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HKGreen-3: A Rhodol-Based Fluorescent Probe for Peroxynitrite

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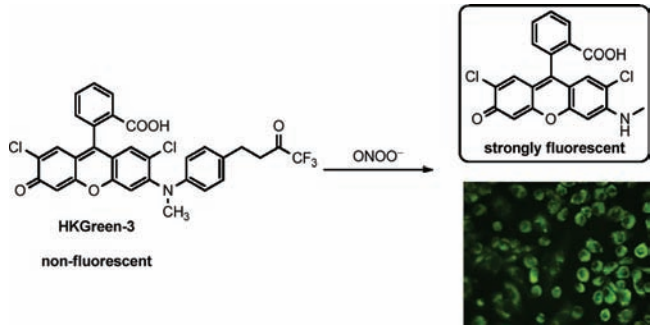
ABSTRACT



A novel fluorescent probe, HKGreen-3, for sensing peroxynitrite is designed on the basis of the rhodol scaffold and a peroxynitrite-specific oxidation reaction. The probe turns out to be highly sensitive and selective for detecting peroxynitrite in both chemical and biological systems.

In the last two decades, the in vivo formation of peroxynitrite ($ONOO^-$), a short-lived and highly reactive species produced from the diffusion-controlled reaction of nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$), has been widely considered as an established phenomenon since the first paper suggesting that peroxynitrite could be a potential biological oxidant was published.[1] More and more evidence indicates that peroxynitrite accounts for most of the cytotoxicity attributed to nitric oxide,[2] and its generation contributes to the pathogenesis of many human diseases such as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer, and neurodegenerative disorders.[2,3]

However, the biological relevance of peroxynitrite-dependent reactions is still occasionally controversial mainly due to the difficulty of its direct and unambiguous detection, which actually is extremely significant to push this field forward.[3,4] In this regard, although a number of peroxynitrite-responsible fluorescent probes have been developed,[5] limited sensitivity and specificity may seriously hamper their practicability in the detection of peroxynitrite. Here, we present a novel rhodol-based fluorescent probe, **HKGreen-3**, for highly senstitive and selective detection of peroxynitrite.

Recently, we developed a divergent synthetic route for rhodol fluorophores, and constructed a rhodol library with the intention to screen for novel rhodol-based fluorescent probes.[6] We noticed that two members of this library, i.e., **1** and **2**, possess distinct fluorescence properties (Figure 1). The latter displayed strong fluorescence emission ($\Phi = 0.67$) in aqueous buffer, while the former surprisingly showed almost no fluorescence ($\Phi = 0.0013$). The significant

(1) 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.
(2) Pacher, P.; Beckman, J. S.; Liaudet, L. *Physiol. Rev.* **2007**, *87*, 315–424.
(3) Ferrer-Sueta, G.; Radi, R. *ACS Chem. Biol.* **2009**, *4*, 161–177.
(4) Radi, R.; Peluffo, G.; Alvarez, M. N.; Naviliat, M.; Cayota, A. *Free Radical Biol. Med.* **2001**, *30*, 463–488.
(5) (a) Wardman, P. *Free Radical Biol. Med.* **2007**, *43*, 995–1022. (b) Ueno, T.; Urano, Y.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 10640–10641. (c) Yang, D.; Wang, H. L.; Sun, Z. N.; Chung, N. W.; Shen, J. G. *J. Am. Chem. Soc.* **2006**, *128*, 6004–6005. (d) Yang, D.; Sun, Z. N.; Peng, T.; Wang, H. L.; Shen, J. G.; Chen, Y.; Tam, P. K. H. In *Live Cell Imaging: Methods and Protocols*; Papkovsky, D. B., Ed.; Humana Press: New York, 2009; Vol. 591, pp 93−103. (e) Sun, Z. N.; Liu, F. Q.; Chen, Y.; Tam, P. K. H.; Yang, D. *Org. Lett.* **2008**, *10*, 2171–2174.
(6) Peng, T.; Yang, D. *Org. Lett.* **2010**, *12*, 496–499.

