Activity-Based Fluorescent Reporters for Monoamine Oxidases in Living Cells

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Experimental Section

Synthetic Materials and Methods. Silica gel P60 (SiliCycle) was used for column chromatography. Analytical thin layer chromatography was performed using SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Acros Organics (Morris Plains, NJ) and were used as received. ^{1}H NMR spectra were collected in $CDCl_3$, CD_3OD, or d^6-DMSO (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AV-300 or AV-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 using the peak of residual proton signals of $CDCl_3$, CD_3OD, or d^6-DMSO as an internal reference. Mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley.

***tert*-Butyl-3-chloropropyl(methyl)carbamate (1).** Triethylamine (0.6 mL, 4.1 mmol) was added dropwise to a solution of *N*-methyl-3-chloropropylamine hydrochloride (495 mg, 3.4 mmol) in dichloromethane (1.6 mL) and the resulting solution was stirred for 15 min. Next, a solution of di-*tert*-butyl dicarbonate (749 mg, 3.4 mmol) in dichloromethane (1.6 mL) was added dropwise to the amine solution over a period of 1 h, and the resulting mixture was stirred overnight at room temperature. The reaction was diluted to 10 mL with dichloromethane, washed with 1N HCl (1 × 10 mL), water (2 × 5 mL), saturated aq. $NaHCO_3$ (1 × 10 mL), brine (1 × 10 mL), and the organic layer was separated and dried over Na_2SO_4. The solvent was removed by rotary evaporation to afford compound **1** as a pale yellow oil that was used without further purification (445 mg, 63% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.56 (t, 2H, J = 6.6 Hz), 3.44 (t, 2H, J = 6.6 Hz), 2.85 (s, 3H), 1.97 (m, 2H), 1.44 (s, 9H). LR ESI-MS calculated for

$C_9H_{18}NO_2ClNa$ (MNa^+) 320.1, found 320.1.

***tert*-Butyl-3-chloropropylcarbamate (2).** Triethylamine (1.67 mL, 12 mmol) was added dropwise to a solution of 3-chloropropylamine hydrochloride (1.30 g, 10 mmol) in dichloromethane (4.5 mL) and the resulting solution was stirred for 15 min. Next, a solution of di-*tert*-butyl dicarbonate (2.18 g, 10 mmol) in dichloromethane (4.5 mL) was added dropwise to the amine solution over a period of 1 h, and the resulting mixture was stirred overnight at room temperature. The reaction was diluted to 10 mL with dichloromethane, washed with 1N HCl (1 × 10 mL), water (2 × 5 mL), saturated aq. $NaHCO_3$ (1 × 10 mL), brine (1 × 10 mL), and the organic layer was separated and dried over Na_2SO_4. The solvent was removed by rotary evaporation to give **2** as a colorless oil (1.68 g, 87% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.70 (s, 1H), 3.83 (t, 2H, *J* = 6.6 Hz), 3.29 (m, 2H), 1.96 (m, 2H), 1.52 (s, 9H). HR FAB-MS calculated for $C_8H_{17}NO_2Cl$ (MH^+) 194.0947, found 194.0948.

***tert*-Butyl-methyl-(3-(3-oxo-3H-phenoxazin-7-yloxy)propyl)carbamate (3).** Under a nitrogen atmosphere, resorufin sodium salt (118 mg, 0.5 mmol), *tert*-butyl-3-chloropropyl(methyl)carbamate (**1**) (426 mg, 2.0 mmol), and potassium carbonate (104 mg, 0.75 mmol) were combined in an oven-dried Schlenk flask. Anhydrous DMF (5 mL) was added by syringe and the resulting mixture was heated at 120 ºC for 48 h under nitrogen. The reaction was cooled to room temperature, diluted with 100 mL ethyl acetate, washed with water (4 × 100 mL) and brine (1 × 100 mL), and the organic layer was separated and dried over Na_2SO_4. The solvent was removed by rotary evaporation and purification by flash column chromatography (silica gel, 1:1 hexanes/ethyl acetate) delivered **3** as an orange solid (108 mg, 56% yield). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.77 (d, 1H, *J* = 4.4 Hz), 7.70 (s, 1H), 7.53 (d, 1H, *J* = 4.8 Hz), 7.05 (d, 1H, *J* = 3.3 Hz), 6.98 (s, 1H), 6.85 (d, 1H, *J* = 3.9 Hz), 6.35 (s, 1H), 4.15 (t, 2H, *J* = 5.7 Hz), 3.46 (s, 2H), 2.89 (s, 3H), 2.06 (m, 2H), 1.42 (s, 9H). HR FAB-MS calculated for $C_{21}H_{25}N_2O_5$ (MH^+) 385.1774, found 385.1763.

