Water Soluble Furoxan Derivatives as NO Prodrugs

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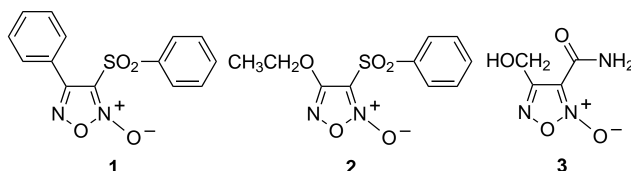
The synthesis, characterization, NO donor properties, and *in vitro* vasodilating activity of a series of water soluble furoxans (**5−14a,b**) are described. All of the compounds released NO when treated with a large excess of cysteine under physiological conditions (pH 7.4; 37 °C). The amount of NO produced after 1 h of incubation was evaluated by detecting nitrites, *via* the Griess reaction. Derivatives **5b**, **7b**, and **14b** were able to release nitric oxide also in the absence of the thiol cofactor. The initial rates of NO release were determined at different concentrations, using a spectrophotometric technique based on the NO-induced oxidation of oxyhemoglobin (HbO_2) to methemoglobin (MetHb). The initial rates of NO release were linearly dependent on the concentrations of the single compounds. The vasodilating potency (EC_{50}) of all the derivatives was assessed on rat aortic strips precontracted with noradrenaline. Correlation between potency and initial NO release rate is discussed.

Introduction

The discovery that nitric oxide is an important physiological mediator in cardiovascular, immune, central, and peripheral nervous systems has given rise to a great interest in NO prodrugs, those derivatives able to release this messenger under physiological conditions.[1] The classical employment of NO donors is in the treatment of cardiovascular diseases, but a variety of new therapeutic possibilities are emerging.[2,3]

Recently it has been shown that a few derivatives of 1,2,5-oxadiazole 2-oxide (furoxan, furazan oxide) are able to increase the level of cytosolic cGMP in human platelets[4] and to activate rat liver guanylate cyclase in the presence of thiols.[5] This behavior is explained by the finding that furoxans can release nitric oxide under the action of thiol cofactors.[5] With furoxancarboxamides as models, nitroso thiols were isolated in release.[5] Generally speaking the overall reaction of NO donation by furoxans appears to be complex. Some mechanisms have been hypothesized.[5,6] They take into account not only the intermediacy of nitroso thiols but also direct release of nitroxyl anion (NO^-) and NO$^•$. The involvement of the ring 3-position has been pointed out.[6] Anyway the subject still needs a specific study. Recently we synthesized and tested for their antiaggregatory and vasodilating properties many new derivatives of the furoxan system.[7] CHF 2206 (**1**)[8] and CHF 2363 (**2**)[9] merited further investigation.



Simultaneously, researchers at the Cassella Institute undertook a wide study on furoxancarboxamides from which CAS 1609 (**3**) was selected as a candidate for further preclinical work.[10] The furoxan system is also a flexible tool in the design of hybrid drugs with mixed α_1-antagonist properties and NO dependent vasodilating activity.[11]

Most of the furoxans we have studied so far are poorly water soluble. This limits the study of their NO donor capability under physiological conditions. This article describes the preparation of a series of water soluble furoxans and a study of their NO donor properties as well as of their *in vitro* vasodilating activity. The correlation between the initial rates of the NO release and the vasodilating potencies of the compounds is also analyzed.

Chemistry

A classical strategy to obtain water soluble derivatives of a ring system is the direct attachment to it of basic side chains containing amine functions.[12] We used this approach to achieve our aim of preparing water soluble furoxans. In a recent paper we have shown that phenylsulfonyl-substituted furoxans are flexible intermediates for the synthesis of new functionalized furoxan derivatives.[13] All of the compounds described in the present work have been synthesized from such starting materials, according to the pathways shown in Scheme 1.

Dimethylaminoalkoxy-substituted phenylfuroxans **5a**, **6a**, and **5b**, **6b** were obtained by action of the appropriate dimethylamino alcohol either on 4-(phenylsulfonyl)-3-phenylfuroxan (**4a**) or on its 4-phenyl isomer **4b**. The reaction was run in tetrahydrofuran (THF) in the presence of sodium hydroxide at room temperature. The (aminoalkyl)thio compounds **7a, 8a**, and **7b**, **8b** were prepared by a similar procedure. The [2-(dimethylamino)ethyl]sulfonyl-substituted phenylfuroxan isomers **9a,b** were easily synthesized by the action of potassium permanganate on acetic acid solutions of **8a** and **8b**, respectively. We obtained the phenylsulfonyl-substituted furoxans **11−13** from **10** under conditions similar to those employed for the synthesis of the phenyl analogues. With the amino alcohols we isolated only the 3-phenylsulfonyl isomers (**11−13**), but with 2-(dimethylamino)ethanethiol as a reagent we obtained the two possible isomers **14a** and **14b** in the ratio ∼1:4. It is noteworthy that the major product in the reaction with the thiol is that formed by substitution of the

