A Red-Emitting Naphthofluorescein-Based Fluorescent Probe for Selective Detection of Hydrogen Peroxide in Living Cells

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Synthetic Materials and Methods. All reactions were carried out under a dry nitrogen atmosphere. Silica gel 60 (230-400 mesh, Fisher) was used for column chromatography. Analytical thin layer chromatography was performed using Fisher 60 F254 silica gel (precoated sheets, 0.25 mm thick). Dichloro[1,1´-bis(diphenylphosphino) ferrocene]palladium (II) dichloromethane adduct, $Pd(dppf)Cl_2 \bullet CH_2Cl_2$, and 1,1'-bis(diphenylphosphino)ferrocene, dppf, were purchased from Strem Chemicals (Newburyport, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. ^{1}H NMR spectra were collected in $CDCl_3$ or DMSO-d_6, (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC using a Bruker AVQ-400 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million. High-resolution mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

Naphthofluorescein (1). This known compound was made by modifying literature conditions.[1-3] 1,6-Dihydroxynaphthalene (6.41 g, 40.0 mmol), phthalic anhydride (2.96 g, 20.0 mmol), and methanesulfonic acid (50 mL) were combined in a 150-mL heavy-walled pressure flask and heated at 135 °C for 48 h. After cooling to room temperature, the dark purple solution was poured into 500 mL of an ice/water slurry and stirred vigorously to precipitate a dark, brick red solid. The solid was collected by vacuum filtration, dried in air, and purified by flash column chromatography (silica gel, 2.5-5% methanol/dichloromethane) to provide **1** as a brick red solid (2.60 g, 30% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.92 (2H, d, J = 4.6 Hz), 8.32 (1H, d, J = 3.8 Hz), 7.97 (1H, m), 7.88 (1H, m), 7.60 (2H, d, J = 4.6 Hz), 7.55 (1H, d, J = 3.6 Hz), 7.21 (2H, m), 7.07 (2H, s), 6.88 (2H, d, J = 4.4 Hz). Phenolic protons were not observed under these conditions. HRFAB-MS: calculated for [MH^+] 433.1078, found 433.1076.

Naphthofluorescein bis-triflate (2). Naphthofluorescein **1** (434.1 mg, 1.0 mmol) was dissolved in anhydrous DMF (3 mL) in a dried 25-mL Schlenk tube and placed under a nitrogen atmosphere. DIPEA (991.7 μL, 6.0 mmol) was added slowly by syringe over 1 min at room temperature, resulting in a considerable darkening of the solution. A solution of *N*-phenyl-bis(trifluoromethanesulfonimide) (1.08 g, 3.0 mmol) in DMF (3 mL) was added dropwise over 5 min, and the reaction stirred for 24 h. The mixture was diluted in ethyl acetate (200 mL), washed with H_2O (3 × 50 mL), brine (1 × 50 mL), and dried over Na_2SO_4. Purification by flash column chromatography (silica gel, dichloromethane) gave **2** as an off-white solid (201.0 mg, 29% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.81 (2H, d, J = 4.6 Hz), 8.17 (1H, d, J = 3.6 Hz), 7.70 (2H, m), 7.61 (2H, d, J = 4.4 Hz), 7.50 (2H, d, J = 4.4 Hz), 7.08 (1H, d, J = 3.4 Hz), 7.02 (2H, s), 6.90 (2H, d, J = 4.4 Hz). HRFAB-MS: calculated for [MH^+] 697.0062, found 697.0062.

