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Study on the photo-induced nitric oxide-releasing ability of 4-alkoxy furoxans

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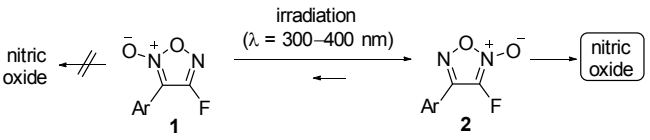
Abstract: Nitric oxide (NO) has a range of biological activities in living systems. Because NO is a gaseous and short-lived molecule, the development of methods to enable a temporally and spatially resolved NO supply has been a challenging task. In this report, 3-aryl 4-alkoxy furoxan is revealed as a potent photo-induced NO-donor. While negligible levels of NO release were observed under ambient fluorescent light, a significant amount of NO was released under UV irradiation (λ = 300–400 nm). The thiol mediator bearing a pendant amino group proved indispensable for the NO release from aryl alkoxy furoxans. Analysis of the NO-releasing reaction of aryl alkoxy furoxan revealed that the main coproduct is the corresponding aryl nitrile derived from furoxan ring-fragmentation. From these results, a plausible photo-induced NO-releasing mechanism was proposed, in which the key steps are nucleophilic attack by the thiol at the 3-position of furoxan leading to the furoxan ring-fragmentation, and the formation of tetrahydrothiazole bearing a *C*-nitroso group. Comparison between fluorofuroxan and alkoxy furoxan is also discussed.

Introduction

Nitric oxide (NO) is a gaseous and short-lived small molecule. It is associated with a number of biological activities, including immune response, vasodilation, neurotransmission, and inhibition of platelet aggregation.[1] NO has played a notable role in therapeutics because it has anticancer effects at higher concentrations.[2] However, gas-based therapy is not practical; thus, extensive studies have been conducted to develop NO donors (NODs) capable of releasing NO under physiological conditions.[3] For the purpose of practical NOD usage, NO must be released from the NODs at the right place, right time, and right quantity. This level of control of NO release with high spatiotemporal and quantitative precision can be achieved by introducing an appropriate switch function into the NODs that is triggered by an external stimulus. Light is an ideal regulating tool because of its ability to exert various otherwise impossible chemical transformations without remaining in the system after the reaction, and it can be modulated by time, amplitude, and wavelength. Accordingly, photo-induced NODs (PINODs) have received considerable attention.[4]

Some furoxans have shown thiol-mediated NO-releasing abilities under physiological conditions.[5] We recently[6] established a synthetic route for both fluorofuroxan regioisomers and found that 3-fluoro-substituted isomer **2** generates a greater amount of NO in the presence of a thiol mediator than the 4-fluoro-substituted isomer **1** (Scheme 1). Furthermore, 3-fluorofuroxan **2** is generated from 4-fluorofuroxan **1** by photochemical isomerization. These data follow that 4-fluorofuroxan **1** is a potential PINOD in the presence of thiol. This is a rare example of furoxan molecules with a switchable NO-releasing ability.[7] The isomerization-based NO release mechanism of 4-fluorofuroxan is distinct from that of conventional PINODs; conventional PINODs are mainly rendered photosensitive by either photolabile protecting groups or a weak covalent bond between NO and its counterpart that is cleavable upon photo-irradiation. In some cases, the former PINODs suffer from a toxicity issue derived from the co-products after the NO-release event,[8] and the latter can be problematic because of its intrinsic instability.[9] Therefore, our strategy based on the photoisomerization of furoxans for the development of PINODs can be a useful complement to the existing methods.



Scheme 1. 4-Flourofuroxan **1** as a PINOD (previous work).

While fluorofuroxans are useful PINODs characterized by a high isomer ratio (**1**:**2** = ~10:90) at the photostationary state (PS), we envisaged that furoxans showing even a low isomer ratio at the PS could also be potential PINODs under continuous photo-irradiation if (a) the thiol-mediated NO release from the post-isomerization isomer is adequately fast and (b) the post-isomerization isomer generates more NO than the pre-isomerization isomer. Continuous photo-irradiation would continuously supply the NO-releasing isomer to maintain the PS as the isomer is consumed by NO release, even at low isomer ratios. This would result in complete NO release until the

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consumption of the starting furoxan. Based on this assumption, we sought a furoxan candidate to meet the above criteria and eventually unearthed 3-aryl-4-alkoxy furoxan as the suitable architecture for PINOD.

Herein, we present the photo-induced NO-releasing abilities of 4-alkoxy furoxans. While 4-alkoxy furoxans showed a negligible NO-releasing capability under ambient fluorescent light, NO was released from 4-alkoxy furoxans in response to UV irradiation. The isomer ratio at the PS varied from 100:0 to 15:85 depending on the substituents on the furoxan ring, but the NO-release abilities under continuous UV irradiation had little correlation with the isomer ratio at the PS. The reaction mechanism for NO release was proposed based on an investigation of the results for the NO-release event and the effect of the thiol structure on the NO-releasing ability. Furthermore, we compared the potential of 4-fluorofuroxans and 4-alkoxy furoxans as PINODs.

