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A selective phosphine-based fluorescent probe for nitroxyl in living cells

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ABSTRACT

A novel fluorescein-based fluorescent probe for nitroxyl (HNO) based on the reductive Staudinger ligation of HNO with an aromatic phosphine was prepared. This probe reacts with HNO derived from Angeli's salt and 4-bromo Piloty's acid under physiological conditions without interference by other biological redox species. Confocal microscopy demonstrates this probe detects HNO by fluorescence in HeLa cells and mass spectrometric analysis of cell lysates confirms this probe detects HNO following the proposed mechanism.

The discovery and establishment of nitric oxide (NO) as an important signaling agent involved in many physiological processes including blood pressure control, neurotransmission, and the immune response initiated the further study of the biological roles of many redox-related nitrogen-containing compounds.[1–5] Nitroxyl (HNO), the one-electron reduced and protonated derivative of NO, possesses distinguishable physiological and pharmacological properties from NO.[6–8] HNO-releasing pro-drugs increase cardiac inotropy and lusitropy and elicit arterial and venous dilation without building tolerance, properties that make these compounds intriguing candidates for the treatment of congestive heart failure.[9–16] These cardiac outcomes occur via selective and covalent thiol modification that increases myocardial calcium cycling and enhances the calcium sensitivity of the myofilament.[17–19] Such biological properties drive the search for new chemical HNO donors as well as the definition of an endogenous biochemical pathway of HNO formation.[20–28]

Development of new HNO donors and understanding endogenous HNO production requires robust HNO detection methods. Early methods of HNO detection (e.g., identification of N_2O, trapping with thiols or ferric heme proteins) either lack the sensitivity or the selectivity to unambiguously demonstrate endogenous HNO formation. These limitations have led to the development of new HNO detection strategies that include a group of Cu^{II}-based fluorescent complexes and HNO-specific electrodes.[29–33]

Organophosphines are suitable for HNO detection based on their documented ligation reaction with HNO, their rapid rate of HNO trapping and lack of cross-reactivity with other physiologically relevant nitrogen oxides, such as NO, nitrite, nitrate, and peroxynitrite.[34–36] In light of this phosphine-mediated ligation chemistry described for HNO, we and others envisioned the development of phosphine probes with fluorophore leaving groups.[37–39] The reaction of HNO with two equivalents of phosphine nucleophiles produces phosphine oxides (**2**) and the corresponding phosphine azaylides (**3**), forming the chemical basis of these newly reported HNO detection strategies (Scheme 1) In the presence of an internal electrophilic ester, these azaylides undergo Staudinger ligation to yield the thermodynamically stable amide (**4**, Scheme 1) and the corresponding ester-derived alcohol. Based on this mechanism, we designed and synthesized **1**, which produces HNO-dependent fluorescence by generating the known fluorophore, fluorescein monomethyl ether (**5**, Scheme 1).

Compound **1** was prepared by the straightforward coupling of 2-(diphenylphosphino)benzoic acid with fluorescein methyl ether, a previously reported fluorescein derivative,[40] in 61% yield (Scheme 2).

We first investigated the feasibility of **1** to detect HNO in buffered solution. In the initial ligation experiment, solutions of **1**

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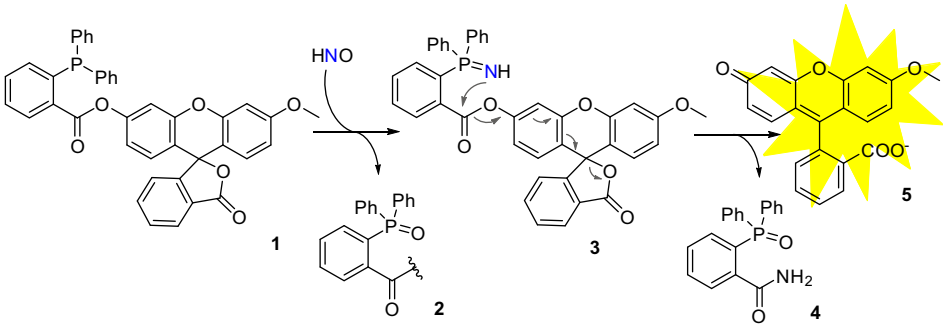
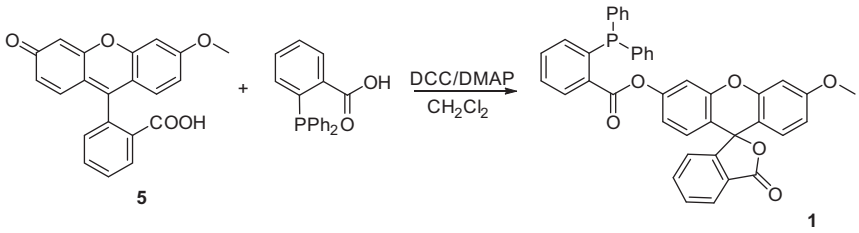


Scheme 1. Proposed mechanism of probe **1** with HNO.



