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Fluorogenic and Chromogenic Rhodamine Spirolactam Based Probe for Nitric Oxide by Spiro Ring Opening Reaction

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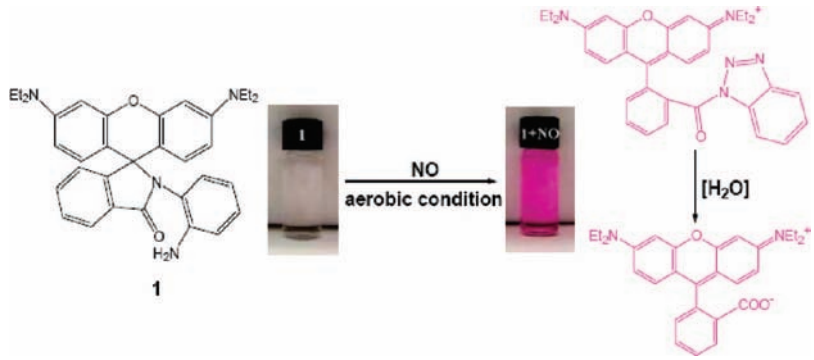
ABSTRACT



A new fluorogenic and chromogenic probe rhodamine B spirolactam (1) was developed that in aqueous solutions exhibited a highly selective and sensitive "turn-on" type spectral response toward NO detection following a NO-induced spiro-ring opening reaction in 1.

Many recent developments have shown that rhodamine spirolactam is a promising structural scaffold for the design of selective chemosensors. Upon metal binding, its structure can undergo a change from the spirolactam to an open ring amide, resulting in a magenta-colored, highly fluorescent compound.[1] Despite the large number of reports, use of this motif has mainly been limited to detection of metallic species. Yang et al. recently reported that rhodamine B hydrazide, a probe originally developed by Czarnik et al. for Cu^{2+},[1a] exhibited a fluorescence response to peroxynitrite.[2] Its reaction mechanism, however, remains unclear. Considering the interesting spectral characteristics of rhodamine spirolactam-based probes in sensing events, there is still room for the development of new spirolactam-based probes for efficient recognition and quantification of small inorganic and biologically important species under physiological conditions.

Nitric oxide (NO) has recently been increasingly recognized as an important biomolecule in a number of physiologically and biologically significant processes. It plays a critical role not only in the cardiovascular system but also in the central and peripheral nervous systems and in the immune systems.[3] Thus, NO is currently considered to be a vital bioregulatory molecule, and research dealing with NO

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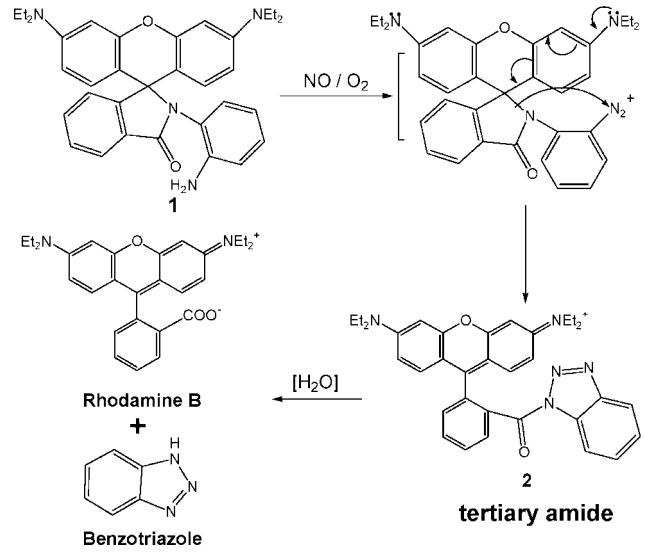
illustrates the need to monitor the NO concentration produced either *in vivo* or *in vitro* both rapidly and sensitively. Although many reactions and a number of techniques[4] have been reported for assaying NO, fluorimetry remains a promising means due to its high sensitivity and its ease of use in biological research, such as fluorescence imaging, for example. NO detection has two main mechanisms based on transition-metal and PET (photoinduced electron transfer) NO-sensitive modulators.[5] The latter method, in which small organic molecules detect reactive NO-derivative species rather than NO itself, have been applied to a range of biological research. Methods utilizing these mechanisms, however, suffer several disadvantages. First, synthesis of the probes involves complicated and low-yield multistep procedures. Second, the presence of blank signals of probes themselves may decrease the sensitivity of NO detection or hinder the performance of imaging research. And third, the probes lack obvious chromogenic behavior toward the target molecule. Therefore, there is still plenty of room for improvement in terms of selectivity, sensitivity, and performance based on a new luminescent mechanism.

