A highly sensitive fluorogenic probe for cytochrome P450 activity in live cells

Melissa M. Yatzeck, Luke D. Lavis, Tzu-Yuan Chao, Sunil S. Chandran, and Ronald T. Raines*

Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA
Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, USA

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Instrumentation. NMR spectra were obtained with a Bruker DMX-400 Avance spectrometer (^{1}H, 400 MHz; ^{13}C, 100.6 MHz; ^{31}P, 161 MHz) at the National Magnetic Resonance Facility at Madison (NMRFAM). Carbon-13 spectra were proton-decoupled. Mass spectrometry was performed with a Micromass LCT (electrospray ionization, ESI) mass spectrometer in the Mass Spectrometry Facility in the Department of Chemistry. Fluorometric measurements were recorded with fluorescence grade quartz or glass cuvettes (Starna Cells) and a QuantaMaster1 photon-counting spectrofluorometer equipped for sample stirring (Photon Technology International). Cells were imaged with a Nikon Eclipse TE2000-U confocal microscope equipped with a Zeiss AxioCam digital camera.

General Synthetic Methods. Silyl ether **2**[1] and morpholinourea–Rh_{110} (**6**)[2] were prepared as described previously. All other reagents were obtained from Aldrich Chemical (Milwaukee, WI) or Fisher Scientific (Hanover Park, IL) and used without further purification. Dimethylformamide (DMF), tetrahydrofuran (THF), and dichloromethane (CH_2Cl_2) were drawn from a Baker CYCLE-TAINER solvent delivery system. Procedures were performed at room temperature (<23 °C) unless indicated otherwise. Reactions were monitored by thin-layer chromatography with aluminum-backed plates coated with silica gel containing F_{254} phosphor and visualized by UV illumination or staining with I_2, ceric ammonium molybdate, or phosphomolybdic acid. Compounds were purified by flash chromatography with open columns loaded with silica gel-60 (230–400 mesh), or on a FlashMaster Solo system (Argonaut Inc., Redwood City, CA) with Isolute Flash Si II columns (International Sorbent Technology Ltd., Hengoed, Mid Glamorgan, UK).

The term "high vacuum" refers to a vacuum (≤1 mm Hg) achieved by a mechanical belt-drive oil pump. The term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water-aspirator pressure (<20 mm Hg) while maintaining the water-bath temperature below 40 °C. The term "concentrated under high vacuum" refers to the removal of solvents and other volatile materials using a rotary evaporator at high vacuum while maintaining the water-bath temperature below 40 °C.

Silyl ether 3. Silyl ether **2**[1] (5.75 g, 17.8 mmol) was dissolved in of Et_2O (120 mL) under Ar(g). Sodium hydride (0.513 g, 21.4 mmol) was added portionwise, and the mixture was allowed to stir for 30 min. Diethyl sulfate (3.30 g, 21.4 mmol) was added dropwise, and the resulting mixture was stirred for 16 h at ambient temperature under Ar(g). The reaction was quenched by the addition of 60 mL of H_2O, the layers were separated, and the aqueous layer was extracted with EtOAc (2 × 60 mL). The combined organic extracts were dried over $MgSO_4$(s) and concentrated under reduced pressure to give a yellow oil, which was purified by column chromatography (silica gel 20% v/v CH_2Cl_2 in hexanes) to give **3** as light yellow oil (3.56 g, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.55 (s, 1H), 6.51 (s, 1H), 4.00 (q, J = 7 Hz, 2H), 3.48 (t, J = 8 Hz, 2H), 2.48 (3H, s), 2.24 (3H, s), 2.18 (2H, t, J = 8 Hz), 1.53 (6H, s), 1.45 (3H, t, J = 7 Hz), 0.855 (9H, s), –0.025 (6H, s). ^{13}C NMR (100 MHz) δ (ppm): 158.92, 137.64, 135.78, 131.19, 127.09, 111.87, 63.97, 61.72, 45.90, 40.00, 32.43 (2C), 26.19 (3C), 26.05, 20.97, 18.49, 15.24, –5.05 (2C). HRMS (ESI): m/z 373.2556 (MNa^+ [$C_{21}H_{38}O_2SiNa$] = 373.2539).

