Notes

Furoxans as Nitric Oxide Donors. 4-Phenyl-3-furoxancarbonitrile: Thiol-Mediated Nitric Oxide Release and Biological Evaluation

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4-Phenyl-3-furoxancarbonitrile (**2**) affords nitric oxide under the action of thiol cofactors. Two principal products were isolated in the reaction with thiophenol: the phenylcyanoglyoxime (**6**) and 5-amino-3-phenyl-4-(phenylthio)isoxazole (**7**). Mechanisms which could account for the formation of these two products are discussed. Compound **2** is an efficient activator of the rat lung soluble guanylate cyclase, displays high vasodilatory activity on strips of rat thoracic aorta precontracted with noradrenaline, and is a potent inhibitor of platelet aggregation.

Introduction

The furoxan (furazan oxide; 1,2,5-oxadiazole 2-oxide) ring is a simple system with an enigmatic and intriguing chemistry.[1,2] Recent results have shown that furoxan derivatives are able to activate the soluble guanylate cyclase[3,4] by releasing nitric oxide (NO) under the action of thiol cofactors.[4] Nitric oxide is an important messenger implicated in a wide range of biological functions including control of vascular tone, platelet inhibition, cell adhesion, neurotransmission, penile erection, enzyme regulation, and immune regulation.[5] Thus, the furoxan system can be useful in the design of NO-releasing drugs.

The mechanism of nitric oxide release by furoxan derivatives is a subject in need of a thorough investigation. At the moment only a speculative mechanism of NO release from furoxancarboxamides has been proposed.[4]

In our laboratory we have been studying the pharmacochemistry of the furoxan ring for many years, and we have described many new unsymmetrically substituted furoxans.[1,2] Recently we began a systematic study of the vasodilator and platelet antiaggregatory activities of about 20 substituted furoxans and their ability both to release nitric oxide and activate the soluble guanylate cyclase under the action of thiol cofactors. One of the most active compounds we found in this screening was 4-phenyl-3-furoxancarbonitrile (**2**). In this paper we report the results of a study of NO release from **2** by the action of thiol cofactors and propose possible mechanisms for the reaction with thiophenol. **2** was selected for this study both for its ability to release large amount of nitric oxide and because, in the reaction with thiophenol, TLC detection showed the presence of few final compounds and not a complex mixture as in the case for any other furoxans, which are efficient NO donors. In addition we discuss the ability of **2** to activate the soluble guanylate cyclase, inhibit platelet aggregation, and relax strips of rat thoracic aorta precontracted with noradrenaline.

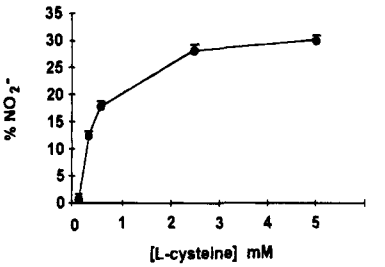
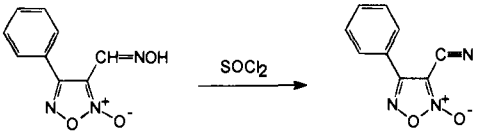


Figure 1. NO_2^- release as function of L-cysteine millimolar concentration; incubation for 5 min; pH 7.4; 37 °C; values are given as means ±SE for four to six replicates.

Scheme 1



1 2

Results and Discussion

Chemistry. The synthesis of **2** has been previously discussed.[6] An alternative and simpler way to prepare this compound is by dehydration of the corresponding oxime derivative **1** by the action of thionyl chloride (Scheme 1).

