

Nitrooxymethyl-Substituted Analogues of Celecoxib: Synthesis and Pharmacological Characterization

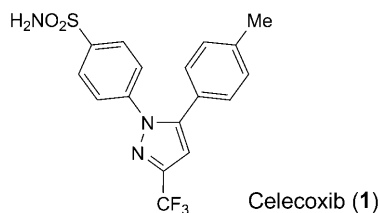
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Nitrooxymethyl-substituted analogues of celecoxib were synthesized and tested for their cyclooxygenase (COX)-inhibiting, vasodilator, and anti-aggregatory activities, as well as for their metabolic stability in human serum and whole blood. The results showed their potency and selectivity in inhibiting the COX isoforms, evaluated in whole human blood, as well as their anti-aggregatory activity to depend closely on the position at which the NO-donor moiety is introduced. All products dilated rat aorta strips precontracted with phenylephrine in a dose-dependent manner through a cGMP-dependent mechanism. They were stable in human serum while, in blood, they were metabolically transformed, principally to the related alcohols.

Introduction. – Cyclooxygenase (COX) is one of the key enzymes implicated in the transformation of arachidonic acid into prostanoids. It exists in at least two isoforms, COX-1 and COX-2. The former is prevalently a constitutive and the latter an inducible isoform. Selective blocking of these isoforms induces specific pharmacological effects which can be exploited in therapy [1]. COX-2-Selective inhibitors (Coxibs) are a new class of drugs recently introduced into the market [2]; they induce anti-inflammatory effects without the gastrotoxic side-effects typical of the classical non-steroidal anti-inflammatory drugs (NSAIDs), which are nonselective COX inhibitors. An interesting aspect of these drugs is their potential use in treating *Alzheimer's* disease and certain forms of cancer [3][4]. The drawback in their use is that they increase the risk of heart attack and stroke [5][6]. Blood vessels and platelets are the major targets of prostanoids such as prostacycline PGI_2 , prostaglandin PGE_2 , and thromboxane TxA_2 in the cardiovascular system. The proaggregatory and vasoconstrictor TxA_2 is mainly synthesized *via* COX-1 in the platelets, while vasodilator (PGI_2 , PGE_2) and antiplatelet (PGI_2) compounds are synthesized mainly in the vascular endothelium. A strategy to improve the benefit–risk profiles of these drugs is to design a multi-target drug by combining COX-2-selective inhibition with nitric oxide (NO)-dependent activities. NO displays a variety of effects in the cardiovascular system, including vasodilation, inhibition of platelet aggregation, modulation of platelet and leukocytes adherence to vessels, and inhibition of smooth muscle cell proliferation [7]. Examples of this type of approach have already been reported [8][9] including by our group [10][11]. As a development of our work in this field, we now describe the synthesis and structural characterization of a new series of NO-donor COX-2 inhibitors obtained by introducing NO-donor nitrooxy functions into the well-known selective COX-2

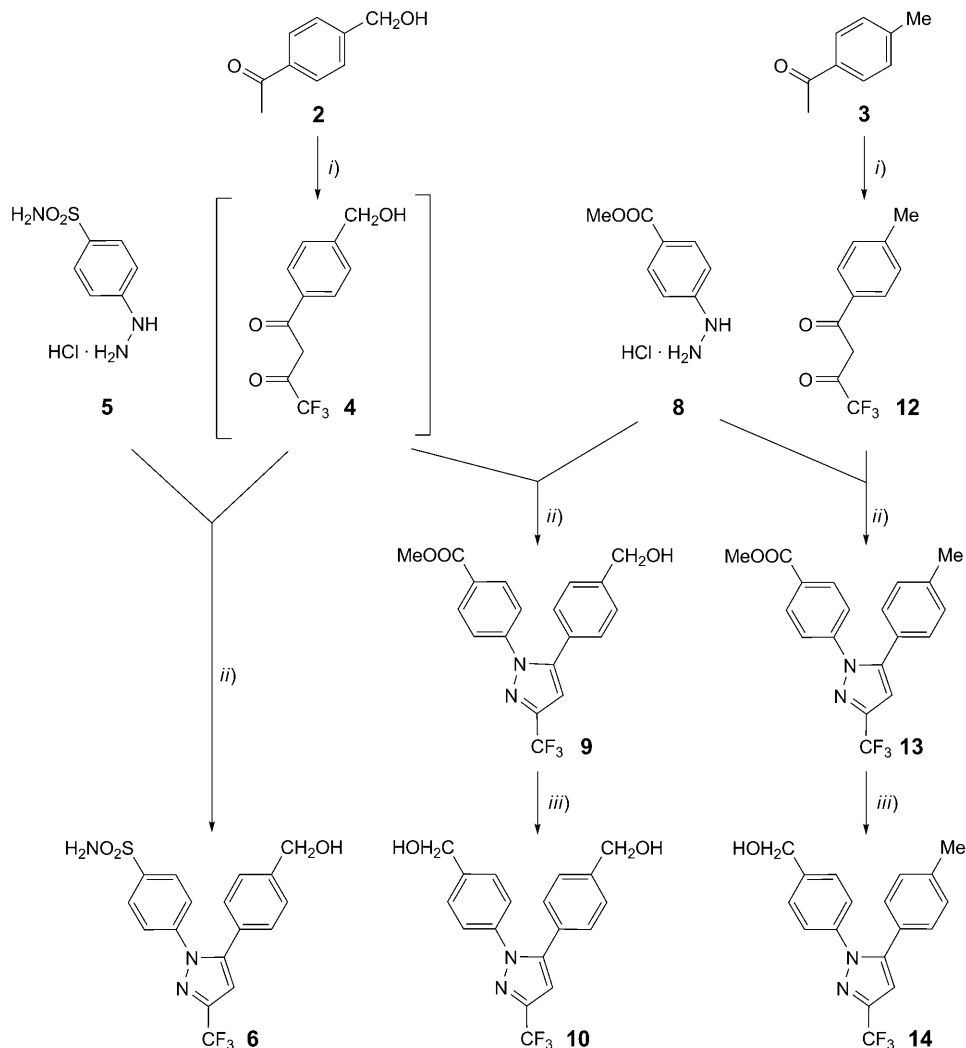
inhibitor celecoxib (**1**) [12] that has recently been placed under surveillance by FDA, following its suspected cardiotoxicity.