Results and Discussion

1. Photo-induced NO-releasing ability of 4-alkoxy furoxans

Alkoxy sulfonyl furoxans and cyanofuroxans, which are often utilized as NO-releasing units in the application-oriented molecules,[10] such as hybrid compounds,[11] would not be suitable as PINODs because both of their regioisomers release non-negligible amounts of NO (Figure 1).[12] Synthetically accessible aryl sulfonyl furoxans have distinct NO-releasing ability difference between the regioisomers, but the NO-releasing yield from the more active regioisomer is rather low (Figure 1).[13] However, aryl alkoxy furoxans bear attractive NO-releasing properties; the regioisomers have a magnificent difference in the NO-releasing abilities, and the more active 3-alkoxy furoxans generate NO in a high yield (up to about 90% mol/mol, Figure 1).[14] Photo-isomerization gave a low isomer ratio at the PS (up to 10–20% for 3-alkoxy furoxan).[15] Nonetheless, we envisaged that continuous photo-irradiation on 4-alkoxy furoxans in the presence of a thiol mediator could continuously supply 3-alkoxy furoxans as long as the 3-alkoxy furoxan is converted to NO at a sufficient reaction rate (vide supra). Thus, controllable NO release would be enabled by modulating the irradiation time and power.

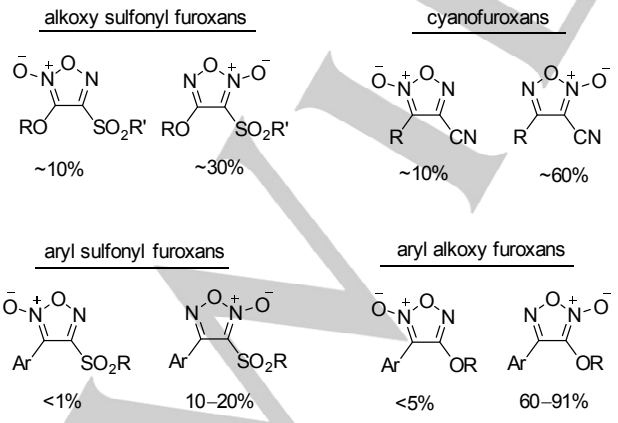
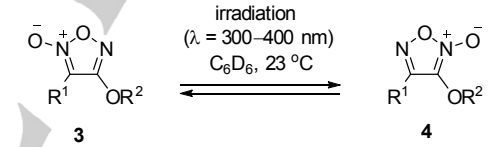
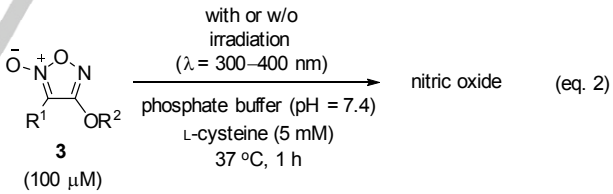


Figure 1. Amount of NO release (mol/mol) in a pH-7.4-buffered solution containing cysteine (0.5–10 mM).

A series of 4-alkoxy furoxans were synthesized from the corresponding 4-nitrofuroxans[16] using a one-step literature method.[17] We then examined the photoisomerization of the synthesized 4-alkoxy furoxans. The 4-alkoxy furoxans were dissolved in C_6D_6 and irradiated using 300–400 nm light at 23 °C until the PS was reached (eq. 1). The regioisomeric ratios are shown in Table 1 (the time profiles are reported in the Supporting Information). In all cases, no significant byproduct formation is observed. In contrast to the photo-isomerization of 4-fluorofuroxans, where the proportion of the post-isomerization isomer (3-fluorofuroxan) reached 90% at the PS,[6] the photo-isomerization of 4-alkoxy furoxans affords a lower proportion of the post-isomerization isomer, 3-alkoxy furoxans (<1–85%). Generally, as the alkoxy substituent (OR^2) becomes more electron-withdrawing and the aryl-substituent (R^1) becomes more electron-donating, 3-alkoxy furoxan **4** becomes more favored than 4-alkoxy furoxan **3**. One of the possible parameters to determine the isomer ratio is the difference between the molar absorption coefficients of the isomers at the irradiation wavelength. However, in the case of alkoxy furoxans, the difference of the integrated absorption intensities of both regioisomers over a range of 300–400 nm is not significant, as exemplified by the UV absorption spectra of regioisomers **3a** and **4a** (Figure 2). The factors to determine the isomer ratio in the photo-isomerization remain to be elucidated.



