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A highly selective turn-on fluorescent probe for hypochlorous acid based on hypochlorous acid-induced oxidative intramolecular cyclization of boron dipyrromethene-hydrazone

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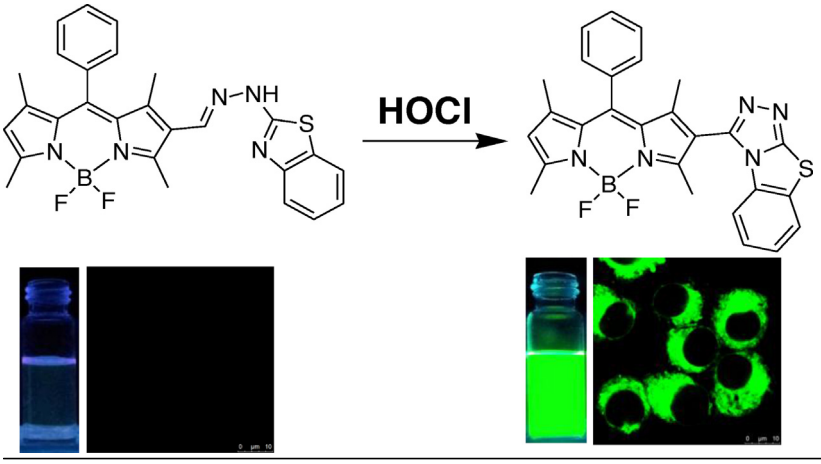
HIGHLIGHTS

- A BODIPY-based fluorescent probe for sensing HOCl was developed.
- The probe utilizes the HOCl-promoted cyclization in response to the amount of HOCl.
- The probe might have application in the investigation of HOCl in biological systems.

GRAPHICAL ABSTRACT



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ABSTRACT

A BODIPY-based fluorescent probe, **HBP**, was developed for the detection of hypochlorous acid based on the specific hypochlorous acid-promoted oxidative intramolecular cyclization of heterocyclic hydrazone in response to the amount of HOCl. The reaction is accompanied by a 41-fold increase in the fluorescent quantum yield (from 0.004 to 0.164). The fluorescence intensity of the reaction between HOCl and **HBP** is linear in the HOCl concentration range of 1–8 μM with a detection limit of 2.4 nM (S/N = 3). Confocal fluorescence microscopy imaging using RAW264.7 cells showed that the new probe **HBP** could be used as an effective fluorescent probe for detecting HOCl in living cells.

1. Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical ($OH^•$) and hypochlorous acid (HOCl), are formed as a byproduct during dioxygen metabolism and play important roles in a wide variety of biological events. Among them, hypochlorous acid is produced as a

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defense tool in the immune system and plays a vital role in killing a wide range of pathogens [1–4]. Endogenous hypochlorous acid is mainly produced from the reaction of chloride ion and hydrogen peroxide catalyzed by the enzyme myeloperoxidase (MPO) in leukocytes including macrophages, monocytes, and neutrophils [5,6]. However, an excess amount of hypochlorous acid causes several human diseases such as arthritis, cancer and neurodegeneration [7–9]. Because of the pathophysiological importance of hypochlorous acid, the development of highly sensitive and selective probes for hypochlorous acid has become essential. Fluorescent probes that can quickly respond to the change in concentration of hypochlorous acid could be used to study the dynamic distribution of hypochlorous acid in living cells.

A common approach towards the design of HOCl fluorescent probes is to connect a HOCl-reactive moiety with an organic fluorophore. HOCl has high oxidation properties. Several functional groups, such as *p*-alkoxyaniline, *p*-methoxyphenol, oxime, selenide, and thiol, have been identified as HOCl-reactive moieties [10–28]. In particular, hypochlorous acid triggers intramolecular cyclization of heterocyclic hydrazone to form 1,2,4-trazole, which can be used for the development of the HOCl fluorescent probe [29].

In this study, a BODIPY-based fluorescent probe **HBP** was designed for HOCl detection. **HBP** displays weak fluorescence with a quantum yield of $\Phi = 0.004$ due to isomerization of the unbridged imine (C=N), which has been known to exhibit a non-radiative decay process in the excited state [10]. The strong fluorescence of BODIPY is restored after the unbridged C=N was changed to bridged C=N by hypochlorous acid-triggered intramolecular cyclization. This new probe exhibits high selectivity and sensitivity towards HOCl over other ROS and reactive nitrogen species (RNS) in aqueous solution. Most importantly, **HBP** shows good cell-membrane permeability and can be successfully applied to image endogenous HOCl in living cells.

2. Experimental

2.1. Materials and instrumentations

All reagents were obtained from commercial sources and used as received without further purification. UV/vis spectra were recorded on an Agilent 8453 UV/vis spectrometer. Fluorescence spectra measurements were performed on a Hitachi F-7000 fluorescence spectrophotometer. NMR spectra were obtained on a Bruker DRX-300 and Agilent Unity INOVA-500 NMR spectrometer. Fluorescent images were taken on a Leica TCS SP5 X AOBS Confocal Fluorescence Microscope.