The results of a preliminary pharmacological screening of these new hybrid products and of their alcoholic metabolites show that their potency and selectivity in inhibiting the COX isoforms, evaluated in whole human blood, is closely dependent on the position at which the NO-donor moiety is introduced. This aspect is discussed, as well as the NO-dependent ability of the products to relax rat aorta strips precontracted with phenylephrine and, for selected compounds, their capacity to inhibit collagen-induced platelet aggregation of human platelet rich plasma (PRP).

Results and Discussion. – 1. *Chemistry.* The general strategy for the synthesis of the nitrooxy substituted celecoxib derivatives **7**, **11**, and **15** is outlined in *Schemes 1* and *2*. The trifluoromethyl β -diketone **4** was obtained by refluxing 4-(hydroxymethyl)acetophenone (**2**) and ethyl trifluoroacetate in MeOH in the presence of MeONa. The intermediate **4** was isolated but not purified, since it was immediately condensed in refluxing EtOH with the hydrochloride of the phenylhydrazine derivative **5** to give, with a fair yield, the 1,5-diphenyl-1*H*-pyrazole derivative **6**, accompanied by a small amount of the 1,3-diphenyl isomer. This is in keeping with reports that 1,5-diarylpyrazole derivatives are almost exclusively produced, when the hydrochloride salt of the phenylhydrazine is condensed with trifluoromethyl β -diketones in refluxing EtOH or MeOH [13]. Treatment of **6** dissolved in MeCN with *N*-bromosuccinimide (NBS) in the presence of Ph_3P and AgNO_3 afforded a crude product that was purified by flash chromatography to give the expected final compound **7**, mononitrooxymethyl-substituted at the 5-phenyl group. When **4** underwent reaction with the hydrochloride salt of phenylhydrazine derivative **8**, it produced the pyrazolylbenzoate **9** in a highly regiospecific manner. Reduction of the ester function of **9** with LiAlH_4 yielded the dimethanol **10**, which was transformed into the final dinitrooxy derivative **11** according to the same procedure used to prepare **7** from **6**. This reaction was accompanied by the formation of small amounts of a mixture of the two isomeric mononitrooxy alcohols **11a** and **11b** in a ratio of *ca.* 1 : 1 (NMR detection). When **8** was allowed to react in refluxing MeOH with the trifluoromethyl β -diketone **12**, obtained from the commercially available 4-methylacetophenone (**3**), the 1,5-diphenylpyrazole derivative **13** was yielded in a moderately regiospecific manner. This product was separated by flash chromatography from a minor amount of 1,3-diphenyl isomer and transformed into the final mononitrooxy derivative **15**, through the intermediate formation of the alcohol **14**, following the sequence of reactions used to prepare **11** from **9**.

