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Photomanipulation of Vasodilation with a Blue-Light-Controllable Nitric Oxide Releaser

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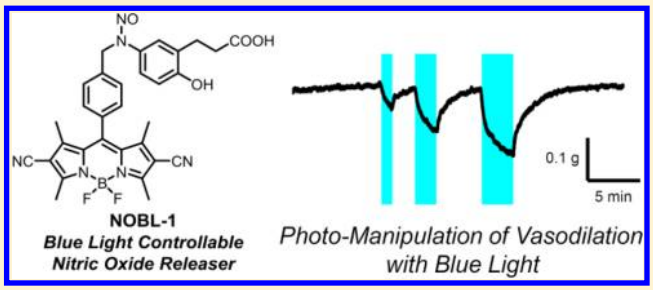
Supporting Information

ABSTRACT: Spatiotemporally controllable nitric oxide (NO)-releasers allow us to analyze the physiological effects of NO, a gaseous mediator that modulates many biological signaling networks, and are also candidate chemotherapeutic agents. We designed and synthesized a blue-light-controllable NO releaser, named NOBL-1, which bears an *N*-nitrosoaminophenol moiety for NO release tethered to a BODIPY dye moiety for harvesting blue light. Photoinduced electron transfer from *N*-nitrosoaniline to the antenna moiety upon irradiation with relatively noncytotoxic blue light (470−500 nm) should result in NO release with formation of a stable quinone moiety. NO release from NOBL-1 was confirmed by ESR spin trapping and fluorescence detection. Spatially controlled NO release in cells was observed with DAR-4M AM, a fluorogenic NO probe. We also demonstrated temporally controlled vasodilation of rat aorta *ex vivo* by blue-light-induced NO release from NOBL-1. This compound should be useful for precise examination of the functions of NO with excellent spatiotemporal control.



INTRODUCTION

Nitric oxide (NO) is synthesized in the human body by nitric oxide synthase (NOS) and is a mediator of various physiological processes,[1] serving as an endothelial derived relaxing factor (EDRF),[2] a neurotransmitter,[3] and an immune response mediator.[4] NO is a colorless gas that is unstable under physiological conditions ($t_{1/2}$ = 0.1−5 s),[1] so that NO releasers,[5] which can store and release NO, have been developed for biological research on the roles of NO or as candidates of chemotherapeutic agents. Among them, light-controllable NO releasers are expected to be particularly useful as precisely controllable chemical tools or as agents for photodynamic therapy, due to the apoptosis-inducing activity of NO in cancer cells.[6] So far, several transition metal-nitrosyl complexes, which contain low-energy metal-nitrosyl bonds as visible light-absorbing chromophores, have been developed as visible-light-controllable NO releasers.[7] However, metal-containing NO releasers often show problematic cytotoxicity due to transition metal. Nonmetal-containing photoresponsive NO releasers have also been developed, but these require relatively cytotoxic UV light as one-photon light sources for NO release.[8] Although near-infrared two-photon excitation methods have been explored to trigger NO release, in order to overcome the disadvantage of UV light,[9] the necessary devices are expensive and not readily available.[10] In this report, we describe the design and synthesis of a nonmetal-containing, blue-light-controllable NO releaser, named NOBL-1, based on application of photoinduced electron transfer. NOBL-1 was confirmed to enable site-specific NO release in living cell cultures, as well as temporal control of NO-induced vasodilation of rat aorta *ex vivo*.

To design a visible-light-controllable NO releaser, we initially focused on previously reported UV-controllable NO releasers, *N*,*N*′-bis(carboxymethyl)-*N*,*N*′-dinitroso-*p*-phenylenediamines (BNNs).[8b] In spite of potential carcinogenesis property of *N*-nitrosoaniline, this structure was taken into the structure due to its advantages as a very efficient NO releaser. BNN was not reported to have obvious toxicity.[8c] On the basis of that work, it appeared that an aminyl radical (Chart 1a) can release NO spontaneously as a result of formation of a stable quinone. We considered that it might be possible to obtain a visible-light-controllable NO releaser, if such an oxidation state could be induced by visible light irradiation. To induce such a redox state, we planned to utilize photoinduced electron transfer (PeT). It is known that certain dyes bearing an electron-rich substituent exhibit single electron transfer from the substituent to the dye after photoexcitation.[11] This results in formation of a short-lived radical pair (Chart 1b). We considered that this phenomenon in combination with the BNN reaction mechanism would provide the basis for a new molecular design strategy for NO donors, as shown in Chart 1c. That is, when a light-harvesting dye (an antenna moiety) is excited by visible light irradiation, single electron transfer from *N*-nitrosoaminophenol to the antenna moiety would occur. In this oxidation state of *N*-nitrosoaminophenol, NO would be

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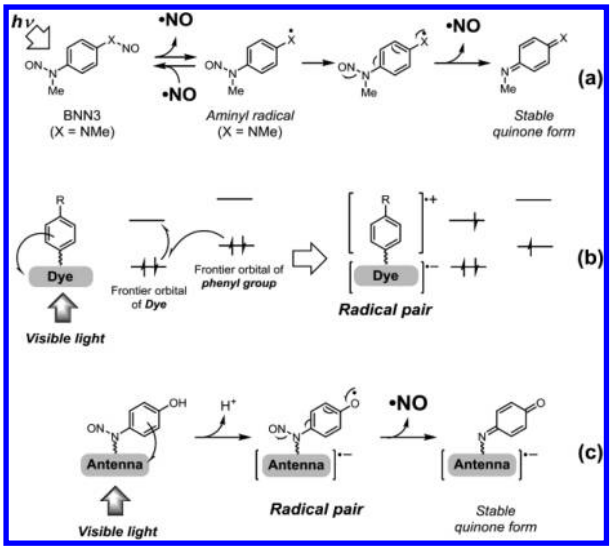
Chart 1. (a) Previously Proposed Mechanism of NO Release from BNN; (b) Photoinduced Electron Transfer Mechanism in Dyes; (c) Concept for an NO Releaser Based on the Photoinduced Electron Transfer Mechanism



readily released to form a stable quinone moiety. Consequently, visible light could trigger NO release.

