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Water-soluble Hantzsch ester as switch-on fluorescent probe for efficiently detecting nitric oxide

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ABSTRACT

A water soluble Hantzsch ester derivative of coumarin, **DHPS**, was synthesized and successfully applied in the fluorescent sensing nitric oxide (NO) in aqueous solution. The fluorescence of probe **DHPS** is extremely weak, while its fluorescence was greatly switched on upon the addition of NO solution and showed high selectivity and sensitivity to NO. The limitation of the detection was calculated to be 18 nM. The NO-induced aromatization of dihydropyridine in **DHPS** to pyridine derivative (**PYS**) proved to be the switching mechanism for the fluorescent sensing process, which was confirmed through spectra characterization and computation study. Cytotoxicity assay demonstrated both **DHPS** and **PYS** are biocompatible, the **DHPS** was successfully applied to track the endogenously produced NO in the RAW 264.7 cells.

1. Introduction

Nitric oxide (NO), endogenously generated by conversion of L-arginine to citrulline through inducible nitric oxide synthase (iNOS) in macrophage or living organisms, is widely recognized as an ubiquitous biomarker and signaling molecule in the peripheral and central nervous systems [1,2], and it acts vital biological functions in numerous metabolic process and physiological systems such as neurotransmission, anticarcinogenic action, and immune systems [3,4]. Moreover, NO also participates in the normal metabolism of plants and pollutes the atmosphere after releasing as exhaust gas by vehicles [5,6]. NO actually played protective and proliferative effect on normal life activity when it exists at low concentration, but high-level NO can trigger severe cell cycle arrest, senescence and apoptosis [7–9]. Therefore, the disorder of NO can induce many disease ranging over cancer, hypertension Parkinson disease, and so on [10]. Owing to its unequivocal significance, the detection of NO has inspired many researchers to exploit new techniques and materials. Up to date, the approaches involved in the NO detection cover electron paramagnetic resonance spectroscopy [11], colorimetric method [12], electrochemical method [13], chemiluminescence [14], and fluorescence [15–17]. Among those developed analytical methods, fluorescence showed tremendous advantages when it comes to high sensitivity and selectivity [18–20]. Additionally, fluorescence can also be used in both solutions and solid phase, and even gas phase. These valuable merits made it be a preferential tool for the detection of NO.

In last few decades, various fluorescent compounds and materials, including quantum dots [21], inorganic complexes [22–24], nanoparticles [25,26], biomacromolecule or cyclodextrin based fluorophores [27,28] and small organic fluorescent molecules [29], have been well established as valid chemosensors to detect NO in vitro and analyze the NO generation and distribution in cells or living organisms. Among them, small organic molecules particularly attracted attentions because of its intrinsic advantages such as easy preparation, good stability and adjustable structures. In these reported small molecules, PET quenching based *o*-phenylenediamine derivatives are the most greeted and studied [1]. When encountering NO together with oxygen, the vicinal diamine was turned into triazole and thus leading to the prohibition of PET process. As a result, the florescence was activated. Following the above rules, many probes were designed and some of them responded specially and rapidly to NO at fairly low levels, as well as directly used as visualization reagents in both living cells and organisms [16,30–34]. However, this developed strategy still has its limitations as follows: (i) Self-oxidation-sensitive electron-abundant *o*-phenylenediamine groups makes it easily react with other reactive oxygen/nitrogen species, lighting up its fluorescence and interfering with the sensing process of NO; (ii) oxidized species rather than NO itself are demanded to react with the vicinal aromatic diamines [1,35,36]. To solve those problems, our group recently reported a highly selective fluorogenic probe for NO sensing based on the Hantzsch ester [37]. As well known, Hantzsch ester is able to quantitatively react with NO to give the corresponding pyridine, a reaction that is independent of oxygen [38,39]. Although this smart and reasonable probe was successfully applied as

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ratiometric fluorescent chemosensor and visualization for NO in solutions and living cells, its pH stability, cytotoxicity, and especially the water solubility need to be further improved.

Taking the above information into consideration, we herein report a new water soluble Hantzsch ester derivatives carrying coumarin fluorophore (**DHPS**, Scheme 1) and its application as efficient NO sensors. The probe is biocompatible and can be applied to detect the in situ generated NO in living cells.

2. Experimental

2.1. General

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III 400 MHz spectrometer (Bruker Co., Germany) at 25 °C using $CDCl_3$, DMSO-d_6, D_2O as solvents and calibrated using tetramethylsilane (TMS) as an internal reference. High resolution mass spectra were acquired on a Waters LCT Premier XE spectrometer (Waters, Milford, MA, USA). Absorption spectra were recorded with Varian Cary-50 UV–Vis spectrophotometer (Agilent Technologies, USA). Fluorescence spectra were recorded with a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA) equipped with a 1 cm × 1 cm quartz cell. FT-IR spectra were taken on an Affinity-1 Fourier Transform Infrared Spectrometer in the range of 4000–400 cm^{-1} (Thermo Fisher Nicolet, USA). All pH measurements were determined by FiveEasy Plus FEP20 pH meter (Mettler-Toledo Instruments, Shanghai Co., LTD.). Water was purified with a Milli-Q Gradient system (Millipore, Molsheim, France). Unless otherwise specified, all reagents were of analytical reagent grade and purchased from Beijing Chemical Reagent Co. (Beijing, China) and used without further purification. All solutions were prepared in ultra-pure water. Error bars were calculated based on three parallel experiments.