Herein we report a new luminescent probe (compound **1**) for the detection of NO by a new intramolecular pathway. The probe exhibits "turn-on" type fluorogenic and chromogenic behavior toward NO in aqueous solution with great sensitivity and selectivity, and it can be used for the sensing of NO under normal physiological conditions. The probe is composed of two moieties: rhodamine B spirolactam as the potential strong fluorophore and chromophore, and *o*-phenylenediamine as an NO-reactive group in a lactam form as a "masked" NO-sensitive modulator. Based on this strategy, a highly sensitive and selective colorimetric and fluorimetric sensing system was developed for NO in aqueous solution. The synthetic procedure of compound **1** is briefly expressed in Figure S1 (see Supporting Information).

The new probe **1** was prepared in high yield from rhodamine B by using a "one-pot" synthetic procedure ($POCl_3$, reflux; then o-phenylenediamine, NEt_3, CH_3CN, 70%). A colorless and nonfluorescent product was obtained after crystallization from CH_2Cl_2/CH_3CN. The resultant compound is also colorless in aqueous solution at pH 7.4, suggesting that the spirocyclic form is predominant. The single crystal structure of **1** was determined by X-ray crystallography (Figure S2, Supporting Information), and it clearly supports this unique spirolactam-ring form.

The sensing process of NO by the proposed probe is briefly expressed in Scheme 1. The sensing mechanism of NO is

Scheme 1. NO-Induced Ring Opening of **1**



remarkably different from that produced by o-phenylenediamine-based PET-processes.[5k–n] As shown in Scheme 1, diazotization of the amino group of compound **1** is caused by the attack of NO, thus inducing the opening of the spiro ring to produce rhodamine B acylbenzotriazole (compound **2**), a species of rhodamine B tertiary amide that displays strong absorption and fluorescence characteristics similar to rhodamine B.[6] Compound **2** is unstable in aqueous solution and further hydrolyzes to form rhodamine B and benzotriazole. As a result, the concentration of NO can be detected by spectrophotometry or fluorimetry. In reality, the reactive species in the above-mentioned attack is thought to be NO^+ equivalent, which is generated by rapid autoxidation of NO under aerobic conditions in the physiological environment.[7] Therefore, compound **1** is a reactive NO-derivative probe for the indirect determination of NO concentration.

As expected, **1** alone in 0.10 M pH 7.40 sodium phosphate buffer solution (H_2O-CH_3CN = 80/20, v /v)[8] exhibited neither absorption nor fluorescence peaks in the longer wavelength range (λ > 400 nm), and existed as a colorless solution due to its spiro-ring structure. Addition of NOC13,

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(8) Complementary experiments showed that probe **1** also worked well toward NO sensing by using DMSO as a co-solvent ($H_2O-DMSO$ = 99/1, v/v).

a NO carrier,[9] to **1** at 37 °C caused development of a strong absorbance peak at ~554 nm and a strong fluorescence peak at $\lambda_{em} \approx 574$ nm, which are similar to the absorption and emission peaks of rhodamine B chromophore (Figure 1), a

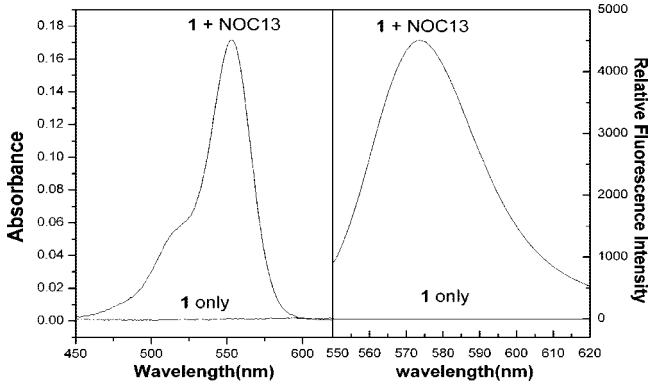

Figure 1. Absorbance spectra and fluorescence emission spectra (excitation at 540 nm) of compound **1** (10 μM) in the presence of NOC13 at 37 °C in 0.1 M sodium phosphate buffer (H_2O-CH_3CN = 80/20, v/v), pH 7.40. The spectra were obtained 15 min after the reaction with NOC13.

similarity that can be ascribed to the delocalized xanthene moiety of rhodamines. This great fluorescence enhancement and the strong color change (from colorless to magenta) due to the reaction of **1** with NO could be easily detected by the "naked eye." These observations indicate that the NO-induced ring-opening reaction takes place at physiological temperature.

