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Two-photon fluorescent probe for peroxynitrite

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ABSTRACT

Peroxynitrite ($ONOO^-$), a strong oxidant found in biological systems, and an increase in its levels is related to numerous diseases, immune responses, and redox regulation of signaling pathways. Herein, we report a new two-photon probe that sensitively and selectively detects $ONOO^-$ among other ROS/RNSs (reactive oxygen/nitrogen species) using peroxynitrite-triggered dearylation reaction.

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Peroxynitrite ($ONOO^-$), 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 $ONOO^-$ levels is related to many diseases, such as cardiovascular, neurodegenerative, inflammatory diseases, metabolic diseases, pain, and cancer.[1] Moreover, $ONOO^-$ has been reported to play important roles in immune responses and redox regulation of signaling pathways.[2] However, the effects of $ONOO^-$ are not clearly understood owing to its short half-life (<20 ms) under typical physiological conditions and low concentration.[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 $ONOO^-$ directly.[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.[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.[11–13] Nevertheless, only few peroxynitrite probes that are two-photon excitable have been reported.[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 probe—**JH-PN4**—that selectively detects $ONOO^-$ 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.[8] **JH-PN4** is highly selective and sensitive to $ONOO^-$ and has better two-photon excitation properties compared to a previously reported probe. [14]

JH-PN4 is derived from 2-methylamino-6-acetylnaphthalene (acedan derivative, compound **1**), a well-known two-photon fluorophore, as a reporting group[15] and *N*-methyl-*p*-hydroxyaniline as a targeting moiety. *N*-Aryl group can quench the fluorescence of the acedan derivative efficiently and be eliminated by $ONOO^-$ with good selectivity among other ROS/RNSs.[8] The fluorescence of compound **1** can be recovered by dearylation, which leads to a 'push–pull' structure of compound **1**. **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 **JH-PN1–3** to perform systematic studies on the N-, O-substituent effects.

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

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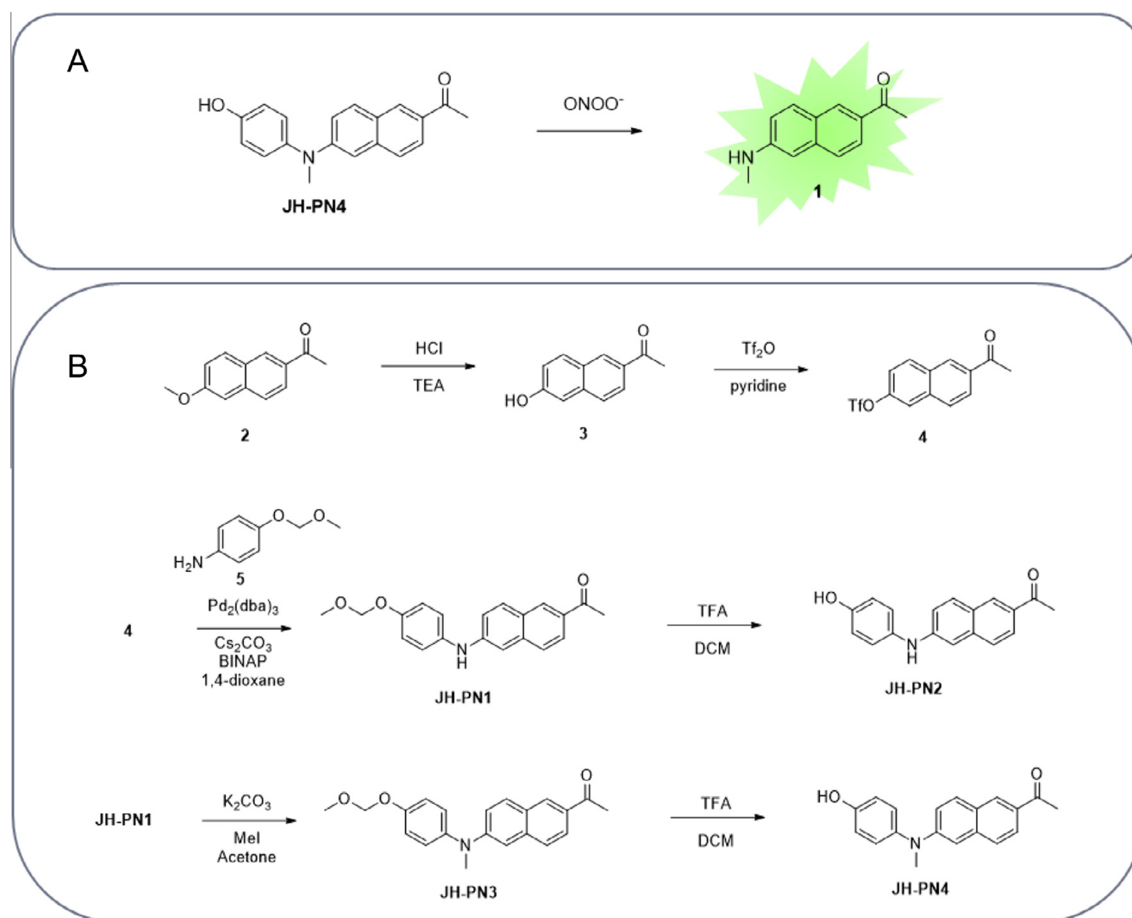

Scheme 1. (A) Sensing mechanism and (B) synthesis of probes **JH-PN1–4**. TEA = trimethylamine, Tf_2O = trifluoromethanesulfonic anhydride, dba = dibenzylideneacetone, BINAP = (±) 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl, TFA = trifluoroacetic acid, DCM = dichloromethane.

the fluorescence intensity upon the addition of $ONOO^-$ or other ROS species (Fig. S1B). This is probably due to oxidation of diarylamine by $ONOO^-$.[16] 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.[17]

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**.[8,16] 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

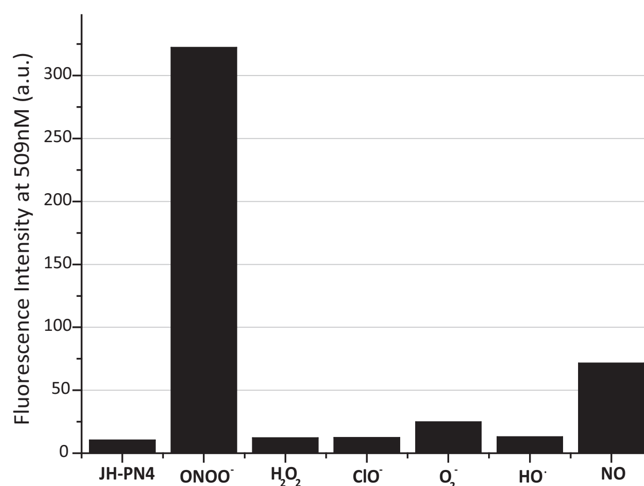

Figure 1. Fluorescence intensity (λ_{em} = 509 nm) of **JH-PN4** (20 μM) with various reactive oxygen/nitrogen species [ROS/RNSs, 2.0 equiv of $ONOO^-$, 10 equiv of other ROSs, and 100 equiv of nitric oxide (NO)]. Data were acquired at 25 °C in 10 mM phosphate buffer with 0.4% dimethyl formamide (DMF) at pH 7.4 with excitation at 360 nm. Reactions were carried out for 1 h at room temperature before the fluorescence intensities of the probe solutions were measured.

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