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Visible light-induced nitric oxide release from a novel nitrobenzene derivative cross-conjugated with a coumarin fluorophore

Kai Kitamura [a], Naoya Ieda [a], Kazuhiro Hishikawa [a], Takayoshi Suzuki [b], Naoki Miyata [a], Kiyoshi Fukuhara [c], Hidehiko Nakagawa [a,*]

[a] Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan
[b] Kyoto Prefectural University of Medicine, 13, Taishogun, Nishitakatsukasa-cho, Kita-ku, Kyoto 403-8334, Japan
[c] School of Pharmacy, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

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ABSTRACT

Nitric oxide (NO) is a well-known free-radical molecule which is endogenously biosynthesised and shows various functions in mammals. To investigate NO functions, photocontrollable NO donors, compounds which release NO in response to light, are expected to be potentially useful. However, most of the conventional NO donors require harmful ultra-violet light for NO release. In this study, two dimethylnitrobenzene derivatives conjugated with coumarins were designed, synthesized and evaluated as photocontrollable NO donors. The optical properties and efficiency of photo-induced NO release were dependent upon the nature of the conjugation system. One of these compounds, **Bhc-DNB** (**1**), showed spatiotemporally well-controlled NO release in cultured cells upon exposure to light in the less-cytotoxic visible wavelength range (400–430 nm).

Nitric oxide (NO) is a small-molecular, gaseous mediator that plays important roles in various physiological processes, including vasorelaxation,[1] neuromodulation[2] and biodefence.[3] It may also be involved in the pathophysiology of diseases such as cancer,[4] Alzheimer's disease[5] and schizophrenia.[6] Therefore, methods for precisely controlled release of NO are required for research purposes and might ultimately be clinically relevant. Since NO is unstable under ambient conditions, compounds that release NO in situ, NO donors, have been developed and employed for NO research.[7] Since physiological NO production by nitric oxide synthase (NOS) is precisely regulated by enzymes and signal transduction machinery, photocontrolled release of NO from a donor molecule seems particularly attractive as a means to achieve spatiotemporally controlled intracellular NO release.

We previously reported on photo-induced NO release from 6-nitrobenzo[*a*]pyrene (6-nitroBaP),[8] and based on this finding, we developed 2,6-dimethylnitrobenzene (2,6-DNB) derivatives as photocontrollable NO donors.[9] These 2,6-DNB derivatives have an aromatic nitro group that is not planar with respect to the benzene ring due to the steric effect of the two methyl groups at *ortho*-positions and this twisted conformation is considered to facilitate photo-induced isomerisation of the nitro group to nitrite ester, which subsequently undergoes homolytic fission to release NO (Fig. 1). As photocontrollable NO donors, these 2,6-DNB derivatives are attractive, because they are stable (easy to handle), not metal-containing (non-cytotoxic) and, above all, unique in their mechanism of NO release. However, they have a serious limitation, in that their maximum absorption band for NO release is mainly in the UV-A range, which is harmful to living cells. To develop novel NO donors suitable for cellular applications, we focused on coumarins as compact fluorophores, particularly those with an electron-donating group at the 7-position, such as 7-hydroxy- and 7-amino-coumarins. The absorption of such coumarin derivatives is expected to be shifted to longer wavelength through intermolecular charge transfer (ICT). Therefore, we designed and synthesized two novel 2,6-DNB derivatives conjugated with coumarin fluorophores, that is, **Bhc-DNB** (**1**) and **DEAMC-DNB** (**2**) (Fig. 2). In **Bhc-DNB** (**1**), the olefin linker to the 2,6-DNB moiety is cross-conjugated[10] with 6-bromo-7-hydroxycoumarin (Bhc)[11] at the 4-position of the coumarin ring, while in **DEAMC-DNB** (**2**), the linker is linearly conjugated with 7-(diethylamino)-4-methylcoumarin at the 3-position. In the latter case, the nitro group of 2,6-DNB is expected to be an efficient electron acceptor for ICT, whereas this is not in the case in **Bhc-DNB** (**1**). Thus, the electron density of the NO-releasing nitro group of **Bhc-DNB** (**1**) in the photo-excited state is expected to be lower than that of **DEAMC-DNB** (**2**).

These molecules were synthesized as shown in Scheme S1 (see Supplementary material). Their chemical structure and purity were

* Corresponding author.