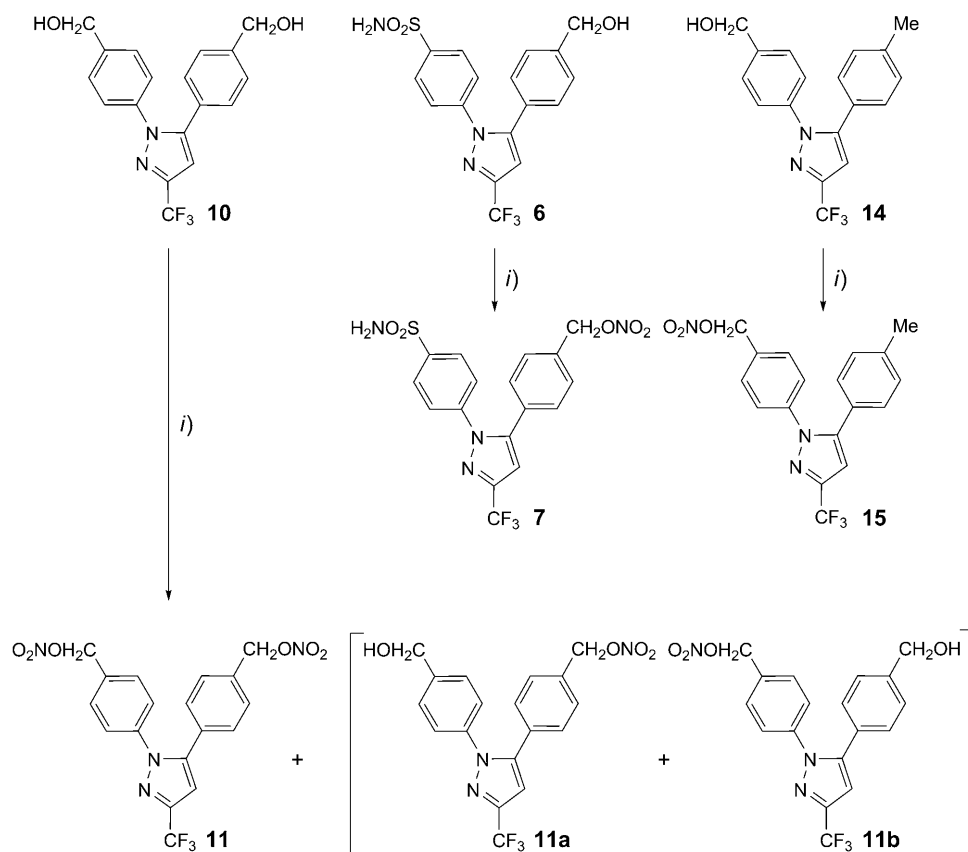
Scheme 1



i) MeONa, CF₃COOEt, MeOH, reflux. *ii*) Refluxing in MeOH (in EtOH for **6**). *iii*) LiAlH₄, THF.

2. Stability in Human Serum and in Human Whole Blood. It is known that nitrates can undergo chemical and enzymatic metabolism [14]. Celecoxib (**1**) and its derivatives were found to be stable following 24 h incubation in human serum. By contrast, derivatives **7**, **11**, and **15** were extensively metabolized when incubated in whole human blood. This transformation was monitored by RP-HPLC. The half-lives ($t_{1/2}$) are reported in Table I. Products **11** and **15** behaved similarly, while **7** surprisingly did not: its $t_{1/2}$ is *ca.* 1 h, vs. 25–30 h of the other compounds, and after 24 h only 4.5% of the original product remained unchanged. Compounds **11** and **15** were principally

Scheme 2



i) Ph_3P , AgNO_3 , *N*-bromosuccinimide (NBS), $-15^\circ \rightarrow \text{r.t.}$

transformed into the corresponding alcohols **10**, **11a/11b** and **14**, respectively (Table 1). These metabolites were identified by comparison of their retention times with those of alcohols **10** and **14**, which were used as intermediates to prepare the final compounds. Similarly, the mixture **11a/11b** was used as reference to identify the two isomeric mononitrooxy alcohols that derive from the biotransformation of **11**. The behavior of

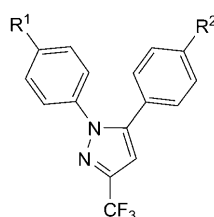
Table 1. Whole Human Blood Stability

Compound	% of unchanged compound after 24 h (\pm SEM)	% of alcoholic metabolites after 24 h (\pm SEM)	$t_{1/2}$ [h]
1	100		
7	4.5 ± 1.1	4.5 ± 0.5 (6)	0.8
11	59.4 ± 4.4	16.2 ± 0.7 of 11a/11b 11.7 ± 1.0 of 10	30.0
15	54.4 ± 2.3	41.2 ± 1.7 (14)	25.4

compound **7** again differed: HPLC analysis, using the alcohol **6** as standard, revealed only 4.5% of this product as detectable metabolite. This indicates that the other metabolites formed are not free in the cytosol, but they are sequestered by components of the blood compartment.

3. *COX Inhibition.* The ability of the products and of their alcoholic metabolites to inhibit the COX enzymes was evaluated in human whole blood according to an established procedure [15]. To assay the extent of COX-2 isoform inhibition, human heparinized whole blood was incubated with lipopolysaccharide (LPS) overnight in the presence of the inhibitors, and plasma reduction of PGE₂ production in comparison with control samples was assayed as a function of COX-2 inhibition. For COX-1 inhibition, whole blood samples without any anticoagulant were incubated with the inhibitors for 1 h; serum was then collected and analyzed for TxB₂ production. The potencies of the products are expressed as *IC*₅₀ (Table 2); when this could not be calculated because inhibition did not reach 50%, inhibition at the maximum concentration tested (100 μM) is reported.

Table 2. COX-1 and COX-2 Inhibition Data for Compounds and Celecoxib (**1**) Taken as Reference



Compound	R ¹	R ²	COX-1		COX-2
			<i>IC</i> ₅₀ ± SE [μM]	% Inhibition ± SE (100 μM ^a)	<i>IC</i> ₅₀ ± SE [μM]
1	SO ₂ NH ₂	Me	14 ± 2	–	1.3 ± 0.4
6	SO ₂ NH ₂	CH ₂ OH	b)	20 ± 13	52 ± 3
7	SO ₂ NH ₂	CH ₂ ONO ₂	b)	6.7 ± 3.8	67 ± 19
10	CH ₂ OH	CH ₂ OH	0.51 ± 0.14	–	12 ± 3
11	CH ₂ ONO ₂	CH ₂ ONO ₂	b)	15 ± 7	19 ± 1
14	CH ₂ OH	Me	0.41 ± 0.08	–	11 ± 3
15	CH ₂ ONO ₂	Me	4.6 ± 1.6	–	31 ± 10

^a) Maximum concentration tested. ^b) Inhibition of control TxB₂ production did not reach 50%.