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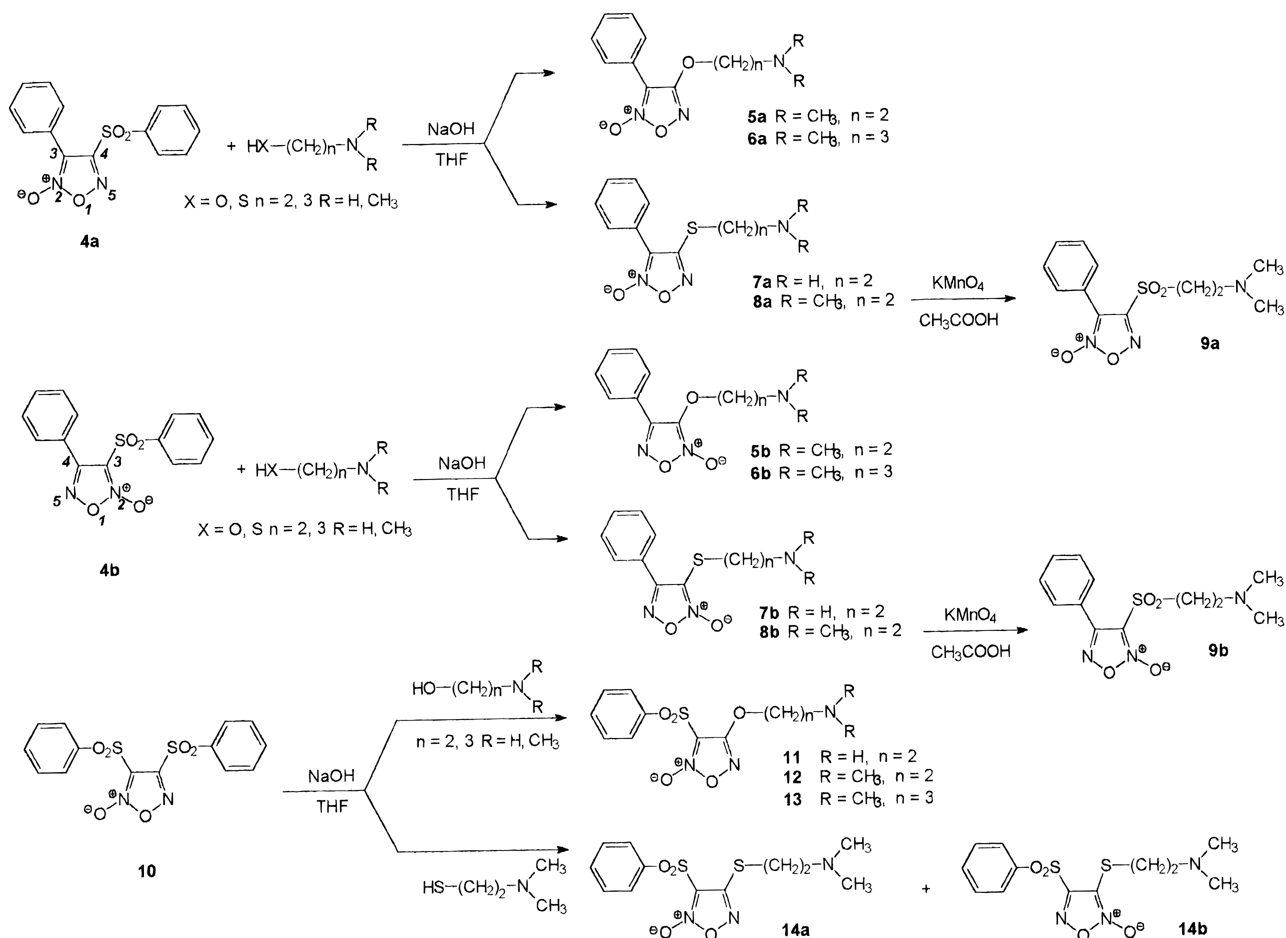
Scheme 1



3-sulfonyl group, the one which is retained in the amino alcohol reaction.

All of the proposed structures were established by ^{1}H and ^{13}C NMR spectroscopy. The structures of the isomer pairs **5**−**9a,b**, in which a phenyl group is directly linked to the furoxan ring, were confirmed on the basis of the well-known shielding effect exerted by the $N^{+}-O^{-}$ moiety both on the C′-1 and, to a lesser extent, on the C′-4 of the 3-phenyl ring compared with the corresponding carbons of the 4-phenyl isomers.[14] This same effect influences also the chemical shifts of hydrogens present in side chains.[15] Indeed, the protons of the aminoalkyl group in the 4-phenyl isomers display small but significant upfield shifts with respect to the corresponding resonances in the 3-phenyl isomers. In a similar manner the structure was assigned to the isomer pair **14a** and **14b**. A more complex situation arises from the 3-phenylsulfonyl derivatives **11**−**13**, for which only one isomer is available. In this case we based our structural assignments on the close resemblance of their spectra to those of 3-(phenylsulfonyl)-4-ethoxyfuroxan. In this last compound the connectivity relative to C-4 and C-3 in the furoxan ring was assigned by using 1D (INEPT) and 2D (HETCOR) polarization transfer techniques.[13]

All of the derivatives we synthesized, isolated as oxalates, are freely water soluble in the concentration range we used for studying NO release (10^{-3}−10^{-6} M). The increase of solubility obtained grafting a basic chain to the furoxan ring is relevant. For example derivative **12**·$H_2C_2O_4$ is water soluble at concentration of about 2.2×10^{-2} M (22 °C); the comparable solubility of the analogue 3-(phenylsulfonyl)-4-ethoxyfuroxan (**2**) is 5.7×10^{-5} M.

Extents and Rates of Nitric Oxide Release. In order to evidence spontaneous release of nitric oxide, each compound was dissolved in pH 7.4 buffered water and kept at 37 °C for 1 and 24 h. The amount of NO released was evaluated by detecting nitrites, which are the oxidative products of nitric oxide, by the Griess reaction. The extent of the thiol-induced NO generation was similarly determined after 1 h of incubation in the presence of a large excess of cysteine (1:50). The results expressed as percent NO_2^- (mol/mol) are summarized in Table 1.

The initial rates of NO release in the presence of cysteine (5-fold molar excess) and, if necessary, in its absence were determined at different concentrations in the range 10^{-3}−10^{-6} M. In this study a spectrophotometric technique, based on the NO-induced oxidation of oxyhemoglobin (HbO_2) to methemoglobin (MetHb), was employed.[16] With the furoxan derivatives unable to release spontaneously NO, no oxidation occurred in the absence of cysteine. The reaction was followed by detecting the increase of absorbance (ΔA) at $\lambda = 401$ nm. The initial rates of nitric oxide donation, expressed as ΔA min^{-1}, were linearly dependent on the concentrations of the single compounds. Correlation coefficients (r) of the regression lines ranged from 0.98 to 0.99. Typical examples are reported in Figure 1. From these data the concentrations able to give $\Delta A = 0.1$ min^{-1} ($C_{0.1}^{1\,\mathrm{min}}$) were evaluated for each member of the series. A