2.2. Preparation of ROS and RNS

Various ROS and RNS including HOCl, $^{\bullet}OH$, H_2O_2, $^1O_2$, NO_2^-, NO_3^-, NO, $ONOO^-$, O_2^- and *t*-BuOOH were prepared according to the following methods. HOCl was prepared from commercial bleach; the concentration of hypochlorite (OCl^-) was determined by using an extinction coefficient of 350 $M^{-1}\,cm^{-1}$ (292 nm) at pH 9.0. Hydroxyl radical ($^{\bullet}OH$) was generated by Fenton reaction on mixing $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ with 10 equivalents of H_2O_2; the concentration of $^{\bullet}OH$ was estimated from the concentration of Fe^{2+}. The concentration of the commercially available stock H_2O_2 solution was estimated by optical absorbance at 240 nm. Singlet oxygen ($^1O_2$) was generated by the addition of NaOCl and H_2O_2 according to the literature [30]. The source of NO_2^- and NO_3^- was from $NaNO_2$ and $NaNO_3$. Nitric oxide (NO) was generated from sodium nitroferricyanide(III) dihydrate. Peroxynitrite ($ONOO^-$) was prepared as the reported method [31]; the concentration of peroxynitrite was estimated by using an extinction coefficient of 1670 $M^{-1}\,cm^{-1}$ (302 nm). Superoxide (O_2^-) is prepared from KO_2. *t*-BuOOH was obtained commercially from Alfa Aesar.

2.3. Synthesis of the probe ***HBP***

2-Benzothiazole hydrazone (93.7 mg, 0.56 mmol) was added to a solution of BODIPY–aldehyde (100 mg, 0.28 mmol) in EtOH (35 mL) at room temperature. The reaction mixture was stirred for 12 h at room temperature. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (hexane:ethyl acetate = 1:1) to give the compound as a dark purple solid. Yield: 119.3 mg (85%). m.p. 279.3 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.06(s, 1H), 7.60 (d, J = 7.5 Hz, 1H), 7.51 (m, 3H), 7.45 (d, J = 6.5 Hz, 1H), 7.29 (m, 3H), 7.15–7.125 (m, 1H), 6.06 (s, 1H), 4.77 (s, 1H), 2.77 (s, 3H), 2.59 (s, 3H), 1.50 (s, 3H), 1.39 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.0, 158.3, 154.8, 146.4, 145.1, 142.3, 140.9, 139.8, 134.6, 132.6, 130.5, 129.4, 129.3, 129.2, 128.1, 127.9, 126.5, 124.6, 122.8, 122.6, 122.3, 121.6, 116.7, 14.8, 14.6, 14.2, 12.3. MS (ESI^+): m/z = 500.3 $[M+H]^+$. HRMS (ESI^+): calcd. for $C_{27}H_{24}BF_2N_5S$ $[M+H]^+$ 500.1892; found 500.1872.

2.4. The oxidized ***HBP*** from the reaction of ***HBP*** and NaOCl

Sodium hypochlorite (0.5 M, 1 mL, 1 mmol) was added to a solution of **HBP** (100 mg, 0.20 mmol) in THF/EtOH/PBS buffer (5 mL/30 mL/30 mL) at room temperature. The reaction mixture was stirred for 30 min. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (hexane:ethyl acetate = 1:1) to give the compound as a red solid. Yield: 63.6 mg (64%). m.p. 225.4 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69–7.67 (m, 1H), 7.52–7.48 (m, 3H), 7.39–7.34 (m, 2H), 7.33 (d, J = 2 Hz, 2H), 7.29–7.27 (m, 1H), 6.13 (s, 1H), 2.63 (s, 3H), 2.50 (s, 3H), 1.45 (s, 3H), 1.30 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 160.8, 156.7, 152.8, 147.2, 143.6, 143.4, 141.4, 135.0, 133.9, 133.2, 131.2, 130.3, 130.2, 130.1, 130.0, 128.5, 128.1, 127.4, 127.0, 125.3, 123.9, 117.8, 114.5, 15.6, 15.4, 14.0, 13.4. MS (ESI^+): m/z = 497.1 $[M]^+$, 498.2 $[M+H]^+$. HRMS (ESI^+): calcd. for $C_{27}H_{22}BF_2N_5S$ $[M+H]^+$ 498.1735; found 498.1713.

2.5. Cell culture for RAW264.7 macrophages

The cell line RAW264.7 was provided by the Food Industry Research and Development Institute (Taiwan). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C under an atmosphere of 5% CO_2. Cells were plated on 18 mm glass coverslips and allowed to adhere for 24 h.

2.6. Cytotoxicity assay

The methyl thiazolyl tetrazolium (MTT) assay was used to measure the cytotoxicity of **HBP** in RAW264.7 cells. RAW264.7 cells were seeded into a 96-well cell-culture plate. Various concentrations (5, 10, 15, 20, 30 μM) of **HBP** were added to the wells. The cells were incubated at 37 °C under 5% CO_2 for 24 h. 10 μL MTT (5 mg mL^{-1}) was added to each well and incubated at 37 °C under 5% CO_2 for 4 h. Remove the MTT solution and yellow precipitates (formazan) observed in plates were dissolved in 200 μL DMSO and 25 μL Sorenson's glycine buffer (0.1 M glycine and 0.1 M NaCl). Multiskan GO microplate reader was used to measure the absorbance at 570 nm for each well. The viability of cells was calculated according to the following equation:

$$\text{Cell viability (\%)} = \left(\frac{\text{mean of absorbance value of treatment group}}{\text{mean of absorbance value of control group}}\right)$$

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