On the basis of this concept, we designed a novel blue-light-controllable NO releaser, NOBL-1, which consists of *N*-nitrosoaminophenol structure as an NO-releasing moiety and BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene structure, as a light-harvesting antenna moiety (Figure 1). BODIPY dyes

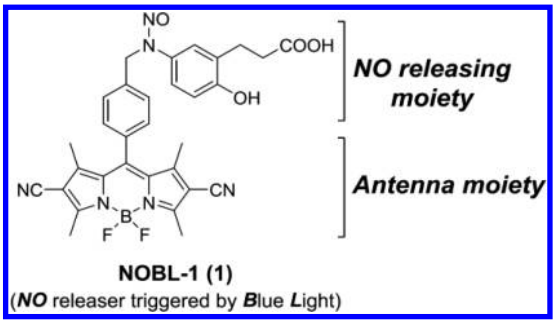


Figure 1. Design of a blue-light-controllable NO releaser, NOBL-1 (**1**).

typically have a large absorption coefficient in the visible light region, and their photophysical properties can easily be tuned by structural modification.[12] We focused on cyano-BODIPY as an antenna moiety in order to stabilize the anion radical, so that electron transfer would occur more easily. We also installed a carboxyl group in order to increase the hydrophilicity, which should make the molecule more suitable for biological application.

RESULT AND DISCUSSION

NOBL-1 was synthesized as shown in Scheme 1. After protection of 2-hydroxy-5-nitrobenzaldehyde (**2**), Wittig reaction gave *t*-butyl cinnamate derivative **4**.[13] Reduction and deprotection with Pd−C/H_2 afforded aminophenol **5**. 3-Cyano-2,4-dimethylpyrrole (**8**) was synthesized as reported.[14] Terephthalaldehydic acid (**6**) was converted into Weinreb amide **7**, which was conjugated with pyrrole **8** in the presence of TFA, followed by oxidation and insertion of fluoroborate to obtain BODIPY dye **9** as an antenna moiety. Compound **9** was reduced to aldehyde with Schwartz's reagent[15] and aminophenol **5** was linked to the antenna moiety by reductive amination. Deprotection and *N*-nitrosation afforded NOBL-1 (**1**). As a reference compound for the BODIPY dye moiety, compound **13** was also synthesized in one pot from benzaldehyde and pyrrole **8**. Its structure and purity were confirmed by ^{1}H NMR, ^{13}C NMR, mass spectrometry, and elemental analysis.

The absorption and fluorescence spectra of NOBL-1 were measured and compared with those of the reference compound, BODIPY **13**, which lacks the NO-releasing moiety. Due to poor solubility of **13** in aqueous solutions, DMSO was used as the solvent in this experiment. It was confirmed that the absorption spectrum of NOBL-1 in water is almost the same as that in DMSO (Figure S2). As shown in Figure 2, although the absorption coefficient of NOBL-1 in the blue light region is comparable to that of **13**, the fluorescence intensity of NOBL-1 was much weaker than that of **13**. Fluorescence is attenuated by PeT, because the relaxation process after PeT is different from that of fluorescence emission.[11] So, this result implies that the fluorescence of the BODIPY moiety of NOBL-1 would be quenched by PeT from the NO-releasing moiety.

To detect NO release from NOBL-1 in response to visible light irradiation, the ESR spin-trapping method with iron and *N*-methylglucamine dithiocarbamate complex (Fe-MGD) was employed. Fe-MGD traps NO efficiently to form NO-Fe-MGD complex, which exhibits a typical three-line signal at around 330 mT in 1 GHz ESR spectrometry.[16] Because NOBL-1 shows a large absorbance in the blue wavelength region, a 470−500 nm band-pass filter was fitted to an apparatus for irradiation (MAX-302, Asahi Spectra). After blue-light irradiation (470−500 nm, 200 mW/cm^2) of a PBS-buffered solution containing Fe-MGD and NOBL-1 (100 μM) for 15 min, the ESR spectrum showed a typical triplet signal assigned to NO-Fe-MGD complex (Figure 3). In the absence of blue-light irradiation, this typical signal was not observed (Figure S3a). This result supports the idea that NOBL-1 releases NO upon blue-light irradiation. To confirm the importance of the antenna moiety, *N*-methyl-*N*-nitroso-4-aminophenol (Figure S1, **14**) was also assessed for its NO-releasing ability in response to blue light. As expected, the typical triplet signal was not observed in the solution irradiated with blue light (Figure S3b), whereas UV irradiation was found to induce NO release (Figure S3c). Additionally, as shown in Figure 2a and Figure S2b, the absorption band in the blue-light region of NOBL-1 was clearly derived from the antenna moiety. These results demonstrate the importance of the antenna moiety for NO release from NOBL-1.

Next, we examined whether NO release from NOBL-1 could be controlled with temporal precision by blue-light irradiation. A buffered solution containing NOBL-1 and DAR-4M, a fluorogenic probe for NO detection,[17] was exposed to the triggering light. As shown in Figure 4, the fluorescence was increased only during irradiation. In the absence of NOBL-1 (Figure S4) or the triggering light (Figure 4D), the fluorescence was not increased. Furthermore, when the triggering light intensity was attenuated, the fluorescence increase was also attenuated. These results indicated that temporally precise control of NO release from NOBL-1 could be achieved. The maximum concentration of NO released from 10 μM NOBL-1 was determined to be 7.6 μM, based on a fluorescence standard curve obtained with DAR-4 M and 1-