The data show that product **7**, derived from substituting the nitrooxymethyl function for the methyl group of celecoxib (**1**), is a feeble COX-2 inhibitor, being *ca.* 50 times less potent than the lead, but that it retains a good degree of COX-2 selectivity: it displayed negligible COX-1 activity when tested at 100 μM concentration. Its alcoholic metabolite **6** behaves similarly. When it is the aminosulfonyl group that is substituted, which is an important determinant for the COX-2 selectivity of **1** [16], **15** is obtained, which is a more potent COX-1 inhibitor than **1**, and which displays a rather good COX-1 selectivity. COX-1 Inhibition potency and selectivity are even more evident in its alcoholic metabolite **14**. Finally, the simultaneous substitution of both the Me and the

aminosulfonyl moiety in **1** provides **11**, which is a fairly potent COX-2 inhibitor, with negligible COX-1 activity when tested at a concentration of 100 μM . By contrast, its dihydroxy metabolite **10** shows a COX-inhibitory profile very close to that of **14**. All compounds were also tested for their ability to inhibit collagen-induced platelet aggregation of human platelet rich plasma (PRP). The products **10** and **14** display antiplatelet action in the μM range (Table 3) in keeping with their ability to inhibit the COX-1 isoform. Also the activity of the NO donor **15** is essentially COX-1- and not NO-dependent, in agreement with the reduced ability of platelets to effect NO release from organic nitrates [17]. Indeed, the anti-aggregatory potency of this compound was not modified by the presence of ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a well-known inhibitor of the soluble guanylate cyclase (sGC).

Table 3. Anti-Aggregatory and Vasodilating Properties of Derivatives

Compound	R ¹	R ²	Anti-aggregatory activity		Vasodilating activity
			<i>IC</i> ₅₀ (95% CL) [μM]	% inhibition \pm SE (100 μM ^a)	<i>EC</i> ₅₀ \pm SE [μM]
1	SO ₂ NH ₂	Me	31 (25–39)	–	–
6	SO ₂ NH ₂	CH ₂ OH	^b)	5.8 \pm 2.1	–
7	SO ₂ NH ₂	CH ₂ ONO ₂	^b)	3.4 \pm 2.6	0.26 \pm 0.05 7.0 \pm 1.3 ^c)
10	CH ₂ OH	CH ₂ OH	8.4 (7.0–10)	–	–
11	CH ₂ ONO ₂	CH ₂ ONO ₂	^b)	7.4 \pm 4.3	0.10 \pm 0.03 26 \pm 9 ^c)
14	CH ₂ OH	Me	11 (10–12)	–	–
15	CH ₂ ONO ₂	Me	48 (44–52) ^d)	–	0.12 \pm 0.01 > 100 ^c)
ISDN ^e)					4.7 \pm 0.6 > 100 ^c)

^a) Maximum concentration tested. ^b) Inhibition of control aggregation effect did not reach 50%. ^c) In the presence of 1 μM ODQ. ^d) Unchanged in the presence of 100 μM ODQ. ^e) Isosorbide dinitrate.

4. *Vasodilation.* The *in vitro* vasodilating activities of this new series of COX inhibitors was assessed on rat aorta strips precontracted with phenylephrine. All products relaxed the contracted tissues in a concentration-dependent manner. Their potencies, expressed as *EC*₅₀, are shown in Table 3. All act as potent vasodilators, their potencies being closely distributed in the submicromolar range. Vasodilating potencies markedly decreased, when the experiments were repeated in the presence of 1 μM

ODQ. This is typical of the classical nitrooxy-containing compounds, such as isosorbide dinitrate (ISDN), which was used as reference.

Conclusions. – Introduction at the *para*-position of the phenyl rings present in celecoxib (**1**) of either the nitrooxymethyl group or the hydroxymethyl group, deriving from its metabolic transformation, gives rise to a complex behavior. When these moieties are linked to the *N*-phenyl ring, the selective and potent COX-1 inhibitors **14** and **15**, endowed with anti-aggregatory activity, are obtained. By contrast, when they are attached to the *C*-phenyl group, the selective but weak COX-2 inhibitors **6** and **7** are generated. Interesting results occurred following the simultaneous introduction of either two nitrooxy or two hydroxymethyl functions on the two phenyl groups. In the former case, the fairly potent and selective COX-2 inhibitor **11**, endowed with a feeble anti-aggregatory activity, was obtained, while, in the latter case, compound **10** was obtained, which is a potent and selective COX-1 inhibitor endowed with a high anti-aggregatory activity. All of the products containing nitrooxy moieties displayed NO-dependent vasodilatory activity. Compound **11** emerges as the most interesting product arising from these chemical manipulations of compound **1**, in terms of the possibility of producing a COX-2-selective inhibitor potentially possessing low cardiotoxicity.

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Experimental Part

General. Compounds **2** [18], **5** [19], **8** [20], and **12** [12] were synthesized according to the methods described in the literature. THF was distilled immediately before use from Na and benzophenone. Anhydrous MgSO_4 was used as drying agent for the organic phases. Flash column chromatography (FC): silica gel (Merck Kieselgel 60, 230–400 mesh ASTM) with the eluents indicated. TLC: 5×20 cm plates with 0.25-mm layer thickness. M.p.: cap. apparatus (Büchi 540); m.p. with decomposition were determined after placing the sample in a bath at a temp. 10° below the m.p.; a heating rate of 1° min^{-1} was used. FT-IR: Perkin-Elmer SPECTRUM BXII. ^1H - and ^{13}C -NMR: Bruker Avance-300. MS: Finnigan-Mat TSQ-700. Analysis (C, H, N) of the new compounds was performed by REDOX (Monza) and the results are within $\pm 0.4\%$ of the theoretical values.