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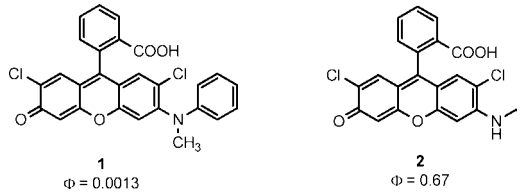
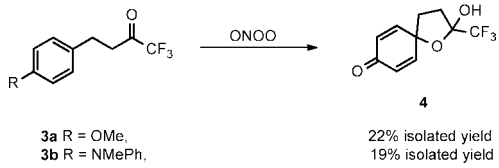


Figure 1. The distinct fluorescence property of *N*-phenylrhodol and *N*-methylrhodol. The fluorescence quantum yields were determined in 50 mM phosphate buffer at pH 8.0, with rhodamine 6G (Φ = 0.76 in water) as reference.

fluorescence difference of these two fluorophores (**1** vs **2**, Figure 1) suggests a strategy to develop a novel fluorescence "turn-on" switch, i.e., to convert **1** to **2** by breaking the covalent bond between the nitrogen and the phenyl ring through a chemical reaction. The cleavage of the covalent bond between the nitrogen atom and an anyl ring is certainly not easy; however, we expected that the strong oxidizing property of peroxynitrite could make it possible.

Previously, our group reported that peroxynitrite could react with anisole- or diarylether-derived activated ketones, **3a** for example, to provide product **4** (Scheme 1).[5c,7] To test

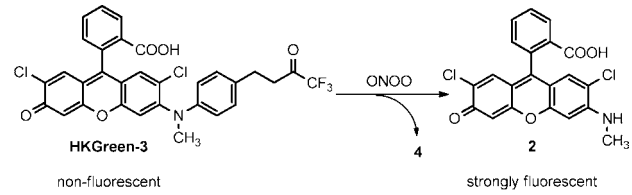
Scheme 1. Oxidations of Anisole- and Diarylamine-Derived Activated Ketones by Peroxynitrite



whether peroxynitrite could break the N−C(phenyl) bond of diarylamine, we first synthesized a diarylamine-derived ketone **3b** bearing the trifluoromethyl ketone unit (Schemes 1 and S1 in the Supporting Information). When **3b** reacted with peroxynitrite, the same dienone product **4** was isolated (Scheme 1), implicating that the N−C(phenyl) covalent bond was indeed cleaved by the oxidation, the mechanism of which may be similar to that we proposed before.[7b] Furthermore, we also found that the reactions of **3b** with other reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as H_2O_2, $^1O_2$, $^{\bullet}NO$, $O_2^{\bullet-}$, $ROO^{\bullet}$, $^{\bullet}OH$, and OCl^-, could not occur in a similar manner as peroxynitrite, suggesting that the diarylamine-derived ketone **3b** reacts with peroxynitrite in a specific way.

With this information in hand, we next designed and synthesized a new fluorescent probe, named **HKGreen-3**, by incorporating the diarylamine-derived ketone into the rhodol scaffold (Scheme 2). We expected that peroxynitrie

Scheme 2. Reaction of **HKGreen-3** with Peroxynitrite



would react with the trifluoromethyl ketone and further lead to *N*-dearylation, along with the release of highly fluorescent compound **2**.

Then we assessed the spectroscopic properties of **HKGreen-3** under simulated physiological conditions (0.1 M phosphate buffer, pH 7.4). The probe solution displays one major absorption peak at 520 nm (Figure S1 in the Supporting Information), indicating that the probe mainly exists as the lactone-open form in aqueous media. In the absence of peroxynitrite, **HKGreen-3** exhibits almost negligible background fluorescence (Φ < 0.0001, Figure 2a) as