Compound **2** (0.1 mM), dissolved in phosphate buffer (pH 7.4) containing L-cysteine, released NO which, in presence of air, was converted into NO_2^-. Nitrites were detected by the Griess reaction. Virtually no formation of NO_2^- was detectable in absence of L-cysteine. NO release was dependent on the concentration of thiol cofactor (see Figure 1) and was fast. Working in 5 mM L-cysteine, after 1 min, 5 min, and 1 h (infinite time), the yield of NO_2^- released was 14 ± 2%, 29 ± 1%, and 40 ± 2%, respectively. When **2** (0.1 mM) was treated with thiophenol (0.5 mM), the reaction was completed after 1 h (yield in NO_2^-: 64 ± 2%). The study of the dependence of NO_2^- release on the thiophenol concentration was limited by solubility problems. This picture is qualitatively in keeping with the findings of Feelisch, who showed that the extent of NO liberated from

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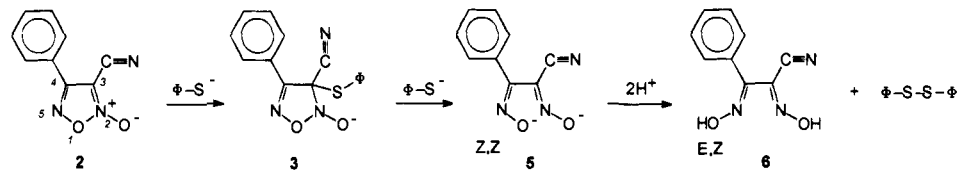
Scheme 2



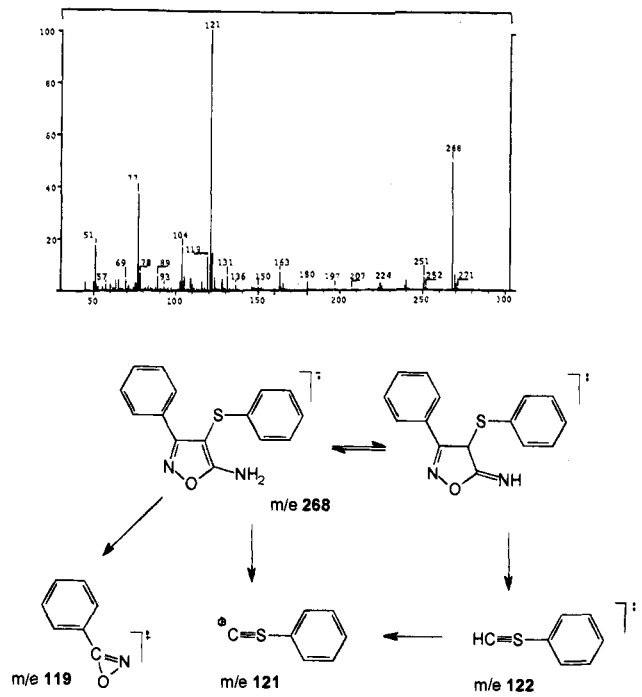


Figure 2. Mass spectrum of **7** and formation scheme of the fragments used to propose its structure.

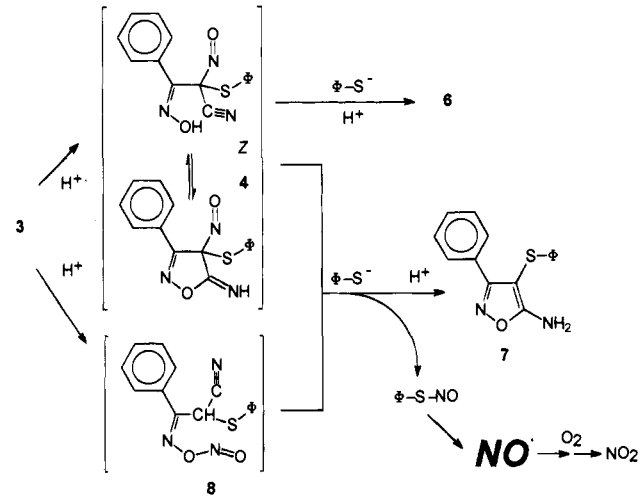
furoxancarboxamides depends on the nature and concentration of the thiol.[4]