4,4,4-Trifluoro-1-[4-(hydroxymethyl)phenyl]butane-1,3-dione (4). 1.5M MeONa in MeOH (5 ml, 7.5 mmol) was added to a soln. of 4-(hydroxymethyl)acetophenone (**2**) (0.72 g, 4.8 mmol) in dry MeOH (4 ml). The mixture was stirred for 5 min, after which ethyl trifluoroacetate (0.7 ml, 7.0 mmol) was added. After refluxing for 24 h, the mixture was cooled to r.t. and concentrated *in vacuo*; 10% HCl (10 ml) was added, and the mixture was extracted with AcOEt. The extracts were dried and concentrated to afford **4** as a brown oil (0.99 g), which was used without further purification.

4-[5-[4-(Hydroxymethyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (6). (4-Sulfamoylphenyl)hydrazine hydrochloride (**5**; 0.93 g, 4.2 mmol) was added to a stirred soln. of **4** (0.99 g) in EtOH (50 ml). The mixture was heated to reflux for 24 h. After cooling to r.t., the mixture was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H_2O and brine, dried, and concentrated *in vacuo* to give an oil that was purified by FC (petroleum ether (PE)/i-PrOH 9:1) to give **6** (0.95 g, 57%). Pale yellow solid. M.p. $157\text{--}158^\circ$ (from benzene). ^1H -NMR (CDCl_3): 7.84 (*d*, $J=8.6$, 2 arom. H); 7.41 (*d*, $J=8.6$, 2 arom. H); 7.34 (*d*, $J=8.1$, 2 arom. H); 7.20 (*d*, $J=8.1$, 2 arom. H); 6.76 (*s*, CH (pyrazole)); 5.31 (*s*, NH_2); 4.69 (*s*, CH_2); 2.06 (*br. s*, OH). ^{13}C -NMR (CDCl_3): 144.9; 144.2 (*q*, $^2J=38$); 142.4; 142.3; 141.5; 129.0; 127.7; 127.5; 127.4; 125.6; 121.0 (*q*, $^1J=268$); 106.6 (*q*, $^3J=2$); 64.5. CI-MS (i-Bu): 398 ($[M+1]^+$). Anal. calc. for $\text{C}_{17}\text{H}_{14}\text{F}_3\text{N}_3\text{O}_3\text{S}$ (397.38): C 51.38, H 3.55, N 10.57; found C 51.68, H 3.66, N 10.52.

Methyl 4-[5-[4-(Hydroxymethyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzoate (9). [4-(Methoxycarbonyl)phenyl]hydrazine hydrochloride (**8**; 0.84 g, 4.2 mmol) was added to a stirred soln. of **4** (0.99 g) in MeOH (40 ml). The mixture was heated to reflux for 3 h. After cooling to r.t., the mixture was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H₂O and brine, dried, and concentrated *in vacuo* to give an oil that was purified by FC (CH₂Cl₂/AcOEt 99:1) to give **9** (0.73 g, 46%). Yellow solid. M.p. 95–97° (dec.; i-Pr₂O). ¹H-NMR (CDCl₃): 8.02 (*d*, *J* = 8.6, 2 arom. H); 7.39 (*d*, *J* = 8.6, 2 arom. H); 7.34 (*d*, *J* = 8.1, 2 arom. H); 7.21 (*d*, *J* = 8.1, 2 arom. H); 6.76 (*s*, CH (pyrazole)); 4.71 (*s*, CH₂); 3.92 (*s*, Me); 2.15 (*br. s*, OH). ¹³C-NMR (CDCl₃): 166.1; 144.8; 143.9 (*q*, ²*J* = 38); 142.7; 142.3; 130.6; 130.0; 129.8; 128.0; 127.2; 125.0; 121.1 (*q*, ¹*J* = 267); 106.3 (*q*, ³*J* = 2); 64.5; 52.4. CI-MS (i-Bu): 377 (*[M+1]*⁺). Anal. calc. for C₁₉H₁₅F₃N₂O₃ (376.34): C 60.64, H 4.02, N 7.44; found: C 60.87, H 4.40, N 7.40.

Methyl 4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzoate (13). Compound **8** (3.41 g, 17.0 mmol) was added to a stirred soln. of **12** (3.87 g, 17.0 mmol) in MeOH (100 ml). The mixture was heated to reflux for 20 h. After cooling to r.t., the mixture was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H₂O and brine, dried, and concentrated *in vacuo* to give an oil that was purified by FC (PE/AcOEt 97:3) to give **13** (2.06 g, 34%). White solid. M.p. 78.5–79.5° (hexane). ¹H-NMR (CDCl₃): 8.03 (*d*, *J* = 8.6, 2 arom. H); 7.39 (*d*, *J* = 8.6, 2 arom. H); 7.16–7.08 (*m*, 4 arom. H); 6.73 (*s*, CH (pyrazole)); 3.92 (*s*, MeO); 2.36 (*br. s*, Me). ¹³C-NMR (CDCl₃): 166.2; 145.2; 143.9 (*q*, ²*J* = 38); 142.8; 139.6; 130.6; 129.8; 129.7; 128.8; 126.1; 125.1, 121.3 (*q*, ¹*J* = 267); 106.1 (*q*, ³*J* = 2); 52.5; 21.4. EI-MS: 360 (100, *M*⁺), 329 (50). Anal. calc. for C₁₉H₁₅F₃N₂O₂ (360.34): C 63.33, H 4.20, N 7.77; found: C 63.95, H 4.13, N 7.81.

