ORGANIC LETTERS

2009
Vol. 11, No. 9
1887−1890

# BODIPY-Based Fluorescent Probe for Peroxynitrite Detection and Imaging in Living Cells

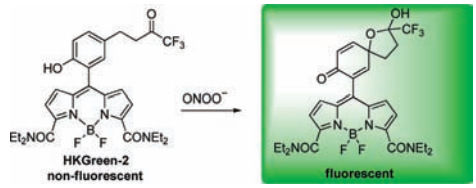
**Zhen-Ning Sun,[†] Hua-Li Wang,[†] Feng-Qin Liu,[‡] Yan Chen,[‡] Paul Kwong Hang Tam,[†] and Dan Yang*,[†]**

*Morningside Laboratory for Chemical Biology, Department of Chemistry, and Department of Surgery, The University of Hong Kong, Pokfulam Road, Hong Kong, People's Republic of China*

*yangdan@hku.hk*

**Received February 9, 2009**

## ABSTRACT




**A fluorescent probe, HKGreen-2, has been developed based on a specific reaction between ketone and peroxynitrite ($ONOO^-$). This probe is highly sensitive and selective for the detection of peroxynitrite not only in abiotic but also in biological systems. With this probe, we successfully detected peroxynitrite generated in murine macrophage cells activated by phorbol 12-myristate 13-acetate (PMA), interferon-$\gamma$ (IFN-$\gamma$), and lipopolysaccharide (LPS). This new probe will be a useful tool for studying the roles of peroxynitrite in biological processes.**

Peroxynitrite ($ONOO^-$), a short-lived reactive oxygen species (ROS) and reactive nitrogen species (RNS), is formed in vivo from a diffusion-controlled reaction between nitric oxide and superoxide in a 1:1 stoichiometry ($k = 0.4-1.9 \times 10^{10}$ $M^{-1}$ $s^{-1}$).[1] Intracellular peroxynitrite formation is associated with elevated NO production and its diffusion to sites of superoxide formation.[1d,2] A well-controlled level of peroxynitrite is essential for cellular integrity and organ homeostasis. However, under pathological conditions such as bacterial infection, lipopolysaccharide (LPS) induces high expression of inducible nitric oxide synthase (iNOS) in macrophages and neutrophils to produce large amounts of $ONOO^-$ in addition to other ROS and RNS.[3] Accumulating evidence suggests that aberrant peroxynitrite activities may contribute to many human diseases including ischemic reperfusion injury, rheumatoid arthritis, septic shock, multiple sclerosis, atherosclerosis, stroke, inflammatory bowel disease, cancer, and several neurodegenerative diseases.[4]

Unfortunately, attempts to investigate the behavior of peroxynitrite in biological systems have been hampered by the difficulty in detecting this highly reactive oxygen species in vivo. To this end, a number of fluorescent probes have been ventured for the detection of peroxynitrite in vitro, but these have proved undependable either because of a lack of

[†] Morningside Laboratory for Chemical Biology, Department of Chemistry.
[‡] Department of Surgery.

(1) (a) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620–1624. (b) Squadrito, G. L.; Pryor, W. A. *Free. Radic. Biol. Med.* **1998**, *25*, 392–403. (c) Fang, F. C. *Nat. Rev. Microbiol.* **2004**, *2*, 820–832. (d) Szabo, C.; Ischiropoulos, H.; Radi, R. *Nat. Rev. Drug Discov.* **2007**, *6*, 662–680.

(2) Trujillo, M.; Naviliat, M.; Alvarez, M. N.; Peluffo, G.; Radi, R. *Analusis* **2000**, *28*, 518–527.

(3) (a) Forehand, J. R.; Johnston, R. B. J.; Bomalaski, J. S. *J. Immunol.* **1993**, *151*, 4918–4925. (b) Fang, F. C. *J. Clin. Invest.* **1997**, *99*, 2818–2825. (c) Muijsers, R. B.; van Den Worm, E.; Folkerts, G.; Beukelman, C. J.; Koster, A. S.; Postma, D. S.; Nijkamp, F. P. *Br. J. Pharmacol.* **2000**, *130*, 932–936. (d) Jacob, A. T.; Ignarro, L. J. *J. Biol. Chem.* **2001**, *51*, 47950–47957. (e) Salonen, T.; Sareila, O.; Jalonen, U.; Kankaanranta, H.; Tuominen, R.; Moilanen, E. *Br. J. Pharmacol.* **2006**, *147*, 790–799.