(eq. 1)



(eq. 2)

Encouraged by the photoisomerization proceeding without the formation of byproducts, we examined the photo-induced NO-releasing abilities of 4-alkoxy furoxans under physiological conditions (eq. 2, Table 1). NO was not released in the absence of the thiol mediator without photo-irradiation (data not shown). In the presence of L-cysteine (5 mM), a marginal amount of NO (0–3% mol/mol) was released from the 4-alkoxy furoxans without photo-irradiation. Then, the 4-alkoxy furoxans were subjected to photo-irradiation. A solution of **3** (100 μM) in a 50-mM phosphate buffer (pH 7.4) containing L-cysteine (5 mM) in a Pyrex vial was warmed to 37 °C with stirring, then subjected to continuous photo-irradiation (λ = 300–400 nm). The amount of released NO was monitored by a Griess test (aliquots were taken at intervals). Selected data are shown in Figure 3 (see the Supporting Information for the other data), and the amounts of NO released after 60 min of UV irradiation are shown in Table 1. The NO-releasing ability could be switched on by photo-irradiation for all

Table 1. Regioisomeric ratio at the photostationary state (eq. 1) and amount of NO released with or without UV irradiation (eq. 2).

Entry	Substrate			Ratio at PS (**3**:**4**)[a]	% NO_2^- (mol/mol) w/o UV-irr.[b]	% NO_2^- (mol/mol) with UV-irr.[c]
	R^1	R^2	**3**			
1	*p*-MeC_6H_4	Me	**3a**	81:19	1	50
2	*p*-MeC_6H_4	Et	**3b**	82:18	1	48
3	*p*-MeC_6H_4	^{i}Pr	**3c**	81:19	1	30
4	*p*-MeC_6H_4	^{t}Bu	**3d**	64:36	<1	53
5	*p*-MeC_6H_4	Ph	**3e**	33:67	<1	39
6	*p*-MeC_6H_4	CH_2CF_3	**3f**	40:60	<1	27
7	*p*-MeC_6H_4	2-Py	**3g**	15:85	2	35
8	*p*-$MeOC_6H_4$	Me	**3h**	54:46	4	58
9	*p*-FC_6H_4	Me	**3i**	>99:<1	3	55
10	$^n C_5H_{11}$	Me	**3j**	>99:<1	2	2

[a] Regioisomeric ratio (**3**:**4**) observed at the photostationary state (PS) after sufficient photo-irradiation, determined by ^{1}H NMR analysis. Furoxan **3** (5 mg) and C_6D_6 (0.8 mL) were added to a Pyrex NMR tube. The solution was irradiated with light (λ = 300–400 nm) at 23 °C (eq. 1). [b][c] Furoxan **3** (100 μM) in 50 mM phosphate buffer (pH 7.4) containing L-cysteine (5 mM) was left under ambient fluorescent light ([b]) or under irradiation with light (λ = 300–400 nm) ([c]) for 1 h at 37 °C (eq. 2). The amount of released NO was evaluated by measuring nitrites using the Griess reagent.

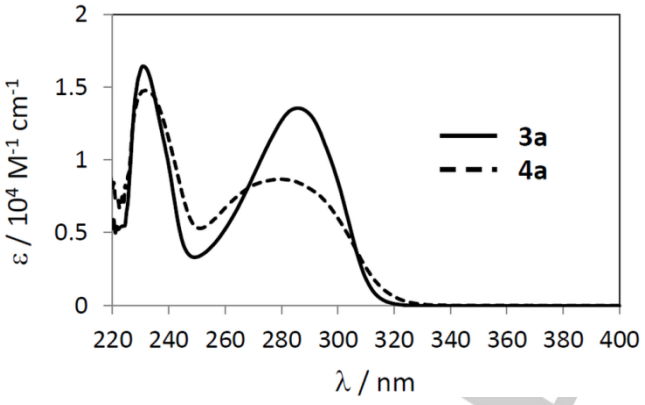


Figure 2. Absorption spectra of **3a** and **4a** in CH_2Cl_2 at 23 °C. ε = molar absorption coefficient

the tested 4-aokoxy furoxans but **3j**. In some cases, the value of released NO is higher than the isomer ratio of 3-alkoxy furoxan at the PS (entries 1, 2, 3, 4, 8, and 9 in Table 1), suggesting that continuous photo-irradiation continuously generates 3-alkoxy furoxan as it is consumed with NO release, in line with our expectation. One drastic instance is the case of 4-methoxyfuroxan **3i** (entry 9 in Table 1), where a significant amount of NO is released under photo-irradiation while the post-isomerization isomer is not detected by ^{1}H NMR analysis at the PS. Glutathione, the most abundant biothiol with a concentration of several millimolars in living systems,[18] also functions as a thiol mediator in the photo-induced NO release (Figure 3). Taken together, 4-alkoxy furoxans could be potential PINODs in living systems without the extraneous addition of thiol.