Table 1. Vasodilating Potency and NO Generation Properties of Water Soluble Furoxans[d]

compd	EC_{50} (μM)	EC_{50} (μM) + HbO_2 10 μM	$C_{0.1}^{1min}$ (M)	% NO_2^- (mol/mol)	% NO_2^- (mol/mol) L-Cys 50X
5a	7.35 ± 2.16	68.0 ± 8.2	8.64×10^{-3} [a]	0	3.9 ± 0.2
5b	1.23 ± 0.23	2.22 ± 0.64	5.00×10^{-4}	6.3 ± 0.2 (97 ± 2)[c]	62.9 ± 3.1
6a	14.3 ± 3.8	124.4 ± 17.7	–	0	3.9 ± 0.2
6b	1.03 ± 0.15	3.63 ± 0.42	7.19×10^{-4}	0	91.1 ± 0.9
7a	15.2 ± 1.4	78.0 ± 9.5	–	0	2.8 ± 0.1
7b	3.48 ± 0.65	15.0 ± 1.7	1.01×10^{-3} [a]	4.5 ± 0.2 (56 ± 1)[c]	37.9 ± 0.5
8a	48.1 ± 3.7	123.8 ± 12.1	–	0	2.9 ± 0.2
8b	1.55 ± 0.02	11.5 ± 1.8	7.07×10^{-4}	0	35.0 ± 2.1
9a	0.101 ± 0.012	0.43 ± 0.02	1.59×10^{-3} [a]	0	8.8 ± 0.3
9b	0.0826 ± 0.0075	1.01 ± 0.14	1.82×10^{-4}	0 (33 ± 1)[c]	36.3 ± 1.2
11	0.013 ± 0.003	0.046 ± 0.009	1.64×10^{-5}	0	19.7 ± 0.6
12	0.0041 ± 0.0009	0.048 ± 0.010	2.04×10^{-5}	0	58.9 ± 0.4
13	0.030 ± 0.0040	0.13 ± 0.03	1.53×10^{-5}	0	20.2 ± 0.6
14a	0.0520 ± 0.0066	0.35 ± 0.05	6.30×10^{-5}	0	27.9 ± 0.4
14b	0.0557 ± 0.0066	0.33 ± 0.06	6.07×10^{-5} [b]	95.3 ± 2.3 (100 ± 1)c	58.5 ± 1.5
SNP	0.0215 ± 0.0026	0.0481 ± 0.0071	2.05×10^{-4}	1.5 ± 0.6	58 ± 2
GTN	0.0224 ± 0.0039	–	–	0	45 ± 1

[a] Values extrapolated. [b] 3.63×10^{-5} in the absence of L-cysteine. [c] % NO_2^- (mol/mol) after 24 h. [d] Tested as oxalates.

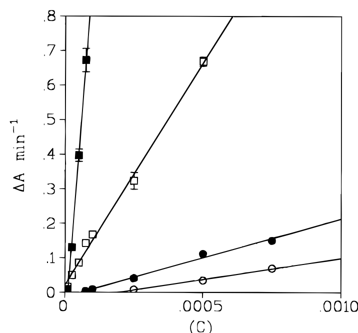


Figure 1. Examples of correlation curves between initial NO formation rate (ΔA min^{-1}) and drug concentration (C): (■) **12**, (□) **14b**, (●) **5b**, (○) **7b**.

variation in the optical density $\Delta A = 0.1$ was chosen in order to have both sufficiently precise experimental data and the most part of $C_{0.1}^{1min}$ values intrapolated on the lines. Using derivative **14b** as a source of NO, a molar extinction coefficient $\Delta\epsilon = \epsilon_{401MetHb} - \epsilon_{401HbO2} = 38.8 \pm 0.1$ mM^{-1} cm^{-1} was evaluated for the oxidation reaction. On the basis of this value, in line with previous results reported in literature,[16] $\Delta A = 0.1$ min^{-1} corresponds to 2.6 μmol L^{-1} of NO developed in 1 min.

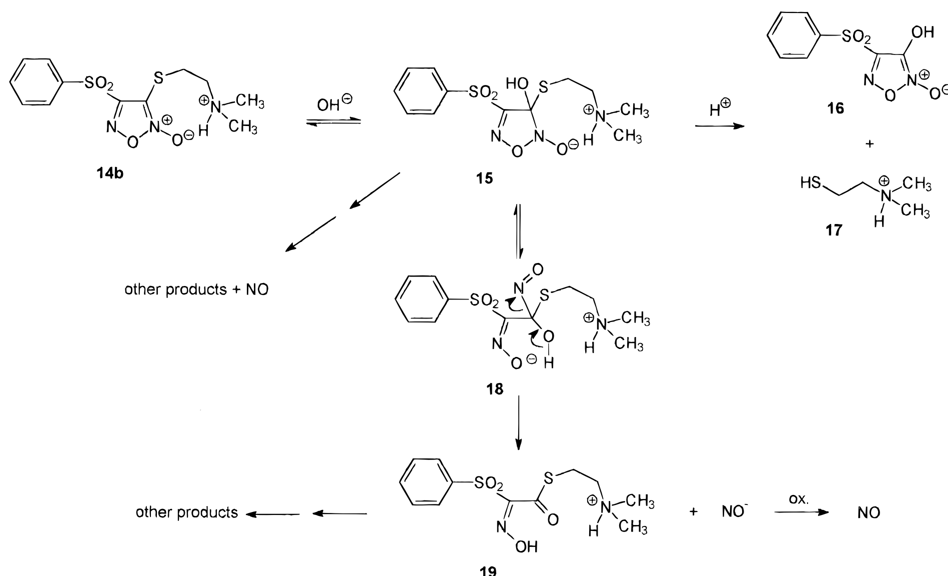
Vasodilating Activities. Vasodilating effects of all of the derivatives were assessed on endothelium-denuded strips of rat aorta precontracted with noradrenaline. The compounds were able to bring about a concentration-dependent relaxation of the strips. Concentration–response curves were also evaluated in the presence of HbO_2 (10 μM), a well-known NO scavenger. EC_{50} values are reported in Table 1. With all of the compounds, at the maximum concentrations tested, only a partial recovery (60–85%) of the noradrenergic tone occurred, after 1 h of washing the aortic preparations with fresh Krebs solution. This effect seems to be concentration dependent. Indeed a detailed study performed with the pair of isomers **9a,b** shows that it decreases with the concentration of the compounds and disappears at concentration below 1×10^{-7} M. In addition it is not specific to noradrenaline since we obtained similar results working with K^+ precontracted rat aorta strips. Whether this effect is common to other smooth muscle preparations, it could be matter of further investigations.

