concentrations (0.01–1 μM). At the end of the incubation, the supernatants were collected for the measurement of prostaglandin E_2 (PGE_2) levels by a radioimmunoassay (RIA). To evaluate COX-2 activity, cells were stimulated for 24 h with *Escherichia coli* lipopolysaccharide (LPS, 10 μg/mL) to induce COX-2, in the absence or presence of test compounds (0.01–10 μM). The supernatants were collected for the measurement of PGE_2 by means of 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 with GraphPad Instat, and the data fit was performed using the sigmoidal dose–response equation (variable slope) (GraphPad).

5.2.2. Ex vivo vasorelaxing activity

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86–609. The effects of nitro-oxy compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding. The aortae were immediately excised, freed of extraneous tissues, and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. The 5 mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; glucose 5.5) thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3) connected to a computerized system (Biopac). After an equilibration period of 60 min, the endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM) precontracted vascular rings. A relaxation of <10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation of ≥10% (i.e., significant presence of the endothelium), were discarded. From 30 to 40 min after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM). When the contraction reached a stable plateau, 3-fold increasing concentrations of compounds (1 nM–10 μM) were added. Preliminary experiments showed that the KCl (30 mM) induced contractions remained in a stable tonic state for at least 40 min. The same experiments were carried out also in the presence of a well-known GC inhibitor: 1 μM ODQ which was incubated in aortic preparations after the endothelium removal confirmation. The vasorelaxing efficacy was evaluated as maximal vasorelaxing response (E_{max}), expressed as a percentage of the contractile tone induced by 30 mM KCl. When the limit concentration of 10 μM (the highest concentration that could be administered) of the tested compounds did not produce the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage of the contractile tone induced by 30 mM KCl, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as the negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by 30 mM KCl. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as the mean ± standard error for 6–10 experiments. Two-way ANOVA was used for statistical analysis, and $P < 0.05$ was considered representative of significant statistical differences. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism, version 4.0).

5.2.3. Determination of the formation of nitrites and nitrates, in rat liver homogenate

The experimental procedures were carried out following the guidelines of the European Community Council Directive 86–609. The liver homogenates were obtained from male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding. The portal vein was immediately cannulated and the liver was perfused with 4 °C-cold homogenization buffer (composition: K_2HPO_4 100 mM; EDTA 1 mM; KCl 15 mM; sucrose 0.25 M and Ethanol 0.1%, pH 7.4). After about 5 min of slow perfusion, the liver was minced with scissors and washed with cold homogenation buffer. Then, it was dried with filter paper, weighted and resuspended (1:5 w/v) in cold homogenation buffer. The sample was finally homogenated in Turrax. In a vial, 400 μL of the above homogenate were mixed with 500 μL of assay buffer (composition: K_2HPO_4 100 mM; EDTA 1 mM; pH 7.4), containing glutathione, NADH and NADPH (all at the final concentration of 1 mM). The vials were then thermostated at 37 °C, and 100 μL of a DMSO solution of the tested NO-donor were added (final concentration of the tested compound = 1 mM), allowing the release of NO, which in turn is rapidly converted to the stable inorganic metabolites, i.e. nitrites and nitrates. Aliquots (100 μL) of the above medium were collected at selected intervals (5, 15, 30, 60 and 120 min) and added into a beaker containing 1.9 mL of an aqueous solution of H_2SO_4 (0.1 M) and KI (0.1 M). This solution allow nitrite ions to be exhaustively and instantaneously reduced to NO, which is amperometrically titrated by a NO-selective electrode connected to an Apollo 4000 free radical analyser (World Precision Instrument), allowing us to determine the concentration of the nitrites previously formed in the biological sample. In parallel experiments, a Nitrate Reductor (World Precision Instrument) was constantly kept immerged into the vial. This tool allowed nitrates to be constantly converted to nitrites. The concentration of nitrites in these samples is defined as NOx, which reflects the sum of all the nitrites and nitrates previously derived from NO. Opportune calibration curves were previously obtained with standard solutions of sodium nitrite.

5.2.4. In vivo analgesic and anti-inflammatory study

In vivo anti-inflammatory activity of the new compounds was also assessed. Male Swiss albino mice (23–25 g) and Sprague–Dawley or Wistar rats (150–200 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 paw pressure test was performed by inducing an inflammatory process by the intraplantar (ipl) carrageenan administration 4 h before the test. The carrageenan induced paw oedema test was also performed, evaluating the paw volume of the right hind paw 4 h after the injection of carrageenan and comparing it with saline/carrageenan-treated controls. The analgesic activity of compounds was also assessed by performing the abdominal constriction test, using mice into which a 0.6% solution of acetic acid (10 mL/kg) had been injected intraperitoneal (ip). The number of stretching movements was counted for 10 min, starting 5 min after administration. *Statistical analysis*. Results were expressed as the means ± S.E.M. 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 analysed using the "Origin 7.5" software.

5.3. Solubility assessment in phosphate buffered saline (PBS) and simulated gastric fluid (SGF)

Stock solutions of compounds **1a**, **1c**, **18** and **19** of concentration 20 mM were prepared in DMSO. A standard phosphate buffered saline (PBS) 0.01 M (pH 7.4) was used to determine the solubility in neutral pH conditions; SGF 0.05 M solution without pepsin (pH was adjusted to 1.5 using concentrated HCl) was instead used to check the solubility of compounds in acid medium. Seven point calibration standards (1, 5, 10, 25, 50, 100 and 200 μM) were prepared from each 20 mM solution stock by serial dilution. 2 μL of each test compound (stock solution 20 mM in DMSO) were added to 198 μL of PBS and SGF in two duplicate wells in multiscreen solubility filter plate, covered and shaken for 90 min at 300 rpm using a Multipulse Vortexer (Glas-Col). The sample was filtered using a Millipore manifold filter assembly and collected in an acceptor plate and then analysed on HPLC together with the standards solutions (HPLC-Waters Separations Module 2695 with photodiode array detector).

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