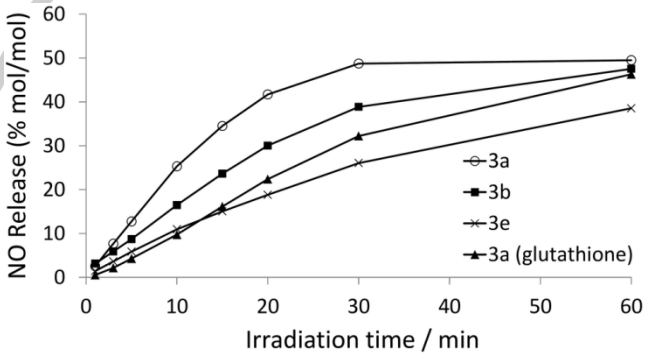


Figure 3. NO-releasing profile of selected 4-alkoxy furoxans (**3**) as a function of photo-irradiation time (λ = 300–400 nm). A buffered aqueous solution of **3** (100 μM) containing L-cysteine (5 mM) or glutathione (5 mM) was irradiated in a Pyrex vial at 37 °C.

2. Mechanistic study

2-1. Examination of possible intermediates in photo-induced thiol-mediated NO release from 4-alkoxy furoxans

Although 4-alkoxy furoxans are PINODs in the presence of a thiol mediator, these results should be interpreted with care because

the heteroatom substituent on a furoxan ring can be susceptible to nucleophilic substitution. One needs to clarify the active intermediates for NO release to elucidate the whole reaction mechanism for photo-induced thiol-mediated NO release from 4-alkoxy furoxans. The possible mechanisms for NO release from 4-alkoxy furoxans under photo-irradiation are delineated in Figure 4. 4-Alkoxy furoxan **3** may undergo nucleophilic substitution by action of a thiol group, affording 4-sulfanyl furoxan **5**. Then, **5** isomerizes by light irradiation to its regioisomer **6**, which may release NO (path A). Alternatively, 4-alkoxy furoxan **3** may undergo photo-isomerization to afford 3-alkoxy furoxan **4**, which is converted to 3-sulfanyl furoxan **6** and releases NO (path B), or **4** may release NO through a path that avoids the intermediacy of **6** (path C).

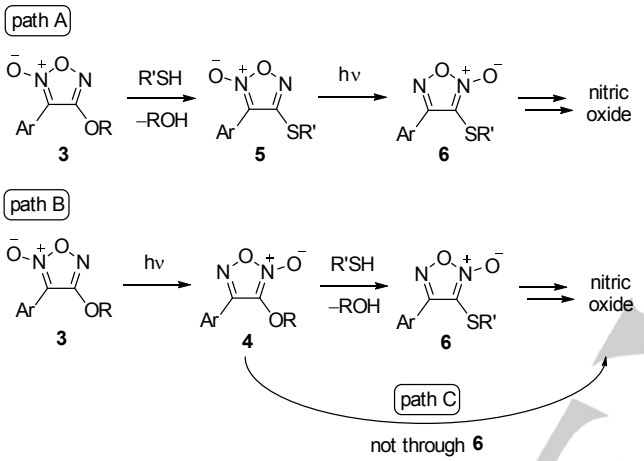
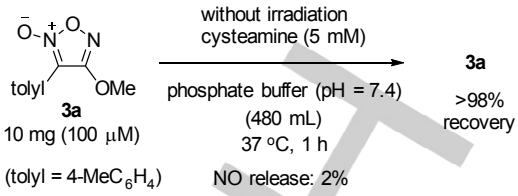


Figure 4. Possible mechanism for NO release from 4-alkoxy furoxans under photo-irradiation.

We first examined the susceptibility of **3a** to nucleophilic substitution (Scheme 2). A solution of **3a** was incubated in a phosphate buffer (pH 7.4) containing cysteamine (5 mM) for 1 h without UV irradiation. The highly diluted conditions (in general ~0.1 mg furoxan in 6 mL buffered solution) employed for the standard experiments on thiol-mediated NO release from furoxans made in-situ NMR analysis of the reaction mixture difficult; thus, the NO-releasing reactions were conducted on a 10-mg scale (substrate) in ~0.5 L of solvent. The residue obtained after work-up was analyzed by ^{1}H NMR spectroscopy, and the product yields were determined using an internal standard (durene). Instead of L-cysteine, cysteamine was used as a thiol mediator (because it is less polar) to assure that the generated organic moved to the organic phase without loss. ^{1}H NMR analysis revealed that the starting material, **3a**, was entirely recovered. The corresponding 4-sulfanyl furoxan **7** was not detected. This result indicates that path A in Figure 4 is not probable.

We measured the NO-releasing abilities of **4a** and 3-sulfanyl furoxan **8** in the presence of cysteamine to examine the possibility of the intermediacy of **6** in the NO-release event (entries 3 and 5, Table 2).[19] There was a significant difference between **4a** and **8**

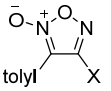


Scheme 2. Stability investigation of 4-methoxyfuroxan **3a** in a cysteamine-containing buffered solution without UV irradiation.

