J|A|C|S COMMUNICATIONS

Published on Web 05/13/2009

Photoinduced Nitric Oxide Release from a Hindered Nitrobenzene Derivative by Two-Photon Excitation

Kazuhiro Hishikawa,[†] Hidehiko Nakagawa,*[,†] Toshiaki Furuta,[§] Kiyoshi Fukuhara,[‡] Hiroki Tsumoto,[†] Takayoshi Suzuki,[†] and Naoki Miyata*[,†,‡]

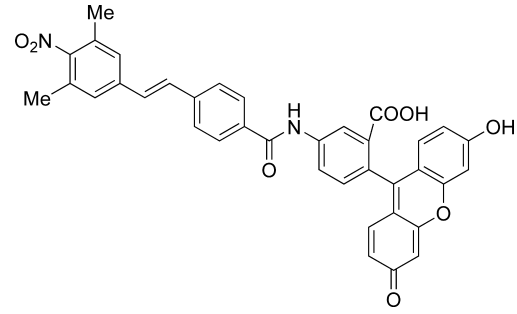
Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori Mizuho-ku, Nagoya, Aichi 467-8603, Japan, Department of Biomolecular Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 247-8510, Japan, and the Division of Organic Chemistry, National Institute of Health Sciences, Setagaya, Tokyo 158-8501, Japan

Received December 1, 2008; E-mail: deco@phar.nagoya-cu.ac.jp; miyata-n@phar.nagoya-cu.ac.jp

Nitric oxide (NO) has pleiotropic roles in mammals including roles in blood pressure regulation, neuromodulation, and biodefense.[1] Since NO, a free radical, is unstable under ambient conditions, various NO donors have been developed and employed for biological studies.[2] Although the NO donors have been useful research tools, many of them release NO via spontaneous decomposition, so that it is difficult to control NO release. Considering that NO formation in vivo is precisely regulated by enzymes and signal transduction machinery, controllable NO donors seem indispensable for investigation of NO physiology and for developing potential theraputic agents.

We have previously reported on photoinduced NO release from 6-nitrobenzo[*a*]pyrene (6-nitroBaP).[3] It was suggested that the nitro group is not planar with respect to the pyrene group due to the hydrogen atoms at the two *peri*-positions and that the consequent twisted conformation would facilitate isomerization reaction of the nitro group to nitrite ester, which is the key reaction for subsequent NO release. Based on this finding, we then found that 4-substituted-2,6-dimethylnitrobenzenes can also release NO upon photoirradiation.[4] In the latter compounds, the steric effect of the two methyl groups at the *ortho*-positions causes the nitro group to take a twisted conformation with respect to the benzene ring, and thereby facilitates isomerization of the nitro group. It is noteworthy that those dimethylnitrobenzene derivatives are unique as NO donors in their mechanism of NO release. However, their maximum absorption band for NO release is in the UV-A range, which could be harmful to living cells. To overcome this limitation, it would be very advantageous to develop compounds working at longer wavelengths, ideally in the range 700−900 nm, where photoirradiation is less biologically harmful and penetrates deeper into tissues. To realize this advantage, we adopted the two-photon excitation (TPE) technique. TPE, which is already used for fluorescence bioimaging, etc., offers advantages not only in excitation at longer wavelengths but also in high spatial resolution. Though several NO releasers utilizing the TPE technique have been reported,[5] these molecules contain a nitrosyl-chelated metal ion, which is potentially harmful to living cells and is usually metabolically unstable. Here, we report a novel 2,6-dimethylnitrobenzene-based NO donor which can be controlled by the TPE technique and which does not contain metal ions. Our novel NO donor, **Flu-DNB** (**1**), was designed by connecting the 2,6-dimethylnitrobenzene moiety with a two-photon excitation moiety through a styryl and weakly conjugated amide linker (Chart 1). We adopted fluorescein as the two-photon absorbing moiety; it is well-known to yield fluorescence by TPE.[6] **Flu-DNB** (**1**) was synthesized as shown in Scheme 1 and characterized by ^{1}H NMR, ^{13}C NMR, mass spectroscopy, and elemental analysis.