Scheme 2. Synthesis of probe **1**.

(40 μM) were incubated with increasing concentrations of Angeli's salt (AS, 0–5 equiv) in 1:3 MeCN/PBS (containing 0.1 mM EDTA, pH 7.4) at ambient temperature and excitation at 465 nm led to a concentration-dependent increase in emission intensity at 520 nm with a 73.8-fold maximum response observed at 5 equiv AS (Fig. 1, panel A). The solution changed from clear to yellow over 20 min with a maximum fluorescence achieved at 40 min the and fluorescence intensity remained unchanged after 2 h (Fig. 1, panel B and SI Fig. S4). Such a time course may suggest that ligation is the rate determining step in fluorescence generation. Use of higher amounts of AS results in more intense fluorescence (Fig. 1). Control experiments with increasing amounts of nitrite, the by-product of AS decomposition, and **1** (40 μM) do not generate a fluorescence response, illustrating that the response depends on the HNO-mediated release of the fluorophore.

The commercial availability, water solubility and rapid HNO release rate make AS the donor of choice for most chemical and biological studies, including recent studies regarding HNO detection with both phosphine and Cu^{II}-based fluorophores.[30–33,37–39] Treatment of **1** with *p*-bromo Piloty's acid, a structurally distinct HNO donor, also results in fluorescence enhancement proportional to *p*-bromo Piloty's acid concentration (SI Fig. S5). The slower continual release of HNO from this compound[41] may better mimic its endogenous production and may result in the slightly decreased observed fluorescence response. These results demonstrate HNO detection by **1** from a source besides AS.

Before attempting HNO detection in cells, **1** was assessed for the selectivity toward HNO compared to other biological redox species. Figure 2 shows the comparative fluorescence response of **1** to excess amounts of NO, NO_2^-, NO_3^-, H_2O_2, H_2S, GSH, *S*-nitrosoglutathione

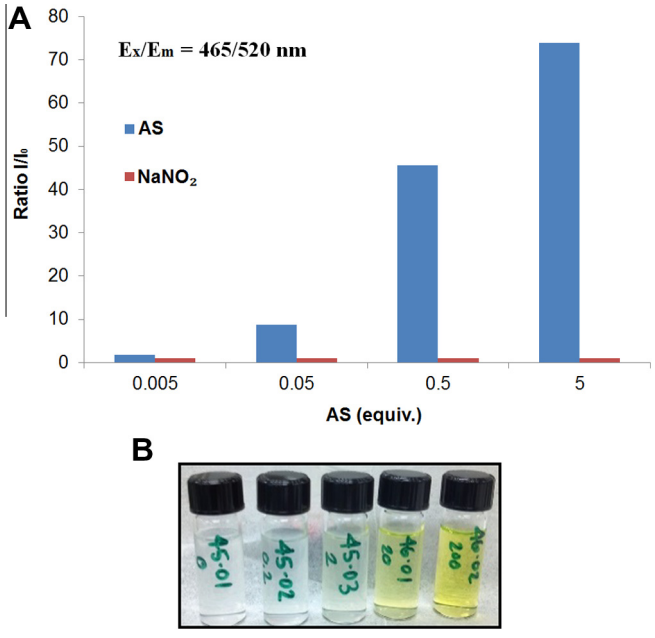


Figure 1. Panel A: fluorescence responses of **1** (40 μM) to 0.005, 0.05, 0.5, 5 equiv of AS or $NaNO_2$ in CH_3CN/PBS after 2 h incubation at room temperature. Panel B: the ligation induces a distinct color change.

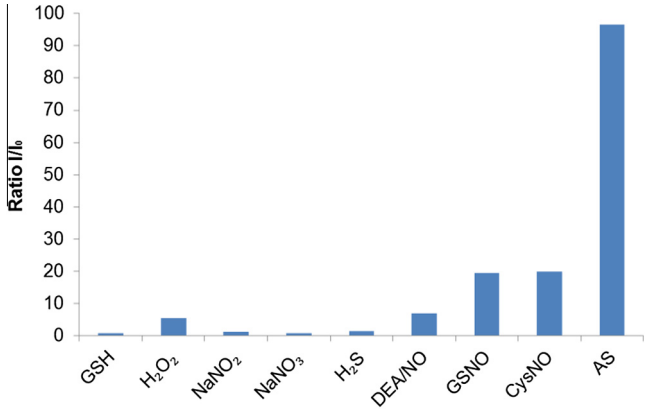


Figure 2. Fluorescence responses of **1** (40 μM) in CH_3CN/PBS at room temperature for 2 h after addition of 200 equiv of GSH, H_2O_2, $NaNO_2$, $NaNO_3$, Na_2S, DEA/NO, GSNO, CysNO, AS.