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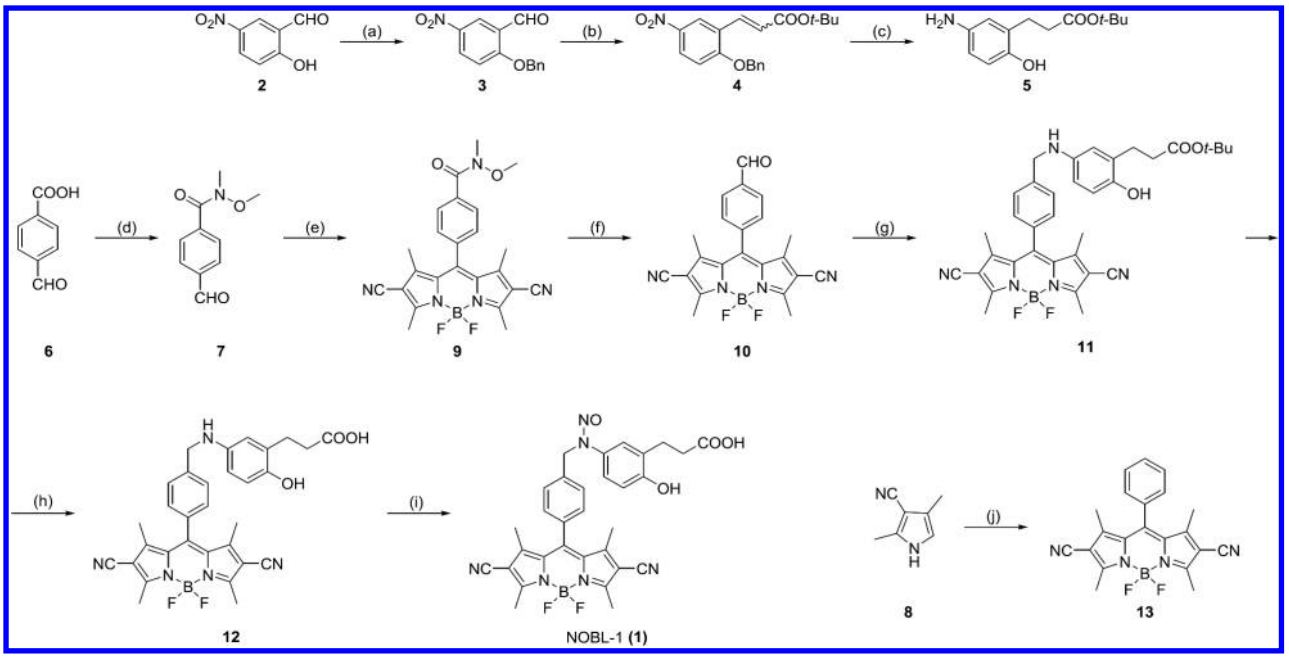
Scheme 1. Synthesis of NOBL-1 (1) and BODIPY 13[a]



[a]Reagents and conditions: (a) BnBr, K_2CO_3, DMF, rt, 90%; (b) $BrCH_2COOt$-Bu, PPh_3, sat. $NaHCO_3$, THF, rt, quant.; (c) Pd−C, MeOH, H_2, rt, 79%; (d) NHMeOMe·HCl, EDCI·HCl, *N*-methylmorpholine, CH_2Cl_2, rt, 67%; (e) 8, TFA, CH_2Cl_2, rt; then, DDQ; then, BF_3·OEt_2, DIPEA, 0 °C, 64%; (f) Cp_2ZrHCl, THF, rt, 70%; (g) **5**, AcOH, CH_2Cl_2, rt; then, $NaBH(OAc)_3$, rt, 83%; (h) HCl, AcOEt, rt; (i) $NaNO_2$, AcOH, H_2O, 0 °C, 44% (2 steps); (j) benzaldehyde, TFA, CH_2Cl_2, rt; then, DDQ, rt; then, BF_3·OEt_2, DIPEA, rt, 48%.

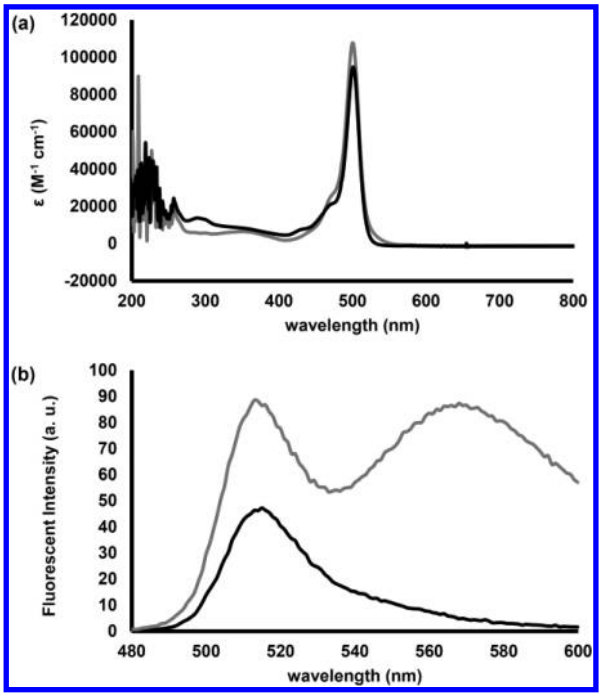


Figure 2. Spectral comparison of NOBL-1 and **13**. (a) Absorbance spectra of NOBL-1 (dark line) and **13** (gray line) in DMSO. (b) Fluorescence spectra of NOBL-1 (dark line) and **13** (gray line) in DMSO solution (10 μM, excitation at 500 nm).

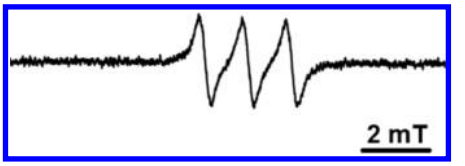


Figure 3. ESR spectra of a solution containing NOBL-1 (100 μM), $FeSO_4$ (1.5 mM), and *N*-methylglucamine dithiocarbamate (6 mM) in PBS (10 mM, pH 7.0, containing 1% DMF) after photoirradiation with blue light (470−500 nm, 200 mW/cm^2).