(4) Pacher, P.; Beckman, J. S.; Liaudet, L. *Physiol. Rev.* **2007**, *87*, 315–424.

10.1021/ol900279z CCC: $40.75 
**Published on Web 03/30/2009**

specificity toward peroxynitrite or incompatibility with living cells and more complex natural environments.[5]

Our overall strategy is to develop more sensitive fluorescent probes on the basis of a specific reaction for peroxynitrite discovered by our group. We previously developed a fluorescent probe, **HKGreen-1**, which can detect peroxynitrite selectively.[6] The poor yield of the reaction between **HKGreen-1** and peroxynitrite, however, limits the probe's ability to detect peroxynitrite in vivo. As shown in Figure 1a, only 21% yield (based on 80% conversion) of the desired

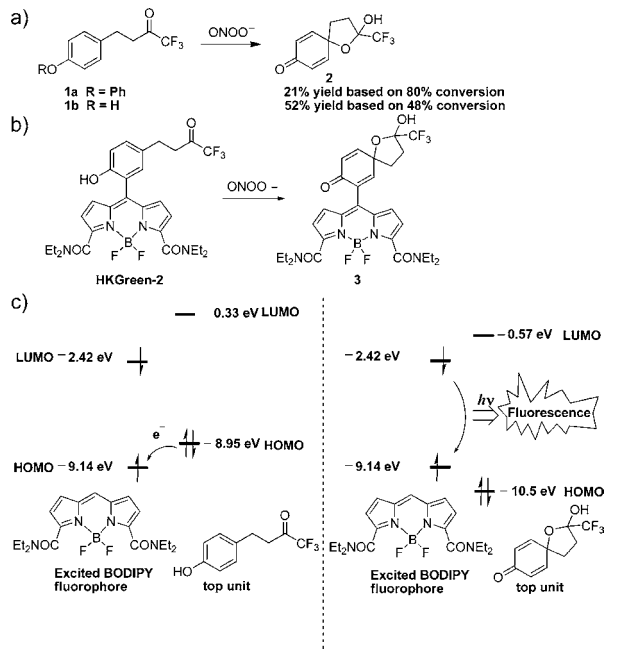



**Figure 1.** (a) Model reactions of **1a** and **1b** toward peroxynitrite. (b) Reaction between **HKGreen-2** and peroxynitrite. (c) HOMO and LUMO energies of BODIPY units (fluorophore) and the top units of both **HKGreen-2** and its oxidation product. The values were obtained from semiempirical PM3 calculation.

compound **2** is obtained in the model reaction between peroxynitrite and phenoxyphenyl-derived ketone **1a**. We reasoned that the poor yield of this reaction was due to the difficulty in the cleavage of the aryl-oxygen bond. Thus, we modified compound **1a** by removing the aryl group to improve the reaction yield. Gratifyingly, ketone **1b** gave a better yield (52% yield based on 48% conversion) in its reaction with peroxynitrite.

With this modified moiety, we designed a new probe, **HKGreen-2**, that would be suitable for peroxynitrite detection based on a photoinduced electron transfer (PET) mechanism (Figure 1b and c).[7] We first investigated the reactivity of **HKGreen-2** toward peroxynitrite in an abiotic system. As indicated in Figure 2a, the probe is nonfluorescent