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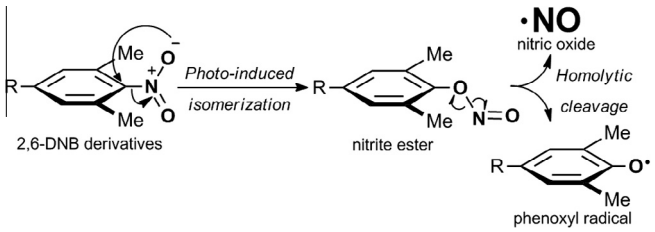

Figure 1. Proposed mechanism of photo-induced NO release from 2,6-dimethylnitrobenzene (2,6-DNB) derivatives.

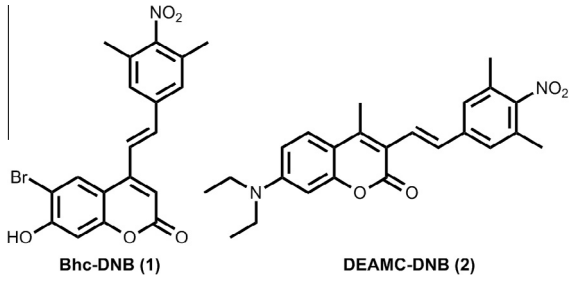

Figure 2. Chemical structures of **Bhc-DNB** (**1**) and **DEAMC-DNB** (**2**).

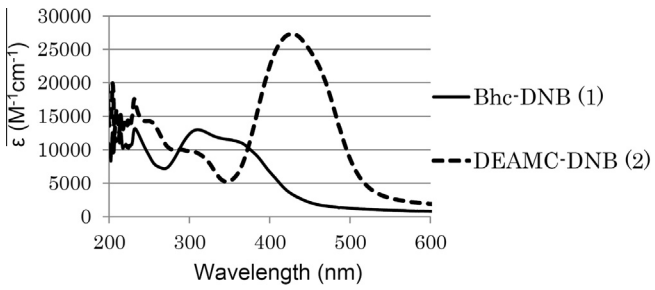

Figure 3. UV–visible absorption spectra of **Bhc-DNB** (**1**) and **DEAMC-DNB** (**2**); $\varepsilon_{360\ nm}$ of **Bhc-DNB** (**1**) is 11217 $M^{-1}\,cm^{-1}$, $\varepsilon_{426\ nm}$ (λ_{max}) of **DEAMC-DNB** (**2**) = 47,345$M^{-1}\,cm^{-1}$.

confirmed by ^{1}H NMR, ^{13}C NMR, mass spectrometry and elemental analysis.

First of all, we measured the ultraviolet-visible absorption spectra to examine the effect of the difference in conjugation system. As we had expected, **DEAMC-DNB** (**2**) showed a quite elongated absorption band at around 400–500 nm owing to efficient ICT, whereas **Bhc-DNB** (**1**) showed an absorption band at around 350–430 nm, which corresponds to that of Bhc itself.[11] In other words, the absorption band of **Bhc-DNB** (**1**) was not shifted, presumably due to the cross-conjugated structure (Fig. 3).

Next, to confirm NO release from the compounds we adopted an ESR spin trapping method with ferrous *N*-methylglucamine dithiocarbamate (Fe^{2+}–MGD_2) complex, which traps NO to yield an NO–Fe^{2+}–MGD_2 complex showing typical triplet signals at around 330 mT in 1 GHz ESR spectroscopy. Aqueous solutions (50% DMSO, 2.5 mM potassium phosphate buffer, pH 7.35) of the compounds in the presence of 1.5 mM Fe^{2+}–MGD_2 complex were photoirradiated under the indicated conditions, and the solutions were subjected to ESR analysis. As shown in Figure 4, solutions of **Bhc-DNB** (**1**) irradiated at 325–385 nm (UV-A light) or 400–430 nm (violet light) showed a typical triplet signal assigned to NO–Fe^{2+}–MGD_2 complex in the ESR spectrum, confirming NO release from the compound (Fig. 4A and B). In contrast, a solution of **DEAMC-DNB** (**2**) irradiated with 430–460 nm light did not show the triplet signal in the ESR spectrum, which means that NO is not released by photoirradiation in this wavelength range (Fig. 4C). Thus, we found that **Bhc-DNB** (**1**) released NO upon light irradiation around its absorption band, whereas **DEAMC-DNB** (**2**) did not. This interesting result presumably reflects the difference in conjugation systems, namely the difference of electron density of the NO-releasing nitro group in the excited state. It appears that ICT in the **DEAMC-DNB** (**2**) molecule gives a double-bond character to the C—N bond of the NO-releasing nitro group, which prevents isomerisation of the nitro group to nitrite ester because of the increase of its planarity (Fig. S2, Supplementary material). In order to confirm the expectation, the nature of the nitro group was examined by infrared spectroscopy, which showed that the property of the nitro group of **DEAMC-DNB** (**2**) is similar to that of nitrobenzene, which has no steric effect of methyl groups, than that of 2,6-DNB such as 2-nitromesitylene and **Bhc-DNB** (**1**). (Fig. S3 and Table S1, Supplementary material) The observation that **Bhc-DNB** (**1**) could release NO in response to visible light suggested that it is potentially available as an NO donor controllable with visible light.