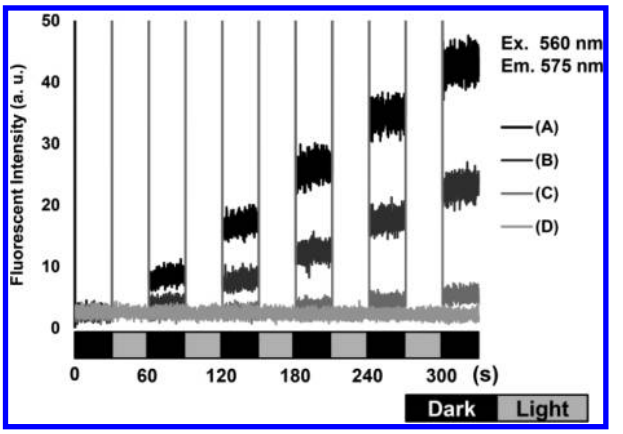


Figure 4. Fluorescence measurement for detection of NO generation from NOBL-1 (10 μM in 10 mM PBS, pH 7.4, containing 0.3% DMSO) using DAR-4M. The fluorescence intensity was determined at 575 nm with excitation at 560 nm. Irradiation light was not attenuated (A) or was attenuated to 50% (B), 20% (C), or 0% (D).

hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7),[18] a spontaneous nitric oxide releaser which in principle releases two equivalents of NO (Figure S5).

Next, photoinduced NO release from NOBL-1 in HEK293 cells was examined with acetoxymethyl DAR-4M (DAR-4M AM), an ester that permeates through the cell membrane and is hydrolyzed intracellularly to DAR-4M, a fluorogenic NO probe.[17] HEK293 cells were treated with NOBL-1 and DAR-4M AM and then irradiated with blue light (470−500 nm, 25

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mW/cm^2) for 5 min. Fluorescence intensity was clearly increased in the irradiated cells, as observed by fluorescence microscopy (Figure 5a). In the absence of the triggering light

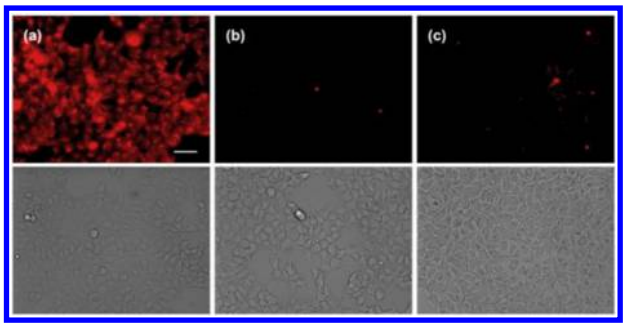

Figure 5. Fluorescence imaging of NO release from NOBL-1 in HEK293 cells using DAR-4M AM. Cultured HEK293 cells were treated with DAR-4M AM (5 μM) and NOBL-1 (10 μM). The dishes were then photoirradiated with blue light (470−500 nm, 25 mW/cm^2 for 5 min) or incubated in the dark for 15 min. The dishes were observed with a differential interference microscope. (a) After photoirradiation with NOBL-1, (b) after incubation with NOBL-1 in the dark, (c) after photoirradiation without NOBL-1. Upper: fluorescence images. Lower: DIC images. Scale bar represents 50 μm.

(Figure 5b) or NOBL-1 (Figure 5c), little fluorescence was observed. These results suggested that NOBL-1 enabled visible-light-mediated control of NO release in cells. To confirm that NO release could be confined to a specified area, HEK293 cells were treated with NOBL-1 and DAR-4M AM, and then the cells within a specified region were stimulated with an argon laser (488 nm) via a confocal fluorescence microscope. Cell images were obtained by confocal fluorescence microscopy before and after irradiation. As shown in Figure 6, the fluorescence intensity was increased only within and around the irradiated area, and no fluorescence increase was observed

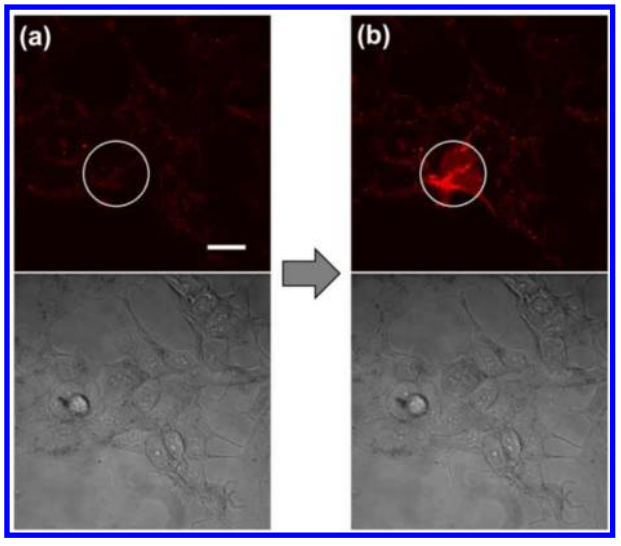

Figure 6. Fluorescence imaging of NO release from NOBL-1 in HEK293 cells in the presence of DAR-4M AM. Cultured HEK293 cells were treated with DAR-4M AM (5 μM) and NOBL-1 (10 μM). The dish was then photoirradiated inside the indicated circle using a confocal microscope equipped with an argon laser. The dish was observed with the confocal microscope. (a) Before photoirradiation, (b) after photoirradiation. Upper: fluorescence images. Lower: bright-field images. Scale bar represents 20 μm.

in the absence of NOBL-1 (Figure S6). Thus, NOBL-1 enabled site-specific control of NO release in cells with noncytotoxic blue light.