Alcohol 4. A solution containing silyl ether **3** (3.525 g, 10.05 mmol), THF (35 mL), H_2O (20 mL), and AcOH (60 mL) was stirred for 4 h. The reaction mixture was concentrated under high vacuum to give a light-yellow oil, which was purified by flash chromatography on a silica gel column (4:1 Hexanes/EtOAc) to give **4** as a yellow oil (2.327 g, 98%). ^{1}H NMR δ (ppm): 6.57 (1H, s), 6.53 (1H, s), 4.02 (2H, q, J = 7 Hz), 3.55 (2H, t, J = 7 Hz), 2.50 (3H, s), 2.24 (3H, s), 2.21 (2H, t, J = 7 Hz), 1.55 (6H, s), 1.46 (3H, t, J = 7 Hz). ^{13}C NMR (100 MHz) δ (ppm):

158.83, 137.64, 136.16, 130.86, 127.31, 112.14, 64.09, 61.59, 45.80, 39.97, 32.48 (2C), 26.02, 20.94, 15.23. HRMS (ESI): *m*/*z* 259.1663 (MNa^+ [$C_{15}H_{24}O_2Na$] = 259.1674).

Acid 5. Alcohol **4** (2.327 g, 9.846 mmol) was dissolved in 10 mL of acetone, and the resulting solution was cooled to 0 °C with an ice bath. Jones reagent (prepared from 0.148 g of CrO_3, 12.6 mL of H_2SO_4, and 6 mL of water) was added slowly and with stirring. The reaction mixture was allowed to stir for 1 h at 0 °C under Ar(g), quenched with isopropanol (2 mL), and stirred at room temperature for an additional 20 min. The reaction mixture was concentrated under reduced pressure to give the crude product as a dark green solid. EtOAc (20 mL) and water (20 mL) were added and, the organic layer was separated. The aqueous solution was extracted with EtOAc (2 × 20 mL). The combined organic extracts were washed with saturated NaCl(aq) (2 × 20 mL), dried over $MgSO_4$(s), and filtered. The solution was concentrated under reduced pressure to give a yellow oil, which was purified by flash chromatography on a silica gel column (90:5:5 hexanes/CH_2Cl_2/acetone), to give **5** as a light yellow crystalline solid (1.498 g, 34%). ^{1}H NMR δ (ppm): 6.58 (1H, s), 6.54 (1H, s), 4.02 (2H, q, J = 7 Hz), 3.06 (2H, s), 2.52 (3H, s), 2.26 (3H, s), 1.63 (6H, s), 1.46 (3H, t, J = 7 Hz). ^{13}C NMR (100 MHz) δ (ppm): 158.10, 137.47, 135.98, 129.77, 127.09, 111.66, 104.99, 63.75, 47.00, 39.49, 31.58 (2C), 25.59, 20.79, 14.90. HRMS (ESI): *m*/*z* 273.1465 (MNa^+ [$C_{15}H_{22}O_3Na$] = 273.1467).