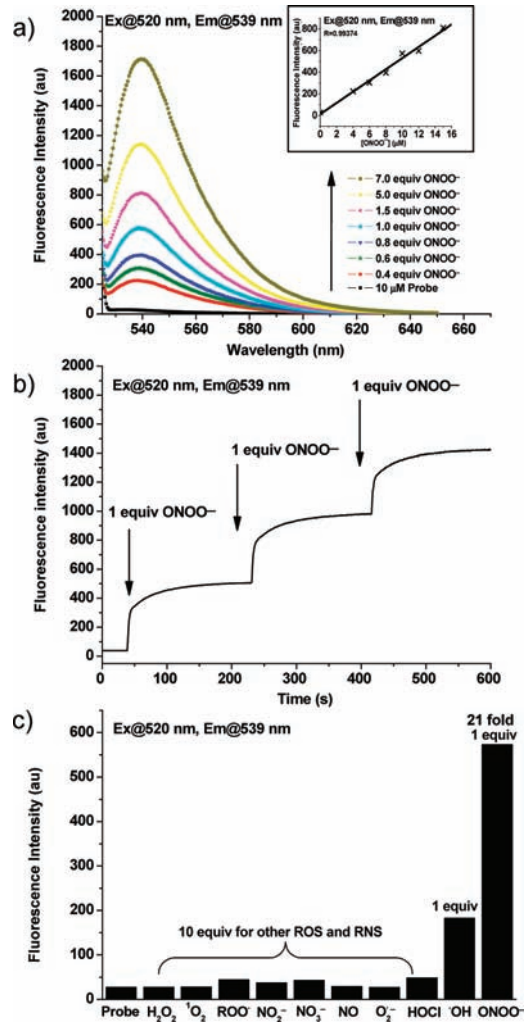



**Figure 2.** (a) Fluorescence emission spectra of **HKGreen-2** (10 $\mu$M) in 0.1 M potassium phosphate buffer (pH 7.4), recorded 0.5 h after reaction with various amounts of $ONOO^-$ (ranging from 0 to 7 equiv). (Insert) Linear correlation between the emission intensity and the concentration of peroxynitrite. (b) Real-time detection of $ONOO^-$. The real-time fluorescence emission spectrum of **HKGreen-2** (10 $\mu$M) in 0.1 M potassium phosphate buffer (pH 7.4) was recorded during addition of 1 equiv of $ONOO^-$ (repeating three times). (c) Fluorescence intensity of **HKGreen-2** (10 $\mu$M) in various ROS and RNS-generating systems (described in the Supporting Information).

prior to its reaction with peroxynitrite; intensity of the fluorescence signal rises dramatically with increasing peroxynitrite concentration. Upon treatment with 7 equiv of peroxynitrite, **HKGreen-2** exhibits a 69-fold surge in fluorescence intensity—a much higher sensitivity than that displayed by our previous probe, **HKGreen-1**. A linear

(5) (a) Setsukinai, K.; Urano, Y.; Kakinuma, K.; Majima, H. J.; Nagano, T. *J. Biol. Chem.* **2003**, *278*, 3170–3175. (b) Ueno, T.; Urano, Y.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 10640–10641.

(6) (a) Yang, D.; Wong, M. K.; Yan, Z. *J. Org. Chem.* **2000**, *65*, 4179–4184. (b) Yang, D.; Wang, H. L.; Sun, Z. N.; Chung, N. W.; Shen, J. G. *J. Am. Chem. Soc.* **2006**, *128*, 6004–6005.

(7) (a) Kollmannsberger, M.; Gareis, T.; Heinl, S.; Breu, J.; Daub, J. *Angew. Chem., Int. Ed.* **1997**, *36*, 1333–1335. (b) Gabe, Y.; Urano, Y.; Kikuchi, K.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 3357–3367. (c) Ueno, T.; Urano, Y.; Setsukinai, K.; Takakusa, H.; Kojima, H.; Kikuchi, K.; Ohkubo, K.; Fukuzumi, S.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 14079–14085. (d) Ziessel, R.; Ulrich, G.; Harriman, A. *New J. Chem.* **2007**, *31*, 496–501. (e) Loudet, A.; Burgess, K. *Chem. Rev.* **2007**, *107*, 4891–4932. (f) Ulrich, G.; Ziessel, R.; Harriman, A. *Angew. Chem., Int. Ed.* **2008**, *47*, 1184–1201.