2.2. Theoretical calculation

To gain further understanding of the sensing mechanism, the quantum chemical calculations were carried out using the Gaussian 09 program package [40]. The possible ground state structures have been optimized with density functional theory (DFT) at B3LYP/6-31 + G (d) level [41–43]. On the basis of optimized configuration for the ground state, TD-DFT calculations were performed using the B3LYP (Becke's three-parameter hybrid exchange-correlation (x-c) functional) method without any symmetry restraint to get the necessary excitation energies and oscillator strengths.

2.3. Cell toxicity

The cytotoxicity of **DHPS** and **PYS** toward Raw 264.7 cell lines were tested by a standard MTT assays. The cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Gibco) containing 10% FBS (fetal bovine serum, v/v 1/1) in 5% CO_2 at 37 °C. After incubating for 48 h in the medium, the cells were seeded in 96-well plates in 100 μL medium per well with a density of 5000 cells. After culturing for another 24 h, different concentrations of **DHPS** and **PYS** in 100 μL DMEM were added to each well. After incubation of 4 h, the medium was replaced with 20 μL MTT (5 mg/mL) for another 4 h. Finally, by removing the MTT and adding 200 μL DMSO, and oscillating for 10 min, the formed formazan crystals were fully dissolved. The absorptions of the purple crystals was recorded on a Thermo Scientific Multiskan Go at 490 nm. The relative viability of the cells was calculated based on the data of five parallel tests by using following formula eqn.

$$\text{Cell viability} = \frac{OD_{490}(\text{Sample}) - OD_{490}(\text{Blank})}{OD_{490}(\text{Control}) - OD_{490}(\text{Blank})} \times 100\%$$

where OD_{490} (Sample) represents the optical density of the well treated with various concentrations of the compounds, OD_{490} (Control) represents that of the wells treated with DMEM + 10% FBS and OD_{490} (Blank) represents that of blank wells treated with pure DMSO. The finally reported percent cell survival values are relative to the untreated control cells.

2.4. Cell imaging

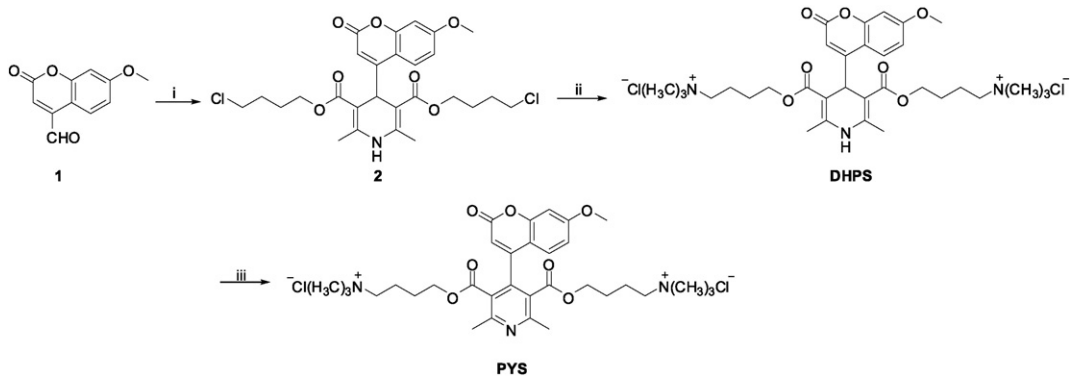
Raw 264.7 cells were cultured in DMEM medium supplemented with 10% FBS in a humid atmosphere containing 5% CO_2 at 37 °C. For live cell imaging studies, the cells were seeded in Glass Bottom Cell Culture Dishes at 2×10^5 cells per dish and cultured for 24 h. To induce NO production, Raw264.7 cells were stimulated with 0.5 μg mL^{-1} Lipopolysaccharide (LPS) for 4 h. Probe **DHPS** was then subsequently added and the cells was incubated for another 16 h at the same conditions. Finally, the cells were washed with 1 mL PBS 3 times and then 1 mL of PBS was added to each well, observed by using Zeiss Abserver A1 Inverted Fluorescence Microscopy equipped with an EM-CCD camera (Hamamatsu) and an X-Cite 120 metal halide lamp (EXFP). Bright filed and fluorescence images were taken with a 40× objective lens.

2.5. Synthesis

The synthetic route is shown in Scheme 1. Compound **1** [37] and 4-chlorobutyl 3-oxobutanoate [44] were synthesized according to the reported literatures.

2.5.1. Synthesis of *2*

Compound **1** (380 mg, 2.0 mmol) and 4-chlorobutyl 3-oxobutanoate (1.15 g, 6.0 mmol) were dissolved in 20 mL of EtOH, then ammonium hydroxide (210 mg, 6.0 mmol) was added. The mixture was refluxed for 4 h, the solvent was evaporated under reduced pressure after cooling



Scheme 1. Synthetic route for the target compound **DHPS** and its pyridine product **PYS**. i) 4-chlorobutyl 3-oxobutanoate, $NH_3 \cdot H_2O$, EtOH, reflux; ii) trimethylamine, EtOH, reflux; iii) NO, EtOH, room temperature.