Discussion

All of the investigated furoxans released nitric oxide when treated under physiological conditions with an excess of cysteine (1:50). The extent of release covers a wide range (3–91%). Derivatives **5b**, **7b**, and **14b** are able to donate NO also in the absence of the thiol cofactor. After 1 h the yield of NO spontaneously released by **14b** was 95%, while in the case of **5b** and **7b** only 6% and 5% were released, respectively. After 24 h the yields increase to 100%, 97%, and 56%, respectively. Also derivative **9b** over 24 h behaves as a spontaneous NO donor (yield 33%). With the interesting exception of the derivative **14b**, the presence of cysteine increases the extent of NO donation. In all of these compounds, the basic chain at the 3-position, ionizable under physiological conditions, is essential for non-thiol-mediated release. Indeed their analogues **5a**, **7a**, **9a**, and **14a**, with the same chain at the 4-position do not spontaneously release NO, not even over 24 h. Also the suppression of the basic function on the lateral chain cancels spontaneous donation, as observed in 4-phenylfuroxans 3-EtX-substituted (X = S, O, SO_2) and in 4-(phenylsulfonyl)-3-ethylthiofuroxan.[17]

Comparison of the data reported in Table 1 indicates that, in the phenylfuroxan series, 4-phenyl isomers are markedly better NO donors than the corresponding 3-phenyl isomers. This is in line with our previous findings on other phenylfuroxan derivatives.[18] Phenylfuroxans bearing (aminoalkyl)oxy moieties at the 3-position release an amount of NO larger than the corresponding thio and sulfonyl analogues. In the couple of homologues **5b** and **6b**, both the members are good NO donors but **5b** only is able of spontaneous release. The introduction of two methyl groups on the amino function present on the lateral chain of **7b** to give **8b** does not modify the extent of the release induced by thiol cofactor but removes the spontaneous donation. In the 3-phenylsulfonyl series all of the compounds display good NO-donating properties. The best NO donor is the derivative **12** in which either the increase of the length of the lateral chain or the elimination of the methyl groups

Scheme 2



present on the amino nitrogen, as well as the substitution of sulfur for oxygen at the 4-position to afford **13**, **11**, and **14a**, respectively, decreases the extent of the release. The sole member of the 4-phenylsulfonyl series at our disposal, the 4-(phenylsulfonyl)-3-((2-(dimethylamino)ethyl)thio)furoxan (**14b**) shows strong NO donor character both in the presence and in the absence of cysteine. The behavior of this compound is worthy of comment since NO-releasing capabilities of all of the previous studied furoxans were highly dependent on the presence of thiol cofactors. One could speculate that **14b** undergoes attack at the 3-position by hydroxyl anion with consequent nucleophilic displacement of 2-(dimethylamino)ethanethiol (**17**), through the intermediacy of the adduct **15** (Scheme 2). Thus thiol **17** could be the ultimate responsible for NO release. Although other data do not seem to support this hypothesis (e.g., the behavior of **5b** and **9b**), we incubated, under physiological conditions, **14b** (10^{-4} M) with *N*-ethylmaleimide (NEM), a well known agent able to trap thiols.[19] No change in the extent of nitrite ion was detectable. In order to exclude the influence in the release by traces of contaminating heavy metals, we repeated the experiment also in the presence of chelators (EDTA, 0.1 mM). Again no change in the amount of NO_2^- was observed. A more attracting explanation of the behavior of **14b** is that **15** does not evolve to the substitution product **16** but gives the intermediate **18** since thiolate anion could be, in this system, a poor leaving group. Some formally correct mechanisms able to justify NO formation from **18** can be envisaged. One apparently straightforward implies the loss of nitroxyl anion (NO^-) (see Scheme 2) which, in turn, should react either with molecular oxygen to yield NO and then NO_2^- or dimerize to $N_2O_2^-$ with subsequent generation of N_2O. If this is the case, the extent of NO_2^- production (96%) indicates that, under the experimental conditions used, the former pathway have to be strongly predominant otherwise other schemes of NO generation must operate or be paramount. Anyway, these results, as other previous findings, indicate that the overall mechanism of NO donation by furoxans is quite complex and that the matter is in need of specific experimental studies.

Substitution of the phenyl ring for phenylsulfonyl moiety in **14b** to give **8b** suppresses the spontaneous release, perhaps because the beneficial effect of an electron-withdrawing function in the 4-position is lost. In this compound NO release requires, as in most furoxans, the attack at the 3-position by thiolate anion, a stronger nucleophile than OH^-, to give an adduct which is a reasonable intermediate on the route of thiol-mediated NO donation.[5,6] The finding that the suppression of the two methyl groups present on the amino function of **8b** affords the weak spontaneous NO donor **7b** suggests that charged lateral chain plays fine roles in NO donation. The substitution in **8b** either of the ((dimethylamino)ethyl)oxy chain or of the ((dimethylamino)ethyl)sulfonyl chain for the ((dimethylamino)ethyl)thio one affords **5b** and **9b**, respectively. These compounds give rise to slow spontaneous release of different extent. This suggests that the introduction in such models at the 4-position of the phenylsulfonyl group should afford potent spontaneous donors. The lack of spontaneous release moving from **5b** to **6b** could be partially due to the "damping effect" of the methylene group on the electron-withdrawing influence of the charged dimethylamino function. In conclusion these data show that the first step on the route of NO production, under physiological conditions, by furoxans, without the assistance of thiol cofactors, could be the result of attack at the 3-position by OH^- group, a consequence of a complex balance of electronic and steric factors exerted by the substituents at the ring. On the basis of these considerations, studies are in progress to obtain new classes of spontaneously NO-releasing furoxans.

