Accepted Manuscript

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PII: S1001-8417(18)30345-0
DOI: https://doi.org/10.1016/j.cclet.2018.08.019
Reference: CCLET 4640

To appear in: *Chinese Chemical Letters*

Please cite this article as: He H, He T, Zhang Z, Xu X, Yang H, Qian X, Yang Y, Ring-restricted *N*-nitrosated rhodamine as a green-light triggered, orange-emission calibrated and fast-releasing nitric oxide donor, *Chinese Chemical Letters* (2018), https://doi.org/10.1016/j.cclet.2018.08.019

Communication

Ring-restricted *N*-nitrosated rhodamine as a green-light triggered, orange-emission calibrated and fast-releasing nitric oxide donor

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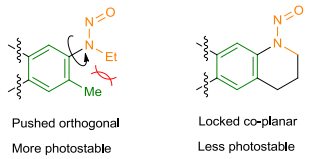
Graphical abstract



Locking the *N*-nitrosamine coplanar with the fluorophore facilitates photo-triggered NO release.

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords: NO donor
Photo-trigger
Photo-calibrate
Platelet aggregation inhibition
Antimicrobial

ABSTRACT

Nitric oxide (NO) donors are versatile tools for nitric oxide biology. The biological response of NO is dependent on the transient concentration and the sustained duration. *N*-nitrosated rhodamines are photo-triggered and photo-calibrated NO donors. We recently discovered that suppression of the dihedral angle between the *N*-nitroso fragment with the rhodamine scaffold facilitates NO release. Inspired by this discovery, we developed a fast-releasing NO donor (**NOD575**) suitable for biological applications, *e.g.*, the pulmonary arterial smooth muscle cells (PASMCs).

Release of nitric oxide (NO) in a spatially or temporally controlled fashion facilitates investigation of the mechanism of NO in biological processes [1] and can also be harnessed as therapeutic or biomanipulative applications [2,3]. Compared to various chemo-triggers, *e.g.*, enzymes [4], reducing agents [5], oxidative species [6] and nucleophiles [7], light is particularly desirable due to its versatility, non-invasiveness, and feasibility for biological settings. The flux and the dose of NO are critical to its biological outcome [8]. Modulating the intensity of the photo-irradiation may offer a convenient way to adjust the photo-decomposition rate of our photo-triggered and photo-calibrated NO donors. However, in cases where the light intensity is not readily adjusted, NO donors of varying releasing kinetics could be a desirable alternative.

The existing photo-triggered NO donors include photo-caged spontaneous NO donors [9], *ortho*-substituted nitrobenzene [10], and metal-nitroso complexes [11]. One limitation of the aforementioned photo-triggered NO donors is the lack of a mechanism to calibrate the dose and kinetics of NO release. Commercial NO probes were employed to monitor the NO generation characteristics. However, they detect NO by scavenging NO and therefore prevent NO from eliciting its biological functions. We have recently devised a series of NO donors, which are *N*-nitrosated push-pull fluorophores (Fig. 1) and addressed this dilemma [12-14]. Their release of NO is not only triggered by light, but also calibrated by simultaneous release of a fluorophore, whose fluorescence signifies the release of NO in a real-time fashion. In particular, **NOD560** is interesting because it can be photo-activated by a wide-selection of laser lines, in the spectral range of 375-556 nm and the resulting rhodamine dye is a superior fluorophore for imaging-based applications [14-19]. The only drawback is its relatively slow kinetics. Therefore, development of such NO donors with accelerated NO release rate is an important research topic.

N-Nitrosated secondary amines have been sketched in its *N*-nitroso form (**a**, Fig. 1) following the convention. However, we note that the diazonium oxide (**b**, Fig. 1) is in fact the major contributing resonance structure for *N*-nitrosamine. Upon photoexcitation of the structure **b,** the intramolecular charge transfer occurs and the molecule adopts the structure **a**. The single bond between the two nitrogen

atoms (highlighted in blue) in the form **a** homolyzes to release NO and an anilinyl radical. Based on the above analysis, it becomes obvious that a weak N-N bond is the key to high homolysis tendency. In **NOD545** [12], **NOD550** [13], and **NOD560** [14], the nitrosamine moiety and the chromophore are nearly orthogonal to each. Reducing the dihedral angle in between is expected to effectively promote the electronic delocalization from the nitrogen atom to the dye scaffold, weaken the N-N bond and hence enhance NO release kinetics. Therefore, we designed a novel NO donor with the nitrosamine moiety locked coplanar with the dye scaffold.

