A Selective, Cell-Permeable Optical Probe for Hydrogen Peroxide in Living Cells

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Synthetic Materials and Methods. 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), Pd(dppf)Cl_2, was purchased from Strem Chemicals (Newburyport, MA), anhydrous DMF was purchased from Acros Organics (Morris Plains, NJ), and both reagents were used as received. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. ^{1}H NMR spectra were collected in $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AV-300 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.

3´,6´-Diiodofluoran (1). 3-Iodophenol (5.5 g, 25 mmol), phthalic anhydride (1.9 g, 12.5 mmol), and methanesulfonic acid (12.5 mL) were added to a 75-mL heavy-walled reaction flask and heated at 135 ºC for 48 h. After cooling to room temperature, the dark purple solution was poured into 600 mL of an ice/water slurry and stirred to precipitate a grey solid. The solid was collected by filtration and dissolved in chloroform before passing through a plug of silica to yield a pink solution. The solution was evaporated to dryness and the resulting pale orange solid was recrystallized from dichloromethane to give fluoran **1** a white solid (1.7 g, 25% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.04 (1H, dd, J_1 = 6.6 Hz, J_2 = 2.4 Hz), 8.04 (2H, dd, J_1 = 6.6 Hz, J_2 = 2.4 Hz), 7.70 (2H, d, J = 1.8 Hz), 7.67 (2H, m), 7.39 (2H, dd, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.13 (1H, dd, J_1 = 6.0 Hz, J_2 = 1.5 Hz), 6.55 (1H, d, J = 8.4 Hz). HRFAB-MS: calculated for [MH^+] 552.8798, found 552.8807.

3´,6´-Bis(pinacolatoboron)fluoran (Peroxyfluor-1, PF1, 2). Fluoran **1** (60 mg, 0.11 mmol), bis(pinacolato) diboron (83 mg, 0.33 mmol), potassium acetate (64 mg, 0.65 mg), and Pd(dppf)Cl_2 (8 mg, 0.011 mmol) were dried *in vacuo* in a 50-mL Schlenk flask before adding anhydrous DMF (5 mL) by syringe. The reaction was heated at 80 ºC for 2 h under nitrogen. The dark brown reaction was cooled to room temperature and poured into 50 mL of water. The solid was collected by filtration, redissolved in dichloromethane, and evaporated to dryness. Purification by flash chromatography (silica gel, 1% methanol/dichloromethane) yielded diboronic ester **2** as a white solid (30 mg, 50% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.04 (1H, t, J = 3.9 Hz), 7.74 (2H, s), 7.61 (2H, t, J = 3.9 Hz), 7.44 (2H, dd, J_1 = 7.8 Hz, J_2 = 0.9 Hz), 7.08 (1H, t, J = 3.9 Hz), 6.87 (2H, d, J = 7.8 Hz), 1.36 (24H, s). HRFAB-MS: calculated for [MH^+] 553.2569, found 553.2579.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7. Absorption spectra were recorded on a Varian Cary 50 spectrophotometer (Walnut Creek, CA)

and fluorescence spectra were recorded on 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 (3.5-mL volume, Starna, Atascadero, CA).

Cell Culture. HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), glutamine (2 mM), and penicillin/streptomycin (50 μg/ml, Invitrogen). One day before imaging, cells were passed and plated on 18-mm glass coverslips coated with poly-L-lysine (50 μg/ml). Immediately before the experiments, cells were washed with PBS buffer and then incubated with the probe (5 μM in PBS) for 5 min at 25 °C.

Fluorescence Imaging. Confocal fluorescence imaging was performed with a Zeiss LSM510 META laser scanning microscopy system containing an Axioplan 2 MOT upright microscope and a 40x water-immersion objective lens. Excitation at 488 nm was carried out with an argon ion laser. Emission was collected in a window from 505 nm to 580 nm using a META detection system. PF-1 was incubated with live cell samples for 5 min. Addition of H_2O_2 (10 to 100 μM) to cell samples was performed directly on the microscope stage by bath application to the media. Cell imaging was then carried out after washing cells with PBS.