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Nitrooxymethyl Substituted Analogues of Celecoxib: Synthesis and Pharmacological Characterization

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Abstract

Nitrooxymethyl substituted analogues of Celecoxib were synthesized and tested for their COX inhibiting, vasodilator, and antiaggregatory 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 antiaggregatory 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 onto the market [2]; they induce anti-inflammatory effects without the gastrototoxic side-effects typical of the classical non-steroidal anti-inflammatory drugs (NSAIDs), which are non-selective 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 PGI₂, prostaglandin PGE₂ and thromboxane TxA₂ in the cardiovascular system. The proaggregatory and vasoconstrictor TxA₂ is mainly synthesized via COX-1 in the platelets, while vasodilator (PGI₂, PGE₂) and antiplatelet (PGI₂) 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] (Figure 1), that has recently been placed under surveillance by FDA, following its suspected cardiotoxicity.

Figure 1

The results of a preliminary pharmacological screening on these new hybrid products and on 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, 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 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 Ph3P and AgNO3 afforded a crude product that was purified by flash-chromatography to give the expected final compound 7, mononitrooxyethyl 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 LiAlH₄ yielded the di-alcohol 10, which was transformed into the final dinitrooxy derivative 11 following 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.

2. Stability in human serum and in human whole blood. It is known that nitrates can undergo chemical and enzymatic metabolism [14]. Celecoxib and its derivatives were found to be stable following 24 h incubation in human serum. By contrast, derivatives 7, 11, 15 were extensively metabolized when incubated in whole human blood. This transformation was monitored by RP-HPLC. The half lives (t₁/₂) are reported in Table 1. Products 11 and 15 behaved similarly, while 7 surprisingly did not: its t₁/₂ is about 1 h, versus 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 transformed into the corresponding alcohols 10, 11a/b 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/b was used as reference to identify the two mononitrooxy isomeric alcohols that derive from the biotransformation of 11. The behavior of 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 sequestrated 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 following an established procedure [15]. In order 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 PGE$_2$ 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; plasma was then collected and analyzed for TxB$_2$ 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.

The data show that product 7, derived from substituting the nitrooxymethyl function for the methyl group of Celecoxib, is a feeble COX-2 inhibitor, being about fifty 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, that is a more potent COX-1 inhibitor than 1 and that 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 methyl and the aminosulfonyl moiety in 1 gives rise to 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 antiaggregatory potency of this compound was not modified by the presence of ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC).

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\textsubscript{50}, are 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 \( p \)-position of the phenyl rings present in Celecoxib of either the nitrooxymethyl group or the hydroxymethyl group, deriving from its metabolic transformation, gives rise to a complex behaviour. When these moieties are linked to the N-phenyl ring the selective and potent COX-1 inhibitors 14, 15, endowed with antiaggregatory activity, are obtained. By contrast, when they are attached to the C-phenyl group the selective but weak COX-2 inhibitors 6, 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 antiaggregatory 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 antiaggregatory 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.* M.p. were measured with a capillary apparatus (*Büchi 540*). M.p. with decomposition were determined after placing the sample in a bath at a temperature 10° below the M.p.; a heating rate of 1° min⁻¹ was used. All compounds were routinely checked by FT-IR (*PerkinElmer SPECTRUM BXII*), ¹H and ¹³C-NMR (*Bruker Avance 300*) and mass spectrometry (*Finnigan-Mat TSQ-700*). Flash column chromatography was performed on silica gel (*Merck Kieselgel 60, 230-400 mesh ASTM*) using the eluents indicated. Thin layer chromatography (TLC) was carried out on 5 x 20 cm plates with 0.25 mm layer thickness. Anhydrous MgSO₄ was used as drying agent for the org. phases. Analysis (C, H, N) of the new compounds was performed by REDOX (*Monza*) and the results are within ± 0.4% of the theoretical values. Compounds 2 [18], 5 [19], 8 [20], and 12 [12], were synthesized following methods described in the literature. Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone.

*4,4,4-Trifluoro-1-[4-(hydroxymethyl)phenyl]butane-1,3-dione (4).* 1.5 M 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 extracted with AcOEt. The extracts were dried and concentrated to afford 4 as a brown oil (0.99 g); this was used without further purification.

4-{5-{4-(Hydroxymethyl)phenyl}-3-(trifluoromethyl)-1H-pyrazol-1-yl}benzensulfonamide (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 H2O and brine, dried and concentrated in vacuo to give an oil that was purified by flash chromatography (eluent PE/i-PrOH 9/1) to give 6 as a pale yellow solid (0.95 g, 57% yield). M.p. 157-158° (from benzene).