In order to obtain more insight into the pathway of this release, the reaction with thiophenol was repeated on a semimicroscale using as solvent a mixture of water buffered at pH 7.4/ethanol. After filtration of diphenyl disulfide, we isolated from the reaction mixture two principal products which were separated by column chromatography. Similar results were obtained running the reaction in a water/ethanol mixture containing sodium hydroxide. One of the compounds (R_f = 0.1; yield 20%) was identical (mixed mp, IR) with a sample of phenylcyanoglyoxime (**6**), which must possess an *E,Z* configuration because it gives a 2:1 Ni^{2+} red complex.[7,8] Dioximes are well-known reduction products of benzofuroxans under the action of thiol,[2,9,10] and glyoximes were isolated in fair yield in the reaction between furoxancarboxamides and L-cysteine.[4]

A reasonable mechanism for this transformation is shown in Scheme 2. Nucleophilic attack of the thiophenoxy anion at the C-3 position affords the intermediate **3**. Subsequent nucleophilic attack of the thiolate group on the sulfur center of **3** produces the unstable *Z,Z* glyoxime **5**, which immediately isomerizes to **6**.

Analytical data and the mass spectrum (*m/e* = 268 M) (Figure 2) of the other compound (R_f = 0.31; yield 60%) were in keeping with the molecular formula $C_{15}H_{12}N_2OS$. The high-resolution mass spectrum showed a base peak (*m/e* = 121) compatible with a C_7H_5S composition. Tandem mass spectrometry analysis (parent ions, daughter ions) showed that this ion originates

Scheme 3



from both the molecular ion and the ion at *m/e* = 122 and evidenced in it the presence of a phenyl ring. High resolution of the peak at *m/e* = 119 was in keeping with a C_7H_5NO composition, and mass–mass analysis (daughter ions) evidenced the presence also in this fragment of a phenyl group. On this basis we suggest for the compound the structure of 5-amino-3-phenyl-4-(phenylthio)isoxazole (**7**). In Figure 2 a fragmentation scheme is reported, which accounts for the ions we have used for the structural determination. ^1H-, ^{13}C-, and ^{15}N-NMR and IR spectra are in agreement with our structural assignment, which is conclusively supported by the identity (mixed mp, IR) of compound **7** with an authentic sample of 5-amino-3-phenyl-4-(phenylthio)-isoxazole synthesized according to ref 11. This derivative appears to be a reasonable candidate as a final product on the way of NO releasing (NO_2^- yield 64%, **7** yield 60%). The aromatic character of **7** is probably the driving force for its facile formation.

Possible mechanisms which can account for the product **7** are shown in Scheme 3. We discussed above the formation of **3** by action of the thiophenoxy anion on **2** and its transformation into **6**. Compound **3** could also undergo ring opening to give nitroso derivative **4**. Nucleophilic attack of the thiolate group on the sulfur center of **4**, accompanied by *Z,E* stereoisomerization of the oxime group, could be another pathway to afford **6**. On the contrary, attack of the thiolate group on the nitroso moiety of **4** could afford the final derivative **7** and phenylthionitrite which immediately decomposes to nitric oxide and diphenyl disulfide by radical cleavage. Nitric oxide is thus oxidized to N_2O_3 which hydrolyzes to nitrite.

There is another interesting mechanistic possibility to justify the formation of **7**. This concerns the transfer of one electron from the thiolate anion to **4** with formation of an intermediate radical anion which can afford **7** losing NO. Obviously the two proposed mechanisms can be concomitant. The release from **4** of the