Chart 1. Chemical Structure of **Flu-DNB** (**1**)



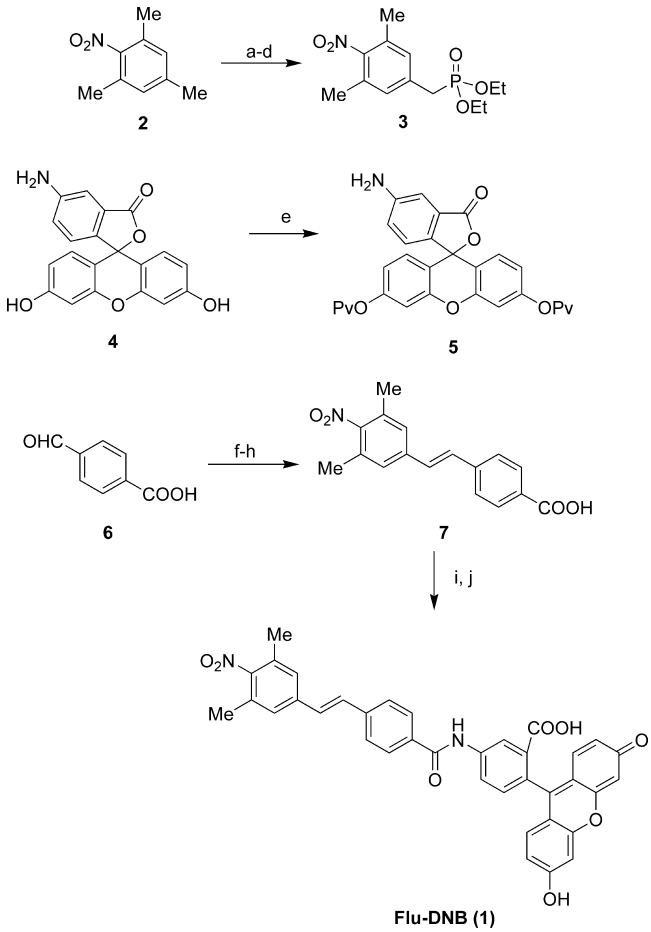
To confirm NO release from **Flu-DNB** via the conventional single photon excitation pathway, a solution of **Flu-DNB** was irradiated with 330−380 nm (UV-A) light (100 W Hg lamp) in the presence of an Fe-MGD complex, which traps NO to yield an Fe-MGD-NO complex showing triplet signals at ∼330 mT in ESR spectrometry. After irradiation, the ESR spectra of the solution showed typical triplet signals assigned to the Fe-MGD-NO complex, suggesting that **Flu-DNB** released NO upon UV-A irradiation (Figure 1). No NO release was observed upon irradiation at 450−480 nm, which is around the maximum absorption band of fluorescein. The decomposition product was separated and collected by HPLC and analyzed by ^{1}H NMR. The ^{1}H NMR spectra of the photodecomposition product was assigned as that of the dimethylphenol form of the compound.

We next investigated the TPE-based decomposition of **Flu-DNB**. A solution of **Flu-DNB** was irradiated with a pulse laser (Tsunami; Spectra-Physics) at 720 to 800 nm, and the products were analyzed by HPLC. At this wavelength range, **Flu- DNB** does not show any conventional single-photon absorption. Nevertheless, a decrease of **Flu-DNB** was observed, depending on the duration of pulse laser irradiation. The two-photon uncaging cross section (δ_u),[7] a parameter of two-photon decomposition efficiency, was calculated, and the results are shown in Figure 2. NO release from **Flu-DNB** by TPE was further confirmed by measuring NO_2^-/NO_3^-, the oxidation products of NO under aerobic conditions. A solution of 100 μM **Flu-DNB** was irradiated with a pulse laser at 720 nm, and the resulting solution was analyzed with a NO_2/NO_3 Assay-Kit FX (Dojindo). The concentration of NO_2^-/NO_3^- in the solution was determined as 8.3 μM after 10-min irradiation with a 720-nm pulse laser. (Table 1) NO release from **Flu-DNB** by TPE was also confirmed by an ESR spin trapping method with the Fe-MGD

[†] Nagoya City University.
[§] Toho University.
[‡] National Institute of Health Science.

10.1021/ja8093668 CCC: $40.75

Scheme 1. Synthesis of **Flu-DNB** (**1**)[a]



[a] Reagents and conditions: (a) CrO_3, AcOH; (b) $NaBH_4$, BF_3 etherate; (c) PBr_3; (d) $P(OEt)_3$, $(n\text{-}Bu)_4NI$, 120 °C; (e) Pv_2O, Cs_2CO_3; (f) $(Boc)_2O$, DMAP, *t*-BuOH; (g) **3**, NaH, 0 °C; (h) TFA, $CHCl_3$; (i) $(COCl)_2$, DMF; then **5**, DMAP; (j) 2N-NaOH, *i*-PrOH, $CHCl_3$; then 2N-HCl. Pv_2O = pivalic anhydride, DMAP = 4-dimethylaminopyridine.