1H-NMR (CDCl3): 7.84 (d, 2H, J = 8.6, Ar); 7.41 (d, 2H, J = 8.6, Ar); 7.34 (d, 2H, J = 8.1, Ar); 7.20 (d, 2H, J = 8.1, Ar); 6.76 (s, 1H, CH pyrazol); 5.31 (s, 2H, NH2); 4.69 (s, 2H, CH2); 2.06 (s, br, 1H, OH). 13C-NMR (CDCl3): 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 C17H14F3N3O2S (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)phenylhydrazine 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 reaction 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 flash chromatography (eluent CH2Cl2/ AcOEt 99/1) to give 9 as a yellow solid (0.73 g, 46% yield). M.p. 95-97° decomp. (i-Pr2O). 1H-NMR (CDCl3): 8.02 (d, 2H, J = 8.6, Ar); 7.39 (d, 2H, J = 8.6, Ar);
7.34 (d, 2H, J = 8.1, Ar); 7.21 (d, 2H, J = 8.1, Ar); 6.76 (s, 1H, CH pyrazol); 4.71 (s, 2H, CH₂); 3.92 (s, 3H, CH₃); 2.15 (s, br, 1H, OH). ¹³C-NMR (CDCl₃): 166.1; 144.8; 143.9 (q, ²J = 38); 142.7; 142.3; 130.6; 130.0; 129.8; 128.0; 127.2; 125.0; 121.1 (q, ¹J = 267); 106.3 (q, ³J = 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-(methyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-1-yl}benzoate (13).*

4-(Methoxycarbonyl)phenylhydrazine hydrochloride (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 flash chromatography (eluent PE/AcOEt 97/3) to give 13 as a white solid (2.06 g, 34% yield). M.p. 78.5-79.5° (hexane). ¹H-NMR (CDCl₃): 8.03 (d, 2H, J = 8.6, Ar); 7.39 (d, 2H, J = 8.6, Ar); 7.16-7.08 (m, 4H, Ar); 6.73 (s, 1H, CH pyrazol); 3.92 (s, 3H, OCH₃); 2.36 (s, br, 3H, CH₃). ¹³C-NMR (CDCl₃): 166.2; 145.2; 143.9 (q, ²J = 38); 142.8; 139.6; 130.6; 129.8; 129.7; 128.8; 126.1; 125.1, 121.3 (q, ¹J = 267); 106.1 (q, ³J = 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) maintained 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, 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 flash chromatography (eluent CH₂Cl₂/AcOEt 8/2) to give 10 as a white solid
(0.70 g, 59% yield). M.p. 107-111° (i-Pr₂O). ¹H-NMR (CDCl₃): 7.35-7.26 (m, 6H, Ar); 7.21 (d, 2H, J = 8.1, Ar); 6.74 (s, 1H, CH pyrazol); 4.70 (s, 4H, two CH₂); 1.98 (s, br, 1H, OH); 1.90 (s, br, 1H, OH). ¹³C-NMR (CDCl₃): 144.5; 143.2 (q, ²J = 38), 141.8; 141.3; 138.3; 128.9; 128.3; 127.4; 127.1; 125.6; 121.2 (q, ¹J = 267); 105.6 (q, ³J = 2); 64.6; 64.4. Cl-Ms (i-Bu) 349 (M⁺). Anal. calc. for C₁₃H₁₅F₃N₂O₂ (348.32): C 62.07, H 4.34, N 8.04; found C 62.45, H 4.42, N 7.94.

4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]phenylethanol (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) maintained 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, the residue was taken up in AcOEt, washed with H₂O and brine, dried and concentrated in vacuo to give a solid (1.91 g, yield 96%) that was purified by crystallization from EtOH/H₂O to give 14 as a white solid. M.p. 109-111° (EtOH/H₂O). ¹H-NMR (CDCl₃): 7.35-7.27 (m, 4H, Ar); 7.11-7.09 (m, 4H, Ar); 6.71 (s, 1H, CH pyrazol); 4.70 (s, 2H, CH₂); 2.34 (s, 3H, CH₃); 1.93 (s, br, 1H, OH). ¹³C-NMR (CDCl₃): 144.8, 143.2 (q, ²J = 38), 141.2, 139.1, 138.5, 129.4, 128.7, 127.3, 126.2, 125.5, 121.3 (q, ¹J = 267), 105.3 (q, ³J = 2); 64.4, 21.3. El-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) maintained under N₂. The mixture was cooled to 0°, and NBS (0.50 g, 2.8 mmol) was added portion-wise. 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 flash chromatography (eluent PE/AcOEt 7/3) to give 7.
as a white solid (0.76 g, 70% yield). $^1$H-NMR ((D$_6$)DMSO): 7.89 (d, 2H, $J$ = 8.6, Ar); 7.58-7.51 (m, 6H, Ar and NH$_2$); 7.39 (d, 2H, $J$ = 8.3, Ar); 7.30 (s, 1H, CH pyrazol); 5.60 (s, 2H, CH$_2$). $^{13}$C-NMR ((D$_6$)DMSO): 144.5; 144.5; 142.2 (q, $^2J$ = 37); 140.9; 133.4; 129.5; 129.2; 129.0; 126.8; 126.0; 121.2 (q, $^1J$ = 267); 106.7 (q, $^3J$ = 2); 74.3. EI-MS: 442 (40%, $M^+$), 397 (40%), 302 (100%). Anal. calc. for C$_7$H$_3$F$_3$N$_4$O$_5$S (442.38): C 46.16, H 2.96, N 12.66; found C 45.96, H 2.89, N 12.29.