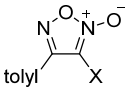
Table 2. NO-releasing abilities of selected furoxans under several conditions.[a]

		% NO_2^- (mol/mol) with thiol additive		
entry	furoxan	no additive	with EtSH	with cysteamine
1	**2a**	–	2	40
2	**3a**	0	–	2
3	**4a**	2	3	81
4	**7**	1	–	5
5	**8**	4	4	32
6	**9**	0	–	1
7	**10**	0	0	11

[a] Furoxan (100 μM) in 50 mM phosphate buffer (pH 7.4, 6 mL) in the absence or presence of thiol additive (5 mM) was left for 1 h at 37 °C. The amount of NO released was evaluated by measuring nitrites using the Griess reagent.



3a: X = OMe
7: X = $SCH_2CH_2NH_2$
9: X = SEt



4a: X = OMe
8: X = $SCH_2CH_2NH_2$
10: X = SEt

in cysteamine-mediated NO release (**4a**: 81%, **8**: 32%), suggesting that path C, where 3-alkoxy furoxan **4** generates NO without the intermediacy of 3-sulfanyl furoxan **6**, is plausible; however, the partial involvement of path B could not be excluded.

2-2. Dependence of NO release from 3-alkoxy furoxan on the structure of the thiol mediator

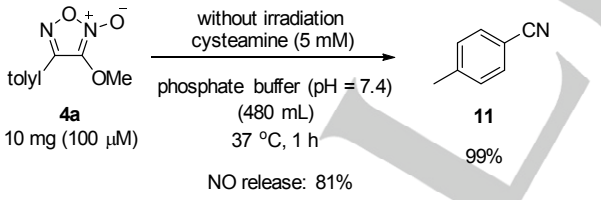
With the knowledge in hand that alkoxy furoxan releases NO without replacing the alkoxy group with any other group, the mechanism of thiol-mediated NO release from 3-alkoxy furoxan is the next question to be solved. The involvement of the thiol mediator in the NO release mechanism from furoxans has been debated.[12a, 14b, 19, 20] We reported that NO release from 3-fluorofuroxans depends on the structure of the employed thiol; 3-fluorofuroxans provided 30–40% yields in the presence of β-aminoethanethiol derivatives (cysteamine and L-cysteine), whereas the use of non-substituted ethanethiol nearly ceased NO

release (2% yield)[6]. However, 3-cyanofuroxan provided a 64% NO yield, even when using PhSH instead of the β-aminoethanethiol derivative as a thiol mediator, as reported by Gasco.[19] Thus, the dependency of the NO release on the structure of the thiol mediator varies according to furoxans and has not been systematically studied.

We examined the NO-releasing ability of 3-methoxyfuroxan **4a** in the presence of ethanethiol and cysteamine (entry 3, Table 2). 3-Methoxyfuroxan releases little NO (3% mol/mol) in the presence of ethanethiol, while a high yield (81% mol/mol) of NO is generated using cysteamine. Thus, the effect of the thiol structure on the NO release from 3-methoxyfuroxan proves significant, similar to that for 3-fluorofuroxan. These results suggest that the amino group on the thiol mediator plays an important role in the mechanism of NO release (vide infra).

2-3. Determination of the co-product in the NO-releasing reaction

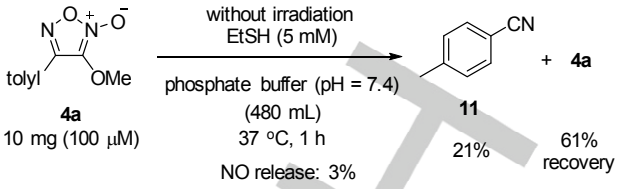
Determination of the co-product generated from furoxans in the thiol-mediated NO-releasing reaction is important to clarify the reaction mechanism. However, it has been rarely discussed;[19] so we investigated the fate of furoxan after the NO release. As shown in Scheme 3, 3-methoxyfuroxan **4a** was incubated in the presence of cysteamine on a 10-mg scale under conditions otherwise identical to that employed when examining the NO-releasing ability on a 0.1-mg scale (which provided 81% mol/mol NO release). As a result, *p*-tolunitrile (**11**) was obtained quantitatively. No other compounds, such as **4a** and **8,** were detected. Despite the small discrepancy in the yields of NO and **11**, this result indicates that the co-product of 3-alkoxy furoxan after the NO-releasing event is the corresponding nitrile, which is in sharp contrast to the case of 3-cyanofuroxan, where cyanoglyoxime and 5-amino-4-sulfanylisoxazole were identified in the NO-releasing reaction.[19]



Scheme 3. Examination of co-product in NO release from 3-methoxyfuroxan **4a**.

Next, 3-methoxyfuroxan **4a** was incubated in the presence of ethanethiol (Scheme 4). The recovery of **4a** was only 61%, and nitrile **11** was obtained in 21% yield, whereas **4a** released NO in only a 3% yield under this condition (entry 3, Table 2). When **4a** was incubated in the absence of thiol, the recovery of **4a** was 88% with less than 3% formation of **11**. Thus, we infer that nitrile formation is caused by the reaction of **4a** and thiol, and should precede the NO-releasing step (vide infra).

2-4. Reaction mechanism for photo-induced NO release from 4-alkoxy furoxan



Scheme 4. Reaction of 3-alkoxy furoxan **4a** with ethanethiol.

