Two-photon fluorescent probe for peroxynitrite

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Peroxynitrite (ONOO\textsuperscript{−}), a strong oxidant found in biological systems, causes oxidation of biomolecules involved in a variety of physiological and pathological processes. Many reports suggest that an increase in ONOO\textsuperscript{−} levels is related to many diseases, such as cardiovascular, neurodegenerative, inflammatory diseases, metabolic diseases, pain, and cancer.\textsuperscript{1} Moreover, ONOO\textsuperscript{−} has been reported to play important roles in immune responses and redox regulation of signaling pathways.\textsuperscript{2} However, the effects of ONOO\textsuperscript{−} are not clearly understood owing to its short half-life (<20 ms) under typical physiological conditions and low concentration.\textsuperscript{1} It is therefore challenging and highly desired to develop novel chemical tools for detecting peroxynitrite.

Fluorescence probes have been developed as powerful tools for peroxynitrite detection since they can detect subcellular ONOO\textsuperscript{−} directly.\textsuperscript{3–9} However, these probes require short excitation wavelengths ranging from ultraviolet (UV) to visible light, limiting their use in tissues or animals owing to cellular autofluorescence, artificial reactive oxygen species (ROS) generation, and shallow tissue penetration depth.\textsuperscript{10}

Over the past decade, two-photon microscopy (TPM), which utilizes two near-infrared (NIR) photons as the excitation source, has become a useful tool for biomedical research, offering several advantages including localized excitation, reduced photodamage, longer observation time, and greater tissue penetration depth.\textsuperscript{11–13} Nevertheless, only few peroxynitrite probes that are two-photon excitable have been reported.\textsuperscript{8,14} They can detect peroxynitrite in live cells and animals using two-photon microscopy. However, a two-photon peroxynitrite probe with improved two-photon absorbing property is still needed for further exploration.

Herein, we report a new two-photon probe that sensitively and selectively detects ONOO\textsuperscript{−} among other ROS/RNSs (reactive oxygen/nitrogen species) using peroxynitrite-triggered dearylation reaction. Peroxynitrite was recently reported to trigger oxidative N-dearylation reaction, which can be used to generate a fluorescence turn-on response.\textsuperscript{8} \textbf{JH-PN4} is highly selective and sensitive to ONOO\textsuperscript{−} and has better two-photon excitation properties compared to a previously reported probe.\textsuperscript{14} \textbf{JH-PN4} is derived from 2-methylamino-6-acetylnaphthalene (acedan derivative, compound 1), a well-known two-photon fluorophore, as a reporting group\textsuperscript{15} and N-methyl-p-hydroxyniline as a targeting moiety. N-Aryl group can quench the fluorescence of the acedan derivative efficiently and be eliminated by ONOO\textsuperscript{−} with good selectivity among other ROS/RNSs.\textsuperscript{8} The fluorescence of compound 1 can be recovered by dearylation, which leads to a ‘push–pull’ structure of compound 1. \textbf{JH-PN4} was synthesized according to Scheme 1B. The detailed synthetic procedures, nuclear magnetic resonance (NMR) spectra and high resolution mass spectrometry (HRMS) spectra are displayed in the Supporting information (Scheme 1A). We also synthesized probes \textbf{JH-PN1–3} to perform systematic studies on the N-, O-substituent effects.

With \textbf{JH-PN1–4}, we tested their selectivity toward ONOO\textsuperscript{−} among other ROS/RNSs in 10 mM phosphate buffer saline (PBS, 0.4% dimethyl formamide (DMF), pH 7.4). As expected, \textbf{JH-PN1} and \textbf{JH-PN3} having a methoxymethyl ether group did not give any remarkable response to ONOO\textsuperscript{−} and other ROS species (See the intensity range in the y-axis of Fig. S1A and C). \textbf{JH-PN2}, having a diarylamino (Ar–NH–Ar) group, showed only a slight increase in

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the fluorescence intensity upon the addition of ONOO⁻ or other ROS species (Fig. S1B). This is probably due to oxidation of diarylamine by ONOO⁻. Different fluorescence responses of JH-PN1–4 to ONOO⁻ can be explained by the N-substituent effects according to a recent systematic study on the fluorescence intensities of acedan derivatives depending on the amine donor structure.

However, the fluorescence intensity of JH-PN4 at 509 nm increased more than 32-fold when 2 equiv of ONOO⁻ were added. There was no significant change with other excess ROS/RNSs (Fig. 1). Although nitric oxide (NO) showed a slight increase in fluorescence intensity, it is negligible because we added a large amount of NO (2 mM), which cannot exist in a biological condition and fluorescence change is small compared to the change with ONOO⁻. Thus, further experiments were performed with JH-PN4.

