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A Fluorescent Naphthalenediimide-Alkoxyfuroxan Photoinduced Nitric Oxide Donor

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Abstract

We describe the design and facile synthesis of a fluorescent alkoxyfuroxan naphthalenediimide (NDI) hybrid nitric oxide (NO) donor molecule which releases NO under spatiotemporal control when irradiated with visible light (420–600 nm). The hybrid also demonstrates cellular uptake made possible by its fluorescence capability. This research consolidates on our recent work into visible light absorbing photosensitised furoxan NO release by addressing the limitations of previous designs. Key points of photoinduced nitric oxide donors are highlighted such as water solubility, NO release, cellular uptake, fluorescence, and longer absorption wavelengths.

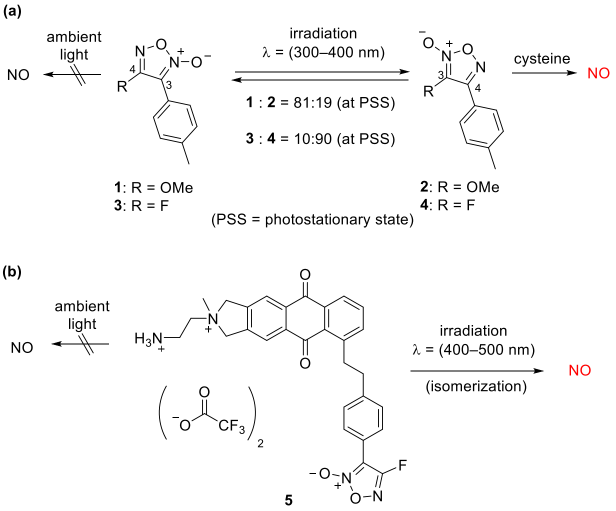
Keywords: Nitric oxide donor, Photoreaction, Furoxan

1. Introduction

Nitric oxide (NO) is a reactive gaseous radical which mediates a variety of biological effects such as vasodilation[1], neuromodulation[2] and inhibition of platelet aggregation[3]. The availability of NO is implicated in a variety of diseases such as cardiovascular disease,[4] male impotence[5] and arthritis.[6] Since NO has a short lifetime and area of effect the administration of NO gas is impractical so stable NO prodrugs have been developed to deliver NO exogenously.[7] Many families of NO donors exist like S-nitrosothiols and NONOates which have led to reliable NO release rates related to their structures.[7-9] However, current NO donors are limited by lack of site specific release. Control over NO release is imperative since the concentration and location of NO release can determine the type of biological response.[10]

Photoswitchable nitric oxide donors (PINODs) address the issues of non-specific donors by releasing NO in a spatiotemporally controlled manner under irradiation. Despite recent progress, there is little variety in PINODs and their release mechanisms, especially those utilizing irradiation wavelengths in the visible or near-IR region.[11-20] Therefore, the development of new PINODs with novel release mechanisms are required in pursuit of controllable discharge of NO under biologically inert conditions.

Recently our research group have made use of the NO donor furoxan and synthesised a series of PINODs.[21,22] 4-Fluoro- and 4-alkoxy-substituted furoxan derivatives displayed isomerization and photoswitchable release of NO under UV irradiation (Scheme 1a). Constrained with the short UV wavelengths required we consolidated on the furoxan PINOD chemistry by tethering a fluorofuroxan to a visible light absorbing water-soluble anthraquinone molecule **5** (Scheme 1b).[23] Compound **5** demonstrated photosensitised NO release under spatiotemporal control. Furthermore, we showed that NO release occurs even in the absence of added thiol under irradiation, in contrast to the general notion that a thiol cofactor is required for the NO release from furoxans.[24] These results led us to suspect that NO release from irradiated furoxan is due to a combination of two distinctive reaction pathways: one is attack of a nucleophile at the more reactive 3-X regioisomer (X = electronegative group) then followed by NO release[25] and the other is discharge of NO from a reactive transient intermediate formed during photochemical isomerization.



Scheme 1. Our previous results. (a) Isomerization of furoxans under UV irradiation and subsequent NO release. (b) Photosensitised isomerization and following NO release from anthraquinone-tethered fluorofuroxan under visible light irradiation.

In retrospect of this research we encountered a few limitations with the design and focused our efforts on the synthesis of an improved molecule. Previously, we were unable to determine whether or where compound **5** was localized within a cell culture system and **5** was only sparingly soluble in phosphate buffer solution. Furthermore, its synthesis presented restrictions due to the labile nature of fluorofuroxan requiring mild conditions during the synthesis scheme. We also decided that although 400 nm irradiation wavelength is largely biologically benign, the absorption wavelength of the sensitiser should be even longer to assist with tissue penetration.

With these previous limitations in mind we sought for an improved sensitiser-furoxan hybrid molecule having the following desirable properties;

1. It is cellular membrane-permeable,
2. It absorbs light of wavelength longer than 400 nm,
3. It is fluorescent,
4. It has improved water solubility,
5. It shows photoswitchable NO release,
6. Its synthesis is facile not limited by furoxan lability.