Morpholinourea–Rh$_{110}$ Trimethyl Lock (1). Acid **5** (0.125 g, 0.499 mmol) was dissolved in 3:2 DMF/pyridine (5 mL). EDC (0.106 g, 0.554 mmol) was added, and resulting mixture was allowed to stir at room temperature for 1 h. Morpholinourea–Rh$_{110}$ (**6**; 0.123 g, 0.277 mmol) was dissolved in 5 mL of 3:2 DMF/pyridine and added to the reaction mixture, which was then covered with aluminum foil and allowed to stir under Ar(g) for two days. The reaction mixture was concentrated under high vacuum to give a dark orange oil, which was dissolved in 75 mL of a 1:1 EtOAc/hexanes. This solution was washed with 1 N HCl (2 × 50 mL) and saturated NaCl(aq) (2 × 50 mL), dried over $MgSO_4$(s), and filtered. The solution was concentrated under reduced pressure to give an off-white solid that was purified by flash chromatography on a silica gel column (1:1 EtOAc/hexanes). Fractions that contained product were combined and concentrated by rotary evaporation under reduced pressure to give **1** as a white crystalline solid (0.088 g, 26% yield): ^{1}H NMR (DMSO) δ (ppm): 9.89 (1H, s), 8.84 (1H, s), 8.01 (1H, d, J = 8 Hz), 7.78 (1H, m), 7.76 (1H, m), 7.72 (1H, m), 7.62 (1H, m), 7.26 (1H, d, J = 8 Hz), 7.16 (1H, m), 7.07 (1H, m), 6.66 (1H, d, J = 9 Hz), 6.64 (1H, d, J = 9 Hz), 6.61 (1H, s), 6.45 (1H, s), 3.98 (2H, q, J = 7 Hz), 3.60 (4H, t, J = 5 Hz), 3.44 (4H, t, J = 5 Hz), 3.33 (9H, s), 2.99 (2H, s), 2.45 (3H, s), 2.16 (3H, s), 1.56 (6H, s), 1.38 (3H, t, J = 7 Hz). ^{13}C NMR (100 MHz) δ (ppm): 170.92, 168.72, 157.74, 154.73, 152.57, 150.81, 142.89, 141.39, 136.58, 135.67, 134.90, 130.66, 130.14, 128.20, 127.85, 126.67, 124.71, 123.96, 115.49, 115.04, 112.66, 111.92, 111.48, 105.95 (2C), 82.26, 65.92 (2C), 63.41, 48.63, 44.14 (2C), 40.91, 40.50, 40.12, 31.37 (2C), 25.36, 20.33, 14.78. HRMS (ESI): *m*/*z* 698.2827 (MNa^+ [$C_{40}H_{41}N_3O_7Na$] = 698.2842).

Kinetics Assays with Purified Enzyme. Kinetic parameters were determined by using microsomes that contained recombinant human cytochrome P450 CYP1A1 isozyme and human NADPH–P450 reductase (Sigma Chemical; product number C3735) as a suspension in 100 mM potassium phosphate buffer, pH 7.4. The enzyme was diluted in phosphate-buffered saline (PBS) before use. PBS (pH 7.4) contained (in 1.00 L) NaCl (8.0 g), KCl (2.0 g), $Na_2HPO_4{\cdot}7H_2O$ (1.15 g), KH_2PO_4 (2.0 g), and NaN_3 (0.10 g).

Kinetic assays were conducted at 37 °C by fluorometric detection of morpholinourea–Rh$_{110}$ (**6**) using excitation and emission wavelengths of 496 and 520 nm, respectively. The reaction mixture (2.00 mL) contained 10 μL of a 0.5 pmol/mL solution of enzyme, 10 μL of 0.8 M

$MgCl_2$, 10 μL of 0.8 M NADPH, and 10 μL of a solution of morpholinourea–Rh_{110} trimethyl lock (**1**) (final concentration: 0.0624–1.00 mM) in PBS. The reaction was initiated by the addition of substrate, and the reaction rate was quantified by comparison to the fluorescence of solutions containing known concentrations of morpholinourea–Rh_{110} (**6**). Values of k_{cat}/K_M and K_M were calculated by standard methods.

Cell Culture and Imaging. Human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37 °C in medium supplemented with fetal bovine serum (10% v/v) and antibiotics, and in the presence of CO_2(g) (5% v/v). Nearly confluent cells were seeded in 8-well chambers and allowed to grow for 24 h. Cells were then incubated for 1 h after the addition of morpholinourea–Rh_{110} trimethyl lock (**1**) (to 10 μM), TCDD (to 10 nM), and resveratrol (to 50 μM). Hoechst 33342 nuclear stain was added 5 min prior to the end of the incubation time and before imaging. In pilot experiments, a range of TCDD, resveratrol, and morpholinourea–Rh_{110} trimethyl lock (**1**) concentrations were tested. The concentrations chosen for the actual studies were the ones causing substantial induction or inhibition without detectable cytotoxicity.

References and Notes

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