NO Donor and Biological Properties of Different Benzofuroxans[1]

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Purpose. To investigate the effect of benzofusion on NO donor properties and related biological activities of the furoxan system. The biological properties considered were the ability to increase the cytosolic levels of cGMP in C6 cells and vasodilation.

Methods. NO donor properties were investigated either in the presence or the absence of cysteine by using the Griess reaction, chemiluminescence, and gas chromatography. Increase of cytosolic cGMP levels were evaluated by radioimmunoassay. Vasodilating activity was assessed on rat aorta strips precontracted with noradrenaline, in the presence and the absence of oxyhemoglobin (HbO_2) and methylene blue (MB), respectively.

Results. Benzofuroxan and its methyl and cyano derivatives were unable to release NO under the experimental conditions. Generally these compounds displayed feeble vasodilating properties and were able to weakly stimulate soluble guanylate cyclase (sGC). By contrast, benzodifuroxan and benzotrifuroxan were able to produce both NO$^{\cdot}$ and its reduced form NO^-, the nitroxyl anion. They displayed potent vasodilating properties and were able to increase cytosolic levels of cGMP in a concentration-dependent manner.

Conclusions. The simple benzofuroxans considered here are devoid of the capability to release NO, they weakly stimulate sGC as well as manifest feeble vasodilating properties by a mechanism that does not involve a thiol-induced NO production. By contrast, benzodifuroxan and benzotrifuroxan behave as typical NO donor furoxans.

KEY WORDS: nitric oxide; NO; benzofuroxans; NO donors.

INTRODUCTION

Furoxans **1** (Fig. 1) represents a recently discovered class of thiol-dependent NO donors in which interest is continuously growing (1,2). In the past few years we here synthesized and tested a number of these derivatives for their NO-releasing properties and for their related biological activities (3,4). We found NO donor capabilities are dependent on the nature, as well as, the position of the substituents at the ring. Generally speaking, electron-withdrawing groups, in particular at the 3-position, adjacent to the $N^+ -O^-$ moiety, enhance the thiol-induced NO release. Moreover, we showed the furoxan system, when appropriately substituted, affords compounds which also behave as NO donors in the absence of thiol cofactors (4). The overall reaction mechanism of NO donation from furoxans appears to be very complex. Either direct NO$^{\cdot}$ release or intermediate formation of nitroxyl anion NO^-, which generates NO$^{\cdot}$ by oxidation, could be involved. Under aerobic conditions the final oxidative metabolites are nitrites and, in much smaller amount, nitrates. Finally we found *in vitro* vasodilating potencies of furoxans are principally dependent on the initial rates of NO release (4). To our knowledge, no study has been devoted to the effect of benzofusion on the NO donor properties and related biological activities of the furoxan system, until now.

In this paper we address this aspect of studying the NO release by benzofuroxan **2**, benzofuroxans **3–6** bearing as substituent the electron-releasing methyl group, or the electron-withdrawing cyano group, as well as by the benzodifuroxan **7** and benzotrifuroxan **8** systems (Fig. 1). The ability of these compounds to increase the cytosolic levels of cGMP in C6 cells and to vasodilate rat aorta strips precontracted with noradrenaline are also discussed. We will show these substances behave quite differently probably due to a different way of interacting with the heme of soluble guanylate cyclase (sGC).

MATERIALS AND METHODS.

Chemistry

Synthesis

It is known that benzofuroxans exhibit the isomerization (or tautomerism) equilibrium **A** (Fig. 2). The thermodynamic equilibrium concentrations depend on the nature and the position of R-substituent at the ring, as well as on the solvent and the temperature. When an isomeric pair of compounds is being considered, it is conventionally referred to by assigning the lowest numerical value of the substituent (5). Benzofuroxan **2** (6), 4-methylbenzofuroxan **3** (7), 5-methylbenzofuroxan **4** (7), benzodifuroxan **7** (8), and benzotrifuroxan **8** (9), were synthesized according to the methods reported in literature. 4-Benzofuroxancarbonitrile **5** and 5-benzofuroxancarbonitrile **6** were synthesized according to Scheme 1, starting from the corresponding oximes **11**, **12**, which were prepared from the related aldehydes **9**, **10** (10).

Melting points of the new compounds were measured on a Büchi 530 capillary apparatus and are uncorrected. Their structure was checked by the usual spectroscopies (IR, Shimadzu FT-IR 8101M; ^1H and ^{13}C NMR, Bruker AC-200) and mass spectrometry (Finnigan-Mat TSQ-700). Elemental analyses were performed by REDOX (Cologno M.) and the results are within ± 0.4% of theoretical values.

