Supporting Information

Design and Synthesis of Fluorescent Probes for Selective Detection of Highly Reactive Oxygen Species in Mitochondria of Living Cells

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Abbreviations

\( \Phi_f \), fluorescence quantum efficiency; HPLC, high-performance liquid chromatography; CH\(_3\)CN, acetonitrile; DMSO, dimethylsulfoxide; DMF, \( N,N \)-dimethylformamide; CH\(_2\)Cl\(_2\), dichloromethane; MeOH, methanol; H\(_2\)O\(_2\), hydrogen peroxide; TFA, trifluoroacetic acid; THF, tetrahydrofuran; AcOEt, ethyl acetate; NaOCl, sodium hypochlorite; OCl\(-\), hypochlorite; •NO, nitric oxide; O\(_2\)•-, superoxide anion; •OH, hydroxyl radical; ONOO\(-\), peroxynitrite; KO\(_2\), potassium superoxide; NOC13: 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene; Fe(ClO\(_4\))\(_2\), iron (II) perchlorate; Na\(_2\)SO\(_4\), sodium sulfate; NaHCO\(_3\), sodium bicarbonate; ROS, reactive oxygen species; hROS, highly reactive oxygen species.
**Materials.** General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co., Dojindo, Acros Organics and Lancaster Synthesis, and were used without further purification. DMF was of fluoreometric grade (Dojindo). All solvents were used after appropriate distillation or purification.

**Instruments.** NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz for $^1$H NMR and at 75 MHz for $^{13}$C NMR. Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuToF for ESI. UV-visible spectra were obtained on a Shimadzu UV-1650. Fluorescence spectroscopic studies were performed on a Hitachi F4500.

**Fluorometric Analysis.** The slit width was 2.5 nm for both excitation and emission. The photon multiplier voltage was 700 V. Relative fluorescence quantum efficiency of MitoAR, MitoHR and HMTMR was obtained by comparing the area under the emission spectrum of the test sample excited at 552 nm with that of a solution of rhodamine B in EtOH, which has a quantum efficiency of 0.97.

**HPLC analysis.** HPLC analysis were performed on an Inertsil ODS-3 (4.6 x 250 mm) column (GL Sciences Inc.) using an HPLC system composed of a pump (PU-980, JASCO) and a detector (MD-2015 or FP-2025, JASCO).

**HPLC preparation.** Preparative HPLC were performed on an Inertsil ODS-3 (10.0 x 250 mm) column (GL Sciences Inc.) using an HPLC system composed of a pump (PU-2080, JASCO) and a detector (MD-2015 or FP-2025, JASCO).

**Fluorescence microscopy.** We used an U-LH100HG confocal laser scanning unit (Olympus) coupled to a IX81FVBF inverted microscope with a PlanApo 60/1.4 objective lens (Olympus). For co-staining experiments, the excitation wavelength was 488 and 543 nm, and the emission was filtered using a 505-525 and 560- nm barrier filter. For hROS imaging, the excitation wavelength was 543 nm, and the emission was filtered using a 560- nm barrier filter.

**Methods of experiments using living cells.** HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified incubator containing 5% CO$_2$ gas. For fluorescence microscopy, HeLa cells were plated in a 35-mm glass-bottom dish (MatTek Corporation) and cultured overnight in DMEM. HL-60 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified incubator containing 5% CO$_2$ in air. For fluorescence microscopy, HL-60 cells were plated in a 35-mm glass-bottomed dish (MatTek Corporation) in Hank’s balanced salts solution (HBSS). For spectrometer, HL-60 cells were diluted to $1 \times 10^6$ cells / mL.

**Method of determination of the concentration of generated superoxide in X/XO system.** Xanthine (10 µM) was added to Cytochrome c (10 µM) and xanthine oxidase (1.73 mU/mL), and the absorbance at 550 nm was measured before and 30 min after addition of xanthine. The concentration of generated superoxide was calculated from the increase of absorbance, with $\varepsilon_{550} = 21.6$ mM$^{-1}$cm$^{-1}$. 
Synthesis and characterization

Scheme S1. Synthetic scheme for MitoAR and MitoHR.