The ability for a molecule to both fluoresce and undergo intersystem crossing for triplet sensitization may initially seem counterintuitive since the quantum yield of sensitization would surely decrease. However, a balance can be obtained whereby part of the absorbed energy is fluoresced and part is used for sensitised NO release. This theranostic property is highly sought after in phototherapeutic applications.[26]

One such sensitiser that displays fluorescent and triplet sensitising character is the naphthalenediimide (NDI). NDIs are attractive because their absorption and fluorescence can be fine-tuned based on lateral core functionalisation.[27] NDIs find application in a variety of fields such as fluorescence spectroscopy, electronic devices, supramolecular structures and ion and pH sensors.[28] Recently water-soluble, core-substituted NDIs have been used as visible light absorbing theranostic sensitisers and demonstrated the generation of singlet oxygen within a cell culture system reducing cancer cell viability by up to 40%.[29,30]

Encouraged by the ability of core-substituted NDIs to sensitise oxygen and our previous findings into triplet state sensitised furoxans, we envisioned NDI-mediated furoxan sensitised NO release by connecting a furoxan to an NDI core (Figure 1).

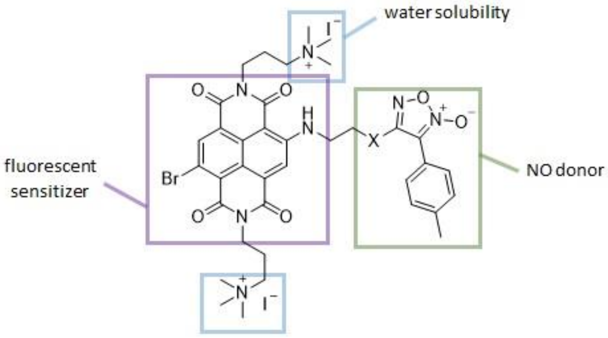


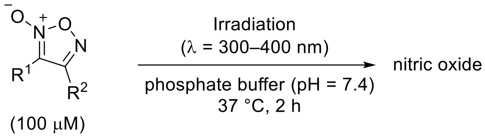
Figure 1. Envisioned working molecule, a water soluble and theranostic core-substituted NDI tethered to a furoxan molecule via heteroatom linker.

In this article we detail the design and synthesis of a fluorescent water-soluble NDI-tethered alkoxyfuroxan, highlight the photophysical properties and demonstrate photoswitchable NO release in pH 7.4 phosphate buffer and cellular uptake.

2. Results and Discussion

Screening of furoxans for NO releasing ability. We began our investigation by selecting a suitably substituted furoxan to be connected to an NDI core. Previously we have shown that sensitised NO release of anthraquinone-tethered fluorofuroxans under visible light irradiation generates comparable quantities of NO as the lone fluorofuroxan moiety does under UV irradiation.[21,23] Therefore, we assumed this may be the case for an NDI-furoxan molecule as well. We screened potential furoxans by irradiating them with 300–400 nm light in 50 mM pH 7.4 phosphate buffer and compared the NO release after 2 hours of irradiation (Table 1). With simplicity in mind we selected furoxans that would be stable to nucleophilic aromatic substitution conditions required for attachment to the NDI core.

Table 1. NO release of furoxan after 2 hours of irradiation (300–400 nm) in 50 mM pH 7.4 phosphate buffer solution.



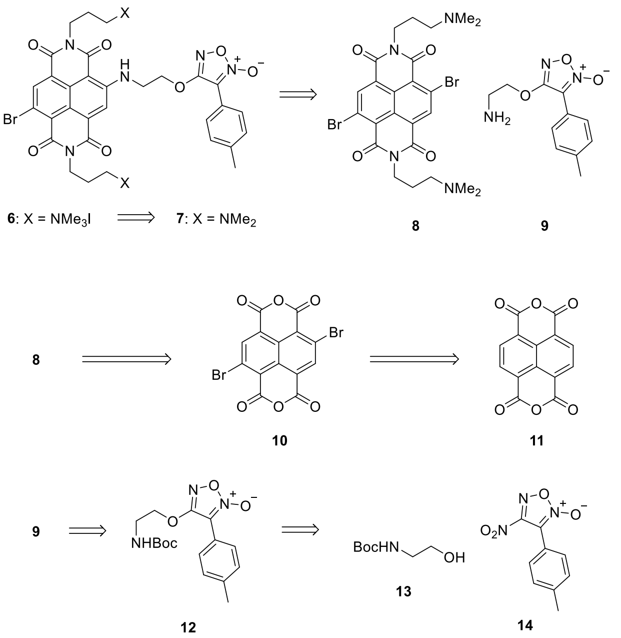
Entry	Substrate R^1	R^2	NO_2^- released (μM) [a)]
1	*p*-MeC_6H_4	F	28
2	*p*-MeC_6H_4	OMe	31
3	*p*-MeC_6H_4	NMe_2	17[b)]
4	*p*-MeC_6H_4	CN	10[c)]
5	*p*-MeC_6H_4	*n*C_6H_{13}	7
6	*p*-MeC_6H_4	CF_3	2
7	Napthalen-2-yl	OMe	12
8	Napthalen-2-yl	NMe_2	12

a) NO_2^- release determined by Griess test. b) 22% after 5 h irradiation. c) 14% after 5 h irradiation.