*Preparation of 4-Benzofuroxanaldehyde Oxime (**11**) and of 5-Benzofuroxanaldehyde Oxime (**12**).* A mixture of the appropriate benzofuroxanaldehyde **9**, **10** (1.64 g, 10 mmol), hydroxylamine hydrochloride (0.76 g, 11 mmol), and pyridine (0.87 g, 11 mmol), in EtOH (50 mL) was refluxed for 30 min and then evaporated *in vacuo*. The residue was treated with 1N HCl and extracted with EtOAc. The combined organic layers were washed with NaCl saturated solution, dried on $MgSO_4$, and evaporated. The crude material obtained was recrystallized from EtOAc/petroleum ether.

11: yield 92%; m.p. 257–258°C; anal. ($C_7H_5N_3O_3$) C,H,N.

12: yield 95%; m.p. 142–143°C; anal. ($C_7H_5N_3O_3$) C, H, N.

*Preparation of 4-Benzofuroxancarbonitrile (**5**) and of 5-Benzofuroxancarbonitrile (**6**).* Thionyl chloride (1.45 mL, 20

[1] In this paper we use NO as the generic family name. When necessary, we specify the redox state of NO we are referring to.

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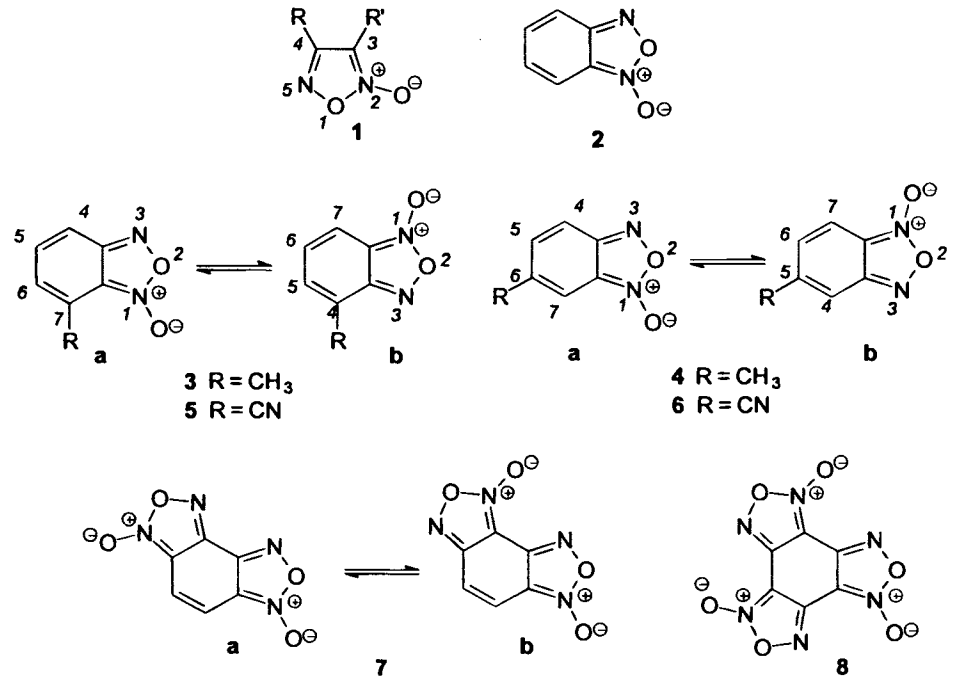
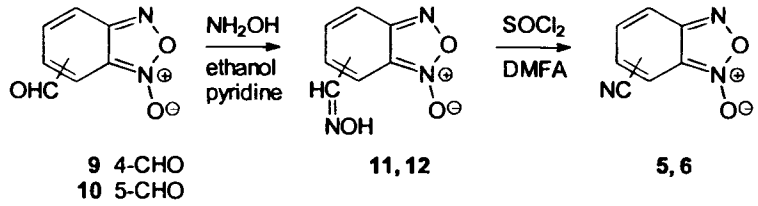


Fig. 1. Chemical structure of furoxans **1** and benzofuroxans **2-8**.



Scheme 1

mmol) was added dropwise to a stirred and ice-water cooled solution of the appropriate benzofuroxanaldehyde oxime **11**, **12** (0.90 g, 5 mmol) in DMFA (15 mL). The stirring was continued for 30 min at 0° and then the reaction mixture was poured into cold water (100 mL). The precipitate formed was collected by filtration, washed with water, dried, and recrystallized from $CHCl_3$/petroleum ether.