4-[1-[4-(Hydroxymethyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-1-yl]phenylmethanol (10). A soln. of **9** (1.26 g, 3.4 mmol) in dry THF (10 ml) was added dropwise to a stirred mixture of LiAlH₄ (0.13 g, 3.4 mmol) in dry THF (10 ml) under N₂. After 30 min, H₂O (1 ml), NaOH 15% (1 ml), and H₂O (1 ml) were added sequentially to the mixture, after which the solid was filtered and washed with MeOH. The filtrate was concentrated *in vacuo*, and the residue was taken up in AcOEt, washed with H₂O and brine, dried, and concentrated *in vacuo* to give an oil that was purified by FC (CH₂Cl₂/AcOEt 8:2) to give **10** (0.70 g, 59%). White solid. M.p. 107–111° (i-Pr₂O). ¹H-NMR (CDCl₃): 7.35–7.26 (*m*, 6 arom. H); 7.21 (*d*, *J* = 8.1, 2 arom. H); 6.74 (*s*, CH (pyrazole)); 4.70 (*s*, 2 CH₂); 1.98 (*br. s*, OH); 1.90 (*br. s*, OH). ¹³C-NMR (CDCl₃): 144.5; 143.2 (*q*, ²*J* = 38), 141.8; 141.3; 138.3; 128.9; 128.3; 127.4; 127.1; 125.6; 121.2 (*q*, ¹*J* = 267); 105.6 (*q*, ³*J* = 2); 64.6; 64.4. CI-MS (i-Bu): 349 (*M+1*)⁺. Anal. calc. for C₁₈H₁₅F₃N₂O₂ (348.32): C 62.07, H 4.34, N 8.04; found: C 62.45, H 4.42, N 7.94.

4-[1-[4-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]phenylmethanol (14). A soln. of **13** (2.18 g, 6.0 mmol) in dry THF (25 ml) was added dropwise to a stirred mixture of LiAlH₄ (0.23 g, 6.0 mmol) in dry THF (25 ml) under N₂. After 30 min, H₂O (1 ml), NaOH 15% (1 ml) and H₂O (1 ml) were added sequentially to the mixture, then the solid was filtered and washed with MeOH. The filtered soln. was concentrated *in vacuo*, and the residue was taken up in AcOEt, washed with H₂O and brine, dried, and concentrated *in vacuo* to give a solid (1.91 g, 96%) that was purified by crystallization from EtOH/H₂O to give **14**. White solid. M.p. 109–111° (EtOH/H₂O). ¹H-NMR (CDCl₃): 7.35–7.27 (*m*, 4 arom. H); 7.11–7.09 (*m*, 4 arom. H); 6.71 (*s*, CH (pyrazole)); 4.70 (*s*, CH₂); 2.34 (*s*, Me); 1.93 (*br. s*, OH). ¹³C-NMR (CDCl₃): 144.8; 143.2 (*q*, ²*J* = 38); 141.2; 139.1; 138.5; 129.4; 128.7; 127.3; 126.2; 125.5; 121.3 (*q*, ¹*J* = 267); 105.3 (*q*, ³*J* = 2); 64.4, 21.3. EI-MS: 332 (100, *M*⁺). Anal. calc. for C₁₈H₁₅F₃N₂O (332.32): C 65.06, H 4.55, N 8.43; found: C 65.12, H 4.53, N 8.33.

4-[1-[4-(Aminosulfonyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-5-yl]benzyl Nitrate (7). AgNO₃ (1.00 g, 5.8 mmol) and Ph₃P (0.75 g, 2.9 mmol) were added to a soln. of **6** (1.00 g, 2.5 mmol) in MeCN (30 ml) under N₂. The mixture was cooled to 0°, and NBS (0.50 g, 2.8 mmol) was added portionwise. Stirring was continued at 0° for 1 h and then at r.t. for 3 h. AcOEt was added to the mixture, and the solid was filtered; the filtrate was washed with H₂O, brine, dried, and concentrated *in vacuo* to give a solid that was purified by FC (PE/AcOEt 7:3) to give **7** (0.76 g, 70%). White solid. ¹H-NMR ((D₆)DMSO): 7.89 (*d*, *J* = 8.6, 2 arom. H); 7.58–7.51 (*m*, 4 arom. H, NH₂); 7.39 (*d*, *J* = 8.3, 2 arom. H); 7.30 (*s*, CH of pyrazol); 5.60 (*s*, CH₂). ¹³C-NMR ((D₆)DMSO): 144.5; 144.5; 142.2 (*q*, ²*J* = 37); 140.9; 133.4; 129.5; 129.2; 129.0; 126.8; 126.0; 121.2 (*q*, ¹*J* = 267); 106.7 (*q*, ³*J* = 2); 74.3. EI-MS: 442 (40, *M*⁺), 397 (40), 302 (100). Anal. calc. for C₁₇H₁₃F₃N₄O₅S (442.38): C 46.16, H 2.96, N 12.66; found: C 45.96, H 2.89, N 12.29.