Several mechanisms have been proposed for NO release from furoxans.[12a, 19, 20, 21] So far, nitrile has not been suggested as the fate of furoxan in any mechanism, and alkoxy furoxans should release NO in a different manner than the conventionally suggested manner. We propose a reaction mechanism for NO release from 4-alkoxy furoxan under photo-irradiation based on the experimental results obtained above (Figure 5). The possibility that 4-sulfanyl furoxan **12** is generated from 4-alkoxy furoxan **3** can be ruled out from the result in Scheme 2. Instead, 4-alkoxy furoxan **3** undergoes photoisomerization to form 3-alkoxy furoxan **4**, albeit to a small extent because of the favored backward reaction from **4** to **3**. **4** reacts with the thiol to give tetrahedral intermediate **13**, which, without forming 3-sulfanyl furoxan **14**, undergoes fragmentation of the 5-membered ring system to generate aryl nitrile and its counterpart, **15**, driven by the resonance-stabilized nature of **15a–c**. In this mechanism, aryl nitrile is formed without concomitant generation of NO, which does not contradict the formation of nitrile that is also observed under non-NO-releasing conditions (Scheme 4). Based on the observation that the β-amino group on thiol is required for NO release, we assume that intermediate **15** is the branching point, from which five-membered tetrahydrothiazole **16** could be generated if X = NH_2. After dehydration, *C*-nitroso compound **17** would be formed. The homolytic cleavage would occur to form NO and radical species **18**, the carbon-centered radical of which is stabilized by the adjacent heteroatoms. Heterolytic cleavage of **17** affording the nitroxyl anion (NO^-) would not likely be a productive pathway for NO release because nitroxyl (HNO) is likely not oxidized to NO in a non-enzymatic process.[12a, 21, 22] The analysis of the sulfur-containing counterpart proved elusive, and some steps in the proposed mechanism are a matter of speculation; a study to further corroborate the proposed mechanism is underway in our laboratory.

2-5. Comparison between fluorofuroxan and alkoxy furoxan

We collected a series of experimental results and can propose a mechanism for photo-induced NO release from 4-alkoxy furoxans. A comparison of the properties and NO-releasing mechanisms of 4-alkoxy furoxan and 4-fluorofuroxan should be worthwhile because of their future applications as chemical tools in biological experiments and novel NO-releasing materials. The reactions of **1a** and **2a** with cysteamine are shown in Figure 6. After incubation in the presence of cysteamine for 5 min, 60% of **1a** was reacted and converted to several products, one of which was identified as 4-sulfanyl furoxan **7** (10–20% yield).[23] Complete conversion of **1a** was observed after 60 min. Less than 2% of NO was released, and 4-sulfanyl furoxan **7** was formed in 90% yield.

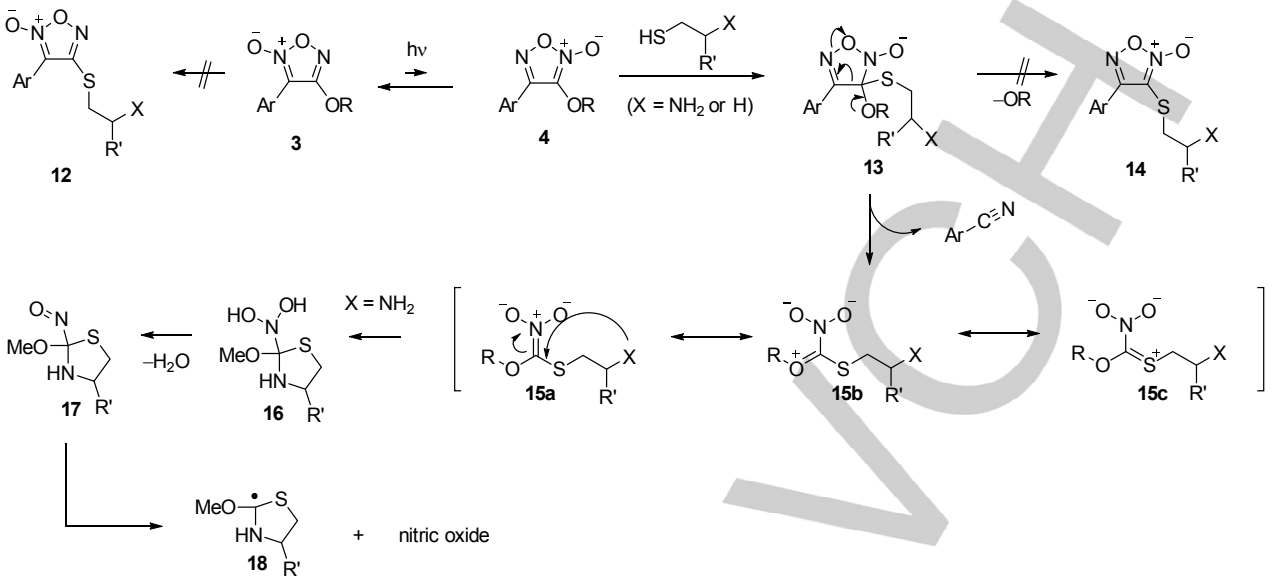


Figure 5. Proposed reaction mechanism for NO release from 4-alkoxy furoxan under photo-irradiation.