The final absorption and fluorescence spectra of the reaction system composed of NOC13 and **1**, and those analogous spectra of rhodamine B in buffer solution were found essentially identical, indicating the release of rhodamine B in the reaction. This conclusion was further supported by the following facts: Because of the easy-hydrolysis of **2** in aqueous solution due to the intrinsic moisture-sensitive characteristic of acylbenzotriazole,[10] the presence of intermediate **2** could only be traced by ESI-MS measurements. After treatment of **1** with NO in dry $CHCl_3$ under aerobic conditions, the resultant solution gave an ESI-MS spectrum (Figure S3, Supporting Information) exhibiting an intense peak at *m*/*z* 544.3 (100% intensity) corresponding to $(\mathbf{2})^+$. Then MS and NMR data further confirm that the final products are rhodamine B and benzotriazole in aqueous solution.

Because rapid NO detection is valuable under physiological conditions, we examined the dynamic absorption and fluorescence spectra of **1** solution for NO (Figure 2). Introducing NO into colorless **1** solution instantly caused a strong magenta color, and the absorbance of the solution at the first detection (30 s) reached a maximun value at ca.

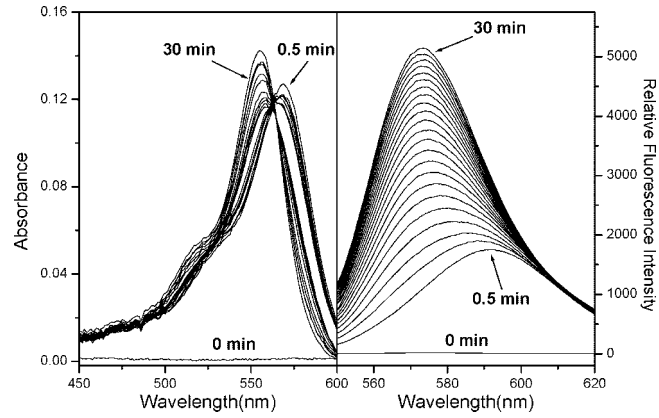

Figure 2. Traces of reaction of **1**(10 μM) with NO at 25 °C in 0.1 M sodium phosphate buffer (H_2O-CH_3CN = 80/20, v/v) of pH 7.4. Excitation wavelength was set to 540 nm.

569 nm, after which the absorption spectrum of the solution exhibited a gradual blue-shift until the equilibration with an absorption peak at 554 nm (~ 30 min). The total blue-shift at this point in time was 15 nm, which agrees well with the absorption wavelength shift between rhodamine B tertiary amide and rhodamine B.[6] Accordingly, the fluorescence emission spectra exhibited a similar blue-shift effect (~ 19 nm) from ca. 592 nm (with a maximum excitation wavelength, λex ≈ 570 nm) to 574 nm (λex = 554 nm). This experimental result indicates that the opening of the spiro-ring in **1** by NO can fully occur within the first 30 s of the reaction. Based on the proposed NO-induced ring opening of **1**, the fluorescence spectra of **1** solution within the first 0.5 min may mainly be attributed to generation of rhodamine B tertiary amide **2** ($\lambda_{ex}/\lambda_{em}$: 570/592 nm), whereas the fluorescence spectra measured after 30 min can be ascribed to the main ingredient of the products, rhodamine B ($\lambda_{ex}/\lambda_{em}$: 554/574 nm), due to the gradual hydrolysis of **2** to release free rhodamine B. As a result, the total fluorescence intensity primarily comes from both rhodamine B tertiary amide **2** and rhodamine B within 30 min of reaction, and the fluorescence measured after 30 min primarily results from rhodamine B. We also noticed that, with the blue-shift of emission wavelength, the fluorescence intensity of this dynamic fluorescence also exhibited an obvious increase, which may be ascribed to the fact that the adopted excitation wavelength (540 nm) is more favorable for the excitation of rhodamine B ($\lambda_{ex} \approx 554$ nm) than for **2** ($\lambda_{ex} \approx 570$ nm).

As pH dependence of fluorescence is generally undesirable in biological applications, the effect of pH on fluorescence was also studied. Our probe **1** shows no fluorescence at pH > 3, which means compound **1** can retain the spirocyclic form over a wide pH range. Importantly, we found that the fluorescence of the reaction product of NO with **1** was also pH-independent when pH was greater than 5. These facts demonstrate that **1** can work well as a NO fluorescent chemosensor over a wide pH range (pH > 5) because it avoids pH-dependency of the fluorescence intensity.