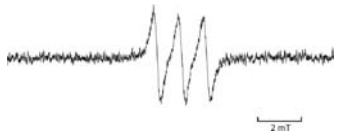


Figure 1. ESR spectra of $[(MGD)_2$-Fe-NO] complex after photoirradiation (330−380 nm) in the presence of Flu-DNB. Samples contained 1 mM Flu-DNB (containing 25% DMSO), 75 mM MGD, and 20 mM $FeSO_4$ in Milli-Q water; ESR spectra were recorded after photoirradiation for 15 min with a modulation width of 1.25 G and a microwave power of 10 mW.

complex, and the NO adduct signal was observed (Supporting Information).

In conclusion, we have demonstrated photoinduced NO generation from a 2,6-dimethylnitrobenzene derivative (**Flu-DNB**) via a TPE process; 8.3% of **Flu-DNB** released NO upon 720 nm pulse laser irradiation for 10 min. Although this release rate is not as large as those of previously reported TPE-active NO donors,[5] the amount of NO released would be enough for biological studies. The slow rate might be caused by the high stability of **Flu-DNB**. This compound is also very stable when stored in the dark. It does not contain any transition metal complex, unlike previous TPE-active NO donors, and this would be highly advantageous for biological applications. This is the first demonstration of two-photon NO release from a non-nitrosyl NO donor. Studies are under way to improve the TPE efficiency of the compound.

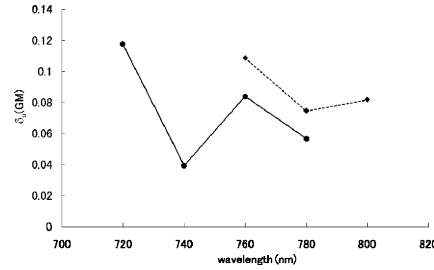


Figure 2. Two-photon uncaging cross section (δ_u) spectra of **Flu-DNB**. The solid line is the cross section value calculated based on the data series indicated by circles, and dashed line is that calculated using the additional data series of ref 6.

Table 1. Concentration of NO_2^-/NO_3^- Generated from **Flu-DNB** (**1**) by Photoirradiation[a]

light	$[NO_2^-]$ (μM)	$[NO_3^-]$ (μM)
330−380 nm (100 W Hg lamp)	4.3	11.7
720 nm pulse laser	0.89	7.4

[a] Reaction conditions are indicated in the Supporting Infomation.

Acknowledgment. This work was supported in part by Grants-in-Aid for Scientific Research (No. 17590089 to H.N.) from the Ministry of Education, Culture, Sports Science, and Technology, Japan, the Kowa Life Science Foundation (H.N.), Takeda Science Foundation (H.N.), and the Nagoya City University Special Fund for Research Promotion (H.N.). We thank Dr. K. Matsumoto, Dr. K. Anzai (both in NIRS, Japan), and Dr. S Mizuochi (Toho University, Japan) for their help in the measurement of ESR spectra in TPE experiments.

Supporting Information Available: Experimental procedure and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265–9269. (b) Palmer, R. M.; Ferige, A. G.; Moncada, S. *Nature* **1987**, *327*, 524–526. (c) Stamler, J. S.; Singel, D. J.; Loscalzo, J. *Science* **1992**, *258*, 1898–1902. (d) Cifone, M. G.; Cironi, L.; Meccia, M. A.; Roncaioli, P.; Festuccia, C.; De Nuntiis, G.; D'Alo, S.; Santoni, A. *Adv. Neuroimmunol.* **1995**, *5*, 443–461.
(2) For a review, see: (a) Wang, P. G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A. J. *Chem. Rev.* **2002**, *102*, 1091–1134.
(3) Fukuhara, K.; Kurihara, M.; Miyata, N. *J. Am. Chem. Soc.* **2001**, *123*, 8662–8666.
(4) Suzuki, T.; Nagae, O.; Kato, Y.; Nakagawa, H.; Fukuhara, K.; Miyata, N. *J. Am. Chem. Soc.* **2005**, *127*, 11720–11726.
(5) (a) Wecksler, S. R.; Mikhailovsky, A.; Korystov, D.; Ford, P. C. *J. Am. Chem. Soc.* **2006**, *128*, 3831–3837. (b) Wecksler, S. R.; Mikhailovsky, A.; Korystov, D.; Buller, F.; Kannan, R.; Tan, L.-S.; Ford, P. C. *Inorg. Chem.* **2007**, *46*, 395–402. (c) Zheng, Q.; Bonoiu, A.; Ohulchanskyy, T. Y.; He, G. S.; Prasad, P. N. *Mol. Pharm.* **2008**, *5*, 389–398.
(6) Xu, C.; Webb, W. W. *J. Opt. Soc.* **1996**, *13*, 481–491.
(7) Furuta, T.; Wang, S. S.-H.; Dantzker, J. L.; Dore, T. M.; Bybee, W. J.; Callaway, E. M.; Denk, W.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1193–1200.

JA8093668