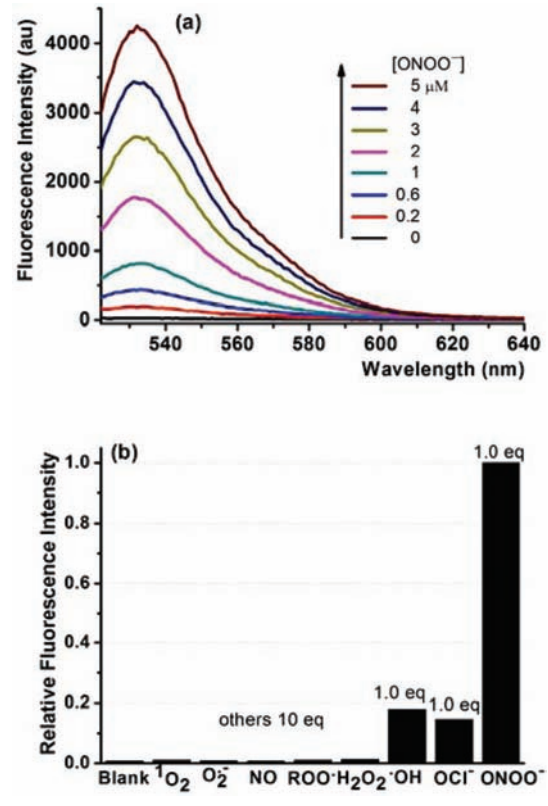


Figure 2. (a) Fluorescence response of 5 μM **HKGreen-3** to different amounts of $ONOO^-$. (b) Relative fluorescence intensity (λ_{em} = 535 nm) of 5 μM **HKGreen-3** in the presence of various ROS and RNS. Data were acquired at 25 °C in 0.1 M phosphate buffer, pH 7.4, with excitation at 520 nm.

expected. The addition of peroxynitrite resulted in dramatic increases of the fluorescence intensity with a maximum at

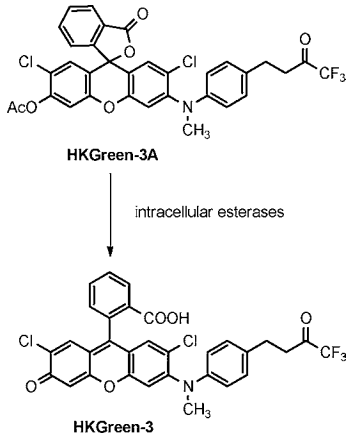
(7) (a) Yang, D.; Tang, Y. C.; Chen, J.; Wang, X. C.; Bartberger, M. D.; Houk, K. N.; Olson, L. *J. Am. Chem. Soc.* **1999**, *121*, 11976–11983. (b) Yang, D.; Wong, M. K.; Yan, Z. *J. Org. Chem.* **2000**, *65*, 4179–4184.

535 nm in a dose-dependent manner (Figure 2a). The reaction of 5 μM **HKGreen-3** with 1 equiv of peroxynitrite triggered a 140-fold fluorescence turn-on response. The fluorescence increases of **HKGreen-3** were not saturated until addition of more than 15 equiv of peroxynitrite (data not shown). A linear relationship between the fluorescence intensity and the peroxynitrite concentration was also observed (Figure S2 in the Supporting Information). The detection limit of the probe for peroxynitrite was estimated to be below 50 nM as 50 nM peroxynitrite could cause a one-fold increase of the fluorescence of the probe. HPLC and LC-MS analyses established that the oxidation of **HKGreen-3** by peroxynitrite indeed generates *N*-methylrhodol **2** as the fluorescent product in 12% yield (Figures S3 and S4 in the Supporting Information).

Owing to the specific reaction between the diarylamine-derived ketone and peroxynitrite, **HKGreen-3** displays highly selective response to peroxynitrite. As shown in Figure 2b, most of the biologically relevant ROS and RNS, including H_2O_2, $^1O_2$, $^{\bullet}NO$, $O_2^{\bullet-}$, and $ROO^{\bullet}$, no matter present in 1 equiv or 10 equiv, only caused negligible fluorescence increases of the probe. Moreover, **HKGreen-3** shows a >6-fold higher response toward peroxynitrite ($ONOO^-$) than toward hydroxyl radical ($^{\bullet}OH$) or hypochlorite (OCl^-).

To demonstrate the ability of **HKGreen-3** to image endogenous peroxynitrite, we chose cultured murine RAW 264.7 macrophages because macrophages are well-known to generate ROS and RNS in immunological and inflammatory processes.[8,9] Considering that the anionic nature of **HKGreen-3** in aqueous media might limit its permeability through cell membrane, we prepared its acetate derivative, **HKGreen-3A** (Scheme 3), anticipating that this more lipophilic derivative would have better permeability and be converted back to **HKGreen-3** by the action of intracellular esterases.[10] RAW 264.7 macrophages were first stimulated with different reagents, and incubated with **HKGreen-3A** (10 μM) for 2 h. Then the cells were monitored using a confocal fluorescence microscope. Without stimulation no fluorescence in the cells was observed (Figure 3a), while strong green fluorescence in the cytoplasm was imaged (Figure 3b) after treatment with stimulants including lipopolysaccharide (LPS, 1 μg/mL), interferon-γ (IFN-γ, 50 ng/mL), and phorbol 12-myristate 13-acetate (PMA, 10 nM). Moreover, we found that the bright fluorescence was suppressed (Figures 3c and 3d) by pretreatment of the cells with either a scavenger of superoxide, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, 100 μM), or an NO synthase inhibitor, aminoguanidine (AG, 1 mM).[11] Since a NO synthase inhibitor can hardly attenuate the generation of H_2O_2,[12] thus that of $^{\bullet}OH$ or OCl^-, and a superoxide scavenger