NO is known to induce vasodilation by activation of soluble guanylate cyclase (sGC), which catalyzes production of cyclic guanosine monophosphate (cGMP).[2] If vasodilation could be controlled by visible-light irradiation of a small-molecular compound, this would be a very useful system for research on the blood circulatory system. Therefore, we examined whether vasodilation of rat aorta *ex vivo* could be controlled by visible light-mediated NO release from NOBL-1. A strip of rat aorta was placed in a Magnus tube filled with Krebs buffer. To block endogenous NO formation by NOS, the aorta was pretreated with $_L$-nitroarginine methyl ester (L-NAME),[19] a NOS inhibitor, and then tensioned by exposure to noradrenaline. After equilibration, NOBL-1 was added and the tube was irradiated with blue light (470−500 nm, 14.5 mW/cm^2). As shown in Figure 7a, relaxation of the aorta was observed, and

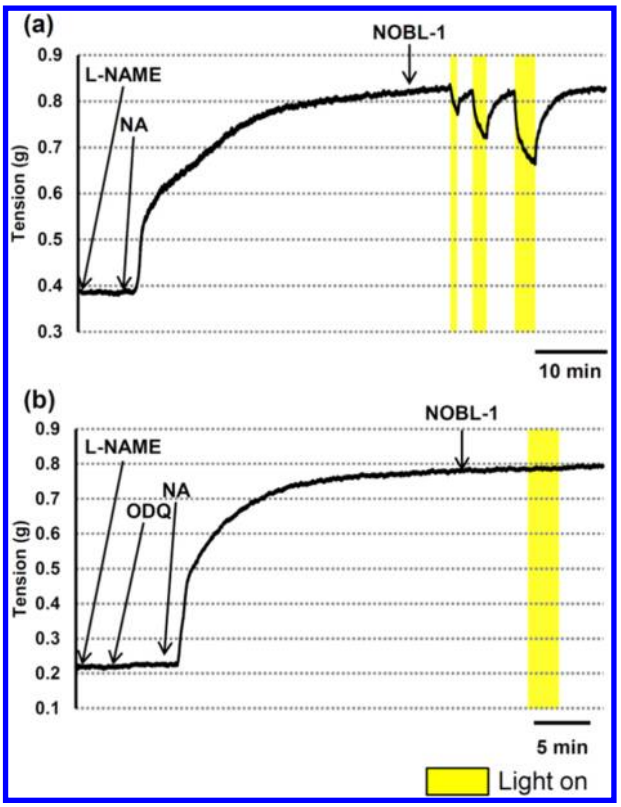

Figure 7. Changes in tension of rat aorta *ex vivo* induced by blue-light-mediated NO release from NOBL-1. Rat aorta was treated with L-NAME (10 μM) and noradrenaline (10 μM), followed by NOBL-1 (1 μM), in Magnus tubes. (a) The tubes were irradiated (470−500 nm, 14.5 mW/cm^2) for 1, 2, and 3 min. (b) ODQ (10 μM) was added before addition of NOBL-1. Light irradiation was conducted for 3 min.

the extent of relaxation was found to be dependent on the irradiation time. Note that the tension of aorta was only decreased during irradiation, and immediately recovered when the light was turned off. To confirm that this relaxation was indeed induced by NO, the aorta was pretreated with 1*H*-1,2,4-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ),[20] a sGC inhibitor, before addition of noradrenaline and NOBL-1. As expected, the relaxation of the aorta was blocked by ODQ treatment (Figure 7b). In the absence of NOBL-1, light irradiation induced only slight relaxation (Figure S7a). To exclude the possibility that

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the vasodilation was induced by photosensitization activity of the antenna moiety, fluorescein, which is reported to have a stronger photosensitization activity than 2,6-dicyano-BODIPY,[21] was added instead of NOBL-1. As shown in Figure S7b, in the presence of fluorescein, the tension of aorta was hardly decreased. Thus, the relaxation mediated by NOBL-1 was not due to photosensitization activity. Furthermore, a higher concentration of NOBL-1 induced a stronger relaxation activity at a given light intensity (Figure S6c). Interestingly, even a familiar device such as a green laser pointer (1 mW, 532 nm) could induce relaxation of aorta in this experimental setting (Figure S7c). Overall, these results indicate that the vasodilation was induced by finely controlled blue-light-induced release of NO from NOBL-1. It should be noted that light-induced vasodilation of coronary arteries with a GaAs diode laser (680 nm) was reported by Prusa et al.[22] They suggested that the vasodilation was caused by release of NO from endogenous nitrosylated biomolecules, such as *S*-nitrosothiol, under light irradiation.[23] Indeed, as shown in Figure S7a, slight vasodilation was induced by light irradiation in the absence of NOBL-1. This slight relaxation might have been triggered by release of NO from endogenous *S*-nitrosothiols, in view of the previous report and our result. However, such light-induced endogenous NO release was weak, and might be abrogated under pathophysiological conditions where the amount of *S*-nitrosothiols is depressed, e.g., due to loss of NOS expression or insufficient O_2 supply.[24] In contrast, NOBL-1 can release larger amounts of NO in a highly controlled manner, regardless of these factors. Therefore, light-induced NO releasers such as NOBL-1 are expected to be valuable research tools, as well as candidate therapeutic drugs.

For biological applications, cytotoxicity is a significant factor. To assess the cytotoxicity of NOBL-1, MTT assay was conducted. In the range of 1−5 μM, within which significant relaxation of rat aorta was induced, NOBL-1 did not show strong cytotoxicity (survival rate at 1 μM, 77%; at 5 μM, 67%). Unfortunately, we could not determine the precise IC_{50} value because the cells were easily detached at higher concentrations on a 96-well plate.

Photodecomposition products of NOBL-1 were explored by LC-ESI-MS examination of an irradiated solution of NOBL-1. Three products (*m*/*z* 403, 567, and 402) were mainly detected (Figure S8). On the basis of the ion mass numbers and structure of NOBL-1, the products were assumed to have been generated via dismutation of the semiquinone, which is expected to be formed after NO release from NOBL-1 (Figure S9).