Next, we investigated the photo-decomposition products of **Bhc-DNB** (**1**) by LC/ESI–MS. It was expected that one of the photo-decomposition products of **Bhc-DNB** (**1**) might be a dimethylphenol compound (**17**) whose phenol group is derived from the nitro group of **Bhc-DNB** (**1**), so we attempted to detect **17** by single ion monitoring at *m*/*z* 389, a mono-isotopic mass derived from the compound containing ^{81}Br. An aqueous solution (50% DMSO, 20 mM potassium phosphate buffer, pH 7.35) of 100 μM **Bhc-DNB** (**1**) was irradiated with 325–385 nm (UV-A) light for 15 min and the resulting solution was subjected to LC/ESI–MS analysis. The retention time of a major photo-decomposition product in the HPLC chromatogram corresponded to that of the peak detected by single ion monitoring (*m*/*z* 389) (Fig. S4, Supplementary material). Moreover, the ESI–MS spectrum of this peak showed *m*/*z* 387, confirming the detection of **17** (Fig. S5, Supplementary material). Thus, **Bhc-DNB** (**1**) is mainly photolyzed to **17** which is a one-electron reduced form of the phenoxyl radical generated by photo-induced NO release.

We next evaluated the decomposition quantum yield (Φ), which is a parameter of photo-decomposition efficiency, of **Bhc-DNB** (**1**) from the decrement of the HPLC peak area after photoirradiation and the photon quantity of the light source measured by utilizing potassium ferrioxalate photo-reduction, whose quantum yield has been reported.[11] It was found that the

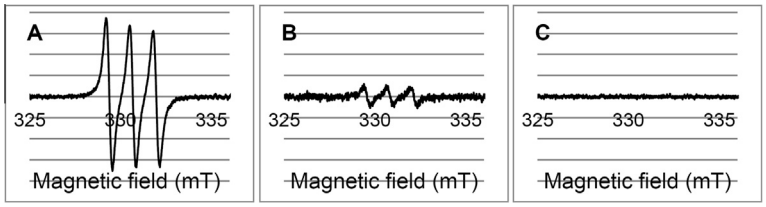

Figure 4. ESR spectra of aqueous solutions (50% DMSO, 2.5 mM potassium phosphate buffer, pH 7.35) containing the test compounds and 1.5 mM Fe^{2+}–MGD_2 complex after photo-irradiation. (A) 100 μM **Bhc-DNB** (**1**), 325–385 nm (Xe lamp, 15 mW/cm^2 at 360 nm, 10 min); (B) 500 μM **Bhc-DNB** (**1**), 400–430 nm (Xe lamp, 190 mW/cm^2 at 415 nm, 15 min); (C) 100 μM **DEAMC-DNB** (**2**), 430–460 nm (Xe lamp, 180 mW/cm^2 at 445 nm, 15 min). Control (blank) data are shown in Figure S1, Supplementary material.