We began with 4-fluoro-3-tolylfuroxan as a control which released 28 μM of NO (Table 1, Entry 1). Changing the fluoro to methoxy group yielded a slightly higher NO release (Table 1, Entry 2). Dimethylaminofuroxan released a reasonable quantity of NO and reached 22 μM after 5 h of irradiation, the lower quantity and slower rate relative to alkoxyfuroxan is possibly due to increased electron donation into the furoxan ring (Table 1, Entry 3). A cyanofuroxan released a moderate quantity of NO (Table 1, Entry 4).[31] *n*-Hexylfuroxan released low quantities of NO (Table 1, Entry 5) and trifluoromethylfuroxan released negligible quantities (Table 1, Entry 6).[32] Increasing the conjugation from tolyl to napthyl reduced the NO release in both alkoxy and amino derivatives possibly due to the formation of a more stable delocalized transient intermediate during isomerization (Entries 7 and 8).

Based on the above results, alkoxyfuroxan was selected in this work as the furoxan counterpart to the NDI sensitiser due to its admirable NO release and stability under nucleophilic substitution conditions. The selection of alkoxyfuroxan further improves upon our previous fluorofuroxan-anthraquinone hybrid since the alkoxyfuroxan enables derivatization in the form of ether linkers, whereas the fluoro-functionality effectively blocks one of the two available functionalizable sites on the furoxan ring.

Synthesis of NDI-alkoxyfuroxan hybrid molecule. With the alkoxyfuroxan selected as the NO donor partner of the NDI sensitiser we designed a fluorescent, sensitizing and water-soluble PINOD (Scheme 2). The desired compound **6** contains a bromine atom at one of the NDI core substitution points, this enables the utilization of spin orbit coupling to induce sensitised triplet energy transfer to the alkoxyfuroxan. The amino core-substitution was carefully selected so as to absorb light in the visible region yet still be able to undergo intramolecular charge transfer, fluoresce and sensitise the furoxan to induce NO release. Compound **6** also contains two terminal quaternary ammonium salts which serve a dual function; quaternarization prevents

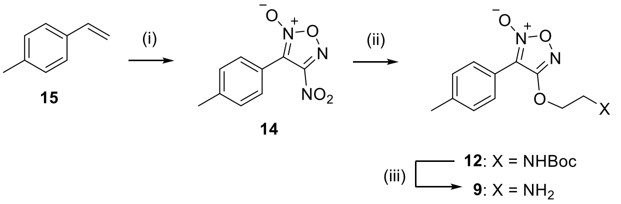


Scheme 2. Retrosynthesis of designed PINOD **6**.

amine from quenching the excited state NDI via single electron transfer and secondly the charged ammonium moieties would enhance water solubility.

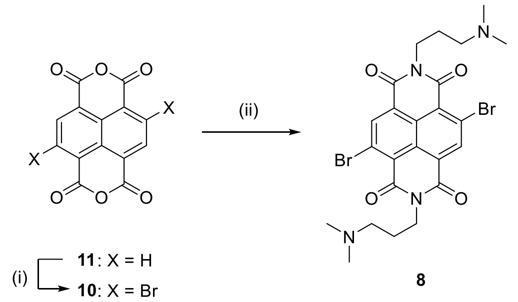
Compound **6** is accessed by methylation of the terminal amines of **7** which in turn is synthesised by nucleophilic aromatic substitution of NDI **8** with amino-pendant alkoxyfuroxan **9**. NDI **8** is synthesised by imidization of dibromo dianhydride **10** with *N*,*N*-dimethylpropylenediamine. Compound **10** can be constructed by dibromination of commercially available dianhydride **11**. Furoxan **9** is synthesised by Boc deprotection of **12** previously furnished by nucleophilic aromatic substitution of the nitrofuroxan **14** with *N*-Boc protected 2-aminoethanol **13**. Nitrofuroxan **14** is synthesised by nitration of 4-methylstyrene (**15**) under acidic conditions. This straightforward and simple synthesis takes advantage of the relative stability of alkoxyfuroxan (in comparison to fluorofuroxan) which enables the key nucleophilic aromatic substitution of NDI **8** to take place without affecting the furoxan ring.

Our convergent synthesis of **6** began with the construction of furoxan intermediate **9** (Scheme 3). Acidic Wieland conditions granted the nitrofuroxan **14** in 27% yield following chromatography and recrystallization.[33] Nucleophilic aromatic substitution of the nitrofuroxan with *N*-Boc protected ethanolamine constructed the protected alkoxyfuroxan **12** followed by TFA deprotection to **9** in high yields.



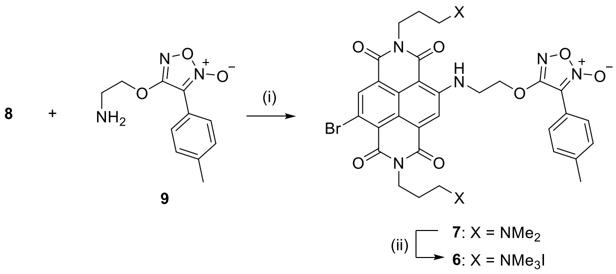
Scheme 3. Synthesis of furoxan **9**. Reagents and conditions: (i) $NaNO_2$, AcOH, DCM, rt, 2 h then 2 M HCl, rt, 14 h, 27%; (ii) *N*-Boc ethanolamine, NaOH, THF, 45 °C, 22 h, 83%; (iii) TFA, DCM, -15 °C to rt, 14 h, 74%.