The quantum yield of photodecomposition, Φ_{PD}, of NOBL-1 was determined with a potassium ferrioxalate actinometer as described previously.[25,26] The value of Φ_{PD} was found to be 0.0019 ± 0.0004 at 488 nm. Although this is quite small, NOBL-1 worked well as a finely controllable NO releaser on irradiation with blue light in our experiments. The reason for this may be that NOBL-1 has a large absorption coefficient in the blue wavelength region ($\varepsilon_{488\ nm}$ = 24 054 $M^{-1}\cdot cm^{-1}$). The efficiency of the photoinduced reaction in such compounds is generally expressed as the product of absorption coefficient and decomposition quantum yield, $\varepsilon\cdot\Phi$. The $\varepsilon\cdot\Phi_{PD}$ value of NOBL-1 at 488 nm was calculated to be 46.7, which is comparable with the values reported for *o*-nitrobenzyl ester caged NOs ($\varepsilon\cdot\Phi$ = 15−70). Furthermore, NOBL-1 can be controlled with relatively noncytotoxic blue light, whereas the caged NOs have to be uncaged with strongly cytotoxic UV light.

CONCLUSION

We have developed a novel blue-light-controllable NO releaser, NOBL-1, composed of *N*-nitroso-4-aminophenol tethered to a BODIPY structure. Release of NO from this compound under blue-light irradiation was confirmed by ESR spin trapping and fluorescence detection of NO. The results of several experiments (comparison of absorbance and fluorescence spectra, NO detection by ESR spin trapping, and examination of photodecomposition by LC-ESI-MS) indicated that photoinduced electron transfer was a key step for blue-light-induced NO release. Fluorescence measurement confirmed that NO release could be controlled with high precision. Spatially controlled NO release in HEK293 cells was achieved by using a confocal fluorescence microscope equipped with an argon laser. Finally, we confirmed that vasodilation of rat aorta *ex vivo* could be temporally controlled by blue-light-induced NO release from NOBL-1. These results indicate that NOBL-1 should be a useful tool for research on the mechanisms of NO action in mammalian tissues, and it may also have potential for phototherapy.

EXPERIMENTAL SECTION

General Methods. Melting point was determined with a Yanaco micro melting point apparatus. Proton nuclear magnetic resonance spectra (^{1}H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a JEOL JNM-A500 spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard, tetramethylsilane. Elemental analysis was performed with Yanaco CHN CORDER NT-5 analyzer, and all values were within ±0.4% of the calculated values. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer. Ultraviolet−visible−light absorbance spectra were recorded on an Agilent 8453 spectrometer. Irradiation was conducted with an Asahi Spectra irradiating apparatus. All other reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, Nacalai Tesque, Kanto Kagaku, Kishida Kagaku, Junsei Kagaku and Dojindo, and used without purification. Flash column chromatography was performed using silica gel 60 (particle size 0.032−0.075 mm) supplied by Taiko Shoji.

Synthesis of 3. To a solution of 2-hydroxy-5-nitrobenzaldehyde (**2**) (1.00 g, 5.99 mmol) and K_2CO_3 (997 mg, 7.21 mmol, 1.2 equiv) in DMF (30 mL) was added BnBr (860 μL, 7.19 mmol, 1.2 equiv). The mixture was stirred for 2 h at room temperature, and further BnBr (215 μL, 1.80 mmol, 0.3 equiv) was added to it. Stirring was continued at room temperature for a further 1.5 h, and the mixture was diluted with AcOEt and washed with water. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 3:1 → 2:1) gave 1.39 g (90%) of **3** as a white solid: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 10.52 (1H, s), 8.73 (1H, d, *J* = 2.8 Hz), 8.41 (1H, dd, *J* = 2.8 HZ, 9.2 Hz), 7.45−7.38 (5H, m), 7.18 (1H, d, *J* = 9.3 Hz), 5.33 (2H, s).

Synthesis of 4. To a slurry of **3** (1.39 g, 5.39 mmol) and *tert*-butyl bromoacetate (1.42 mL, 9.74 mmol, 1.8 equiv) in sat. $NaHCO_3$ (30 mL) and THF (10 mL) was added PPh_3 (2.13 g, 8.13 mmol, 1.5 equiv). The mixture was stirred at room temperature for 1.5 h, then diluted with water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 8:1) gave 2.17 g (q. y.) of **4** as a clear oil: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm, 10:7 mixture of two isomers) (*trans*) 8.44 (1H, d, *J* = 2.8 Hz), 8.18 (1H, dd, *J* = 2.8 Hz, 9.1 Hz), 7.94 (1H, d, *J* = 16.2 Hz), 7.42−7.37 (5H, m), 7.02 (1H, d, *J* = 9.1 Hz), 6.55 (1H, d, *J* = 16.1 Hz), 5.27 (2H, s), 1.53 (9H, s), (*cis*) 8.39 (1H, d, *J* = 2.6 Hz), 8.18 (1H, dd, *J* = 2.8 Hz, 9.1 Hz), 7.42−7.37 (5H, m), 7.03 (1H, d, *J* =

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12.4 Hz), 6.99 (1H, d, J = 9.2 Hz), 6.03 (1H, d, J = 12.4 Hz), 5.21 (2H, s), 1.40 (9H, s).

Synthesis of 5. A slurry of **4** (164 mg, 0.462 mmol) and 5% Pd−C (97 mg) in MeOH (5 mL) was stirred at room temperature under H_2 for 18 h. The reaction mixture was filtered on Celite and the filtrate was evaporated *in vacuo*. The residue was purified by silica gel flash chromatography (*n*-hexane:AcOEt = 1:1) to obtain 86 mg (79%) of **4** as a pale yellow solid: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 6.95 (1H, s), 6.72 (1H, d, J = 8.4 Hz), 6.49 (1H, dd, J = 2.8 Hz, 8.4 Hz), 6.45(1H, d, J = 2.8 Hz), 3.36 (2H, s), 2.77 (2H, t, J = 6.3 Hz), 2.60 (2H, t, J = 6.3 Hz), 1.41 (9H, s).