2-(4-(Diethylamino)-2-hydroxybenzoyl)benzoic acid (**1**) was condensed with 1,2,3,4-tetrahydroquinolin-7-ol (**2**) in concentrated H_2SO_4 at 100 °C for 24 h to furnish the desired rhodamine dye (**3**) in a 91% yield. Compound **3** was then nitrosated to afford **NOD575** in a 94% yield. X-ray diffraction reveals that **NOD575** is in its lactone form in solid state. The dihedral angle between the nitrosamine and the rhodamine dye scaffold was measured to be of 12.66°.

The UV-vis absorption and fluorescence properties of both **NO575** and **3** were acquired in neutral phosphate buffer with 1% DMSO as co-solvent. The maximal absorption of **NOD575** is at 510 nm with two shoulder peaks at 480 nm and 545 nm, respectively, suggesting **NOD575** exists predominantly in its ring-open form (Fig. 2). **NOD575** is not fluorescent. In comparison, the potential decomposition product is highly absorbing at 545 nm and strongly fluorescent with an emission maximum at 575 nm (Φ = 0.58).

The solution of **NOD575** (10 μmol/L) in phosphate buffer (50 mmol/L, pH 7.4) with 1% DMSO was photoirradiated with a laser at 532 nm. The UV-vis absorption and fluorescence emission spectra were recorded intermittently. The absorbance band of **NOD575** gradually decreases with respect to duration of photo-irradiation, the absorption and emission of the product increases concomitantly. The decomposition product was unambiguously confirmed to be **3**, by NMR and MS. It took *ca*. 100 s for its decomposition to complete, compared to *ca*. 2300 s of **NOD560**. **NOD575** expectedly exhibits a much improved decomposition kinetics compared to **NOD560**. This confirms our original hypothesis that reducing dihedral angle can weaken N-N single bond and facilitates homolysis.

Denitrosation of *N*-nitrosamines may occur in reductive environment [20]. We have also previously discovered that reducing agents may accelerate the decomposition of NO donors. Therefore, the chemostability of **NOD575** (10 μmol/L) was tested by incubation with biological thiols and bio-relevant reducing agents, *i.e.*, cysteine, glutathione, and ascorbic acid, in phosphate buffer (50 mmol/L at pH 7.4) with 1% DMSO in the dark at room temperature. The absorbance of each solution at 545 nm remained unchanged in 24 h, suggesting **NOD575** are sufficiently chemostable for practical biological applications (Fig. S3 in Supporting information). Photodecomposition of **NOD575** was then tested in the presence of these three biological relevant reducing agents, *e.g.*, resveratrol, cysteine, and ascorbic acid. Fluorescence turn-on was dramatically improved and completed in as short as 20 s (Fig. 5).

The potentials of **NOD575** for *in vitro* applications were tested. Pulmonary artery smooth muscle cells (PASMCs) were incubated with **NOD575** (20 μmol/L) for 20 min in dark. PASMCs were then washed with phosphate buffer for 3 times. The fluorescence image before and after photolysis by green laser was acquired by a confocal microscopy. Fig. 6 indicated that cells showed no fluorescence without irradiation. After exposure to green light for 60 s, there existed strong red fluorescence from cells. These results showed that **NOD575** could diffuse into PASMCs and it could release NO *in vitro* and the rhodamine dye generated *in situ* could be harnessed for imaging purposes.

In conclusion, we have designed and synthesized a novel NO donor (**NOD575**) following the principle of *N*-nitroso push-pull dyes. It has exhibited photo-triggered release of NO upon irradiation by a green laser line at 532 nm. Also, the release of NO is accompanied by the liberation of a rhodamine fluorophore, whose fluorescence emission (λ_{em} = 575 nm) can be used as a self-calibration mechanism for the NO release. **NOD575** has a small dihedral angle of 12.67° between the *N*-nitroso moiety and the rhodamine scaffold by ring-closure. Such a rational design is engineered into the molecule to weaken the N-N bond and facilitate its hemolysis upon photo-irradiation. Therefore, a short exposure of *ca*. 100 s is sufficient to allow complete photolysis, compared to 2300 s of **NOD560**, a similar analog with a dihedral angle of 82°. Presence of biological thiols or reducing agents does not induce the decomposition of **NOD575** in dark, but can greatly accelerate its photodecomposition. Last, **NOD575** is membrane-permeable and compatible for *in vitro* biological studies.

Acknowledgments

The work is financially supported by the National Natural Science Foundation of China (No. 21572061) and the Fundamental Research Funds for the Central Universities (No. WY1516017).