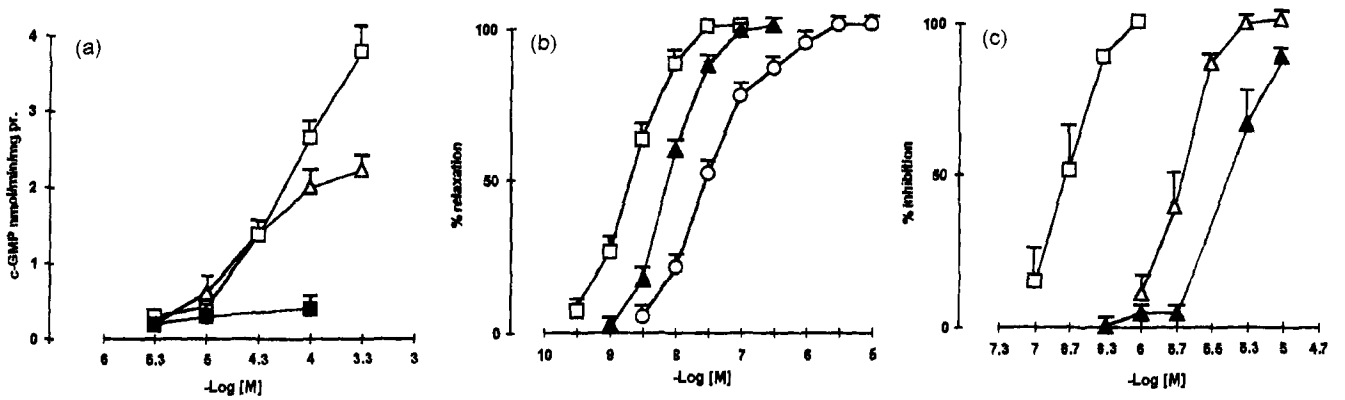


Figure 3. (a) Enzymatic stimulation by compound **2** in the presence (□) and in absence (■) of L-cysteine and by SNP (△) in the presence of L-cysteine as a function of concentration in the standard lung soluble guanylate cyclase test. Basal enzymatic activity was 0.077 ± 0.004 nmol/min/mg of protein. Values are given as means ±SE for six to eleven replicates. (b) Vasorelaxant activity of compound **2** (□), compound **2** + HbO_2 (▲) and GTN (○) as a function of concentration in the standard rat aortic strip test. Values for percent relaxation are given as means ±SE for four to seven replicates. (c) Antiaggregatory activity of compound **2** (□), compound **2** + HbO_2 (▲), and SNP (△) as a function of concentration in the standard *in vitro* human PRP test. Values for percent aggregation are given as means ±SE for two to three replicates.

nitroxyl anion (NO^-), a redox form of nitric oxide of potential biological significance,[5] is formally possible, but it requires as an intermediate a high-energy carbene species.

Finally the production of **7** could also be justified by the formation of **8**, which is one of the intermediates hypothesized to account for the NO release from furoxancarboxamides.[4] The proposed mechanisms apart, these results show that NO release from 4-phenyl-3-furoxancarbonitrile under the action of thiol cofactors is a complex pathway involving the N^+O^- moiety of the furoxan system. More generally they suggest that the NO release from furoxans should be strongly dependent on the nature and the position of the substituents at the heteroaromatic ring. Studies are in progress to obtain more insight into this problem.

Pharmacology. Activation of soluble guanylate cyclase. Partially purified rat lung soluble guanylate cyclase was used in the experiments. After incubation of the enzyme with **2** and L-cysteine, a concentration-dependent increasing of c-GMP over the basal level was observed. The concentration–response curve of the furoxan derivative was partially overlapping that of sodium nitroprusside (SNP) (Figure 3a). Poor activation of the enzyme was observed in the absence of L-cysteine. The stimulatory effect of **2**, at the maximum concentration tested, was 3.74 ± 0.19 nmol/min/mg of protein, which is about 1.7-fold greater than the activation of guanylate cyclase by SNP in the same conditions (2.18 ± 0.11 nmol/min/mg of protein).