***tert*-Butyl-3-(3-oxo-3H-phenoxazin-7-yloxy)propylcarbamate (4).** Under a nitrogen atmosphere, resorufin sodium salt (118.0 mg, 0.5 mmol), *tert*-butyl-3-chloropropylcarbamate (**2**)

(397 mg, 2.0 mmol), and potassium carbonate (104 mg, 0.75 mmol) were combined in an oven-dried Schlenk flask. Anhydrous DMF (5 mL) was added by syringe and the resulting mixture was heated at 120 ºC for 48 h under nitrogen. The reaction was cooled to room temperature, diluted with 100 mL ethyl acetate, washed with water (4 × 100 mL) and brine (1 × 100 mL), and the organic layer was separated and dried over Na_2SO_4. The solvent was removed by rotary evaporation and purification by flash column chromatography (silica gel, 1:1 hexanes/ethyl acetate to 1:3 hexanes/ethyl acetate) furnished **4** as an orange powder (101 mg, 55% yield). ^{1}H NMR (CD_3OD, 300 MHz): δ 7.75 (d, 1H, *J* = 4.5 Hz), 7.70 (s, 1H), 7.52 (d, 1H, *J* = 4.8 Hz), 7.04 (d, 1H, *J* = 4.5 Hz), 6.98 (s, 1H), 6.84 (d, 1H, *J* = 4.95), 6.34 (s, 1H), 4.16 (t, 2H, *J* = 6.0 Hz), 3.26 (t, 2H, *J* = 6.9 Hz), 2.00 (t, 2H, *J* = 6.0 Hz), 1.41 (s, 9H). HR FAB-MS calculated for $C_{20}H_{23}N_2O_5$ (MH^+) 371.1613, found 371.1607.

7-(3-(Methylamino)propoxy)-3H-phenoxazin-3-one (Monoamine Oxidase Reporter 1, MR1, 5). A solution of trifluoroacetic acid (50% v/v, 10 mL) and triisopropylsilane (20 μL) in dichloromethane (10 mL) was added to *tert*-butyl-methyl-(3-(3-oxo-3H-phenoxazin-7-yloxy)propyl)carbamate (**3**) (66 mg, 0.17 mmol). The resulting mixture was stirred at room temperature for 30 min. Removal of the solvent and recrystallization from dichloromethane produced MR1 as a dark red solid (46 mg, 95% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.56 (s, 2H), 7.78 (d, 1H, *J* = 3.3 Hz), 7.52 (d, 1H, *J* = 3.8 Hz), 7.11 (s, 1H), 7.04 (d, 1H, *J* = 2.4 Hz), 6.79 (d, 1H, *J* = 3.0 Hz), 6.26 (s, 1H), 4.22 (t, 2H, *J* = 4.5 Hz), 3.08 (m, 2H), 2.60 (t, 3H, *J* = 3.9 Hz), 2.08 (m, 2H). HR FAB-MS calculated for $C_{16}H_{17}N_2O_3$ (MH^+) 285.1242, found 285.1239.

7-(3-Aminopropoxy)-3H-phenoxazin-3-one (Monoamine Oxidase Reporter 2, MR2, 5). A solution of trifluoroacetic acid (50% v/v, 10 mL) and triisopropylsilane (20 μL) in dichloromethane (10 mL) was added to *tert*-butyl-3-(3-oxo-3H-phenoxazin-7-yloxy)propylcarbamate (**4**) (185 mg, 0.50 mmol). The resulting mixture was stirred at room temperature for 30 min. Removal of the solvent and recrystallization from dichloromethane produced MR2 as an orange solid (129 mg, 95% yield). ^{1}H NMR (DMSO-d_6, 400 MHz): 7.86 (s, 2H), 7.75 (d, 1H, *J* = 3.3 Hz), 7.50 (d, 1H, *J* = 3.8 Hz), 7.08 (s, 1H), 7.02 (d, 1H, *J* = 2.4 Hz),

6.23 (s, 1H), 4.19 (t, 2H, J = 4.5 Hz), 2.95 (m, 2H), 2.01 (m, 2H). HR FAB-MS calculated for $C_{15}H_{15}N_2O_3$ (MH^+) 271.1081, found 271.1083.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 100 mM HEPES buffer (pH 7.4) or Dulbecco's Phosphate-Buffered Saline (DPBS, pH 7.4). 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 fluorescence measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4- or 3.5-mL volume, Starna, Atascadero, CA).