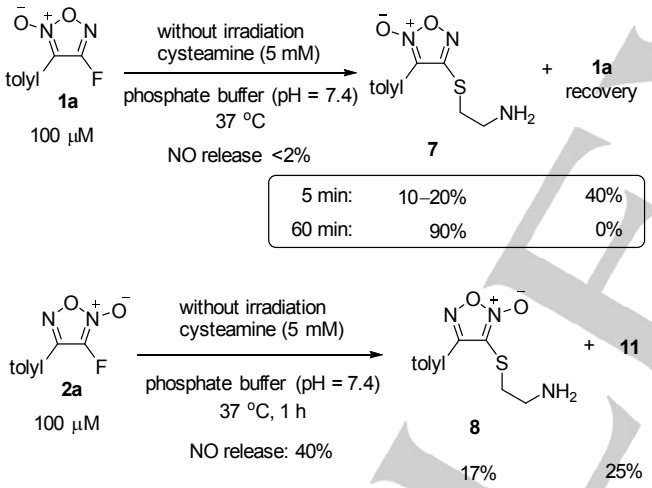


Figure 6. Investigation of the reactivity of fluorofuroxans **1a** and **2a** in a cysteamine-containing buffered solution.

4-Fluorofuroxan **1a** remained intact (>98% recovery) after incubation in a buffered solution in the absence of thiol (data not shown). These results indicate that 4-fluorofuroxan **1** is tolerant to solvolysis but amenable to nucleophilic replacement of fluoride by thiol. The NO-releasing reaction of 3-fluorofuroxan **2a** was analyzed, revealing that it was entirely consumed after 60 min, and that 3-sulfanyl furoxan **8** (17% yield), nitrile **11** (25% yield), and several unidentified compounds were formed.

Based on the above observations, possible pathways for the photo-induced NO release of 4-fluorofuroxan **1** are represented in Figure 7. In contrast to 4-alkoxy furoxan, 4-fluorofuroxan **1** could be converted to 4-sulfanyl furoxan **12**, which would undergo photo-isomerization to **14** and provide NO release (path A, Figure 7). The involvement of this proposed pathway in the NO release from **1** is supported by the relatively facile nucleophilic substitution of **19** by thiol (top scheme in Figure 6). In addition, we confirmed that 4-sulfanyl furoxan **12** releases NO in the presence of L-cysteine (5 mM) under photo-irradiation (30% after 1 h for **7**, Figure S2), which further supports the involvement of path A. Considering the relatively facile photo-isomerization of **1** to **2**, it would be reasonable to think that a part of **1** is converted to **2**. **2** should be converted to NO in the same way as **4** (Figure 5) because 3-fluorofuroxan and 3-alkoxy furoxan show similar NO-releasing dependences on the structure of the thiol mediator (entries 1 and 3, Table 2). Namely, **2** would be subjected to nucleophilic attack by thiol to afford tetrahedral intermediate **19**, followed by cyclization and homolytic cleavage events leading to NO (path C, Figure 7). The formation of 3-sulfanyl furoxan **14** from

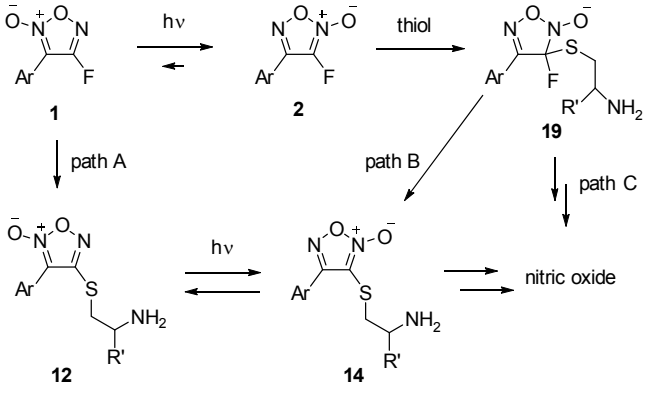


Figure 7. Proposed mechanism for photo-induced NO release of **1**.

19 (path B) should occur besides path A because 3-sulfanyl furoxan **14**(**8**) was observed in the NO-releasing reaction of **2a** (Figure 6, bottom). Overall, the photo-induced NO release from 4-fluorofuroxan is likely to occur by several pathways, in contrast to the case of 4-alkoxy furoxan (Figure 5).