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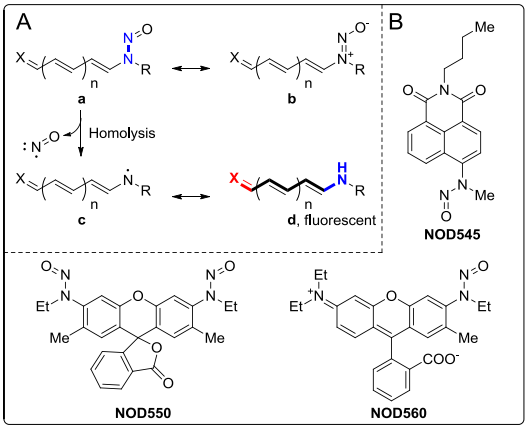

Fig. 1. (A) The resonance structures and NO-releasing mechanism of *N*-nitrosated push-pull dyes. (B) Three representative NO donors of this class.

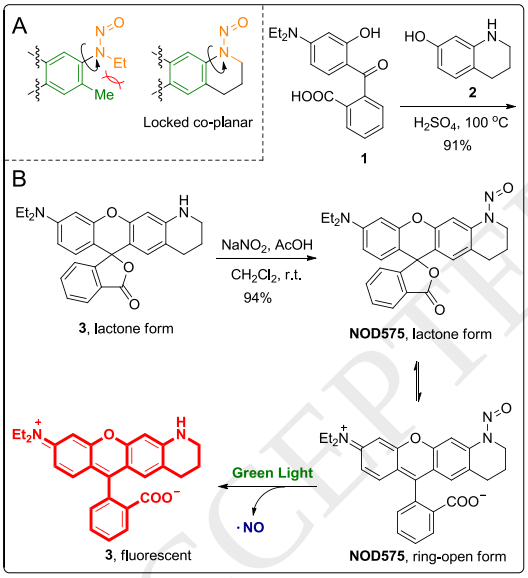

Fig. 2. (A) Dihedral angles between the nitrosamine (orange) and the dye scaffold (green). (B) The synthesis and photo-decomposition of **NOD575**.

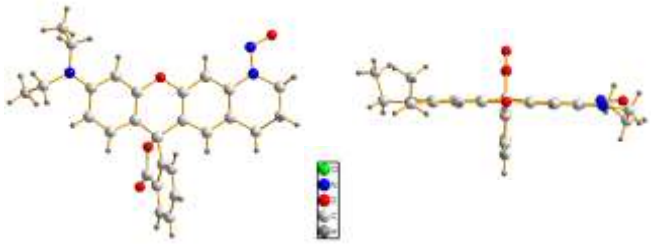

Fig. 3. The two different views of the crystal structure of **NOD575**.

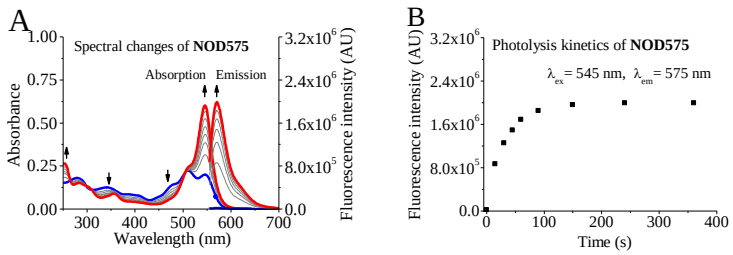

Fig. 4. (A) UV-vis absorption spectral and fluorescence emission spectral changes of **NOD575** solutions upon photo-irradiation by 532 nm. (B) The enhancement of emission intensity at 575 nm of **NOD575** solution with respect to the duration of photo-irradiation.

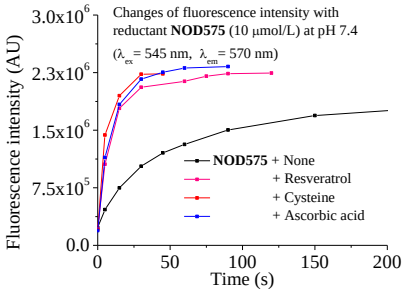

Fig. 5. Spectral monitoring of the photolysis of **NOD575** with 532 nm in phosphate buffer (50 mmol/L, pH 7.4) with 1% DMSO in the presence of various indicated reducing agents.

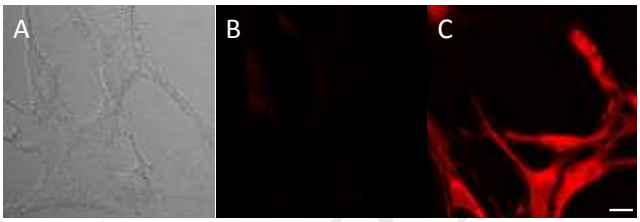

Fig. 6. Confocal fluorescence imaging of PASMCs incubated with 20 μmol/L **NOD575** for 20 min. (A) Bright-field image. (B) Fluorescence image before irradiation. (C) Fluorescence image after 60 s' photo-irradiation. Scale bar: 10 μm. λ_{ex} = 488 nm, λ_{em} =555-650 nm.