5: yield 91%; m.p. 91–92°C; anal. ($C_7H_3N_3O_2$) C,H,N.

6: yield 90%; m.p. 76–77°C, lit. (11) 75–76°C; anal. ($C_7H_3N_3O_2$) C,H,N.

A

Fig. 2. Benzofuroxan tantomerism.

Benzofuroxan Tautomerism

Literature data (5) show that in the methyl derivatives **3** and **4**, the tautomeric ratios were **3b/3a** ca 3:1 ($CHCl_3$, −40°C) and **4b/4a** ca 1:1 ($CHCl_3$, −40°C) respectively.

^{1}H-NMR spectra (Bruker AC-200 spectrometer) show that in cyano substituted benzofuroxans, the **5b** tautomer was favoured over the **5a** one by a factor of ca 5.7:1 (acetone, 0°C), while the **6a** tautomer was favoured over the **6b** one by a factor of ca 2:1 (acetone, −20°C). Benzodifuroxan **7** gave at room temperature a spectrum which can be attributed to the two distinct isomers **7a** and **7b** (**7b/7a** *ca* 1.6:1, acetone) (Fig. 1) (8), while ^{13}C−, ^{15}N−(20°C, acetone) and ^{14}N−(30°C, acetone) spectra of **8** did not show any tautomerism (12).

Detection of Nitrite (13)

A solution of the appropriate compound (20 μL) in DMSO was added to 2 mL of 50 mM phosphate buffer (pH 7.4), containing or not 5 mM L-cysteine. 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-naphthylethyldiamine dihydrochloride (0.2 g), 85%

Table I. NO Release Data[a]

Comp.	NO^-_2%[b] (− L-cys)	NO^-_2%[b] (+ L-cys)	NO%[c] (− L-cys)	NO%[c] (+ L-cys)	N_2O%[d] (− L-cys)	N_2O%[d] (+ L-cys)
2–6	0	0	0	0	0	0
7	0	45 ± 1	0	18.9 ± 0.4	0	11.0 ± 0.5
8	0	96 ± 1	0	21.7 ± 0.9	0	28.1 ± 3.4

Note: For details of each determinaton see section of Materials and Methods.
[a] Yields are reported as percentage mol/mol ± S.E.
[b] Nitrite was determined according to the Griess procedure.
[c] NO radical was determined by chemiluminescence detection.
[d] N_2O was detected by gas-chromatographic method.

phosphoric acid (10 mL) in distilled water (final volume: 100 mL)]. After 10 min at room temperature, absorbance was measured at 540 nm (Perkin-Elmer Lambda 5 spectrophotometer). 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). The ± S.E. data are reported in Table I.

Detection of NO˙ (14)

A solution of the appropriate compound (20 μL) in DMSO was added to 2 mL of 50 mM phosphate buffer (pH 7.4) containing (or not containing) 5 mM L-cysteine, kept in a sealed vial, under argon atmosphere. The final concentration of drug was 10^{-4} M. After 1 h at 37°C, the vial was connected to a NO chemiluminescence detector (Thermo Environmental 42C, modified by AG elettronica industriale, Piacenza, Italy). $KMnO_4$-purified air was used as carrier gas. 1–60 nmol of standard gaseous NO were used for the calibration curve. The yield was expressed as NO% (mol/mol) ± S.E. The results are reported in Table I.

Detection of N_2O (15)

A solution of the appropriate compound (500 μL) in DMSO was added to 1.5 mL of 5 mM phosphate buffer (pH 7.4) containing (or not containing) 0.125 M L-cysteine, kept in a sealed vial, under argon atmosphere. The final concentration of drug was 2.5×10^{-3} M. After 1 h at 37°C, 200 μL of headspace gas were injected from the vial into a gas chromatograph (Carlo Erba FTV 4300, with TCD detector HWD 430; column Hye-SEPQ 80–100 mesh, 6 mm × 2 m; carrier gas Helium 30 ml min^{-1}, isothermal elution at 80°C. Inlet temperature: 150°C; detector temperature: 250°C). 10–1200 nmol of standard gaseous NO were used for the calibration curve. The yield was expressed as N_2O% (mol/mol) ± S.E. The results are reported in Table I.