Also the initial rates of NO release vary widely in the presence of cysteine (5-fold molar excess). The $C_{0.1}^{1\,\mathrm{min}}$ values are spread over the range 1.6×10^{-5} to 8.6×10^{-3} M. 4-Phenyl derivatives are faster release agents than 3-phenyl derivatives. In the former series the $C_{0.1}^{1\,\mathrm{min}}$ values follow the sequence **7b** > **6b** ≃ **8b** > **5b** > **9b**. In the latter series, the sequence is **5a** > **9a**; the other terms display a release rate too low to be measured. Phenylsulfonyl-substituted furoxans are fast-releasing agents, particularly when aminoalkoxy moi-

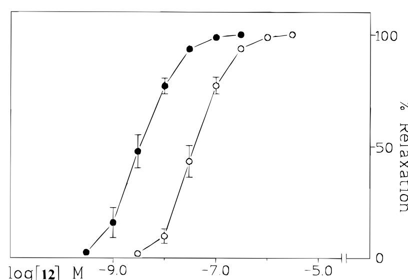


Figure 2. Concentration–response curves for vasodilating activity of compound **12** in the absence (●) and in the presence (○) of 10^{-5} M HbO_2.

eties are present at the 4-position. The couple of isomers **14a** and **14b** show similar donation rates. The spontaneous release by **14b** is slightly faster than that induced by cysteine. Under the experimental conditions used for our study, no clear correlation exists between extent and rate of NO release.

Analysis of EC_{50} values reported in Table 1 indicates that the compounds cover a wide range of vasodilating potency. The EC_{50}s follows the sequence **8a** > **7a** ≃ **6a** > **5a** > **7b** > **8b** ≃ **5b** ≃ **6b** > **9a** > **9b** > **14b** ≃ **14a** > **13** > **11** > **12**. The most active compounds belong to the phenylsulfonyl series. Derivative **12** is about 5 times as potent as glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) taken as references. **11** and **13** display potencies comparable to those of references. The two isomers **14a** and **14b** show the same potency roughly similar to those of GTN and SNP. In the phenylfuroxan series, 4-phenyl isomers are always more potent than the corresponding 3-phenyl ones. 3-((2-(Dimethylamino)ethyl)sulfonyl)-4-phenylfuroxan (**9b**) is the most active member of this group.

In the presence of 10 μM HbO_2, the concentration–response curves were shifted to the right in a parallel manner. A typical example is reported in Figure 2. The increase of EC_{50} values is in keeping with involvement of nitric oxide in the vasorelaxant action.

When the log EC_{50} (M) values are plotted against log $C_{0.1}^{1\min}$ (M) values, the quite satisfactory linear regression equation (1) is obtained and is able to explain 77% of the variance in the data. The significance of the equation was improved by omitting derivative **9a**, which is the sole outlier; in fact its residual (observed minus estimated value) surpasses double the value of *s*. At the moment we have not been able to explain this partly deviant behavior. The clean equation (2) is able to explain 90% of the variance of the data.

$$\log EC_{50} = 0.755\ (\pm 0.129) \log C_{0.1}^{1\min} + 1.42\ (\pm 0.88) \tag{1}$$

$$n = 12 \qquad r = 0.879 \qquad s = 0.44 \qquad F = 33.95$$

$$\log EC_{50} = 0.782\ (\pm 0.083) \log C_{0.1}^{1\min} + 1.50\ (\pm 0.56) \tag{2}$$

$$n = 11 \qquad r = 0.952 \qquad s = 0.30 \qquad F = 74.16$$

In conclusion this work shows that (1) it is possible to modulate over a wide range the NO-donating properties of furoxan derivatives; (2) furoxan system, if appropriately substituted, can behave under physiological conditions as NO donor without the assistance of thiol cofactors (spontaneous release); (3) the *in vitro* vasodilating potencies of furoxans are principally dependent on initial rates of NO release. Selected members of the series described in this work are under investigation in order to evaluate their activity *in vivo* and to clarify if there is some correlation with the corresponding *in vitro* behavior.

Experimental Section

General. All of the compounds were characterized as oxalates which melted with decomposition. Melting points were determined on a Büchi 530 apparatus after introducing the sample into the bath at a temperature 10 °C lower than the melting point. A heating rate of 3 °C min^{-1} was used. The compounds were routinely checked by infrared spectrophotometry (Perkin-Elmer Model 781, Shimadzu FT-IR 8101M) and HPLC (Shimadzu LC10A). ^1H and ^{13}C nuclear magnetic resonance spectra were recorded in dimethyl sulfoxide-d_6 (DMSO-d_6) at 200 and 50 MHz, respectively, with a Brucker AC-200 spectrometer. The ^{13}C-NMR signal of the oxalate anion is normally observed in the range 165–163 ppm. The ^1H-NMR signals of exchangeable protons are very broad and appear in the range 6–11 ppm. Column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM) with the indicated solvent system. Thin layer chromatography (TLC) was carried out on 5 × 20 cm plates precoated with Merck silica gel 60 F_{254}, with a layer thickness of 0.25 mm. Anhydrous $MgSO_4$ was used as drying agent. Solvent removal was achieved under reduced pressure at room temperature. Elemental analyses of the new compounds were performed by REDOX (Cologno M.), and the results are within ±0.4% of the theoretical values. Intermediates **4a**,[13] **4b**,[13] and **10**[21] were synthesized according to literature.

The figures in brackets for the regression lines are standard errors (±SEM).

4-((2-(Dimethylamino)ethyl)oxy)-3-phenylfuroxan (5a) Oxalate. To a stirred solution of **4a** (1 g, 3.3 mmol) in THF (20 mL) were added 2-(dimethylamino)ethanol (0.67 mL, 6.6 mmol) and then 0.79 g of 50% w/w water NaOH solution (9.9 mmol). NaOH solution was added portionwise while the temperature was maintained at 25 °C. The reaction mixture was stirred at room temperature for 6 h and then evaporated *in vacuo*. The residue was treated with water and extracted with CH_2Cl_2. The combined organic layers were dried and concentrated *in vacuo*, and the oily residue was filtered on a short silica gel column (CH_2Cl_2:MeOH, 95:5) to give the title product as an oily free base (0.77 g, 94%). The base was immediately transformed into the corresponding oxalate: mp 157–158 °C dec (MeOH/H_2O); ^1H NMR (DMSO-d_6) δ 2.81 (s, 6H, NCH_3), 3.58 (t, 2H, NCH_2), 4.82 (t, 2H, OCH_2), 8.1–7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 43.1 (NCH_3), 54.9 (NCH_2), 65.9 (OCH_2), 107.8 (C3), 161.9 (C4), 121.9 (PhC1), 126.5/129.1 (PhC2/C3), 130.9 (PhC4). Anal. ($C_{12}H_{15}N_3O_3 \cdot H_2C_2O_4$) C, H, N.