Naphtho-Peroxyfluor-1, (NPF1, 3). Triflate **2** (202.8 mg, 0.29 mmol),

$Pd(dppf)Cl_2 \bullet CH_2Cl_2$ (71.3 mg, 0.09 mmol), dppf (48.4 mg, 0.09 mmol), potassium acetate (285.6 mg, 2.91 mmol), and bis(pinacolato)diboron (369.5 mg, 1.46 mmol) were added to a dried 25-mL Schlenk tube, which was subsequently evacuated and placed under a nitrogen atmosphere. Anhydrous 1,4-dioxane (10 mL) was added by syringe, and the reaction was heated to 100 °C for 24 h. The solvent was removed under reduced pressure, and the crude material purified by flash column chromatography (silica gel, dichloromethane). The off white solid thus obtained was triturated with diethyl ether (3 × 5 mL), furnishing **3** as a bone white solid (50.6 mg, 27% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.77 (2H, d, J = 4.2 Hz), 8.38 (2H, s), 8.12 (3H, m), 7.65 (2H, dd, J_1 = 6.8 Hz, J_2 = 1.6 Hz), 7.58 (2H, d, J = 4.4 Hz), 7.16 (1H, m), 6.87 (2H, d, J = 4.4 Hz), 1.43 (24H, s). HRFAB-MS: calculated for [MH^+] 653.2875, found 653.2882.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. Spectroscopic measurements were performed in 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.5, at 37 °C. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer (Walnut Creek, CA). Fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (1.5-mL volume, Starna, Atascadero, CA).

Various reactive oxygen species (ROS, 100 μM) were administered to NPF1 as follows. Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), and hypochlorite (OCl^-) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical ($^{\bullet}OH$) and *tert*-butoxy radical ($^{\bullet}OtBu$) were generated by reaction of 1 mM Fe^{2+} with 100 μM H_2O_2 or 100 μM TBHP, respectively. Superoxide (O_2^-) was generated enzymatically using a xanthine/xanthine oxidase system. Xanthine sodium salt (Sigma-Aldrich, St. Louis, MO) was prepared at 1.741 mg/mL (10 mM) in H_2O and cooled to 0°C. Catalase (from Bovine Liver, 2860 U/mg solid, Sigma-Aldrich, St. Louis, MO) was prepared separately at 3.5 mg/mL in H_2O and cooled to 0°C. A 2 mL reaction cocktail was prepared by dissolving 2.2 mg xanthine oxidase (from buttermilk, 0.09 U/mg solid, Sigma-Aldrich, St. Louis, MO) in 1944 μL HEPES buffer (20 mM, pH 7.5, 1.0 mM EDTA) at 0°C, to which was added 20 μL catalase mix, 16 μL NPF1 (0.625 mM in EtOH), and 20 μL xanthine oxidase mix sequentially, with gentle mixing after each addition. The final concentrations are 1.1 mg/mL xanthine oxidase, 0.035 mg/mL catalase, 5 μM NPF1, and 100 μM xanthine sodium salt. 1.5 mL of the reaction cocktail was then transferred to a cuvette at 37°C for spectrophotometric measurement. For the experiment described, the production of O_2^- is calculated to be 100 μM within 1 min at 37°C. NO^+ was delivered using *S*-nitrosocysteine (SNOC). NO was delivered using NOC-5.

Preparation and Staining of Cell Cultures. RAW264.7 macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose with GlutaMAX™ (Invitrogen, Carlsbad, CA) and supplemented with 10% Fetal Bovine

Serum (FBS, Hyclone). Cells were split 1/30 twice a week. Two days before assaying, cells were passed and plated in 6-well plates coated with poly-L-lysine (50 μg/mL, Sigma, St. Louis, MO). For all experiments, cellular growth media was removed by aspiration, and a solution of NPF1 (from 2.5 mM stocks in DMSO) in DMEM added. Cells were allowed to incubate with dye at 37 °C, 5% CO_2, for 1 h. H_2O_2 was added by bath application to the medium from a 100 mM aqueous stock. Control samples were treated with an equivalent volume of deionized water. Cells were incubated at 37 °C, 5% CO_2 for 1 h and assayed accordingly.

Flow Cytometry Experiments. Cells were treated as described in Figure 4 and analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with a 635 He-Ne red diode array laser and standard 488 Ar laser. Fluorescence was collected by a 661 nm band pass filter.

1. Lee, L. U.S. Patent 4,933,471, **1990**.
2. Haugland, R. P. *The Handbook: A Guide to Fluorescent Probes and Labeling Technologies*, Spence, M. T. Z. Ed.; Invitrogen Corp: Carlsbad, CA, 2005; 10th Ed.
3. Hilderbrand, S. A.; Weissleder, R. *Tetrahedron Lett.* **2007**, *48*, 4383-4385.