Synthesis of 7. To a slurry of **6** (5.00 g, 33.3 mmol) in CH_2Cl_2 (200 mL) was added *N*-methylmorpholine (3.66 mL, 33.3 mmol, 1.0 equiv), followed by EDC·HCl (7.68 g, 40.1 mmol, 1.2 equiv) and NHMeOMe·HCl (3.60 g, 36.9 mmol, 1.1 equiv). The mixture was stirred at room temperature for 4.5 h, and then evaporated *in vacuo*. The residue was taken up in Et_2O and the mixture was washed with water and brine. The organic layer was dried over Na_2SO_4. Filtration and evaporation *in vacuo* gave 4.31 g (67%) of **7** as a yellow oil: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 10.07 (1H, s), 7.93 (2H, d, J = 8.4 Hz), 7.81 (2H, d, J = 8.2 Hz), 3.54 (3H, s), 3.39 (3H, s).

Synthesis of 9. To a solution of **7** (1.26 g, 6.51 mmol) and **8** (1.55 g, 12.9 mmol, 2.0 equiv) in CH_2Cl_2 (50 mL) was added TFA (0.2 mL). The mixture was stirred at room temperature for 19 h, and then DDQ (2.21 g, 9.71 mmol, 1.5 equiv) was added to it. Stirring was continued for a further 20 min, then BF_3·OEt_2 (8.5 mL, 32.4 mmol, 5.0 equiv) was added, followed by DIPEA (6.0 mL, 34.4 mmol, 5.3 equiv) on an ice−water bath. Stirring was continued for a further 10 min on the ice−water bath, then the reaction mixture was quenched with water and filtered. The organic layer was separated, and the water layer was extracted with $CHCl_3$. The combined organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 1:1 → 1:2) gave 1.92 g (64%) of **9** as a dark red solid: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.88 (2H, d, J = 8.2 Hz), 7.35 (2H, d, J = 8.3 Hz), 4.28 (4H, q, J = 7.1 Hz), 3.51 (3H, s), 3.42 (3H, s), 2.84 (6H, s), 1.68 (6H, s), 1.33 (6H, t, J = 7.1 Hz).

Synthesis of 10. To a solution of **9** (206 mg, 0.447 mmol) in dry THF (10 mL) was added Cp_2ZrHCl (142 mg, 0.551 mmol, 1.2 equiv). The mixture was stirred for 5 min, and then quenched with water. The mixture was extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 2:1) gave 126 mg (70%) of **10** as a red solid: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 10.37 (1H, s), 8.31 (2H, d, J = 8.3 Hz), 7.74 (2H, d, J = 8.1 Hz), 4.51 (4H, q, J = 7.1 Hz), 3.07 (6H, s), 1.87 (6H, s), 1.55 (6H, t, J = 7.1 Hz).

Synthesis of 11. A solution of **5** (130 mg, 0.549 mmol, 1.0 equiv), **10** (221 mg, 0.550 mmol), and AcOH (0.5 mL) in CH_2Cl_2 (5 mL) was stirred at room temperature for 2 h, then $NaBH(OAc)_3$ (353 mg, 1.67 mmol, 3.0 equiv) was added to it and stirring was continued for 10 min. The reaction mixture was quenched with water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt =2:1 → 3:2) gave 283 mg (83%) of **11** as a dark green solid: ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.61 (2H, d, J = 8.1 Hz), 7.22 (2H, d, J = 8.1 Hz), 6.96 (1H, brs), 6.75 (1H, d, J = 8.6 Hz), 6.43 (1H, dd, J = 2.9 Hz, 8.5 Hz), 6.38 (1H, d, J = 2.8 Hz), 4.43 (2H, s), 2.76 (2H, t, J = 6.2 Hz), 2.72 (6H, s), 2.58 (2H, t, J = 6.2 Hz), 1.59 (6H, s), 1.42 (9H, s).

Synthesis of NOBL-1. A solution of **11** (273 mg, 0.438 mmol) in 4 N HCl AcOEt (7 mL) and AcOEt (3 mL) was stirred at room temperature for 19.5 h. The reaction mixture was neutralized with 2 N NaOH and sat. $NaHCO_3$, and extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation, and purification of the residue by silica gel flash chromatography ($CHCl_3$:MeOH = 100:1 → 30:1 → 10:1) gave 247 mg (crude) of **12** as a dark green solid. A solution of **12** (crude 247 mg) in AcOH (6 mL) was added to a solution of $NaNO_2$ (34 mg, 0.493 mmol) in water (6 mL) on an ice−water bath. Stirring was continued for 10 min, then the mixture was diluted with AcOEt and washed with 10% citric acid. The organic layer was evaporated *in vacuo*. The residue was taken up in sat. $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 2:1 → AcOEt only → AcOEt:MeOH = 100:1 → AcOEt:MeOH = 20:1) gave 113 mg (44%) of NOBL-1 as a red solid. This was recrystallized from *n*-hexane and $CHCl_3$ to obtain 75 mg (66%) of green crystals: mp 195.3−198.3 °C; ^{1}H NMR (DMSO-d_6, 500 MHz, δ; ppm) 12.08 (1H, brs), 9.82 (1H, s), 7.36 (2H, d, J = 8.2 Hz), 7.32 (2H, d, J = 8.2 Hz), 7.29 (1H, d, J = 2.4 Hz), 7.15 (1H, dd, J = 2.7 Hz, 8.7 Hz), 6.82 (8.5 Hz), 5.38 (2H, s), 2.75 (2H, t, J = 7.8 Hz), 2.63 (6H, s) 2.48 (2H, t, J = 7.8 Hz), 1.39 (6H, s); ^{13}C NMR (DMSO-d_6, 125 MHz, δ; ppm) 173.7, 158.8, 154.9, 149.1, 147.1, 137.1, 132.0, 130.7, 130.7, 129.2, 127.7, 127.5, 123.3, 120.9, 114.9, 113.3, 105.4, 46.8, 33.1, 25.2, 13.5, 13.3. Anal. Calcd for $C_{31}H_{27}BF_2N_6O_4$·4/3H_2O: C, 60.01; H, 4.82; N, 13.55. Found: C, 60.29; H, 4.79; N, 13.28. MS (FAB): m/z 597 [$(M + 1)^+$].

