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A Highly Selective Fluorescent Probe for the Detection and Imaging of Peroxynitrite in Living Cells

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Peroxynitrite ($ONOO^-$), a highly reactive oxidant that is generated through the spontaneous reaction of nitric oxide (NO) and superoxide ($O_2^{\bullet-}$), has attracted much attention ever since Beckman and co-workers first proposed its formation in vivo in 1990.[1] Peroxynitrite and its secondary metabolites ($^\bullet NO_2$, $CO_3^{\bullet-}$, and $^\bullet OH$) can effect tyrosine nitration[2−6] and oxidation of nucleic acids, sulfhydryls, lipids, and amino acids, all of which may result in serious damage to living cells.[7,8] As a result, the production of peroxynitrite in biological systems is implicated with a series of human diseases, including acute and chronic inflammatory processes,[9] ischemic stroke,[10] diabetes,[11] sepsis,[12] ischemia-reperfusion,[13] atherosclerosis,[14] and neurodegenerative disorders (ALS, Lou Gehrig's disease, and Alzheimer's disease).[15]

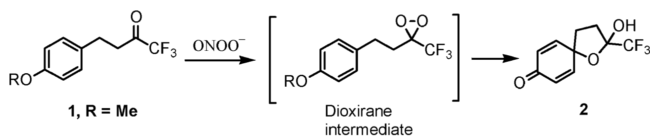
Unfortunately, the short half-life (<20 ms in a typical physiological environment) and the multiple reaction pathways of peroxynitrite in biological systems preclude its direct isolation and detection. To date, no methods are available for the direct detection of peroxynitrite with complete specificity and high sensitivity;[16] indeed, identifying and quantifying peroxynitrite formation necessitates performing tedious and time-consuming control experiments using a combination of scavengers and inhibitors. Therefore, there is a need to develop highly sensitive and specific methods for the detection of peroxynitrite and, more importantly, to allow the direct analysis of peroxynitrite production in vivo.

Our design concept was inspired by the similar chemical behavior displayed by $ONOO^-$ and peroxymonosulfate ($HOOSO_3^-$), the commercial source of which is Oxone. We demonstrated previously that peroxynitrite reacts with activated ketones to form dioxiranes in a manner similar to that by which ketones react with peroxymonosulfate.[17,18] Furthermore, when the anisole-derived ketone **1** was reacted with peroxynitrite, it formed the same dienone product (**2**), albeit in lower conversion and yield (44% yield based on 50% conversion), as did the reaction between **1** and peroxymonosulfate (Scheme 1). This result implies that peroxynitrite oxidized ketone **1** in situ to form dioxirane intermediate, which subsequently oxidized the phenyl ring to afford **2**.

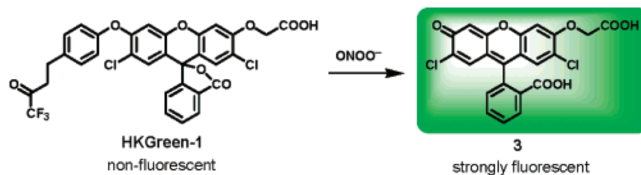
More promisingly, we found that such reactions did not occur between ketone **1** and the other reactive oxygen species (ROS) or reactive nitrogen species (RNS), such as H_2O_2, $^1O_2$, $^\bullet OH$, NO, $O_2^{\bullet-}$, ^-OCl, and $ROO^\bullet$ (i.e., ketone **1** displays considerable specificity toward peroxynitrite). This phenomenon provides a unique opportunity to develop a chemical tool to monitor peroxynitrite in a specific manner. In this regard, fluorescent probes are well suited as reagents that allow the cellular chemistry of peroxynitrite to be examined at the molecular level.

We envisioned that fluorescent probes for peroxynitrite could be designed by replacing the methoxy group of **1** with a fluorescent chromophore. Upon treatment with peroxynitrite in situ, the ketone

Scheme 1. Peroxynitrite-Mediated Oxidation of an Anisole-Derived Ketone via a Dioxirane Intermediate



Scheme 2. Reaction of **HKGreen-1** with Peroxynitrite



unit of the probe would generate a dioxirane that would selectively oxidize the phenyl ring to afford dienone product and, more importantly, release the fluorescent molecule. Dichlorofluorescein (DCF) is an ideal fluorophore because its excitation and emission wavelengths occur in the visible range, it exhibits a high fluorescence quantum yield, and it has good stability toward oxidants, especially peroxynitrite. Thus, we synthesized the probe **HKGreen-1** (Supporting Information), which possesses a ketone unit linked to a DCF moiety through an aryl ether linkage. The introduction of the CH_2COOH group not only decreases the fluorescence background of the probe in cells but also increases the solubility of the probe in aqueous solution. We expected that the virtually nonfluorescent probe **HKGreen-1** (fluorescence quantum yield: $\Phi = 0.05$ at 0.1 M in pH = 7.3 buffer solution) would yield the strongly fluorescent product **3** upon its reaction with peroxynitrite (Scheme 2). In fact, similar to DCF, **3** is a strong fluorophore; it has a quantum yield of 0.46 at 0.1 M in buffer solution (pH = 7.3).