Monoamine Oxidase Kinetics Assays. Kinetics experiments were performed in 96-well fluorescence assay plates (Black/Clear Bottom, BD Biosciences, Franklin Lakes, NJ) and employed recombinant forms of human MAO A (5.0 mg/mL in buffered solution, 150 U/mg, Sigma-Aldrich, St. Louis, MO) or MAO B (5.0 mg/mL in buffered solution, 45 U/mg, Sigma-Aldrich, St. Louis, MO) expressed in baculovirus infected BTI insect cells. Stock solutions of MR1 and MR2 were prepared in DMSO (0 to 2.0 mM) and diluted in enzyme assay buffer (5% glycerol in 100 mM HEPES, pH 7.4) to a final concentration containing 1% DMSO. MAO A or MAO B were added to a final protein concentration of 1 μg enzyme per 100 μL well at 25 ºC, and fluorescence measurements were taken at 5 min intervals from 0 to 3 h on a Molecular Devices Spectramax Gemini XS Microplate Spectrofluorometer with excitation at 544 nm and emission intensity collected at 590 nm. Kinetics experiments with MR1 and MR2 and purified MAO A and MAO B enzymes were performed a minimum of six times apiece.

Preparation of Cell Cultures. PC12 cells (rat pheochromocytoma, adrenal gland) were seeded at a density of 1.8×10^6 cells/well in a 6-well tissue culture dish at the Cell Culture Facility at the University of California, Berkeley and differentiated for 3 to 5 days with Nerve

Growth Factor (2.5S, 30 ng/mL, Invitrogen, Carlsbad, CA) in Dulbecco's Modified Eagle Medium (DMEM 1X, without phenol red, Gibco/Invitrogen, Carlsbad, CA) containing 4.5 g/L D-glucose, 2 mM GlutaMax-1 (Invitrogen), and 10% Fetal Bovine Serum (HyClone, Logan, UT).

Monoamine Oxidase Live-Cell Assays. Immediately before the MAO activity assays, live PC12 cells were washed with DMEM (without L-glutamine, sodium pyruvate, or phenol red, Invitrogen, Carlsbad, CA). The live cells were then treated with either 250 μM of the global MAO inhibitor pargyline hydrochloride (Sigma-Aldrich, St. Louis, MO) in DMEM (0.25% DMSO final concentration) or DMSO in DMEM (vehicle controls, 0.25% DMSO final concentration) for 80 min at 37 ºC in a 5% CO_2 incubator. MR1 or MR2 were then added to final concentrations of 250 μM from 50 mM stock solutions in DMSO (final total DMSO concentration per assay 0.75% v/v) and incubated for 3 or 7 hours at 37 ºC in a 5% CO_2 incubator. Brightfield measurements on cells treated with inhibitor and dye, dye or inhibitor only, or DMSO vehicle control confirm the viability of the cells throughout the experiments. After incubation with the MR activity probes, the media was removed and the cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.4) to remove excess extracellular dye. Next, 1.5 mL DPBS was added and the cells were lifted from the dish surface using a cell scraper. The resuspended cells were immediately assayed for fluorescence using the PTI scanning spectrofluorometer (Lawrenceville, NJ) described previously. Spectra were recorded at 25 °C with excitation at 550 nm, and emission was collected from 560 to 750 nm. Fluorescence experiments with MR1 and MR2 and live PC12 cells with or without pargyline inhibitor were performed in triplicate.