4-((3-(Dimethylamino)propyl)oxy)-3-phenylfuroxan (6a) Oxalate. Prepared as **5a** starting from **4a** (1 g, 3.3 mmol) and 3-(dimethylamino)propanol (0.77 mL, 9.9 mmol) with stirring at room temperature for 4 h: column eluent, CH_2Cl_2:MeOH, 95:5; 0.78 g, 90%; mp of the oxalate 146–147 °C dec (*i*-PrOH/MeOH); ^1H NMR (DMSO-d_6) δ 2.29 (m, 2H, CCH_2) 2.79 (s, 6H, NCH_3), 3.25 (t, 2H, NCH_2), 4.55 (t, 2H, OCH_2), 8.1–7.4 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 23.7 (CCH_2), 42.5 (NCH_3), 53.8 (NCH_2), 68.4 (OCH_2), 107.9 (C3), 162.3 (C4), 122.1 (PhC1), 126.5/129.4 (PhC2/C3), 131.0 (PhC4). Anal. ($C_{13}H_{17}N_3O_3 \cdot H_2C_2O_4$) C, H, N.

3-((2-(Dimethylamino)ethyl)oxy)-4-phenylfuroxan (5b) Oxalate. Prepared as **5a** starting from **4b** (1 g, 3.3 mmol) and 2-(dimethylamino)ethanol (1 mL, 9.9 mmol) with stirring at room temperature for 30 min: column eluent, EtOAc:acetone, 1:1; 0.56 g, 68%; mp of the oxalate 143–144 °C dec (MeOH); ^1H NMR (DMSO-d_6) δ 2.60 (s, 6H, NCH_3), 3.10 (m, 2H, NCH_2), 3.28 (m, 2H, OCH_2), 7.9–7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 43.1 (NCH_3), 55.9 (NCH_2), 67.3 (OCH_2), 131.2 (C3), 151.1 (C4), 125.2 (PhC1), 126.8/129.4 (PhC2/C3), 131.7 (PhC4). Anal. ($C_{12}H_{15}N_3O_3 \cdot H_2C_2O_4$) C, H, N.

3-((3-Dimethylamino)propyl)oxy)-4-phenylfuroxan (6b) Oxalate. Prepared as **5a** starting from **4b** (1 g, 3.3 mmol) and 3-(dimethylamino)propanol (1.16 mL, 9.9 mmol) with stirring at room temperature for 30 min: eluent, CH_2Cl_2:MeOH, 95:5; 0.45 g, 52%; mp of the oxalate 134−135 °C dec (MeOH); ^{1}H NMR (DMSO-d_6) δ 2.15 (m, 2H, CCH_2) 2.71 (s, 6H, NCH_3), 3.12 (t, 2H, NCH_2), 4.52 (t, 2H, OCH_2), 7.9−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 24.7 (CCH$_2$), 42.6 (NCH$_3$), 53.7 (NCH$_2$), 70.3 (OCH$_2$), 131.2 (C3), 151.2 (C4), 125.3 (PhC1), 128.7/129.5 (PhC2/C3), 131.7 (PhC4). Anal. ($C_{13}H_{17}N_3O_3$·$H_2C_2O_4$) C, H, N.

4-((2-Aminoethyl)thio)-3-phenylfuroxan (7a) Oxalate. To a stirred solution of **4a** (1 g, 3.3 mmol) in THF (30 mL) kept under nitrogen were added first 2-aminoethanethiol hydrochloride (0.75 g, 6.6 mmol) and then 1.06 g of 50% w/w water NaOH solution (13.2 mmol), NaOH solution was added portionwise while the temperature was maintained at 25 °C. The reaction mixture was stirred under nitrogen at room temperature for 3 h. *In vacuo* solvent removal afforded a residue which was treated with water and extracted with CH_2Cl_2. The combined organic layers were dried and evaporated *in vacuo*, and the obtained oily free base **7a** (0.70 g, 90%) was transformed into the corresponding oxalate: mp 186−187 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 3.18 (t, 2H, SCH_2), 3.43 (t, 2H, NCH_2), 7.9−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 28.3 (SCH$_2$), 37.8 (NCH$_2$), 114.5 (C3), 154.1 (C4), 121.9 (PhC1), 129.3/127.7 (PhC2/C3), 131.1 (PhC4). Anal. ($C_{10}H_{11}N_3O_2S$·$H_2C_2O_4$) C, H, N.

3-((2-Aminoethyl)thio)-4-phenylfuroxan (7b) Oxalate. Prepared as **7a** starting from **4b** and 2-aminoethanethiol hydrochloride with stirring at room temperature for 30 min: column eluent at first CH_2Cl_2 and then CH_2Cl_2:MeOH, 95:5; (0.31 g, 40%); mp of the oxalate 193−194 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 2.97 (m, 2H, SCH_2), 3.20 (m, 2H, NCH_2), 7.9−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 28.6 (SCH$_2$), 39.2 (NCH$_2$), 111.1 (C3), 157.9 (C4), 125.9 (PhC1), 129.2/128.0 (PhC2/C3), 131.5 (PhC4). Anal. ($C_{10}H_{11}N_3O_2S$·$H_2C_2O_4$) C, H, N.

4-((2-(Dimethylamino)ethyl)thio)-3-phenylfuroxan (8a) Oxalate. Prepared as **7a** starting from **4a** and 2-(dimethylamino)ethanethiol hydrochloride with stirring at room temperature for 1 h: column eluent CH_2Cl_2:MeOH, 95:5 (0.79 g, 80%); mp of the oxalate 166−167 °C dec (MeOH/H_2O); mp of the hydrochloride 201 °C dec;[21] ^{1}H NMR (DMSO-d_6) δ 2.69 (s, 6H, NCH_3), 3.29 (t, 2H, SCH_2), 3.58 (t, 2H, NCH_2), 7.8−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 42.8 (NCH$_3$), 25.7 (SCH$_2$), 55.1 (NCH$_2$), 114.5 (C3), 154.2 (C4), 121.9 (PhC1), 129.3/127.7 (PhC2/C3), 131.1 (PhC4). Anal. ($C_{12}H_{15}N_3O_2S$·$H_2C_2O_4$) C, H, N.