Initially, we investigated the reactivity of **HKGreen-1** toward peroxynitrite in an abiotic system. Various amounts of 20 μM peroxynitrite in 0.1 M sodium hydroxide solution were added to buffer solutions of **HKGreen-1** that were maintained in the dark. After 15 min, the fluorescence emission of each reaction mixture was measured using a fluorescence spectrometer. As indicated in Figure 1A, the fluorescence intensity increased gradually upon increasing in peroxynitrite concentration (e.g., a ca. 7- to 8-fold enhancement in fluorescence intensity occurred after adding 15 equiv of peroxynitrite to **HKGreen-1** solution; Figure 1A). Moreover, we observed a linear correlation between the emission intensity and the concentration of peroxynitrite (Figure 1A, inset). This result demonstrates that, in abiotic systems, our probe **HKGreen-1** could detect peroxynitrite both qualitatively and quantitatively.

To investigate the specificity of **HKGreen-1** toward peroxynitrite, we tested its response toward various oxidative species that are present in biological systems, including NO, $^1O_2$, $O_2^{\bullet-}$, $^\bullet OH$,

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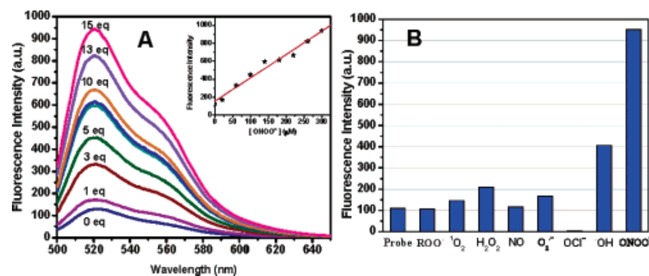

Figure 1. (A) Fluorescence response of 20 μM **HKGreen-1** to $ONOO^-$. Spectra were acquired in 0.1 M potassium phosphate buffer at pH 7.3 (λ_{ex} = 490 nm). Inset: A linear correlation between emission intensity and concentrations of $ONOO^-$. (B) Fluorescence intensity of **HKGreen-1** (20 μM) in various ROS/RNS (300 μM) generating systems at 25 °C for 1 h. The fluorescence intensity was determined at 521 nm with excitation at 490 nm (slit width 2.5 nm).

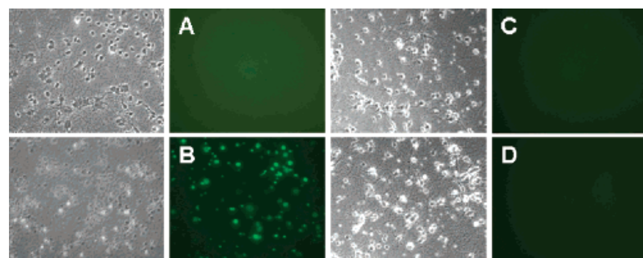

Figure 2. Fluorescence imaging of primary cultured neuronal cells. Left: phase contrast imaging. Right: fluorescence imaging. The neuronal cells were incubated with **HKGreen-1** (20 μM) for 15 min and then subjected to different treatments. (A) Control. (B) 10 μM of SIN-1. (C) 10 μM of SNAP. (D) 100 μM of xanthine and 0.1 IU of xanthine oxidase.

$ROO^{\bullet}$, ^{-}OCl, and $ONOO^-$. Figure 1B indicates that a 7- to 8-fold increase in fluorescence intensity was observed when **HKGreen-1** reacted with 15 equiv of peroxynitrite, whereas the reactions with NO, $^1O_2$, $O_2^{\bullet-}$, and H_2O_2 each led to an increase that was less than 1-fold. Although the presence of $^{\bullet}OH$ induced a ca. 3-fold increase of fluorescence intensity, this response was weaker than that which occurred in the presence of peroxynitrite. Thus, **HKGreen-1** appears to be a highly selective fluorescent probe for the detection of peroxynitrite.

Next, we used primary cultured neuronal cells to investigate the potential of **HKGreen-1** for use in the detection of peroxynitrite in living cells.[19] We monitored the fluorescence images from neuronal cells treated with different oxidants, namely the peroxynitrite donor SIN-1, the NO donor SNAP, and the superoxide donor xanthine/xanthine oxidase. The neuronal cells were incubated with **HKGreen-1** (20 μM) for 15 min and then washed three times with PBS buffer. As expected, we did not observe any fluorescent cells in the absence of stimulants (Figure 2A). Strong fluorescence in the neuronal cells was induced after treatment with SIN-1 (10 μM) (Figure 2B), but not with either SNAP (Figure 2C) or xanthine/xanthine oxidase (Figure 2D). Thus, we conclude that **HKGreen-1** can be used for the selective detection of peroxynitrite produced in cultured cells.

In summary, we have developed a new fluorescent probe **HKGreen-1** that is effective for the highly selective detection of peroxynitrite in living cells. We anticipate that this simple, sensitive fluorescent probe will be of great benefit for biomedical researchers investigating the effects of peroxynitrite in biological systems.

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Supporting Information Available: Experimental details on the synthesis and characterization of **HKGreen-1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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