The NO release from 3-sulfanyl furoxan **14** is worth discussing. One might think that the low NO-releasing level of **8** (4% mol/mol) in the presence of ethanethiol would be unreasonable (entry 5, Table 2) because **14** bears an amino group capable of participating in the cyclization event. This observation could be explained by considering the facile exchange of the sulfanyl substituents, which results in the formation of furoxan **10** that has no capability to release NO using ethanethiol as a thiol mediator. The relatively lower NO-releasing ability of **10** in the presence of cysteamine (11% mol/mol, entry 7 in Table 2) compared to **8** (32% mol/mol, entry 5 in Table 2) could be rationalized by assuming a slower nucleophilic attack by cysteamine because of the lack of 3-position activation by the β-amino group on the sulfanyl substituent.[24]

2-6. Comments in light of the future application of 4-alkoxy furoxans and 4-fluorofuroxans as PINODs

Given that 4-alkoxy furoxans are tolerant to nucleophilic degradation and have high NO-releasing abilities under photo-irradiation, they would find application as experimental biological tools enabling spatiotemporal control of NO doses by taking advantage of light modulation by amplitude, timing, and location. Furthermore, the two substituents are available for further functionalization of the molecule, such as the attachment of a fluorophore or a specific site-targeting agent, in contrast to fluorofuroxans, where only one substituent on the furoxan ring is open to functionalization and a fluorine atom occupies the other. Facile introduction of the alkoxy group into furoxan by nucleophilic substitution renders alkoxy furoxans further attractive. However, 4-fluorofuroxans are appealing because of their exceptionally high photo-isomerization ratio in favor of the NO-releasing congener. Therefore, in the absence of thiol, the photo-irradiation information, such as location and amplitude, is "memorized" as a form of 3-fluorofuroxan and then "decoded" on demand upon exposure to thiol, enabling a controlled NO release. Thus, we envisage that fluorofuroxans would have utility in a static form on certain supports, such as a polymer. The investigations for these applications are ongoing in our laboratory.

3. Cytotoxicity evaluation of 4-alkoxy furoxans

We examined the potential in-vitro cytotoxic activity of the synthesized 4-alkoxy furoxans. HeLa cells were exposed to each compound at various concentrations (7.8–250 μg/mL) and screened for viability (%) using the MTT assay. The IC_{50} values for most of the tested 4-alkoxy furoxans were above 250 μg/mL (Table 3). Thus, 4-alkoxy furoxans would be viable for application as photo-induced NO-releasing experimental tools or pharmaceuticals in photodynamic therapies because of their negligible acute toxicity.

Table 3. IC_{50} in-vitro cytotoxicity of 4-alkoxy furoxans against HeLa cell lines.

Compound	IC_{50} cytotoxicity (μg/mL)[a]
3a	>250
3b	>250
3c	>250
3d	200~250
3e	>250
3f	44.3 ± 0.76
3g	145.3 ± 1.49
3h	35.3 ± 1.51
3i	>250
3j	>250

[a] Mean ± standard error of the mean.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), IC_{50} (50% inhibitory concentration), HeLa cell (human cervical cancer cell)

Conclusion

We showed the potential of 4-alkoxy furoxans as photo-induced NO donors. 4-Alkoxy furoxans, which have marginal NO-releasing abilities, are photo-isomerized under physiological conditions to the corresponding 3-alkoxy furoxans, acquiring a thiol-mediated NO-releasing capability. The NO-releasing ability of 3-alkoxy furoxans highly depends on the structure of the thiol mediator, and the co-product in the NO release proved to be the corresponding nitrile. Based on these experimental results, a plausible reaction mechanism for NO release was proposed.

Experimental Section

Experimental details are given in the Supporting Information. These include details of the synthetic procedures, spectroscopic data, copies of the ^{1}H and ^{13}C NMR spectra, and biological assays.

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Kawanishi Memorial ShinMaywa Education Foundation, and Daiichi Sankyo Foundation of Life Science for financial support.

Keywords: furoxans • heterocycles • photochemistry • mechanism • nitrogen oxides

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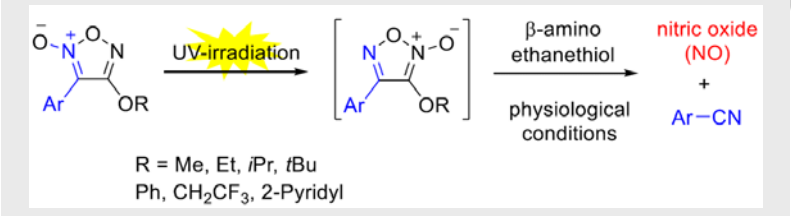

The development of methods to enable a temporally and spatially resolved NO supply has been a challenging task. In this report, 3-aryl 4-alkoxy furoxan is revealed as a potent photo-induced NO-donor. The NO-release switching mechanism is based on photochemical isomerization and the distinct difference of NO-releasing abilities between the regioisomers. The proposed NO-release mechanism is also described.

Prof. Dr. Ryosuke Matsubara, Saori Takazawa, Akihiro Ando, Prof. Dr. Masahiko Hayashi, Rei Tohda, Prof. Dr. Motonari Tsubaki*

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Study on the photo-induced nitric oxide-releasing ability of 4-alkoxy furoxans