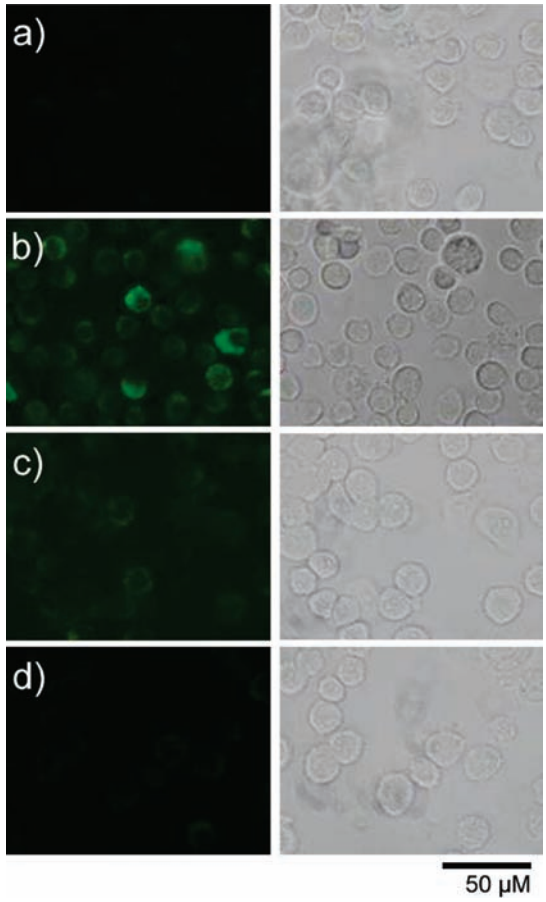



**Figure 3.** Fluorescence imaging of macrophage cells (left: fluorescence imaging; right: phase contrast imaging). Macrophage cells were treated with various stimulants and then incubated with **HKGreen-2** (20 $\mu$M) for 1 h. (a) Control. (b) LPS (1 $\mu$g/mL) and IFN-$\gamma$ (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h. (c) AG (1 mM), LPS (1 $\mu$g/mL), and IFN-$\gamma$ (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h. (d) TEMPO (100 $\mu$M), LPS (1 $\mu$g/mL), and IFN-$\gamma$ (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h.

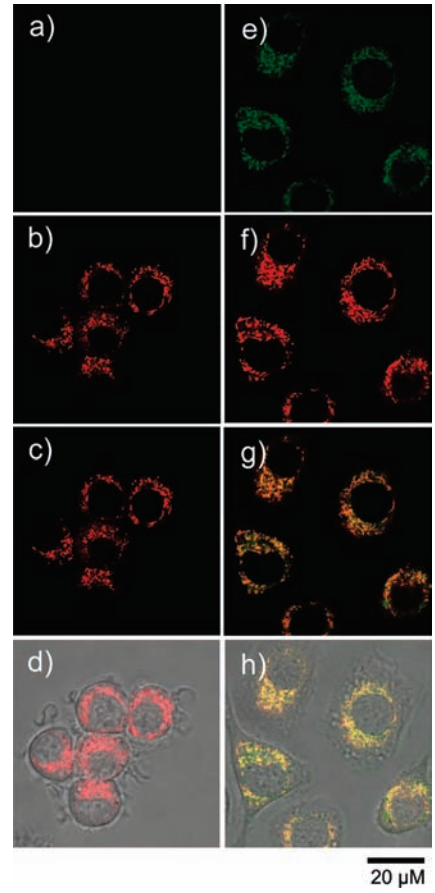



**Figure 4.** Confocal fluorescence images of live J774.1 macrophage cells incubated with **HKGreen-2** and MitoTracker Red CMXRos. (a−d) Controls; (e−h) treated with LPS (1 $\mu$g/mL) and IFN-ã (50 ng/mL) for 4 h then PMA (10 nM) for 0.5 h. (a) **HKGreen-2**. (b) MitoTracker Red CMXRos. (c) **HKGreen-2** and MitoTracker Red CMXRos (overlay). (d) Brightfield, **HKGreen-2** and MitoTracker Red CMXRos (overlay). (e) **HKGreen-2**. (f) MitoTracker Red CMXRos. (g) **HKGreen-2** and MitoTracker Red CMXRos (overlay). (h) Brightfield, **HKGreen-2** and MitoTracker Red CMXRos (overlay).

correlation exists between the emission intensity and the concentration of peroxynitrite when the concentration of $ONOO^-$ ranges from 0 to 15 $\mu$M (Figure 2a insert graph). The real-time emission spectrum shows rapid increases (within 2 s) in fluorescence signal after each addition of peroxynitrite (Figure 2b). Moreover, we have confirmed the formation of compound **3** through an ESI-MS measurement.[8] Therefore, **HKGreen-2** can detect peroxynitrite in an abiotic system in terms of a dose-dependent increase of fluorescence.