Biology and Pharmacology

cGMP Determination in C6 Cells

C6 cells (rat glioma) grown to confluence in 35 mm-diameter Petri dishes were washed twice with a phosphate buffered solution (PBS: 150 mM NaCl, 6.6 mM Na_2HPO_4, pH 7.4) and then incubated for 30 min at 37°C in 1 ml of Ham's F-12 medium containing 0.5 mM isobutylmethylxantine (IBMX). Derivatives under study were dissolved in DMSO, diluted at different concentrations and immediately added to the dishes. The final volume of DMSO was always ≤0.5% of total volume. Incubation was stopped after 15 minutes by aspirating the supernatant and adding 300 μl of ethanol. When ethanol was evaporated, 0.3 ml of Tris-EDTA buffer (Tris-HCl 50 mM, EDTA 4 mM, pH 7.5) were added. After 10 minutes 100 μl supernatant were tested for the level of cGMP by radioimmunoassay with a cGMP [^{3}H]-assay system (Amersham, Bucks, UK). The results, expressed as pmol of cGMP/mg of protein ± S.E. are reported in Table II.

Vasodilating Activities

Thoracic aortas were isolated from male Wistar rats (180–200 g) anesthetized with CO_2 and sacrified by decapitation. All animals were dealt with in a humane way in accordance with recognized guidelines on experimentation. The vessels were helically cut, the endothelium 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-bicarbonate solution (NaCl 112; KCl 5.0; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.0; $NaHCO_3$ 12; glucose 11 mM) maintained at 37°C and gassed with 95% O_2–5% CO_2 (pH 7.4). The aortic strips were allowed to equilibrate for 1 hour and then were contracted with 1 μM noradrenaline, which caused a submaximal response. During this first contraction the absence of intact endothelium was verified by adding 1 μM acetylcholine, which was found not to induce relaxation. The preparations were then extensively washed with Krebs-bicarbonate buffer and after equilibration for 2.5 h from the beginning, a second contraction was evoked by 1 μM noradrenaline. When the response reached its plateau, cumulative concentrations of the vasodilating agent were added. Effects of oxyhemoglobin (HbO_2), a scavenger of NO, and methylene blue (MB), an inhibitor of guanylate cyclase, were evaluated in two separate series of experiments. In the first case, aortic strips, precontracted with 1 μM noradrenaline, were exposed to 10 μM HbO_2 before contraction plateaued, while in the second case 10 μM MB was added to the organ bath 5 minutes before contraction with noradrenaline. The results, expressed as percentage vasodilation or EC_{50} (μM) ± S.E., are reported in Table II.

RESULTS AND DISCUSSION

Results show benzofuroxan **2** does not release NO, neither in the absence nor in the presence of L-cysteine (Table I). It displays very feeble vasodilating properties when tested at 3

Table II. Pharmacological Data

Comp.	% Vasodilation (30 μM)[b]	cGMP[a] (pmol/mg prot.) (100 μM)[b]	(10 μM)[b]	(1 μM)[b]	EC_{50} (μM)	(+ HbO_2 10 μM)	(+ MB 10 μM):
2	28.7 ± 2.7	4.5 ± 1.5	[e]	[e]	[d]	[e]	[e]
3	22.4 ± 4.3	5.8 ± 0.6	[e]	[e]	[d]	[e]	[e]
4	44.8 ± 1.6	4.8 ± 0.4	[e]	[e]	[d]	[e]	[e]
5	[c]	8.6 ± 1.5	[e]	[e]	0.77 ± 0.08	[e]	18.0 ± 2.0
6	34.7 ± 4.4	4.5 ± 1.0	[e]	[e]	[d]	[e]	[e]
7	[c]	49.9 ± 9.5	22.7 ± 0.7	17.5 ± 0.2	0.012 ± 0.002	0.057 ± 0.006	0.91 ± 0.06
8	[c]	40.6 ± 1.4	22.3 ± 0.4	11.8 ± 1.3	0.0041 ± 0.0007	0.075 ± 0.010	3.3 ± 0.5
SNP	[c]	22.0 ± 1.7	16.7 ± 1.8	9.3 ± 1.4	0.022 ± 0.003	[e]	[e]

[a] Basal cGMP level: 1.8 ± 0.8.
[b] Concentration tested.
[c] The compound at this concentration was able to induce complete vasodilation.
[d] Not determined owing to low activity of the compound.
[e] Not tested.