Vasodilator Activity. Vasodilator effects of **2** were assessed on endothelium-denuded strips of rat aorta precontracted with noradrenaline. The furoxan derivative was able to display a concentration-dependent relaxation of the strips. The concentration-response curve of **2** (EC_{50} = 2.3 ± 0.4 × 10^{-9} M) was left-shifted compared to that of glyceryl trinitrate (GTN) (EC_{50} = 2.2 ± 0.4 × 10^{-8} M) (Figure 3b). At the maximum concentration tested, only a partial recovery (66 ± 2%, mean ± SE) of the noradrenergic tone occurred after 0.5 h of washing the aortic preparations with fresh Krebs buffer. A complete recovery was obtained after 1 h of washing. This behavior, absent in the reference compounds (GTN, SNP), was dose-dependent (92 ± 1% at 3 × 10^{-9} M concentration, 85 ± 2% at 1 × 10^{-8} M concentration) and suggests a potential toxicity problem. In the presence of 10 μM oxyhemoglobin (HbO_2) the concentration–response curve shifted to the right in a parallel manner (EC_{50} = 9.5 ± 0.8 × 10^{-9} M) (Figure 3b), in keeping with the involvement of nitric oxide in the vasorelaxant action.

Inhibition of Platelet Aggregation. Compound **2** was able to inhibit in a concentration-dependent manner the platelet aggregation induced by collagen in human platelet-rich plasma (PRP). The concentration–response curve of **2** (IC_{50} = 1.99 ± 0.06 × 10^{-7} M) was left-shifted compared to that of SNP (IC_{50} = 2.2 ± 0.2 × 10^{-6} M) (Figure 3c). In presence of 30 μM oxyhemoglobin (HbO_2), the concentration–response curve was shifted to the right in a parallel manner (IC_{50} = 4.2 ± 0.2 × 10^{-6} M), in keeping with the involvement of nitric oxide in the antiaggregatory action. In conclusion the pharmacological profile of 4-phenyl-3-furoxancarbonitrile is in line with that of a potent NO donor.

Experimental Section

Chemistry. Melting points were measured with a capillary apparatus (Büchi 530) and are uncorrected. All compounds were checked by IR (Perkin-Elmer 781 spectrometer), NMR (Bruker AC-200 spectrometer), and mass spectrometry (Finnigan-Mat TSQ-700 spectrometer). MS-MS spectra were obtained on the same instrument using argon as collision gas. High-resolution mass spectra were carried out on a VG TS70-250 instrument (Mario Negri Institute, Milan). HPLC analyses were performed on a Shimadzu system equipped with two LC10A pumps, a SPD-M10A diode array detector, and Class LC10 software. Silica gel (Merck kieselgel 100), 70–230 mesh ASTM, was employed for column chromatography. TLC was carried out on plates precoated with Merck kieselgel 60 F_{254}, layer thickness 0.25 mm. Anhydrous magnesium sulfate was used as drying agent for the organic layers. Microanalyses were performed by REDOX (Cologno M.) and are within ±0.4% of theoretical values. Compounds **1**,[1] **6**,[7] and **7**,[11] were synthesized according to the literature.

4-Phenyl-3-furoxancarbonitrile (2). To a stirred and ice–water-cooled solution of **1** (200 mg, 0.97 mmol) in anhydrous DMF (2 mL) was added thionyl chloride (0.3 mL). When the reaction was completed (TLC), the mixture was poured into ice. The precipitated product was filtered, washed with water, and dried (yield 90%). The title compound was recrystallized from petroleum ether: bp 40–60 °C, mp 73–74 °C (lit.[6] 73–74 °C); ^{13}C-NMR ($CDCl_3$) δ 95.0 (C-3), 123.2, 126.2, 129.2, 132.2 (C-Ar), 153.6 (C-4), 105.8 (CN).