The NDI core was synthesised from commercially available dianhydride according to literature protocol (Scheme 4).[34,35] Dibromination of dianhydride **11** was made possible by the use of dibromoisocyanuric acid in concentrated sulphuric acid. Due to the insoluble nature of the product the crude mixture was used following filtration and washing with water and hot methanol. Imidization with *N*,*N*-dimethylpropylenediaminegranted the NDI core **8** in moderate yield following silica chromatography and filtration after stirring the product in hot ethanol.



Scheme 4. Synthesis of NDI **8**. Reagents and conditions: (i) dibromoisocyanuric acid, H_2SO_4, 130 °C, 18 h, quant.; (ii) *N*,*N*-dimethylpropylenediamine, AcOH, 130 °C, 0.5 h, 35%.

With both amino alkoxyfuroxan and NDI core in hand we synthesised the hybrid molecule (Scheme 5). To our delight the alkoxyfuroxan was stable to the reaction conditions and connection with **8** granted core-substituted hybrid molecule **7** in high yield following silica chromatography. Simple quaternarization of the pendant amines with iodomethane completed the synthesis of **6**.



Scheme 5. Synthesis of NDI-alkoxyfuroxan hybrid **6**. Reagents and conditions: (i) DMF, 60 °C, 14 h, 83%; (ii) MeI, MeCN, 35 °C, 24 h, 99%.

PINOD capability of hybride molecule 6. Gratifyingly, compound **6** presented sufficient water-solubility (the solubility is >100 μM) so we obtained the absorption and fluorescence of

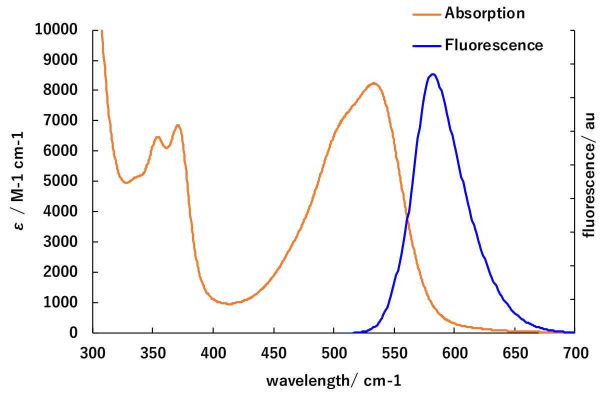


Figure 2. Absorption and fluorescence of **6** (100 μM) in 50 mM pH 7.4 phosphate buffer solution at 25 °C. Fluorescence measurement was conducted with λ_{ex} of 500 nm.

6 in 50 mM pH 7.4 phosphate buffer solution (Figure 2). Compound **6** showed a broad absorption above 400 nm; the intramolecular charge transfer was observed as an intense broad peak with a λ_{max} at 533 nm. The fluorescence spectrum has a λ_{max} of 581 nm (λ_{ex} = 500 nm) and extends up to 650 nm which is useful for identifying cellular uptake locations. The fluorescence quantum yield of **6** in air-saturated water was determined to be 0.10, which is bright enough for the biological experiment.

The NO releasing ability of compound **6** under photoirradiation was next tested. Compound **6** (100 μM) was dissolved in 50 mM pH 7.4 phosphate buffer and irradiated continuously at 37 °C for 4 hours with either 420–600 nm or 500–600 nm light. Aliquots were taken at intervals and the NO% release determined spectroscopically by treatment with Griess reagent (Figure 3). NO was released up to 16 μM after 2 hours of irradiation with 420–600 nm light. When irradiating with a longer wavelength (500–600 nm) the NO release rate is comparatively more gradual, reaching 10 μM over 4 hours. Under ambient lighting conditions there is no significant NO release observed after 4 hours.

We have attempted to directly confirm the photoinduced generation of NO radical from compound **6** using a spin-trapping method with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) as a radical-trapping reagent,[36] but failed to detect the EPR signal of the corresponding imino nitroxide product. Therefore, it is of note that at present we have not confirmed that the NO species detected by the Griess test are only in the NO redox form, it may be possible that NO_2^- or NO^- are also released.

The mechanism of NO release is currently thought to be similar to previous anthraquinone-tethered fluorofuroxan **5**,[23] that is, due to the energy transfer from the excited NDI to the alkoxy furoxan that causes rapid isomerization of the excited furoxan leading to discharge of NO either via solvent molecule interaction with the transient intermediate or an at present unknown photochemical pathway. Another possible mechanism, the electron transfer, is considered. The reduction (E_{red}) and oxidation potentials (E_{ox}) of 4-methoxy-3-tolylfuroxan were determined to be –1.35 V and +1.60 V vs Fc/Fc^+, respectively. According to the literature,[27] E_{red} and E_{ox} of the NDI having the bromo and amino groups at the 2,6-positions are approximately –1.0 V and +1.2 V vs Fc/Fc^+, respectively. Therefore, E_{red} and E_{ox} of the NDI excited state (NDI*) can be speculated to be <+1.2 V and >–1.0 V vs Fc/Fc^+, respectively. Based on the above data, neither the electron transfer from NDI* to alkoxy furoxan nor from alkoxy furoxan to NDI* is likely.