$\times 10^{-5}$ M concentration and its ability to increase the cytosolic levels of cGMP in C6 cells is low and close to the control (Table II). The introduction either at 4-position or at 5-position of the electron-releasing methyl group to obtain compounds **3** and **4** respectively, does not modify this situation. Also the 5-cyano substituted benzofuroxan **6** behaves similarly. On the contrary the 4-cyano isomer **5** significantly increases cytosolic levels of cGMP and displays fairly good potency as vasodilator. This potency is decreased in the presence of MB. On these bases we are induced to conclude this series of compounds is able to weakly stimulate sGC and to manifest feeble vasodilating properties by a mechanism which does not involve a thiol-induced NO production. Since we found all of the furoxans described in the present work are able to slowly oxidize the heme iron of HbO_2 (data not shown), this mechanism could involve interaction of the structures **2–6** with the Fe^{2+} of the sGC heme, with its consequent displacement from the porphyrin plane and activation of the enzyme. Alternative explanation of the lack of correlation between *in vitro* NO release and ability to increase intracellular cGMP could be the need for the drug to be metabolized by the cell to release NO.

A few pharmacological properties of compounds **7** and **8** are known in literature. Benzodifuroxan **7** is a potent MAO inhibitor and vasodilating substance (16). Common hydrophobic and hydrophilic centers between **7**, glyceryl trinitrate (GTN) and 5-hydroxytryptamine (5-HTP) have been claimed to explain these activities. Data in Table II confirm **7** is a potent vasodilator, twice as potent as nitroprusside (SNP) taken as reference, and show **8** is a still more potent vasodilating derivative. Nitric oxide is principally involved in the vasodilating action of both compounds, since a strong decrease in the potency occurs when the experiments are performed in the presence of HbO_2 or MB.

Since, as previously pointed out, the furoxan derivatives studied in the present work are able to slowly oxidize the heme iron, the lower activity observed in the presence of HbO_2 could be partly due to this interaction. Both the substances increase the cytosolic cGMP levels in a concentration-dependent manner. The ability to stimulate sGC is still evident at 1 μM concentration. These compounds produce large amounts of nitrite in the presence of L-cysteine (Table I). No formation of nitrite was observed in the absence of thiol cofactor. In order to clarify which kind of NO redox species is formed by the substances we determined the amount of $NO^{\bullet}$ and NO^- produced under anaerobic conditions (argon atmosphere). $NO^{\bullet}$ was detected by the chemiluminescence method, while N_2O, which is the product derived from dimerization of nitroxyl (HNO) (Eq. 2), was detected by gas-chromatography. This latter species at physiological pH exists in the predominant form of NO^-. Data in Table I show NO donation is due to simultaneous $NO^{\bullet}$ and nitroxyl formation. It is known (1) $NO^{\bullet}$, in air under physiologically conditions, is principally transformed into nitrites according to Eq. 1)

$$1)\ 4\,NO^{\bullet} + O_2 + 2H_2O \rightarrow 4\,H^+ + 4\,NO_2^-$$

$$2)\ H_2N_2O_2 \rightarrow H_2O + N_2O$$

$$\uparrow$$

$$3)\ HNO \xrightarrow{O_2} NO \xrightarrow{O_2} NO_2^-$$

$$\downarrow$$

$$4)\ RS\text{-}SR + NH_2OH$$

On the other hand, nitroxyl, under these conditions, could react with molecular oxygen to yield nitrites likely by the intermediate formation of NO, (Eq. 3) or dimerize and decompose to N_2O (Eq. 2) (17,18). In addition, in the presence of thiol cofactors, nitroxyl could generate NH_2OH, according to Eq. 4) (1). This reaction certainly has different importance in aerobic and anaerobic conditions. This complex whole of interrelated reactions makes the discussion of the relative amounts of each NO-redox species released difficult.

Additional problems in discussing these figures arise from the fact furoxan thiol-induced NO donation could involve also intermediate nitrosothiol formation (18). A recent paper by Wong *et al.* (19) shows these species can generate nitroxyl, in the presence of thiols. On the light of these considerations it is difficult to solve if nitroxyl is directly or indirectly formed by furoxans under the action of thiol cofactors.

In conclusion, benzodifuroxan (**7**) and benzotrifuroxan (**8**), unlike the simple benzofuroxans **2–6**, behave as NO donors. This is probably due to the strong electron-withdrawing properties of the furoxan moiety (20). Since electron-withdrawing groups activate the furoxan ring to thiol-induced NO release (1,3), the extensive NO production by compound **8** and, in less degree, by compound **7** could be justified by the presence in

these structures of three and two joined furoxan rings respectively. In the simple benzofuroxans considered here, NO formation is undetectable because this activation is not operating.

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