5-Amino-3-phenyl-4-(phenylthio)isoxazole (7) and Phenylcyanoglyoxime (6). Compound **2** (200 mg, 1.07 mmol) was dissolved at 37 °C under stirring in 45 mL of a 2:1 mixture of ethanol/7.4 pH phosphate buffer (1.07 mmol). Thiophenol (588 mg, 544 μL, 5.35 mmol) was added to this solution. The reaction was checked by HPLC (250 × 4.6 mm, 5 μm particles, RP-C18 column; eluent: methanol/water in a gradient). After 1 h the reaction mixture was allowed to reach room temperature and the diphenyl disulfide precipitated was filtered off. Ethanol was removed *in vacuo*, and the residue was extracted with diethyl ether. The combined organic layers were dried and filtered. From the residue obtained after solvent removal, compounds **7** (R_f = 0.31; yield 60%) and **6** (R_f = 0.1; yield 20%) were separated by flash chromatography (eluent: petroleum ether, bp 40–60 °C/ethyl acetate, 80/20). Compound **7** was recrystallized from a mixture of benzene/cyclohexane: mp 123–124 °C dec (lit.[11] mp 126 °C); IR (KBr) 3490, 3390, 1625; ^{1}H-NMR ($CDCl_3$) δ 7.0–7.9 (m, 10H), 5.0 (br s, 2H); ^{13}C-NMR ($CDCl_3$) δ 77.1 (C-4), 125.1, 125.4, 127.8, 128.4, 128.5, 129.1, 129.9, 136.8 (C-Ar), 164.0 (C-3), 171.7 (C-5); ^{15}N-NMR DMSO-d_6, ref CH_3NO_2) δ −33.5 (N-2), −315.5 (NH_2). Anal. ($C_{15}H_{12}N_2OS$): C, H, N, S.

Compound **6** was recrystallized from chloroform: m.p. 150–151 °C (lit.[7] mp 150–151 °C); ^{1}H-NMR (DMSO) δ 7.4–7.5 (m, 5H), 12.4 (s, 1H), 13.8 (s, 1H); ^{13}C-NMR (DMSO) δ 132.0, 151.4 (C=NOH), 128.0, 129.2 (splitted on a 400 MHz spectrometer), 129.5 (C-Ar), 109.2 (CN).

Similar results were obtained by treating compound **2** (1.07 mmol) with sodium hydroxide (1.07 mmol) and thiophenol (5.35 mmol) in ethanol/water. In this case, the reaction mixture was first, extracted with diethyl ether and, after pH adjustment with hydrochloric acid, again extracted with the organic solvent.

Quantitative Nitrite Detection. A solution of **2** (20 μL) in DMSO was added to 2 mL of 50 mM phosphate buffer (pH 7.4), containing the appropriate amount of thiol cofactor (see text). The final concentration of **2** was 10^{-4} M. After 1 h at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of the Griess reagent sulfanilamide (4 g), *N*-naphthylethylenediamine dihydrochloride (0.2 g), and 85% phosphoric acid (10 mL) in distilled water (final volume: 100 mL). After the mixture stood for 10 min at room temperature, absorbance was measured at 540 nm; 10–80 nmol/mL sodium nitrite standard solutions were used for the calibration curve. The yield in nitrite was expressed as % NO_2^- (mol/mol) ± SE.

Guanylate Cyclase Assay. Soluble guanylate cyclase was partially purified from rat lungs according to the literature.[12] The guanylate cyclase activity was measured by preincubating the enzyme (10 μg) for 5 min at 37 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 10 mM EGTA, in the absence or presence of 5 mM L-cysteine and of various concentrations of **2** in a final volume of 300 μL.