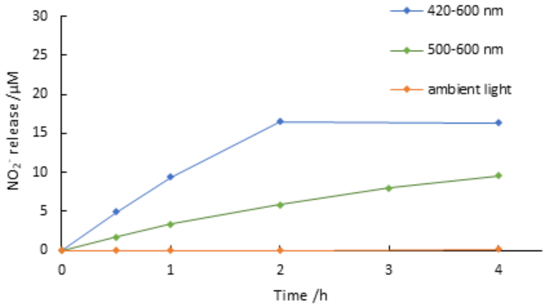


Figure 3. NO-release curve of **6** (100 μM) in 50 mM phosphate buffer under 420–600 nm, 500−600 nm or ambient light irradiation at 37 °C.

Compound **6** underwent photobleaching when irradiated; almost full photobleaching was observed within 3 hours of continuous irradiation (Figure 4a). Furthermore, the fluorescence spectrum underwent a hypsochromic shift from λ_{max} 581 nm to λ_{max} 521 nm with a significant increase in fluorescence intensity under prolonged irradiation (Figure 4b). Despite the possibility of limiting NO release, this can be a useful property since the molecule behaves as a fluorometric indicator to signal when the NO release event has finished. The change in absorption of compound **6** with irradiation shows an overall decrease in intensity at both 533 nm and 371 nm with prolonged irradiation time. The decrease at 371 nm is attributed to a perturbation of the NDI core conjugation, perhaps due to decomposition of the diimide moieties. The decrease at 533 nm could also be attributed to cleavage of the core substituted NDI-amino bond shutting down the intramolecular charge transfer.

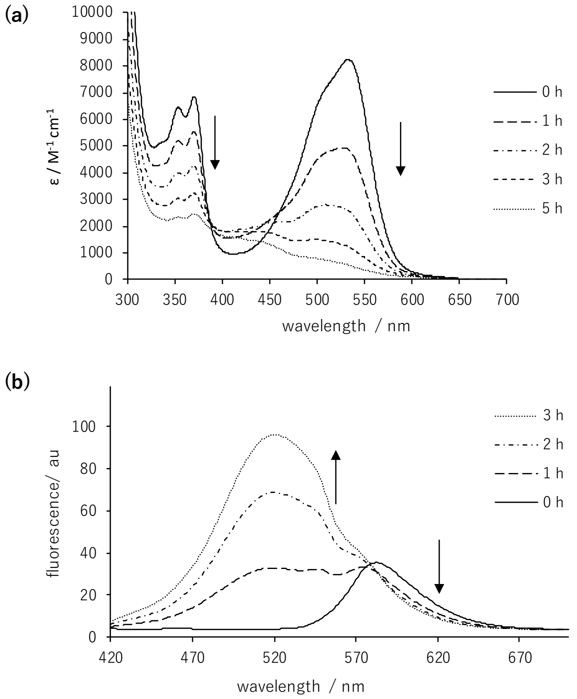


Figure 4. Photobleaching of compound **6** under 420–600 nm continuous irradiation in 50 mM pH 7.4 phosphate buffer solution at 37 °C. At intervals a 2 mL aliquot was taken. (a) Change in the absorption spectra measured at 25 °C. (b) Change in fluorescence spectra measured at 25 °C with λ_{ex} of 420 nm.

With photoswitchable NO release confirmed we tested the cellular uptake of **6** into HeLa cancer cells. Previous research had shown cell membrane permeability for the NDI molecules with tri- and tetrasubstituted quaternary ammonium moieties.[30,37] We were pleased to see that hybrid molecule **6**, even with the dual quaternary ammonium ions and the tethered furoxan molecule, permeated the cell membrane (Figure 5). Compound **6** was uniformly observed in the cytoplasm and sparsely in the nucleus, and may be localized in Golgi apparatus

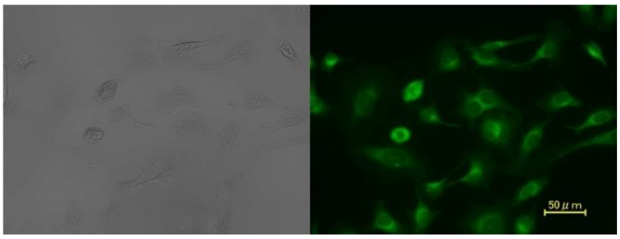


Figure 5. Fluorescence imaging of compound **6** in cultured living HeLa cells. Left: bright field, right: fluorescence image.

slightly more than other organelles. Although the uptake mechanism is not fully understood, it has been suggested that similarities between tetracationic porphyrins and NDIs indicate endocytosis as a possible pathway.[30]

We determined the amount of singlet oxygen ($^1O_2$) that was catalytically generated by compound **6** under the non-deaerated aqeuous conditions. The quantification of $^1O_2$ conducted by an absorption spectroscopic analysis using 1,3-diphenylisobenzofuran (DPBF), a $^1O_2$ scavenger,[38] revealed that three orders of magnitude more $^1O_2$ than NO is released. Although this result indicates that compound **6** may be a good candidate of a photodynamic therapy reagent for cancer disease because both $^1O_2$ and NO have anticancer capabilities,[39] the practical biological use of compound **6** as a pure NO donor is limited at present. Nevertheless, there is still a large hope that the ratio of NO to $^1O_2$ may be improved by taking advantage of intramolecularity of energy transfer from NDI* to the furoxan moiety in contrast to the intermolecular sensitization of molecular oxygen by NDI. The modification of molecular structure such as the tether length and furoxan substituent would dramatically change the sensitization kinetics. Thus, based on this proof-of-concept work, a better NO donor can be designed on demand in the future.

