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Synthesis and evaluation of NO-release from symmetrically substituted furoxans

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Abstract—A series of symmetrically substituted dibenzoyl furoxans were synthesized and investigated for their potential to release nitric oxide, which plays a key role in the nervous and cardiovascular systems. Cysteine was employed to promote nitric oxide release from furoxan via the formation of an *S*-nitrosothiol intermediate. Transition metal ion-mediated *S*-nitrosocysteine decomposition liberates nitric oxide that, in aqueous aerobic solutions, is converted to reactive nitrogen oxide species. The percent nitric oxide released was quantified colorimetrically by the Griess reagent system.

Furoxans (1,2,5-oxadiazole-2-oxides, Fig. 1) are biologically active compounds that are capable of releasing nitric oxide in the presence of thiols. The reaction of thiols, such as cysteine or glutathione, with furoxans is believed to involve the formation of *S*-nitrosothiols.[1] Furoxans, therefore, are potential NO-releasing prodrugs.

Initial investigations of the biological activities of furoxans focused on benzofuroxans.[2] During the past decade, 'single-ring' furoxans, in which the oxadiazole ring is not fused to an aromatic ring system, have garnered more attention from pharmaceutical and medicinal chemists. Indeed, a variety of asymmetrically substituted furoxans have been shown to generate NO in the presence of thiols.[3] The mechanism for nitrosothiol formation, however, is still a matter of investigation. During the course of our research, we became aware of a simple procedure for synthesizing symmetrically substituted furoxans. Surprisingly, the reactions of these compounds with thiols have received little attention. Symmetrically substituted furoxans offer simpler models for probing structure–activity relationship of nitrosothiol formation than the more elegantly designed furoxans appearing in recent reports.

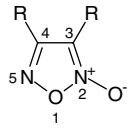
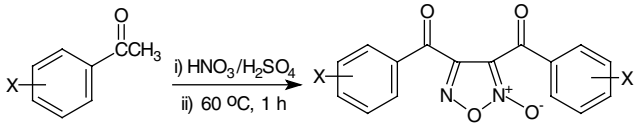


Figure 1. Structure of furoxan heterocyclic ring system.



1, X = H
2, X = 4'-F
3a, X = 2'-Cl, **3b,** X = 4'-Cl
4a, X = 2'-CH_3, **4b,** X = 4'-CH_3
5a, X = 2'-OCH_3, **5b,** X = 4'-OCH_3
6a, X = 3'-CN, **6b,** X = 4'-CN

Scheme 1. Nitric acid oxidation of acetophenone to furoxan.

To gain further insight into the reactivity of 3,4-substituted furoxans with thiols, we synthesized a number of symmetrically substituted dibenzoylfuroxans by treating substituted acetophenones with nitric acid distilled from sulfuric acid (Scheme 1).[4] The heterocyclic products were characterized by their melting points, IR and NMR spectroscopy.[5]

Thiol-mediated nitric oxide release from furoxans has been previously reported.[1a] In the presence of L-cysteine, Gasco reported the % NO released by 3-cyano-4-phe-

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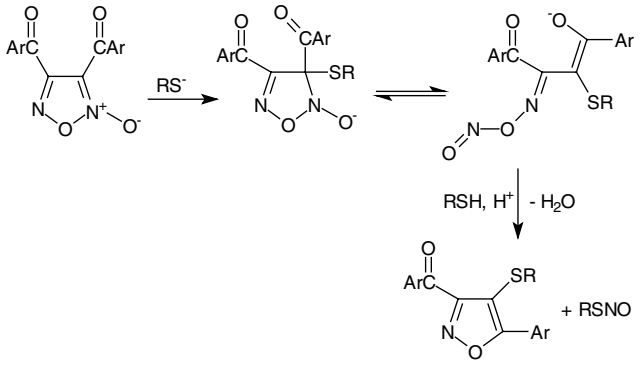
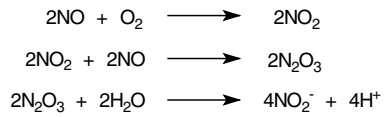


Scheme 2. Mechanism for nitrosothiol formation by furoxans in the presence of thiols.