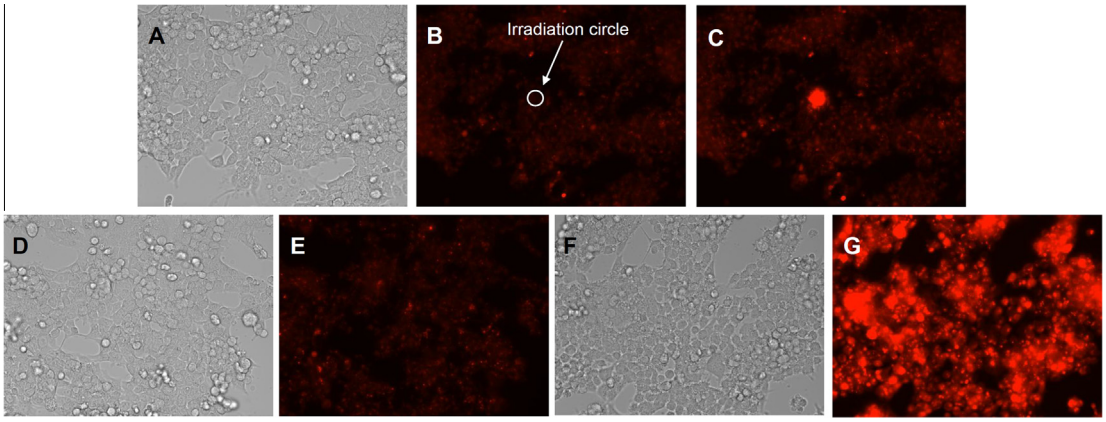

Figure 5. Fluorescence detection images of NO released from **Bhc-DNB** (**1**) in HCT116 cells. Cultured HCT116 cells were treated with 25 μM **Bhc-DNB** (**1**) and 10 μM DAR-4 M AM. Before and after photoirradiation, the cells were observed with a fluorescence microscope. (A) is a differential interference contrast (DIC) image of (B) and (C); (B) is the fluorescence image before photoirradiation; (C) is the fluorescence image after photoirradiation (330–380 nm, 10 min) within the irradiation circle indicated in (B); (D) and (F) are DIC images of (E) and (G), respectively; (E) is the fluorescence image before photoirradiation; (G) is the fluorescence image after photoirradiation (400–430 nm, 15 min) observed in the same the dish as that used for (E). (The control is shown in Fig. S6, Supplementary material.)

decomposition quantum yield at 358 nm ($\Phi_{358\ nm}$) was 0.053 ± 0.04, which is comparable with that of other photo-caged compounds, including some compounds bearing an *o*-nitrobenzyl group,[12] a well-known photo-removable protecting group, and Bhc derivatives.[13]

Finally, we applied **Bhc-DNB** (**1**) to living cells and also explored its cellular localization. To evaluate the NO-releasing ability of **Bhc-DNB** (**1**) in cells, we adopted DAR-4M AM, which is a cell-permeable red fluorescent probe for NO developed by Nagano et al.[14] HCT116 human colon cancer cells were loaded with **Bhc-DNB** (**1**) and the probe. After UV-A (330–380 nm) light irradiation of cells within the indicated irradiation circle under microscope observation, the cells were observed by means of fluorescence microscopy. As shown in Figure 5, red fluorescence of the NO probe was observed in the photoirradiated area. Furthermore after visible-light (400–430 nm) irradiation of the whole cell culture dish, an increase of red fluorescence was also observed, while no such increase was observed in cells without **Bhc-DNB** (**1**) (Fig. S6, Supplementary material). The compound appeared to be distributed in cytoplasm. These results confirmed that **Bhc-DNB** (**1**) is a novel NO donor which is cell-applicable and controllable with visible light (Fig. S6, Supplementary material).

In conclusion, we designed and synthesized two dimethylnitrobenzene derivatives conjugated with coumarin fluorophores, **Bhc-DNB** (**1**) and **DEAMC-DNB** (**2**). **Bhc-DNB** (**1**) released NO in response to not only UV-A, but also visible light (400–430 nm), while **DEAMC-DNB** (**2**) did not release NO in response to irradiation at the wavelength of the absorption maximum. These results are considered to reflect the difference in their conjugation systems. Thus, **Bhc-DNB** (**1**), a cross-conjugated compound, is available as an NO donor from which NO release is controllable with visible light in living cells. The major photo-decomposition product of **Bhc-DNB** (**1**) is a dimethylphenol compound (**17**). **Bhc-DNB** (**1**) is expected to be superior to existing photocontrollable NO donors for cellular studies that require precise, photocontrolled intracellular NO release, because release can be achieved with light in the non-cytotoxic visible wavelength range.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.075.

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