3-((2-(Dimethyamino)ethyl)thio)-4-phenylfuroxan (8b) Oxalate. Prepared as **7a** starting from **4b** and 2-(dimethylamino)ethanethiol hydrochloride with stirring at room temperature for 40 min: column eluent CH_2Cl_2:MeOH, 95:5; (0.67 g, 77%); mp of the oxalate 169−170 °C dec (MeOH); ^{1}H NMR (DMSO-d_6) δ 2.56 (s, 6H, NCH_3), 3.07 (m, 2H, SCH_2), 3.26 (m, 2H, NCH_2), 7.9−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 42.8 (NCH$_3$), 26.3 (SCH$_2$), 56.4 (NCH$_2$), 111.2 (C3), 158.0 (C4), 126.0 (PhC1), 129.2/128.1 (PhC2/C3), 131.5 (PhC4). Anal. ($C_{12}H_{15}N_3O_2S$·$H_2C_2O_4$) C, H, N.

Preparation of 4-((2-(Dimethylamino)ethyl)sulfonyl)-3-phenylfuroxan (9a) Oxalate and the 4-Phenyl Isomer (9b) Oxalate. To a stirred and water-cooled solution of the appropriate thio derivative **8a** or **8b** (1 g, 3.8 mmol) in acetic acid (10 mL) potassium permanganate (1.2 g, 7.6 mmol) was added portionwise. The mixture was stirred at room temperature for 6 h. Acetic acid was removed at room temperature *in vacuo*. The residue was treated with water, and the excess potassium permanganate and the formed manganese dioxide were destroyed by sodium sulfite. The water solution was basified with sodium carbonate and extracted with EtOAc. The combined organic layers were dried and concentrated *in vacuo* to give an oil which was purified by flash chromatography (CH_2Cl_2:MeOH, 95:5) to give the title products as free bases. **9a** (0.66 g, 58%): mp of the oxalate 150−151 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 2.57 (s, 6H, NCH_3), 4.13 (t, 2H, SO_2CH_2), 3.29 (t, 2H, NCH_2), 7.8−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 42.9 (NCH$_3$), 49.7/50.5 ($SO_2$$CH_2$/N$CH_2$), 113.4 (C3), 156.9 (C4), 120.6 (PhC1), 129.8/128.9 (PhC2/C3), 131.5 (PhC4). **9b**: stirring at room temperature for 4 h (0.85 g, 75%); mp of the oxalate 159−160 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 2.38 (s, 6H, NCH_3), 3.97 (t, 2H, SO_2CH_2), 3.06 (t, 2H, NCH_2), 7.7−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 43.6 (NCH$_3$), 50.3 ($SO_2$$CH_2$/N$CH_2$, coincident signals), 117.4 (C3), 155.4 (C4), 124.8 (PhC1), 129.8/128.5 (PhC2/C3), 131.5 (PhC4). Anal. ($C_{12}H_{15}N_3O_4S$·$H_2C_2O_4$) C, H, N.

3-(Phenylsulfonyl)-4-((2-(dimethylamino)ethyl)oxy)-furoxan (12) Oxalate. To a stirred solution of **10** (1 g, 2.7 mmol) in THF (25 mL) was first added 2-(dimethylamino)-ethanol (0.55 mL, 5.5 mmol) and then, keeping the temperature at 25 °C with water cooling, 0.65 g of 50% w/w water NaOH solution (8.1 mmol). The reaction mixture was kept under stirring for 30 min at room temperature. The solvent was removed *in vacuo*, and the residue was treated with water and extracted with CH_2Cl_2. The combined organic layers were dried and concentrated *in vacuo*. The concentrated solution was treated with carbon and filtered on a Celite bed and then evaporated *in vacuo* to give the title compound as a white solid (0.51 g, 60%) which was immediately transformed into the corresponding oxalate: mp 158−159 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 2.83 (s, 6H, NCH_3), 3.52 (t, 2H, NCH_2), 4.78 (t, 2H, OCH_2), 8.1−7.7 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 43.2 (NCH$_3$), 54.8 (NCH$_2$), 66.8 (OCH$_2$), 110.8 (C3), 158.6 (C4), 137.0 (PhC1), 130.1/128.5 (PhC2/C3), 136.3 (PhC4). Anal. ($C_{12}H_{15}N_3O_5S$·$H_2C_2O_4$) C, H, N.

3-(Phenylsulfonyl)-4-((3-dimethylamino)propyl)oxy)-furoxan (13) Oxalate. Prepared as **12** starting from **10** and 3-(dimethylamino)propanol with stirring at room temperature for 1 h (0.71 g, 80%): mp of the oxalate 141−142 °C dec (MeOH, *i*-PrOH); ^{1}H NMR (DMSO-d_6) δ 2.20 (m, 2H, CCH_2), 2.77 (s, 6H, NCH_3), 3.13 (t, 2H, NCH_2), 4.49 (t, 2H, OCH_2), 8.1−7.7 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 23.3 (CCH$_2$), 42.4 (NCH$_3$), 53.5 (NCH$_2$), 68.9 (OCH$_2$), 110.6 (C3), 158.8 (C4), 137.1 (PhC1), 130.1/128.6 (PhC2/C3), 136.2 (PhC4). Anal. ($C_{13}H_{17}N_3O_5S$·$H_2C_2O_4$) C, H, N.

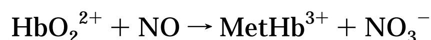
3-(Phenylsulfonyl)-4-((2-amino)ethyloxy)furoxan (11) Oxalate. Prepared as **12** starting from **10** and 2-aminoethanol with stirring at 15−20 °C for 30 min. The concentrated solution was saturated with NaCl and extracted with CH_2Cl_2. The free base was purified by flash chromatography (EtOAc: MeOH, 95:5) (0.43 g, 55%): mp of the oxalate 183−184 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 3.30 (t, 2H, NCH_2), 4.65 (t, 2H, OCH_2), 8.1−7.7 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 37.7 (NCH$_2$), 68.4 (OCH$_2$), 111.0 (C3), 159.0 (C4), 137.0 (PhC1), 130.1/128.8 (PhC2/C3), 136.3 (PhC4). Anal. ($C_{10}H_{11}N_3O_5S$·$H_2C_2O_4$) C, H, N.