MitoAR

(a) 4-Benzylideneaminophenol, K$_2$CO$_3$ / DMF, 80 °C, y. 62.4 % (b) (i) i-PrMgCl / THF, - 20 °C (ii) 3,6-Bis(N,N,N',N'-tetramethylamino)xanthone / THF, - 20 °C → r.t. (iii) MeOH, 2N HCl, y. 53.8 % (c) 4-tert-Butoxyphenol, K$_2$CO$_3$ / DMF, 80 °C, y. 64.4 % (d) (i) i-PrMgCl / THF, - 20 °C (ii) 3,6-Bis(N,N,N',N'-tetramethylamino)xanthone / THF, - 20 °C → r.t. (iii) MeOH (e) CF$_3$SO$_3$H / CF$_3$CH$_2$OH, y. 3.6 %

Synthesis of Benzylidene-[4-(2-iodobenzyloxy)phenyl]amine

A suspension of 2-iodobenzyl bromide (891 mg, 3.00 mmol), 4-benzylideneaminophenol (592 mg, 3.00 mmol), and potassium carbonate (829 mg, 6.00 mmol) in 30 mL of DMF was stirred overnight at 80 °C. After cooling, the solvent was removed by evaporation. The residue was then taken up in sat. NH$_4$Cl, and extracted with CH$_2$Cl$_2$. The solvent was removed in vacuo again. Purification of the residue by column chromatography (silica gel, 1:9 AcOEt / hexane) provided pure product as a yellow oil (0.773 g, 1.87 mmol, 62.4 % yield).

$^1$H-NMR (300 MHz, CDCl$_3$) δ: 5.06 (s, 2H), 7.00-7.04 (m, 3H), 7.24 (dt, 2H, $J = 9.5, 2.8$ Hz), 7.37 (td, 1H, $J = 7.57, 1.16$ Hz), 7.46-7.47 (m, 3H), 7.53 (dt, 1H, $J = 9.3, 3.4$ Hz), 7.87-7.89 (m, 3H), 8.48 (s, 1H).

$^{13}$C-NMR (75 MHz, CDCl$_3$) δ: 69.5, 97.2, 115.5, 122.2, 128.4, 128.6, 128.7, 128.7, 129.5, 131.1, 136.4, 139.1, 139.3, 145.5, 157.1, 158.7.
MitoAR
To a flame-dried flask flushed with argon, benzylidene-[4-(2-iodobenzyloxy)phenyl]amine (248 mg, 0.599 mmol), distilled THF (5 mL), and 2.0 M i-PrMgCl (0.299 mL, 0.599 mmol) were added. The solution was stirred at – 20 °C for 2 h. 3,6-Bis(N,N-dimethylamino)xanthone (33.8 mg, 0.120 mmol) dissolved in THF (10 mL) was added, and the reaction mixture was stirred at r.t. overnight. The reaction was quenched by addition of MeOH (10 mL), treated with 2 N HCl (10 mL) and stirred at r.t. for 20 min. Saturated NaHCO3 was added, and the whole was extracted with CH2Cl2. The organic layer was dried over Na2SO4 and evaporated. Purification of the residue by column chromatography (silica gel, AcOEt, 1:4 MeOH/CH2Cl2, stepwise) and HPLC provided pure MitoAR as a deep purple solid (32.3 mg, 0.0646 mmol, 53.8 % yield).

1H-NMR (300 MHz, CDCl3) δ: 3.36 (s, 12H), 4.72 (s, 2H), 6.20 (dt, 2H, J = 9.66, 2.84 Hz), 6.50 (dt, 2H, J = 9.66, 2.75 Hz), 6.82 (d, 2H, J = 2.38 Hz), 6.91 (dd, 2H, J = 9.54, 2.38 Hz), 7.13 (d, 2H, J = 9.54 Hz), 7.23 (dd, 1H, J = 7.43, 1.01 Hz), 7.57 (td, 1H, J = 7.43, 1.28 Hz), 7.66 (td, 1H, J = 7.52, 1.28 Hz), 7.73 (dd, 1H, J = 7.79, 0.83 Hz).

13C NMR (75MHz, CDCl3): δ: 41.2, 68.9, 96.7, 113.7, 114.3, 115.3, 116.6, 128.6, 129.4, 129.6, 130.5, 131.1, 131.4, 136.2, 140.2, 150.8, 157.4, 157.5, 157.8.

HRMS (ESI-Tof) m/z Found 464.2350 (M-Cl)+, calculated 464.2338 for C30H30N3O2 (1.15 mmu).

4-tert-Butyl 4-(2-iodobenzyloxy)phenyl ether
A suspension of 2-iodobenzyl bromide (0.891 g, 3.00 mmol), 4-tert-butoxyphenol (0.499 g, 3.00 mmol), and potassium carbonate (0.829 g, 6.00 mmol) in 30 mL of DMF was stirred overnight at 80 °C. After cooling, the solvent was removed by rotary evaporation. The residue was taken up in sat. NH4Cl, and extracted with CH2Cl2. The solvent was removed by evaporation again. Purification of the residue by column chromatography (silica gel, 1:9 ethyl acetate/hexane) provided pure product as a colorless oil (0.738 g, 1.93 mmol, 64.4 % yield).