Synthesis of 13. To a solution of benzaldehyde (100 μL, 0.981 mmol) in CH_2Cl_2 (10 mL) was added **8** (118 mg, 0.982 mmol, 2.0 equiv), followed by TFA (3 drops). The reaction mixture was stirred at room temperature for 12 h, and DDQ (223 mg, 0.982 mmol, 2.0 equiv) was added to it. Stirring was continued at room temperature for 20 min, and then DIPEA (854 μL, 4.90 mmol, 10 equiv) was added, followed by BF_3·OEt_2 (1.31 mL, 4.91 mmol, 10 equiv). Stirring was continued at room temperature for 10 min, and the reaction mixture was quenched with water. The mixture was extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 8:1) gave 87 mg (48%) of **13** as a dark green solid. This solid was washed with AcOEt to obtain 58 mg of purified **13** as a brick-colored solid: mp 279.6−282.5 °C; ^{1}H NMR ($CDCl_3$, 500 MHz, δ; ppm) 7.62−7.61 (3H, m), 7.27 (2H, m), 2.73 (6H, s), 1.58 (6H, s); ^{13}C NMR ($CDCl_3$, 150 MHz, δ; ppm) 159.7, 149.5, 147.1, 132.6, 131.5, 130.6, 130.1, 127.0, 113.5, 106.5, 13.8. Anal. Calcd for $C_{21}H_{17}BF_2N_4$: C, 67.40; H, 4.58; N, 14.97. Found: C, 67.24; H, 4.75; N, 14.75. MS (EI): m/z 374 [M^+].

ESR Analysis Using Iron Dithiocarbamate Complex. A solution (total volume 200 μL) of $FeSO_4$·$6H_2O$ (1.5 mM), *N*-methylglucamine dithiocarbamate (6 mM) and NOBL-1 (100 μM) in PBS (pH 7.0, DMF 1%) was photoirradiated (470−500 nm, 200 mW/cm^2) at room temperature for 15 min as a sample for ESR studies. ESR spectra were taken on a JES-RE2X spectrometer (JEOL Co. Ltd., Tokyo, Japan). The measurement conditions were follows: microwave power, 10 mW; frequency, 9.4200 GHz; field, 330 mT; sweep width, 7.5 mT; sweep time, 4 min; modulation width, 0.125 mT; time constant; 0.10 s.

Fluorescence Measurement of DAR-4M. Sample solutions (total volume 2 mL) containing NOBL-1 (10 μM) and DAR-4M (10 μM) in PBS (pH 7.4, containing 0.3% DMSO) were irradiated or incubated in the dark. The fluorescence intensity was determined with an RF5300-PC (Shimadzu) at 575 nm, with excitation at 560 nm. The slit width was set to 1.5 nm for both excitation and emission.

Detection of NO in HEK293 Cells. HEK293 cells were plated on 3.5 cm glass dishes at 2.0 × 10^5 cells/dish with 2 mL of Dulbecco's Modified Eagle Medium (DMEM). The cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air for 2 days. The medium was replaced with 2 mL of fresh DMEM. The cells were incubated with 10 μM NOBL-1 (DMSO 0.1%) or DMSO for 1 h under the above conditions. Then, the medium was replaced with 2 mL of fresh DMEM, and the cells were washed with 2 mL of DMEM twice and treated with 5 μM DAR-4M AM (DMSO 0.1%) for 30 min under the above conditions. The medium was replaced with 2 mL of Dulbecco's PBS containing Mg^{2+} and Ca^{2+} (D-PBS). The cells were washed with 2 mL of D-PBS and irradiated with a MAX-302 (470−500 nm, 25 mW/cm^2) for 5 min. After irradiation, the cells were examined under a differential interference contrast microscope (Olympus, IX71).

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Spatial Control of NO Release in HEK293 Cells. HEK293 cells were plated on 3.5 cm glass dishes at 2.0×10^5 cells/dish with 2 mL of DMEM. The cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air for 2 days. The medium was replaced with 2 mL of fresh DMEM. The cells were treated with 10 μM NOBL-1 (DMSO 0.1%) or DMSO for 1 h under the above conditions. Then, the medium was replaced with 2 mL of fresh DMEM, and the cells were washed with 2 mL of DMEM twice and treated with 5 μM DAR-4M AM (DMSO 0.1%) for 30 min under the above conditions. The medium was replaced with 2 mL of Dulbecco's PBS containing Mg^{2+} and Ca^{2+} (D-PBS). The cells were washed with 2 mL of D-PBS, and subjected to confocal fluorescence microscopy (LSM 510, Carl Zeiss Japan Co. Ltd., Tokyo, Japan). A region of interest of a dish was selected and bleached with an argon laser (488 nm, output 70%, number of scans 1000, light intensity attenuated to 30%).

Modulation of Vasodilation by NOBL-1 under Blue-Light Irradiation. A rat aortic strip was placed in a Magnus tube filled with Krebs buffer at 37 °C. The tension was recorded on a LabChart 7 (ADInstruments). The strip was treated with L-NAME (10 μM) or ODQ (10 μM) before addition of noradrenaline (10 μM). After equilibration, NOBL-1 (1 μM or 10 μM), DMSO (10 μL), or fluorescein (1 μM) was added, and the tube was irradiated with a MAX-302 (14.5 mW/cm^2) or a 1 mW green laser pointer.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures, synthesis of **8**, quantitative determination of NO, photodecomposition determination, and MTT assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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