Preparation of 3-(Phenylsulfonyl)-4-((2-(dimethylamino)ethyl)thio)furoxan (14a) Oxalate and the 4-Phenylsulfonyl Isomer (14b) Oxalate. To a stirred solution of **10** (2 g, 5.5 mmol) in THF (25 mL) kept under nitrogen was added first 2-(dimethylamino)ethanethiol hydrochloride (1.54 g, 11 mmol) and then, portionwise, 1.75 g of 50% w/w water NaOH solution (22 mmol). The temperature during the addition was kept at 25−30 °C. The reaction mixture was stirred at room temperature for 1 h. The solvent was removed *in vacuo*, and the water solution of the residue was extracted with CH_2Cl_2. The combined organic layers were dried and evaporated *in vacuo* to afford a mixture of the two title isomers. The mixture was resolved by flash chromatography (EtOAc: MeOH, 95:5); first eluted **14b** (0.82 g, 45%), second eluted **14a** (0.22 g, 12%). Both the isomers were transformed into the corresponding oxalates. **14a**·$H_2C_2O_4$: mp 143−144 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 2.72 (s, 6H, NCH_3), 3.55 (m, 2H, NCH_2), 3.32 (m, 2H, SCH_2), 8.1−7.6 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 42.6 (NCH$_3$), 54.7 (NCH$_2$), 24.8 (SCH$_2$), 117.3 (C3), 152.8 (C4), 136.4/136.5 (PhC1/PhC4), 130.2/128.5 (PhC2/C3). **14b**·$H_2C_2O_4$: mp 144−145 °C dec (MeOH/H_2O); ^{1}H NMR (DMSO-d_6) δ 2.59 (s, 6H, NCH_3), 3.23 (t, 2H, NCH_2), 2.95 (t, 2H, SCH_2), 8.1−7.8 (m, 5H, C_6H_5); ^{13}C NMR (DMSO-d_6) δ 42.5 (NCH$_3$), 56.1 (NCH$_2$), 26.0 (SCH$_2$), 108.9 (C3), 159.7 (C4), 136.0/136.4 (PhC1/PhC4), 130.3/129.3 (PhC2/C3). Anal. ($C_{12}H_{11}N_3O_4S$·$H_2C_2O_4$) C, H, N.

Quantitative Nitrite Detection. A solution of the ap-

propriate furoxan (20 μL) in distilled water was added to 2 mL of 50 mM phosphate buffer (pH 7.4) containing 5 mM L-cysteine or in its absence. The final concentration of drug 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 10 min at room temperature, the 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 percent NO_2^- (mol/mol) ± SEM.

NO Release Kinetic Study. The rate of NO release was determined using a spectrophotometric technique[16] based on the oxidation of oxyhemoglobin (HbO_2) to methemoglobin (MetHb) according to the equation



$$HbO_2^{2+} + NO \rightarrow MetHb^{3+} + NO_3^-$$

The formation of MetHb was followed by recording the absorbance increase (ΔA) at $\lambda = 401$ nm using a Perkin-Elmer λ5 spectrophotometer and a thermostated (37 °C) cuvette.

The reaction was started by adding drug water solutions of at least three different concentrations (final concentration 10^{-5} to 7.5×10^{-4} M) to a 4 μM HbO_2 solution in 50 mM phosphate buffer (pH 7.4) containing a 5-fold molar excess of cysteine as to the furoxan solute. The increase of the absorbance (ΔA) was recorded over the first 3 min. The initial rates were calculated from the slope of the straight line portion of each curve. Every NO-releasing rate is the average of at least three determinations. The molar extinction coefficient $\Delta\epsilon = \epsilon_{401MetHb} - \epsilon_{401HbO_2}$ was determined by quantitative oxidation of five different concentrations (2−6 μM) of HbO_2 in pH 7.4 phosphate buffer with a 7.5×10^{-5} M water solution of **14b**. The slope ($\Delta\epsilon$) of the straight line ($r = 0.999$) obtained plotting the increase of the absorbance ΔA at $\lambda = 401$ nm against the HbO_2 concentrations was 38.8 ± 0.1 mM^{-1} cm^{-1}. Using $K_3Fe(CN)_6$ as oxidant, a similar value of 39.9 ± 1.4 mM^{-1} cm^{-1} was obtained.

Vasoactivity Determinations. Thoracic aortas were isolated from male Wistar rats weighing 180−200 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-Heinseleit solution (NaCl, 137; KCl, 2.68; $MgCl_2$, 0.50; $CaCl_2$, 5.44; NaH_2PO_4, 0.54; $NaHCO_3$, 8.93; glucose, 8.30; ascorbic acid, 0.10 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 were 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−Heinseleit 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. The effects of oxyhemoglobin on relaxation were evaluated in a separate series of experiments by exposing aortic strips, precontracted with 1 μM noradrenaline, to oxyhemoglobin 10 μM for at least 10 min before addition of vasodilating agent.

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 a solution of commercially available hemoglobin in 50 mM phosphate saline buffer, pH 7.4, to an excess of the reducing agent sodium dithionite ($Na_2S_2O_4$) at 4 °C and was then protected from the light. Hemoglobin (64.5 mg) was gently dissolved into 1 mL of distilled water; 53.6 mg of sodium dithionite was then added to the solution which was loaded onto a chromatographic column (Sephadex G-25, Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer. Two Pasteur pipets were used as columns for 1 mL of this solution.

The purity of the oxyhemoglobin solution was determined spectrophotometrically (600−360 nm) ($\lambda_{max} = 576$ nm, 541 nm; ϵ = 14 600, 13 800) by dissolving 10 μL of the eluted solution into 3 mL of buffer (concentrated oxyHb (μM) = $A \times 2290$). The solutions were freshly prepared daily.

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