1H-NMR (300 MHz, CDCl3) δ: 1.31 (s, 9H), 5.00 (s, 2H), 6.86-6.95 (m, 4H), 7.02 (td, 1H, J = 7.61, 1.53 Hz), 7.36 (td, 1H, J = 7.52, 1.10 Hz), 7.52 (dd, 1H, J = 7.61, 1.38 Hz), 7.86 (dd, 1H, J = 7.89, 0.92 Hz).

13C NMR (75MHz, CDCl3): δ: 28.7, 74.3, 78.1, 97.1, 115.0, 125.3, 128.3, 128.6, 129.4, 139.2, 139.3, 149.1, 154.6.

MitoHR
To a flame-dried flask flushed with argon, 4-tert-butyl 4-(2-iodobenzyloxy)phenyl ether (234 mg, 0.613 mmol), distilled THF (5 mL), and 2.0 M i-PrMgCl (0.307 mL, 0.613 mmol) were added. The solution was stirred at – 20 °C for 2 h. 3,6-Bis(N,N-dimethylamino)xanthone (34.6 mg, 0.123 mmol) dissolved in THF (10 mL) was added and the reaction mixture was stirred at r.t. overnight. The reaction was quenched by addition of MeOH (10 mL), treated with 2 N HCl (10 mL) and stirred at r.t. for 20 min. Saturated NaHCO3 was added, and the whole was extracted with CH2Cl2. The organic layer was dried over Na2SO4 and evaporated. Purification of the residue by column chromatography (silica gel, AcOEt, 1:10 MeOH/CH2Cl2, stepwise) to afford a deep purple solid. This solid was dissolved in CF3CH2OH (10 mL), then CF3SO3H (0.5 mL) was added to the solution at 0 °C, and the mixture was stirred 30 min. Next H2O was added and the whole was extracted with CH2Cl2. The organic layer was dried over Na2SO4 and evaporated. Purification of the residue by HPLC provided pure MitoHR as a deep purple solid (2.19 mg, 0.00437 mmol, 3.6 % yield).

1H-NMR (300 MHz, CH3CN) δ: 3.24 (s, 12H, h), 4.74 (s, 2H), 6.26 (dt, 2H, J = 9.96, 3.03 Hz), 6.49 (dt, 2H, J = 9.96, 3.03 Hz), 6.79 (d, 2H, J = 2.57 Hz), 6.93 (dd, 2H, J = 9.54, 2.38 Hz), 7.11 (d, 2H, J = 9.35 Hz), 7.30 (dd, 1H, J = 7.43, 1.19 Hz), 7.59 (td, 1H, J = 7.43, 1.47 Hz), 7.68 (td, 1H, J = 7.52, 1.41 Hz), 7.75 (dd, 1H, J = 7.70, 1.10 Hz).

13C NMR (75MHz, CD3OD): δ: 28.7, 74.3, 78.1, 97.1, 115.0, 125.3, 128.3, 128.6, 129.4, 139.2, 139.3, 149.1, 154.6.

HRMS (ESI-Tof) m/z Found 465.2148 (M-Cl)+, calculated 465.2178 for C30H29N2O3 (-3.04 mmu).
Figures and Tables

Table S1. Photochemical properties of MitoAR, MitoHR and HMTMR.

<table>
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<th>Emmax</th>
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<th>Fluorescence quantum efficiency (Φfl)</th>
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<tr>
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Figure S1. HPLC chromatogram of (A) MitoAR or (B) MitoHR 10 µM, after reaction with •OH, ONOO⁻, or OCl⁻. Chromatograms of unreacted dye and of HMTMR are also shown. Elution was done with a linear gradient (eluent, 0 min, 20 % CH₃CN/0.1 % TFA aq. ~ 30 min, 80 % CH₃CN/0.1 % TFA aq.; flow rate = 1.0 mL/min). Detection wavelength was 553 nm.
Figure S2. Detection of hROS generation in mitochondria. (a), (b) Brightfield and fluorescence images of HeLa cells loaded with MitoAR (1 μM) for 15 min. (c), (d) Fluorescence images at 10 min and 30 min after addition of H₂O₂ (1 mM).

Figure S3. Relative fluorescence intensity (RFI) measured with a spectrometer at 30 min after stimulation of 1 μM MitoAR-loaded HL-60 cells with various concentrations of H₂O₂ (n = 5). Excitation and detection wavelengths were 553 / 574 nm.
**Figure S4.** Time course of relative fluorescence intensity (RFI) measured with a spectrometer. 1 µM MitoAR-loaded HeLa or HL-60 cells were stimulated with H$_2$O$_2$ (100 µM) at 100 s. Excitation and detection wavelengths were 553 / 574 nm.

**References**