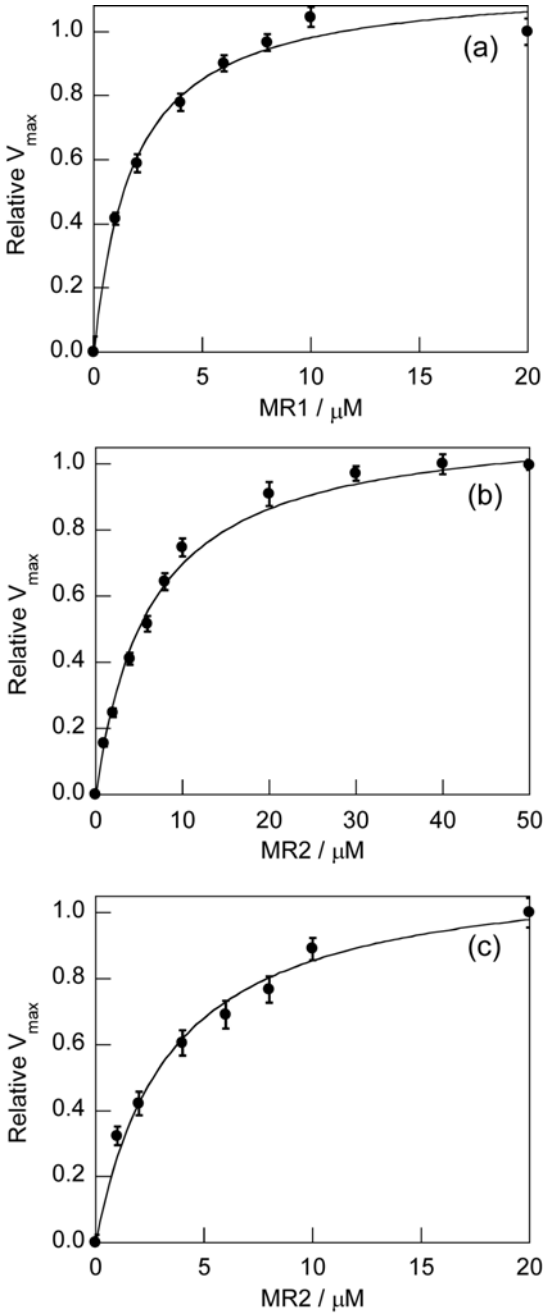

Figure S1. Enzyme kinetics plots of reactions of MR1 with MAO B (panel a), MR2 with MAO A (panel b), and MR2 with MAO B (panel c). Data show relative reaction rates measured over a range of MR1 concentrations, and each data point represents the average of at least six independent experiments. Reactions were performed at 25 °C in enzyme assay buffer (100 mM HEPES, pH 7.4 with 5% glycerol and 1% DMSO) with MAO A or MAO B at a final protein concentration of 10 μg/mL. Fluorescence excitation was provided at 544 nm and emission intensity collected at 590 nm.

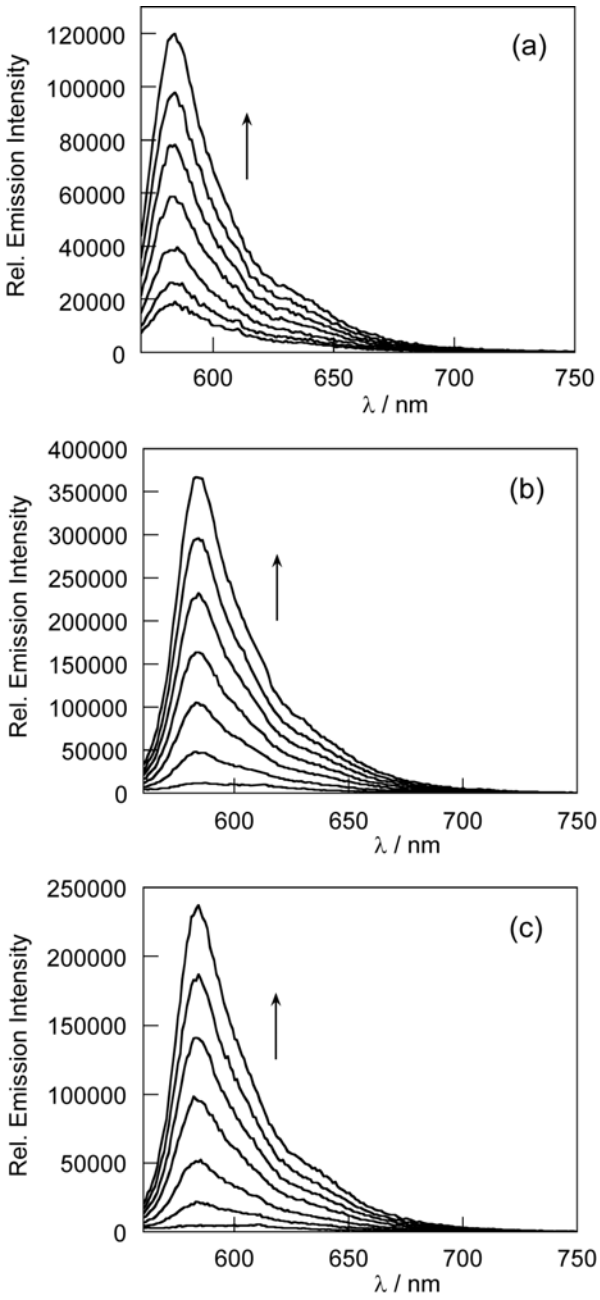

Figure S2. Direct fluorometric detection of MAO activity with MR1 and MR2. Panel (a) shows the fluorescence response of MR1 to MAO B at its K_m value (1.8 μM probe). Panels (b) and (c) show the analogous response of MR2 to MAO A (6.3 μM probe) and MAO B (K_m = 3.4 μM probe). Enzyme reactions were performed at 25 °C in assay buffer (100 mM HEPES, pH 7.4 with 5% glycerol and 1% DMSO) with MAO A or MAO B at a final protein concentration of 50 μg/mL. Fluorescence excitation was provided at 550 nm, and traces are shown after 0, 10, 20, 30, 40, 50, and 60 min after adding MR1 or MR2 to the MAO enzyme. Deprotections of MR1 with MAO A or B under these conditions are not complete at these early time points.