To determine the sensitivity of JH-PN4, fluorescence titration was performed with ONOO⁻ (Fig. 2). As expected, the fluorescence intensity of JH-PN4 at 509 nm increased more than 57-fold when 5 equiv of ONOO⁻ were added (Fig. 2A), which resulted from the elimination of a quencher by dearylation. The detection limit of JH-PN4 was estimated to be as low as 359 nM (Figs. 2B and S2), which means that JH-PN4 has high sensitivity to ONOO⁻. However, the fluorescence intensity decreased when more than 5 equiv of ONOO⁻ were added. This phenomenon is probably due to further oxidation of 1 generated by ONOO⁻ triggered dearylation of JH-PN4. Compound 1, having a secondary amine, can be oxidized with excess ONOO⁻, which reduces the fluorescence of 1. The maximum fluorescence intensity of JH-PN4 upon addition of 5 equiv ONOO⁻ is weaker than the fluorescence intensity of compound 1 itself. Since the emission spectra of the mixture of JH-PN4 and ONOO⁻ are almost identical to that of 1 itself, we can assume that 1 is produced by the dearylation of JH-PN4, but the dearylation of...
Figure 2. (A) Fluorescence spectra and (B) fluorescence intensity at 508 nm of JH-PN4 (20 μM) with ONOO⁻ (0–8 equiv). Data were acquired at 25 °C in 10 mM phosphate buffer (0.4% dimethyl formamide (DMF)) at pH 7.4 with excitation at 360 nm. Reactions were carried out for 30 min at room temperature before the fluorescence intensities of the probe solutions were measured.

Figure 3. (A) Time-dependent fluorescence change. (B) Time course of fluorescence intensity (λem = 508 nm) of JH-PN4 (10 μM) in 10 mM phosphate buffer saline (PBS) (0.2% dimethyl formamide (DMF), pH 7.4) in the presence of 1.0 equiv ONOO⁻ (λex = 360 nm).

Figure 4. (A) Two-photon fluorescence spectra of JH-PN4 (10 μM) with ONOO⁻. Inset shows two-photon fluorescence titration curve for JH-PN4 with ONOO⁻. The excitation wavelength was 740 nm. (B) Two-photon action cross section spectrum of probe JH-PN4 in the absence (■) and in the presence (○) of ONOO⁻. These data were measured in 10 mM phosphate buffer saline (PBS).
**JH-PN4** by ONOO$^-\,$ is not efficient enough to recover the original fluorescence intensity of compound 1.

Next, we tested time-dependent fluorescence change in **JH-PN4** in the presence of 1 equiv ONOO$^-\,$ (Fig. 3). **JH-PN4** rapidly responded to ONOO$^-\,$ within 3 s and saturation time was less than 10 min (Fig. 3B) showing that **JH-PN4** can be a useful tool for detecting ONOO$^-\,$ whose half-life in biological conditions is very short.

We also investigated the fluorescence responses of **JH-PN4** to ONOO$^-\,$ with two-photon excitation mode (Fig. 4). As expected, fluorescence turn-on response to ONOO$^-\,$ was observed with two-photon excitation (Fig. 4A). Maximum two-photon absorption cross section ($\sigma_{\text{max}}$) of **JH-PN4** with 3 equiv of ONOO$^-\,$ was 8.8 GM at 740 nm, which is an improved value compared with a previous reported probe. Moreover, the value of two photon action cross section increased about 4 times with 3 equiv of ONOO$^-\,$ (Fig. 4B). Two-photon excited fluorescence responses with ONOO$^-\,$ support that **JH-PN4** can be a useful tool for ONOO$^-\,$ imaging with two-photon microscopy.

To test the usability of **JH-PN4** as a ONOO$^-\,$ probe, the fluorescence of **JH-PN4** and product 1 was measured in PBS at various pH values ranging from 4 to 9 (Fig. 5). As shown in Figure 5, significant change was not observed under various pH conditions. The fluorescence intensity of **JH-PN4** at 509 nm slightly increased at basic condition (>pH 9), but it was negligible compared to the change in fluorescence intensity with ONOO$^-\,$ addition. The fluorescence intensity of product 1 decreased at pH 4, but it was also negligible. This suggests that **JH-PN4** has good stability under various pH ranges and usability in physiological condition.

In conclusion, we developed a two-photon ONOO$^-\,$ turn-on sensor using peroxynitrite-triggered dearylation. **JH-PN4** shows good sensitivity and selectivity for ONOO$^-\,$ among other ROS/RNSs and improved two-photon excitation properties compared with previous reported probes. These results show that **JH-PN4** can be a useful tool for peroxynitrite imaging in biosystems.

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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.tetlet.2016.01.025](http://dx.doi.org/10.1016/j.tetlet.2016.01.025).

**References and notes**