While **HKGreen-2** is highly sensitive to peroxynitrite, it is important to investigate its selectivity toward various ROS and RNS. As shown in Figure 2c, **HKGreen-2** exhibits a 21-fold increase in fluorescence intensity upon reaction with peroxynitrite, whereas a much weaker response is observed with other ROS and RNS such as HOCl, $^{\bullet}OH$, $H_2O_2$, $^1O_2$, $NO_2^-$, $NO_3^-$, NO, $O_2^{\bullet-}$ and $ROO^{\bullet}$. The fluorescence response toward $^{\bullet}OH$ can be significantly suppressed when 0.1% DMSO (a known scavenger of $^{\bullet}OH$) was used as a cosolvent.[8] These results suggest that, in an abiotic system, **HKGreen-2** displays high selectivity toward peroxynitrite over other ROS and RNS.

Our ultimate goal is to use **HKGreen-2** to detect endogenous $ONOO^-$ production in cells under physiological conditions. We chose the murine macrophage cell line J774.1 as a testing model, because exposure of macrophage cells to stimuli such as LPS/IFN-$\gamma$ (interferon-$\gamma$) and phorbol myristate acetate (PMA) activates the generation of ROS and RNS.[3c,e] We stimulated J774.1 macrophage cells with various reagents, incubated the cells with **HKGreen-2** (20 $\mu$M) for 1 h, and then washed them three times with PBS buffer (Figure 3). We observed no fluorescence in the absence of a stimulant (Figure 3a) but strong fluorescence after treatment with LPS (1 $\mu$g/mL) and interferon-$\gamma$ (IFN-$\gamma$; 50 ng/mL) for 4 h followed by additional stimulation with phorbol 12-myristate 13-acetate (PMA; 10 nM) for 30 min (Figure 3b). However, after the cells were pretreated with aminoguanidine

(8) See Supporting Information.

(AG), an inhibitor of nitric oxide synthase,[3c] much weaker fluorescence was observed in stimulated cells (Figure 3c). In principle, the amount of $^{\bullet}$OH produced in stimulated cells should not be affected by the presence of AG. Thus, the much weaker fluorescence in Figure 3c indicated that the strong fluorescence in Figure 3b should be induced by peroxynitrite rather than $^{\bullet}$OH. Furthermore, after pretreatment of cells with 2,2,6,6-tetramethyl-piperidinooxy (TEMPO), a scavenger of superoxide,[3c] almost no fluorescence was observed in stimulated cells (Figure 3d). These results reveal that **HKGreen-2** can be used to visualize peroxynitrite selectively in biological systems.

Finally, we sought to utilize **HKGreen-2** to probe the subcellular locations of endogenous peroxynitrite in J774.1 macrophage cells by using confocal fluorescence microscopy. Very weak green fluorescence in the cytoplasm of macrophage cells was observed when the cells were loaded with **HKGreen-2** (20 $\mu$M) (Figure 4a). Costaining with MitoTracker Red CMXRos (50 nM), a commercially available mitochondrial fluorescent probe, showed the location of active mitochondria in the cytoplasm of these living cells (Figures 4b−d). After treating the cells with the same stimulants as we used in Figure 3b, we observed much stronger green fluorescence (Figure 4e) compared to the control (Figure 4a), corresponding to an increase of peroxynitrite level in the cytoplasm. Again, by costaining with MitoTracker Red CMXRos and overlaying with brightfield images, we found that the green fluorescence is retained in the cytoplasm (Figures 4f-h). These data indicate that **HKGreen-2** can be used for imaging intracellular peroxynitrite selectively in biological systems.

In summary, on the basis of a specific reaction of peroxynitrite and a PeT mechanism, we have developed a new BODIPY-type fluorescent probe, **HKGreen-2**, which is highly sensitive for the specific detection of peroxynitrite in abiotic and living-cell systems. This probe will be a useful tool for elucidating the role of $ONOO^-$ in various biological processes.

**Acknowledgment.** This work was supported by the University of Hong Kong and the Hong Kong Research Grants Council (HKU7060/05P). Z.N.S. is a recipient of a University Postdoctoral Fellowship. We also thank the Faculty of Medicine at HKU for the Confocal Microscopy and Live Cell Imaging Facility.

**Supporting Information Available:** Synthesis, experimental details, and characterization of **HKGreen-2**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL900279Z