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To evaluate the performance of **1** for the detection of NO, a fluorescence titration of NO with NOC13 (13.7 min half-life for releasing NO[9]) was conducted by titrating a 10 μM solution of **1** at 37 °C in 0.10 M sodium phosphate buffer (pH 7.40) with NOC13 and measuring the fluorescence intensity 30 min after reaction. Upon the addition of 1.0 equiv of NOC13, the fluorescence intensity of the solution of **1** increased ca. 2400-fold with an emission maximum at 574 nm, and the detection limit of NOC13 was calculated to be 3.0 nM. Though **1** showed higher reactivity, its selectivity for NO over other oxygen-containing species such as H_2O_2, NO_2^-, NO_3^-, O_2^-, and •OH, still remained rather high. With the exception of NO, these oxygen-containing substances did not produce detectable fluorescence from compound **1** (see Figure 3). The same selectivity was also exhibited in its

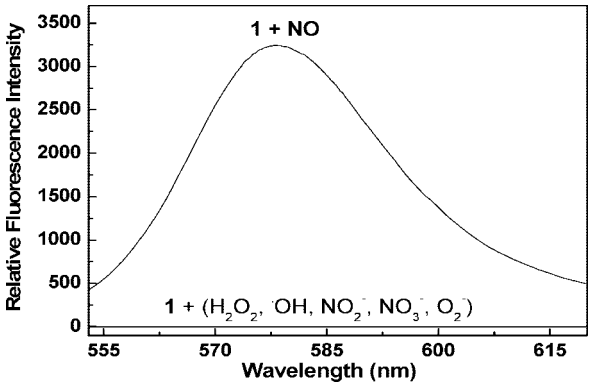

Figure 3. Effects of various reactive oxygen-containing species including H_2O_2 (1.0 mM), •OH (produced by the reaction of 1.0 mM H_2O_2 with 100.0 μM Fe^{2+}), NO_2^- (100 μM), NO_3^- (100 μM), and O_2^- (100 μM KO_2) on the fluorescence intensity of **1** (10 μM) in sodium phosphate buffer (0.1M, pH 7.4, H_2O-CH_3CN = 80/20, v/v). The fluorescence intensities were measured with an excition wavelength at 540 nm.

spectrophotometric behavior. We also noticed that $ONOO^-$ solution showed a similarly strong reaction with **1** in pH 7.4 solution. $ONOO^-$ is generated through the spontaneous reaction of nitric oxide (NO) and O_2^- with the extra short half-life (<20 ms) in a physiological environment, which may undergo the reversible dissociation to NO and O_2^-.[11] As probe **1** can not react with O_2^-, to validate this reaction was caused either by $ONOO^-$ itself or by the decomposed NO in solution, control experiments were thus conducted. Because $ONOO^-$ is fairly stable in alkaline solutions (pH > 10),[11b,12] probe **1** was treated with NO and $ONOO^-$ solution at pH 12.4 (0.10 M KCl-NaOH buffer), respectively. Results showed that **1** produced strong absorbance/fluorescence toward NO solution, whereas little absorbance/fluorescence for stable $ONOO^-$ solution (see Supporting Information), indicating that **1** did not react with $ONOO^-$ ion. Therefore, we can conclude that under physiological environment (pH 7.4), **1** actually reacts not with $ONOO^-$ itself but with the dissociated NO in solution, which further approves the selectivity of **1** for NO.

In summary, we have developed a "switch-on" chromogenic and fluorogenic NO probe employing a novel luminescent mechanism based on an NO- induced ring-opening reaction of a rhodamine spirolactam scaffold. Compared with reported NO fluorescent probes, the proposed probe exhibits the following advantage: a quick, simple, and convenient synthesis. Furthermore, the solution of the probe itself is nonfluorescent and colorless ("clear blank"); thus, greater fluorescence enhancement and remarkable color change toward NO can be obtained, which is greatly favorable to improving the sensitivity of NO detection and to the performance of biological imaging research.

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Supporting Information Available: Synthesis and X-ray crystallography of **1**, ^1H and ^{13}C NMR characterization of **1** and the reaction products of **1** with NO, the pH effect, absorption and fluorescence spectral responses of **1** for NOC13 and $ONOO^-$. This material is available free of charge via the Internet at http://pubs.acs.org.

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