3. Conclusion

We have consolidated on visible light-absorbing photoswitchable furoxan NO donors by addressing the limitations of previous designs. We synthesised a water soluble fluorescent NDI-alkoxyfuroxan hybrid molecule that released NO under in a photoswitchable mannerat longer absorption wavelengths than previous models. Furthermore, the molecule demonstrated cellular uptake into HeLa cells and was observable due to the fluorescence capability. Facile synthesis was made possible by use of a terminal amine-tethered alkoxyfuroxan which was inert to aromatic nucleophilic substitution conditions. The obtained results represent the proof-of-concept of our initial working hypothesis. For the practical biological use, improving the photostability, rate and quantity of NO release and further lengthening the wavelength of sensitiser absorption is necessary and part of the ongoing research within our laboratory.

4. Experimental

General. Unless otherwise noted, all reactions were carried out in well cleaned glasswares with magnetic stirring. Operations were performed under an atmosphere of dry argon using Schlenk and vacuum techniques, unless otherwise noted. All starting materials were obtained from commercial sources or were synthesised using standard procedures. Melting points were measured on a Yanaco MP-500D and are not corrected. ^{1}H and ^{13}C NMR (400 and 100 MHz, respectively) were recorded on a Bruker Avance III HD 400 using TMS (0 ppm) and $CDCl_3$ (77.0 ppm) as an internal standard. The following abbreviations are used in connection with NMR; s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, sep = septet, and m = multiplet. The X-band EPR measurements were carried out using a Bruker EMX system. Mass spectra were measured using a JEOL JMS-T100LP (DART method, ambient ionization) or a LTQ Orbitrap Elite (Thermo Fisher Scientific, Brehmen, Germany) with an electrospray ionization (ESI) ion source. Preparative column chromatography was performed using Kanto Chemical silica gel 60 N (spherical, neutral), Fuji Silysia BW- 4:10MH silica gel or YMC_GEL Silica (6 nm I-40−63 μm). Thin layer chromatography (TLC) was carried out on Merck 25 TLC silica gel 60 F254 aluminum sheets. Nitrofuroxan **14** and naphthalenediimide **8** were prepared according to previously reported literature protocols.[29, 33]

Measurement of NO-releasing ability of furoxans with UV-irradiation (Table 1). A 100 μM stock solution of furoxan was prepared by adding 100 μL of a 10 mM DMSO solution of furoxan into a Pyrex vial containing 10 mL of 50 mM phosphate buffer solution (pH 7.4). The Pyrex vial was placed in a 37 °C oil bath and then irradiated using 300–400 nm light (a 300W Xenon lamp, Asahi Spectra MAX-303 equipped with a 300- to 600-nm ultraviolet-visible module and a 400-nm short-pass filter). At intervals, aliquots (0.5 mL) were taken out, to which the Griess reagent (40 μL) was added. After the samples were stood for 30 min, the absorbance of the samples at 520 nm was measured. The percent nitrite (NO_2^-) (mol/mol) was determined from a calibration curve prepared in advance by using $NaNO_2$ standard solutions (20−100 μM) treated with the Griess reagent.

The Griess reagent was prepared by diluting a mixture of 1 g of sulfanilamide, 50 mg of N-naphthylethylenediamine dihydrochloride, and 2.5 mL of 85% phosphoric acid with distilled water to a final volume of 25 mL.

Absorption and fluorescence spectra. UV–visible spectra were recorded on a Shimadzu UV-1800 spectrometer with a quartz absorption cuvette (light path: 1 cm). Photoluminescence spectra were recorded on a spectrofluorometer (Jasco FP-6500) with a quartz absorption cuvette (light path: 1 cm).

Determination of fluorescence quantum yield of 6. The fluorescence quantum yield of **6** in air-saturated water was determined via the relative determination method with fluorescein as a reference. See the supporting information for the details.

Cellular uptake (Figure 5). HeLa cells (Riken BioResource Center) were cultured in high glucose DME medium (Sigma-Aldrich) containing 10 %(v/v) FBS, 1 %(v/v) Penicillin-Streptomycin (P/S) (Sigma-Aldrich) at 37 °C in a CO_2 incubator (5% CO_2). Then, the HeLa cells were seeded at a density of 1×10^5 cells/mL in DME medium in a 35 mm glass bottom dish. After 24 hours of culture, the cells were incubated with 20 μM **6** for 20 min in a CO_2 incubator (5 % CO_2) at 37 °C. After the incubation, the cells were washed thrice with PBS buffer. The fluorescence derived from **6** in the cells was then analyzed using a fluorescence microscope (BZ-X700; KEYENCE) equipped with BZ-X filter GFP and a 40x objective lens with the exposure time of 1/3 second.

Measurement of the NO-releasing ability of compound 6 with visible light-irradiation (Figure 3). A 100 μM solution of **6** was prepared by adding 10 mL of 50 mM phosphate buffer (pH 7.4) into a Pyrex vial containing **6**, which was placed in a 37 °C oil bath and then irradiated using either 420−600 nm or 500–600 nm light (a 300W Xenon lamp, Asahi Spectra MAX-303 equipped with a 300- to 600-nm ultraviolet-visible module, and a 420-nm long-pass and 500-nm long-pass filter, respectively). At intervals, two aliquots (0.5 mL) were taken, to one of which the Griess reagent (40 μL) was added. After the samples were stood for 30 min, the difference in absorbance of the samples at 520 nm was measured. The percent nitrite (NO_2^-) (mol/mol) was determined from a calibration curve prepared in advance by using $NaNO_2$ standard solutions (20−100 μM) treated with the Griess reagent.