Scheme 3. Oxidation of NO to reactive nitrogen oxides and nitrite ions.

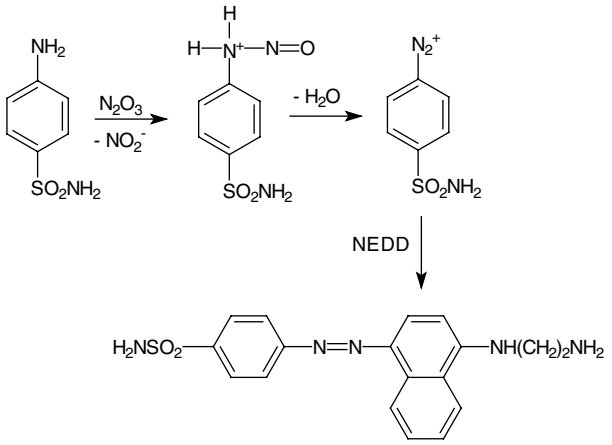
nylfuroxan to be of the order of 40%. Release of nitric oxide is dependent on the concentration of the thiol cofactor. Gasco employed the Griess reagent system to investigate nitric oxide production by 4-phenyl-3-furoxancarbonitrile and gain insight into the mechanism of NO release.[1b]

Thiols or other nucleophilic species are necessary to promote release of NO from the oxadiazole ring.[1a,3e] Nucleophilic attack by thiol on the furoxan results in formation of a nitrosothiol (RSNO) as shown in Scheme 2.

Decomposition of nitrosothiols to liberate NO can be effected thermally or photochemically or, in aqueous systems, by transition metals.[6] Saville, Grisham, and others have shown that the Hg^{2+} promotes the release of NO from RSNO to form reactive nitrogen oxide species.[6] For example, in aqueous aerobic solvents, nitric oxide reacts with molecular oxygen to form various nitrogen oxide species shown in Scheme 3.[7]

Nitrous acid, formed by treating nitrite ions with a strong acid, is capable of nitrosating amines, such as sulfanilamide which is used in the Griess reagent system, to generate diazonium salts. Release of NO in aqueous systems can therefore be quantitatively determined by measuring the formation of a diazo compound that forms when the diazonium ion couples with *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD) in the Griess reagent system[8] (Scheme 4).

To evaluate NO release from the substituted 3,4-dibenzoylfuroxans, 5 M equiv of L-cysteine was added to the furoxan to form *S*-nitrosocysteine. Mercuric (II) chloride was subsequently added to promote decomposition of the RSNO intermediate. The solution was then treated with sulfanilamide in 5% phosphoric acid



Scheme 4. Formation of sulfanilamide diazononium ion in the presence of reactive nitrogen oxide species and formation of diazo compound.

followed by the addition of NEDD to generate the diazo compound that has an absorbance maximum at 540 nm. The signal intensity of the dye is proportional to the amount of NO released. To quantify % NO, a standard RSNO curve was made by measuring the change in absorbance of various concentrations of sodium nitrite solutions treated with L-cysteine and the Griess reagent system. The calibration curve was linear over 4 orders of magnitude with an R^2 value of 0.998. The results from various symmetrically substituted dibenzoylfuroxans are shown in Table 1. Most of the substituted 3,4-dibenzoylfuroxans released similar molar percentages of NO, of the order of 5% NO. Curiously, the 4′-methyl-substituted compound, 3,4-di(4′-methylbenzoyl)furoxan, resulted in significantly higher levels of NO production (18.5%) as determined by nitrite ion concentration. The 4′-methoxy derivative, on the other hand, gave the same NO yield (4.9%) as dibenzoylfuroxans bearing cyano or halogen substituents. The strongly acidic conditions of the Griess reagent system (5% H_3PO_4), however, could result in protonation of the methoxy oxygen. The protonated aryl ether would be expected to exhibit behavior similar to those of cyano-substituted compounds.