4-[1-[4-(Nitrooxymethyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-5-yl]benzyl Nitrate (**11**). AgNO₃ (1.03 g, 6 mmol) and Ph₃P (1.31 g, 5 mmol) were added to a soln. of **10** (0.7 g, 2 mmol) in MeCN (20 ml) under N₂. The mixture was cooled to –15° under N₂, and NBS (0.89 g, 5 mmol) was added portionwise. Stirring was continued at –15° for 1 h and then at r.t. for 12 h. AcOEt was added to the mixture, and the solid was filtered; the filtrate was washed with H₂O, brine and dried, and concentrated *in vacuo* to give an oil which was purified by FC (hexane/AcOEt 9:1). The first eluted compound was **11** (0.42 g, 48%). White solid. M.p. 52–53° (i-Pr₂O/PE). ¹H-NMR (CDCl₃): 7.43–7.25 (*m*, 8 arom. H); 6.78 (*s*, CH (pyrazole)); 5.44, 5.43 (2*s*, 2 CH₂ONO₂). ¹³C-NMR (CDCl₃): 143.9; 143.7 (*q*, ²*J*=38); 139.8; 133.4; 132.7; 130.0; 129.8; 129.4; 129.4; 125.7; 121.1 (*q*, ¹*J*=268); 106.3 (*q*, ³*J*=2); 73.9; 73.6. EI-MS: 438 (30, *M*⁺), 345 (48), 316 (100). Anal. calc. for C₁₈H₁₅F₃N₄O₆ (438.32): C 49.32, H 2.99, N 12.78; found: C 49.38, H 2.95, N 12.56.

The second eluted fraction was the mixture of the two mononitrate products **11a** and **11b**, obtained as a transparent oil. ¹H-NMR (CDCl₃): 7.41–7.20 (*m*, 16 arom. H); 6.75, 6.77 (2*s*, 2 CH (pyrazole)); 5.42, 5.43 (2*s*, 2 CH₂ONO₂); 4.73, 4.72 (2*s*, 2 CH₂OH); 1.79 (*br. s*, 2 OH). ¹³C-NMR (CDCl₃): 144.6; 143.8; 143.4 (*q*, ²*J*=38); 142.1; 141.6; 140.0; 138.2; 133.1; 132.5; 130.3; 129.7; 129.3; 129.2; 129.0; 128.1; 127.5; 127.2; 125.7; 125.6; 121.4 (*q*, ¹*J*=268); 106.0 (*q*, ³*J*=2); 105.9 (*q*, ³*J*=2); 73.9; 73.7; 64.6; 64.4. EI-MS: 393 (97, *M*⁺), 345 (100), 317 (99). Anal. calc. for C₁₈H₁₄F₃N₃O₄ (393.32): C 54.97, H 3.59, N 10.68; found: C 55.44, H 3.94, N 10.30.

4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzyl Nitrate (**15**). AgNO₃ (0.68 g, 4 mmol) and Ph₃P (0.92 g, 3.5 mmol) were added to a soln. of **14** (0.66 g, 2 mmol) in MeCN (30 ml) under N₂. The mixture was cooled to –15° under N₂, and NBS (0.62 g, 3.5 mmol) was added portionwise. Stirring was continued at –15° for 1 h and then at r. t. for 4 h. AcOEt was added to the mixture, and the solid was filtered; the filtrate was washed with H₂O, brine, dried, and concentrated *in vacuo* to give a residue that was purified by FC (PE/AcOEt 9.5:0.5) to give **15** (0.52 g, 68%). White solid. M.p. 106–107° (MeOH). ¹H-NMR (CDCl₃): 7.41–7.34 (*m*, 4 arom. H); 7.16–7.09 (*m*, 4 arom. H); 6.72 (*s*, CH (pyrazole)); 5.43 (*s*, CH₂); 2.37 (*s*, Me). ¹³C-NMR (CDCl₃): 144.9; 143.5 (*q*, ²*J*=38); 140.2; 139.4; 132.3; 130.0; 129.7; 128.7; 126.1; 125.7; 121.2 (*q*, ¹*J*=267); 105.8 (*q*, ³*J*=2); 73.8; 21.3. EI-MS: 377 (100, *M*⁺), 331 (85), 300 (90). Anal. calc. for C₁₈H₁₄F₃N₃O₃ (377.32): C 57.30, H 3.74, N 11.14; found: C 57.30, H 3.91, N 10.82.

Evaluation of Stability in Human Serum and in Whole Human Blood. Blood samples for both stability, COX selectivity, and platelet aggregation assays were obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers, who were treated according to the Helsinki protocol for biomedical experimentation, gave their informed consent to the use of blood samples for research purposes.

Compounds **7**, **11**, and **15** were incubated in human serum (sterile-filtered from human male AB plasma, *Sigma-Aldrich*) and in heparinized human blood at 100 μM concentration. The resulting solns. were maintained at 37±0.5°, and, at appropriate time intervals, the mixtures were diluted 1:2 with MeCN containing 0.1% CF₃COOH in order to deproteinize and precipitate cells, proteins, and enzymes. Samples were sonicated, vortexed, and then centrifuged for 15 min at 2150g. The clear supernatant was filtered by 0.45 μm PTFE filters (*Alltech*) and analyzed by RP-HPLC.