4-[[1-(4-(Nitrooxy)methyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-5-yl]benzyl nitrate (11). AgNO$_3$ (1.03 g, 6 mmol) and Ph$_3$P (1.31 g, 5 mmol) were added to a soln. of 10 (0.7 g, 2 mmol) in MeCN (20 ml) maintained under N$_2$. The mixture was cooled to -15° under N$_2$, and NBS (0.89 g, 5 mmol) was added portion-wise. 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 H$_2$O, brine and dried and concentrated in vacuo to give an oil that was purified by flash chromatography (eluent Hexane/AcOEt 9/1). The first eluted compound was 11 which was obtained as a white solid (0.42 g, 48% yield). M.p. 52-53° (i-Pr$_2$O/PE). $^1$H-NMR (CDCl$_3$): 7.43-7.25 (m, 8H, Ar); 6.78 (s, 1H, CH pyrazol); 5.44, 5.43 (2 s, 4H, CH$_2$ONO$_2$). $^{13}$C-NMR (CDCl$_3$): 143.9; 143.7 (q, $^2J = 38$); 139.8; 133.4; 132.7; 130.0; 129.8; 129.4; 129.4; 125.7; 121.1 (q, $^1J = 268$); 106.3 (q, $^3J = 2$); 73.9; 73.6..EI-MS: 438 (30%, $M^+$), 345 (48%), 316 (100%). Anal. calc. for C$_{19}$H$_{13}$F$_3$N$_4$O$_6$ (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. $^1$H-NMR (CDCl$_3$): 7.41-7.20 (m, 16H, Ar); 6.75, 6.77 (2s, 2H, CH pyrazol); 5.42, 5.43 (2 s, 4H, CH$_2$ONO$_2$); 4.73, 4.72 (2 s, 4H, CH$_2$OH); 1.79 (s, br, 2H, OH). $^{13}$C-NMR (CDCl$_3$): 144.6; 143.8; 143.4 (q, $^2J = 38$); 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, $^1J = 268$); 106.0 (q, $^3J = 2$); 105.9 (q, $^3J = 2$); 73.9; 73.7; 64.6; 64.4. EI-MS: 393 (97%, $M^+$), 347 (100%), 317
(99%). Anal. calc. for C_{18}H_{14}F_{3}N_{3}O_{4} (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). AgNO_{3} (0.68 g, 4 mmol) and Ph_{3}P (0.92 g, 3.5 mmol) were added to a soln. of 14 (0.66 g, 2 mmol) in MeCN (30 ml) maintained under N_{2}. The mixture was cooled to -15° under N_{2}, and NBS (0.62 g, 3.5 mmol) was added portion-wise. 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 H_{2}O, brine, dried and concentrated in vacuo to give a residue that was purified by flash chromatography (eluent PE/AcOEt 9.5/0.5) to give 15 as a white solid (0.52 g, 68% yield). M.p. 106-107° (MeOH). ¹H-NMR (CDCl₃): 7.41-7.34 (m, 4H, Ar) 7.16-7.09 (m, 4H, Ar), 6.72 (s, 1H, CH pyrazol), 5.43 (s, 2H, CH₂), 2.37 (s, 3H, CH₃). ¹³C-NMR (CDCl₃): 144.9; 143.5 (q, ²J = 38); 140.2; 139.4; 132.3; 130.0; 129.7; 128.7; 126.1; 125.7; 121.2 (q, ¹J = 267); 105.8 (q, ³J = 2); 73.8; 21.3. EI-MS: 377 (100%, M⁺), 331 (85%), 300 (90%). Anal. calc. for C_{18}H_{14}F_{3}N_{3}O_{3} (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 soln.s were maintained at 37 ± 0.5 °C and at appropriate time intervals the mixtures were diluted 1:2 with MeCN containing 0.1% trifluoroacetic acid in order to deproteinize and precipitate cells, proteins and enzymes. Samples were
sonicated, vortexed, and then centrifuged for 15’ at 2150 g. The clear supernatant was filtered by 0.45 µm PTFE filters (Alltech) and analyzed by RP-HPLC.