Measurement of the NO-releasing ability of compound 6 without visible light-irradiation (Figure 3). A 100 μM solution of **6** was prepared by adding 10 mL of 50 mM phosphate buffer (pH 7.4) into a Pyrex vial containing **6**, which was placed in a 37 °C oil bath. At intervals, two aliquots (0.5 mL) were taken out, to one of which the Griess reagent (40 μL) was added. After the samples were stood for 30 min, the difference in absorbance of the samples at 520 nm was measured. The percent nitrite

(NO_2^-) (mol/mol) was determined from a calibration curve prepared in advance by using $NaNO_2$ standard solutions (20−100 μM) treated with the Griess reagent.

Synthesis of naphthalenediimide 8 (Scheme 4). 2,6-Dibromonaphtalene-1,4,5,8-tetracarboxylic dianhydride (10): Compound **10** was synthesised according to the reported method.[35] To a solution of dibromoisocyanuric acid (9.56 g, 33.3 mmol) in concentrated H_2SO_4 (0.3 M) was added portionwise 1,4,5,8-naphthalenetetracarboxylic dianhydride (4.47 g, 16.7 mmol) at room temperature, following complete addition the reaction was heated to 130 °C for 18 h. Upon completion the reaction was cooled, poured into ice water and stirred until yellow precipitate formed. The precipitate was filtered and washed with water and hot MeOH then dried to yield a yellow crude mixture containing **10** (7.37 g, 17.3 mmol, quant.). Due to the insoluble nature of **10**, the mixture is used without further purification.

***N,N’*-Bis((dimethylamino)propylamino)-2,6-dibromonaphthalene-1,4,5,8- tetra-carboxylic acid bisimide (8):** Compound **8** was synthesised according to the reported method.[34] To a solution of compound **10** (7.00 g, 16.4 mmol) in acetic acid (210 mL) was added dropwise *N,N*-dimethylpropylenediamine (5.17 mL, 41.1 mmol) and the reaction heated to 130 °C for 1 h. Upon reaction completion the mixture was poured into ice water, basified with K_2CO_3 (pH 8) and extracted 6 times with $CHCl_3$. The organics were dried over Na_2SO_4, filtered and solvent removed in vacuo. The crude solid was partially purified by silica chromatography (5% MeOH, 1% Et_3N in $CHCl_3$). The product was then stirred in EtOH (400 mL) at 60 °C for 30 min and allowed to cool then filtered under vacuum and dried to yield **8** as an orange solid (3.46 g, 5.82 mmol, 35%). The ^1H and ^{13}C NMR spectra were in accordance with the reported data.

Synthesis of furoxan 9 (Scheme 3). *tert*-Butyl 2-{[3-(4-methylphenyl)furoxan-4-yl]oxy}ethylcarbamate (12): To a solution of 3-(4-methylphenyl)-4-nitrofuroxan (1.0 g, 4.52 mmol) in THF (0.5 M) was added *N*-Boc-ethanolamine (948.1 mg, 5.88 mmol) followed by NaOH (235 mg, 5.88 mmol) and stirred at 45 °C for 22 h. Upon reaction completion the mixture was diluted with water and extracted thrice with EtOAc. The organics were dried over Na_2SO_4, filtered and solvent removed in vacuo, the crude was purified by silica chromatography (CH_2Cl_2) to yield **12** as an off white solid (1.25 g, 3.75 mmol, 83%). ^1H NMR (400 MHz, $CDCl_3$) δ_H(ppm) 8.01 (2H, d, J = 8 Hz), 7.32 (2H, d, J = 8 Hz), 4.84 (1H, br s), 4.55 (2H, t, J = 5.2 Hz), 3.67 (2H, apparent q, J = 5.2 Hz), 2.42 (3H, s), 1.44 (9H, s); δ_C (100 MHz, $CDCl_3$); 162.2, 155.7, 141.0, 129.6, 126.1, 119.4, 107.7, 80.0, 70.0, 39.5, 28.4, 21.6; IR (neat) 3349, 2983, 2927, 1681, 1604, 1556, 1537, 1519, 1481, 1442, 1383, 1359, 1332, 1314, 1287, 1274, 1253, 1163, 1115, 1046, 1030, 998, 966, 860, 839, 822, 631 cm^{-1}; HRMS (DART) *m/z* $[M+H]^+$ Calcd for $C_{16}H_{22}N_3O_5$ 336.1566; Found 336.1560; mp 157–159 °C.