The RP-HPLC procedure allowed separation and quantitation of remaining compounds and metabolites. HPLC Analyses were performed with an *HP1100* chromatograph system (*Agilent Technologies*, Palo Alto, CA, USA) equipped with a quaternary pump (model *G1311A*), a membrane degasser (model *G1379A*), a diode-array detector (DAD; model *G1315B*) integrated in the *HP1100* system. Data were analyzed using an *HP ChemStation* system (*Agilent Technologies*). The anal. column was a *ZORBAX Eclipse XDB-C8* (4.6 × 150 mm, 5 μm; *Agilent*). The mobile phase consisted of MeCN/H₂O (70:30) with 0.1% CF₃COOH, and the flow-rate was 1.2 ml/min. The injection volume was 20 μl (*Rheodyne*, Cotati, CA). The column effluent was monitored at 226 and 254 nm. Quantitation was achieved by comparison of peak areas with standards chromatographed under the same conditions.

Cyclooxygenase Inhibition Studies. A whole blood assay [15] was performed to evaluate the ability of synthesised compounds to inhibit COX-1 and COX-2.

Blood samples were divided in two aliquots to test COX-1 and COX-2 inhibition. The COX-2 aliquots were treated with 10 IU/ml of sodium heparine, 10 μg/ml acetylsalicylic acid, and 10 μg/ml

lipopolysaccharide (LPS) from *E. coli*. Methanolic solns. of the tested compounds at different concentrations were prepared, 10- μ l aliquots were distributed in incubation tubes, and the solvent was evaporated. The residues were dissolved by vortexing either in 1 ml of heparinized blood to test COX-2 inhibition, or in 1 ml of untreated blood to test COX-1 inhibition. The final concentrations of the tested compounds were, therefore, diluted 100 times in the incubation tubes. The COX-1 aliquots were incubated in glass tubes for 1 h at 37°, which is sufficient to complete coagulation, then centrifuged at 2000g for 10 min, after which the serum was ready to be tested for platelet TxB₂ production. % Inhibition in samples treated with the test compounds was evaluated in comparison with control samples with basal TxB₂ production.

The COX-2 aliquots were incubated in polyethylene tubes for 24 h at 37° to allow COX-2 expression in monocytes and maximal PGE₂ production. They were then centrifuged at 2000g for 10 min after which the plasma was ready to be tested for PGE₂ production. Basal PGE₂ production in blood untreated with LPS was subtracted from values for each sample, and % inhibition in samples incubated with tested compounds was calculated vs. control samples with maximal PGE₂ production.

Prostanoid production was evaluated by enzyme immunoassay, following the specific instructions provided by *Cayman Chemical*, based on a competitive reaction, for COX-1, between TxB₂ and a TxB₂-acetylcholinesterase conjugate (TxB₂ tracer) for a specific TxB₂ antiserum, and, for COX-2, between PGE₂ and PGE₂-acetylcholinesterase (PGE₂-tracer) for a specific PGE₂ antiserum. Standard curves with known concentrations of TxB₂ and of PGE₂ were used to determine prostanoid concentrations in the sample wells. % Inhibition in compound-treated samples was calculated by comparison with untreated controls. The concentration of the tested compounds causing 50% inhibition (*IC*₅₀) was calculated from the concentration–inhibition response curve (5–6 experiments).

Inhibition of Platelet Aggregation in vitro. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200g for 20 min. Aliquots (500 μ l) of PRP were added into aggregometer (*Chrono-log 4902D*) cuvettes, and aggregation was recorded as increased light transmission under continuous stirring (1000 rpm) at 37° for 10 min after addition of the stimulus. Collagen (1.0 μ g/ml) was used as platelet activator in PRP. The inhibitory activity of the compounds was tested by addition of drug to PRP 10 min before addition of the stimulus (collagen). Drug vehicle (\leq 0.5% DMSO) added to PRP did not affect platelet function in control samples. At least, five experiments were performed for each compound. The potential role of NO and sGC in the inhibitory effect of **15** was verified using the sGC inhibitor, ODC (100 μ M).

The anti-aggregatory activity of the test compounds was evaluated as % inhibition of platelet aggregation compared to controls (5–6 experiments), and *IC*₅₀ values were calculated by nonlinear regression analysis.

Vasodilating Activity Assay. Thoracic aortas were isolated from male *Wistar* rats weighing 180–200 g. The endothelium was removed, and the vessels were helically cut: three strips were obtained from each aorta. The tissue was placed in organ baths containing 30 ml of *Krebs*-bicarbonate buffer of the following composition (mM): NaCl 111.2, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.0, NaHCO₃ 12, glucose 11.1 maintained at 37° and continuously gassed with 95% O₂/5% CO₂ (pH 7.4). The aortic strips were allowed to equilibrate for 120 min and then contracted with 1 μ M (–)-phenylephrine. When the response to the agonist reached a plateau, cumulative concentration–response curves to compounds **7**, **11**, and **15** were determined. The effect of 1 μ M ODC was evaluated in a separate series of experiments in which the ODC was added 5 min before contraction. *EC*₅₀ Values are means of 5–9 determinations. Responses were recorded by an isometric transducer connected to the *MacLab System PowerLab*®.

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