Wink et al.[6b] demonstrated that reactive nitrogen species, formed in the buffered phosphate solutions,

Table 1. Percent NO released by substituted furoxans as determined by the Griess reagent system

3,4-Dibenzoylfuroxan	% NO released (±SE)[a]
1, X = H	<1.0 (.2)
2, X = F	3.8 (.2)
3a, X = 2′-Cl	4.4 (.1)
4a, X = 2′-CH_3	NA[b]
4b, X = 4′-CH_3	18.5 (.2)
5a, X = 2′-OCH_3	3.4 (.2)
5b, X = 4′-OCH_3	4.9 (.2)
6a, X = 3′-CN	5.0 (.3)
6b, X = 4′-CN	5.0 (.2)

[a] Expressed as % NO_2^- (mol/mol) ± SE.
[b] Note: unable to determine due to poor solubility.

are capable of nitrosating amines to generate diazonium salts. Unlike the Griess reagent system for nitrite ion detection, these reactions occur in neutral environments and are dependent on the formation of a reactive nitrogen oxide intermediate as shown below (Eq. 1).

$$4NO + O_2 \rightarrow 2N_2O_3 \quad (1)$$

It should be noted that in this equation, 2 equiv of NO is necessary to generate one nitrosation species. In an effort to determine whether the poor NO release exhibited by the methoxy-substituted dibenzoylfuroxan was due in part to positive charge on a protonated aryl ether, the 4-methoxy and 4-methyl derivatives were run in acetonitrile solutions of sulfanilamide and NEDD in the absence of phosphoric acid (Table 2).

As seen in Table 2, the release of NO for the 4′-methyl derivative is only slightly less (16% vs 18.5%) than the % NO generated in acidic environments typically used in the Griess reagent system. However, the NO generation by the 4′-methoxy derivative has increased significantly under neutral conditions (from 4.9% to 14.0%). In the neutral environment, the nitrosating agent is the reactive nitrogen oxide intermediate generated from the reaction of nitric oxide with molecular oxygen.

Investigations by Feelisch and Gasco support nucleophilic attack by the sulfide on carbon-3 of the oxadiazole ring system (Scheme 2). More recently, QSAR studies on a series of biologically active furoxans demonstrated a correlation between cytotoxicity and their electronic properties.[9] Furoxans exhibiting greatest cytotoxic activity showed higher contribution from nitrogen-2 of the heterocyclic ring on the LUMO energy (E_{LUMO}) enhancing the electrophilicity of carbon-3. Notably, the most cytotoxic furoxans had electrophilic carbons (CHO and CN) or carbons with good leaving groups (CH_2Cl and CH_2SPh) attached to carbon-3 of the oxadiazole ring. In these systems, competition for nucleophilic attack by the thiol between the imino carbons of the furoxan ring and the carbonyl carbons is expected to occur. For the 3,4-dibenzoylfuroxans investigated in this study, nucleophilic attack might occur at the carbonyl carbon. Sulfide addition to the carbonyl should not result in nitrosothiol formation and thus would reduce the yield of NO. While electron-withdrawing groups on the benzene rings are expected to increase electron deficiency in the furoxan ring, they have a greater effect on the electron density of the benzoyl carbons. Increased electrophilicity of the carbonyl carbon relative to carbon-3 of the furoxan ring increases the likelihood that the thiol will react at the benzoyl position rather than the furoxan imino carbon. As a result, dibenzoylfuroxans bearing electron-withdrawing substituents on the phenyl rings result in lower NO yield.

Electron-donating groups on the benzene rings might be expected to reduce the overall electrophilicity of furoxans. However, it can be postulated that the 4′-methyl or methoxy substituents have a stabilizing electronic effect on the carbonyl carbon making it less susceptible to nucleophilic attack and the furoxan ring more likely to react with the thiol. Further investigations into the substituent effects on the reactivity of symmetrically 3,4-disubstituted oxadiazole-2-oxides are currently underway. Theoretical investigations of substituted dibenzoylfuroxans are also being conducted to gain further insight into the electronic contributions of the substituents.

Table 2. Percent NO released by methyl and methoxy-substituted furoxans in neutral acetonitrile solutions

3,4-Dibenzoylfuroxan	% NO released (±SE)[a]
4b, X = 4′-CH_3	16.0 (.2)
5b, X = 4′-OCH_3	14.0 (.2)

[a] Expressed as % N_2O_3 (2 mol/mol) ± SE.

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