Scheme 3. Conversion of Cell Membrane Permeable **HKGreen-3A** to **HKGreen-3** by Intracellular Esterases



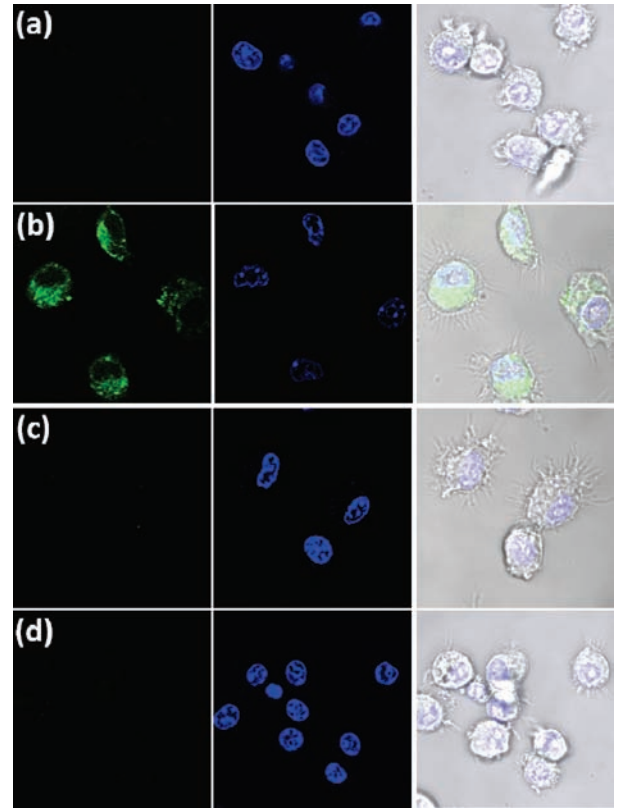


Figure 3. Confocal fluorescence imaging of live RAW 264.7 macrophage cells. The cells were treated with various stimulants, and then stained with **HKGreen-3A** (green channel) and Hoechst 33342 (blue channel). (a) Cells without stimulation. (b) Cells stimulated with LPS, IFN-γ, and PMA. (c) Cells pretreated with TEMPO, and then stimulated with LPS, IFN-γ, and PMA. (d) Cells pretreated with AG, and then stimulated with LPS, IFN-γ, and PMA.

(8) Muijsers, R. B. R.; 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.

(9) Salonen, T.; Sareila, O.; Jalonen, U.; Kankaanranta, H.; Tuominen, R.; Moilanen, E. *Br. J. Pharmacol.* **2006**, *147*, 790–799.

(10) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446–2453.

(11) Muijsers, R. B. R.; 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.

would not affect the production of simple RNS, apparently it is peroxynitrite, rather than other ROS or RNS, which induces the strong fluorescence in the macrophages. Similarly, **HKGreen-3A** was also used for imaging peroxynitrite in J744.1 macrophages (Figure S5 in the Supporting Information). Besides, nuclear staining with Hoechst 33342 (Figure 3) and MTT assays (Figure S6 in the Supporting Information)[13] revealed that **HKGreen-3A** of concentrations below 20 μM does not exhibit obvious cytotoxicity.

In summary, we present herein a novel fluorescent probe **HKGreen-3** and its ester derivative **HKGreen-3A** for the highly sensitive and selective detection and cellular imaging of peroxynitrite. These probes were designed on the basis of a peroxynitrite specific reaction and the rhodol scaffold. We anticipate that the probe with excellent spectroscopic properties will find its applications in understanding the actions of peroxynitrite in biological systems.

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

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(12) Domaille, D. W.; Que, E. L.; Chang, C. J. *Nat. Chem. Biol.* **2008**, *4*, 168–175.

(13) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–64.