The reverse-phase 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 analytical column was a ZORBAX Eclipse XDB-C8 (4.6 x 150 mm, 5 µm) Agilent. The mobile phase consisted of MeCN/H2O (70/30) with 0.1% trifluoroacetic acid 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 nm and 254 nm. Quantitation was done 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 lipopolysaccaride from E.coli (LPS). Methanolic soln.s of the tested compounds at different concentrations were prepared, 10 µl aliquots were distributed in incubation tubes and the solvent was evaporated. The residues were dissolved by vortexing either in 1 ml heparinized blood to test COX-2 inhibition, or in 1 ml 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 2000 g 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 2000 g 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 versus 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. Percent inhibition in compound-treated samples was calculated by comparison with untreated controls. The concentration of the tested compounds causing 50% inhibition (IC₅₀) 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 200 g for 20 minutes. Aliquots (500 µl) 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 minutes after addition of the stimulus. Collagen (1.0 µg/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 (≤ 0.5 % DMSO) added to PRP did not affect platelet function in control samples. At least 5 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, ODQ (100 μM).

The antiaggregatory 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 non-linear 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, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.0, NaHCO$_3$ 12, glucose 11.1 maintained at 37° and continuously gassed with 95% O$_2$ – 5% CO$_2$ (pH = 7.4). The aortic strips were allowed to equilibrate for 120 min and then contracted with 1 μM (-) 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 μM ODQ was evaluated in a separate series of experiments in which the ODQ 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®.

**REFERENCES**

Table 1. *Whole human blood stability*

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of unchanged compound after 24 h (± SEM)</th>
<th>% of alchoholic metabolites after 24 h (± SEM)</th>
<th>(t_{1/2}) (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>4.5 ± 1.1</td>
<td>4.5 ± 0.5 (6)</td>
<td>0.8</td>
</tr>
<tr>
<td>11</td>
<td>59.4 ± 4.4</td>
<td>16.2 ± 0.7 of 11a/b</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.7 ± 1.0 of 10</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>54.4 ± 2.3</td>
<td>41.2 ± 1.7 (14)</td>
<td>25.4</td>
</tr>
</tbody>
</table>
Table 2. *COX-1 and COX-2 inhibition data for compounds and Celecoxib 1, taken as reference.*

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50 ± SE&lt;/sub&gt; μM</th>
<th>% inhibition ± SE (100 μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50 ± SE&lt;/sub&gt; μM</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>SO₂NH₂</td>
<td>Me</td>
<td>14 ± 2</td>
<td>-</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>6</td>
<td>SO₂NH₂</td>
<td>CH₂OH</td>
<td>b) 20 ± 13</td>
<td>52 ± 3</td>
<td></td>
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<tr>
<td>7</td>
<td>SO₂NH₂</td>
<td>CH₂ONO₂</td>
<td>b) 6.7 ± 3.8</td>
<td>67 ± 19</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>CH₂OH</td>
<td>CH₂OH</td>
<td>0.51 ± 0.14</td>
<td>-</td>
<td>12 ± 3</td>
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<tr>
<td>11</td>
<td>CH₂ONO₂</td>
<td>CH₂ONO₂</td>
<td>b) 15 ± 7</td>
<td>19 ± 1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>CH₂OH</td>
<td>Me</td>
<td>0.41 ± 0.08</td>
<td>-</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>15</td>
<td>CH₂ONO₂</td>
<td>Me</td>
<td>4.6 ± 1.6</td>
<td>-</td>
<td>31 ± 10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Maximum concentration tested. <sup>b</sup> Inhibition of control TxB₂ production did not reach 50%.
Table 3. Anti-aggregatory and vasodilating properties of derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Anti-aggregatory activity</th>
<th>Vasodilating activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (95% CL) μM</td>
<td>% inhibition ± SE (100 μM)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>SO&lt;sub&gt;2&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Me</td>
<td>31 (25 – 39)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>SO&lt;sub&gt;2&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>b) 5.8 ± 2.1</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>7</td>
<td>SO&lt;sub&gt;2&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;ONO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>b) 3.4 ± 2.6</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>8.4 (7.0 - 10)</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;ONO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;ONO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>b) 7.4 ± 4.3</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>Me</td>
<td>11 (10 - 12)</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;ONO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Me</td>
<td>48 (44 – 52)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
</tr>
</tbody>
</table>

*<sup>a</sup>) Maximum concentration tested. *<sup>b</sup>) Inhibition of control aggregation effect did not reach 50 %. *<sup>c</sup>) In the presence of 1 μM ODQ. *<sup>d</sup>) Unchanged in the presence of 100 μM ODQ.
Figure 1.

![Chemical Structure Image]
Scheme 1: i) MeONa, CF₃COOEt, MeOH, refluxing; ii) refluxing in MeOH, (EtOH for 6); iii) LiAlH₄, THF.
Scheme 2: i) Ph$_3$P, AgNO$_3$, NBS, - 15° → r. t.