4-(2-Aminoethoxy)-3-(4-methylphenyl)furoxan (9): A stirred solution of **12** (1.15 g, 3.45 mmol) in CH_2Cl_2 (0.1 M) was cooled to –15 °C and added to it TFA (7.5 mL) dropwise and the reaction allowed to warm to rt over 14 h. The solvent was removed in vacuo and the mixture was diluted with K_2CO_3 basified water (pH 8) and extracted thrice with EtOAc. The organics were dried over $MgSO_4$, filtered and solvent removed in vacuo to yield an orange solid (598.3 mg, 2.54 mmol, 73%). The product was used without further purification. ^1H NMR (400 MHz, $CDCl_3$) δ_H(ppm) 8.01 (2H, d, J = 8.4 Hz), 7.32 (2H, d, J = 8.4 Hz), 4.53 (2H, t, J = 5.2 Hz), 3.23 (2H, t, J = 5.2 Hz), 2.41 (3H, s); δ_C (100 MHz, $CDCl_3$) 162.4, 141.0, 129.6, 126.1, 119.4, 107.7, 72.8, 40.8, 21.6; IR (neat) 3386, 2954, 2922, 2862, 1691, 1597, 1550, 1516, 1441, 1396, 1310, 1159, 1123, 990, 857, 818, 797, 732 cm^{-1}; HRMS (DART) *m/z* $[M+H]^+$ Calcd for $C_{11}H_{14}N_3O_3$ 236.1034; Found 236.1035; mp 97–99 °C.

Synthesis of alkoxyfuroxan tethered NDI 6 (Scheme 5). *N,N*’-Bis(3-(dimethylamino)propylamino)-2-bromo-6-(2-{[3-(4-methylphenyl)furoxan-4-yl]oxy}ethylamino)-1,4,5,8-naphthalenetetracarboxylic bisimide (7): Compound **7** was synthesised according to the reported method.[37] NDI **8** (1.0 g, 1.68 mmol) and furoxan **9** (514.5 mg, 2.19 mmol) were stirred in DMF (0.02 M) at 60 °C for 14 h. The reaction mixture was cooled and poured into Na_2CO_3 (pH 8) basified water, diluted with EtOAc and washed gently 5 times with an excess of aq. Na_2CO_3. The organics were dried over Na_2SO_4, filtered and solvent removed in vacuo. The crude was purified by silica chromatography (5% MeOH, 1% Et_3N, 94% $CHCl_3$) to yield a red solid (1.04 g, 1.39 mmol, 83%). ^1H NMR (400 MHz, $CDCl_3$) δ_H(ppm) 10.44 (1H, t, J = 7.2 Hz), 8.84 (1H, s), 8.28 (1H, s), 7.89 (2H, d, J = 8.4 Hz), 7.15 (2H, d, J = 8.4 Hz), 4.86 (2H, t, J = 5.2 Hz), 4.24–4.14 (6H, m), 2.46 (4H, q, J = 6.8 Hz), 2.33 (3H, s), 2.27 (6H, s), 2.26 (6H, s), 1.95–1.86 (4H, m); δ_C (100 MHz, $CDCl_3$) 166.0, 161.9, 161.7, 161.7, 161.2, 151.7, 141.0, 138.5, 129.5, 128.4, 127.5, 126.0, 123.6, 123.4, 121.6, 121.1, 119.6, 119.1, 107.6, 101.1, 68.7, 57.1, 45.3, 45.2, 41.5, 39.8, 38.9, 25.8, 25.6, 21.6; IR (neat) 2937, 2812, 2759, 1707, 1686, 1662, 1633, 1604, 1580, 1439, 1312, 1256, 1256, 1218, 1178, 1162, 1016, 817, 791 cm^{-1}; HRMS (ESI) m/z $[M+H]^+$ Calcd for $C_{35}H_{39}BrN_7O_7$ 750.2068; Found 750.2076; mp 163 °C (decomposed).

***N,N’*-Bis(3-(trimethylamino)propylamino)-2-bromo-6-(2-{[3-(4-methylphenyl)furoxan-4-yl]oxy}ethylamino)-1,4,5,8-naphthalenetetracarboxylic bisimide diiodide (6):** To a solution of compound **7** (75.0 mg, 100 μmol) in MeCN (0.01 M) was added iodomethane (14.3 μL, 230 μmol) and the reaction heated to 35 °C for 24 h. Upon completion the solvent was removed in vacuo and the solid filtered and washed with hexane, Et_2O and $CHCl_3$ to yield a magenta solid (102.4 mg, 99.2 μmol, 99%). ^1H NMR (400 MHz, D_2O) δ_H(ppm) 8.41 (1H, s), 7.97 (1H, s), 7.37 (2H, d, J = 8.2 Hz), 6.78 (2H, d, J = 8.2 Hz), 4.91–4.88 (2H, m), 4.21–4.15 (4H, m), 4.04 (2H, apparent t, J = 7.2 Hz), 3.56–3.52 (4H, m), 3.22 (9H, s), 3.19 (9H, s), 2.28–2.15 (4H, m), 2.09 (3H, s); δ_C (100 MHz, acetonitrile-d_3) 166.9, 163.2, 162.9, 162.7, 162.2, 153.4, 141.9, 138.1, 130.1, 129.5, 128.2, 126.7, 124.6, 124.4, 122.6, 121.4, 120.4, 108.7, 101.5, 70.7, 65.2, 65.0, 53.9, 42.0, 38.7, 37.9, 22.8, 22.5, 21.4; IR (neat) 3435, 2954, 2917, 2847, 1709, 1670, 1637, 1584, 1517, 1475, 1444, 1311, 1267, 1241, 1191, 1163, 1124, 960, 785, 738, 720 cm^{-1}; HRMS (ESI) m/z $[M–2I]^{2+}$ Calcd for $C_{37}H_{44}BrN_7O_7$ 389.6227; Found 389.6232; mp 188 °C (decomposed).

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Supporting Information

Method for the quantum yield determination and copies of the ^{1}H and ^{13}C NMR spectra of all new compounds are available.

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