The reaction was started by addition of GTP (final concentration: 0.2 mM) and terminated after 20 min at 37 °C, by heating for 3 min at 90 °C. Cyclic GMP was measured by a radio immunoassay kit using [^{3}H]c-GMP (Amersham). The generation of c-GMP was linear with time (10–30 min), and cyclic GMP-phosphodiesterase inhibitors did not affect enzymatic activity, so they were not added to reaction mixture. Compound **2** did not show cross-reactivity in the c-GMP radio immunoassay. Protein was calculated according to the literature.[13]

Vasoactivity Determinations. Thoracic aortas were isolated from male Wistar rats, weighing 200–250 g. The vessels were helically cut, the endothelium was removed, and two strips were obtained from each aorta. The tissues were mounted under 1*g* tension in organ baths containing 30 mL of Krebs–Heinslet solution (NaCl, 137; KCl, 2.68; $MgCl_2$, 0.5; $CaCl_2$, 5.44; NaH_2PO_4, 0.5; $NaHCO_3$, 8.93; glucose, 8.3; ascorbic acid, 0.1 mM) at 37 °C and gassed with 95% O_2–5% CO_2 (pH 7.4). The aortic strips were allowed to equilibrate for 1 h and then contracted with noradrenaline (1 μM), which causes a submaximal response. During this first contraction, the absence of intact endothelium was verified by adding acetylcholine (1 μM), which was found not to induce relaxation. The preparations were then extensively washed with Krebs–Heinslet solution, and a second contraction was evoked by noradrenaline (**1** μM). When the response to the agonist plateaued, cumulative concentrations of the vasodilating agent were added. When maximal vasodilation was obtained, aortic strips were washed repeatedly and a third contraction was induced by noradrenaline (1 μM), so as to verify the reversibility of vasodilation. Effects of oxyhemoglobin on relaxation were evaluated in a separate series of experiments, by exposing aortic strips, precontracted with noradrenaline (1 μM), to oxyhemoglobin (10 μM) for at least 10 min before addition of vasodilating agent.

Platelet Aggregation. Blood was collected from normal healthy volunteers who had not taken any drugs during the previous 2 weeks and transferred to tubes containing 0.1 volume of a 3.8% (w/v) trisodium citrate solution. PRP was prepared by centrifugation (18 min) at 160*g* at room temperature. Platelet-poor plasma (PPP) was obtained by further centrifugation at 2000*g* of blood remaining after removal of PRP. Platelet aggregation was studied photometrically in PRP samples,[14,15] using an Elvi aggregometer (Elvi Logos, Milan) connected to a linear recorder.

An aliquot of PRP (500 μL containing approximately 300 000 platelets/μL) was incubated in a cuvette at 37 °C and stirred. The compound being tested was added to PRP dissolved in DMSO, 1 min before collagen (0.8–1.75 μg/mL; Mascia Brunelli, Milan). Control sample received in parallel the same volume of DMSO (1 μL), an amount which did not interfere with the platelet assay. For each PRP, the minimal concentration of collagen capable of producing an irreversible aggregation was selected, which produced at least a 70–80% decrease in optical density in 5 min. This concentration was defined as the threshold aggregating concentration (mean ± SE, n = 15, collagen: 1.17 ± 0.47 μg/mL). Aggregation was recorded as the percent change in light transmission. The aggregation base line (10% light transmission) was set by using PRP and DMSO which were added in equivalent concentrations to the test system. Full transmission (100%) was set by using PPP. IC_{50} values were generated from regression analysis of the concentration–response curve.

Preparation of Oxyhemoglobin. Bovine hemoglobin type 1 (H-2500; Sigma Chemical Co.) contains a mixture of oxyhemoglobin and the oxidized derivative methemoglobin. Pure oxyhemoglobin was prepared by adding to a solution of commercially available hemoglobin in phosphate saline buffer, pH 7.4, a 10-fold mole excess of the reducing agent sodium dithionite ($Na_2S_2O_4$), at 4 °C, and was then protected from the light. Within a few minutes, the mixture was centrifuged at 1000*g* for 10 min, at 4 °C, and the supernatant loaded onto a chromatographic column (Sephadex G-25; Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer. The purity of the oxyhemoglobin solution was determined spectrophotometrically (λ_{max} = 576 and 541 nm; ε = 14 600 and 13 800), and the solutions were frozen in aliquots at −20 °C and stored for 15 days.

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