A Suite of Activity-Based Probes for Human Cytochrome P450 Enzymes

Aaron T. Wright, Joongyu D. Song, and Benjamin F. Cravatt*

SUPPORTING INFORMATION

Synthesis and Characterization of New Activity-Based Probes.

Probe 2. **12** (30 mg, 0.21 mmol), HOBt (33 mg, 0.25 mmol), EDCI (47 mg, 0.25 mmol), and NMM (0.05 mL, 0.41 mmol) were dissolved in CH_3CN/DMF (2/1, 1.6 mL) at rt. After 15 min hex-5-yn-1-amine (30 mg, 0.23 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (4 mL), washed with 10% citric acid (2 × 5 mL), $NaHCO_3$ (sat) (2 × 5 mL), and brine (2 × 5 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate (75/25)) yielding **2** (34 mg, 0.15 mmol, 71%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.62 (m, 2H), 1.75 (m, 2H), 1.98 (t, *J* = 3.5, 1H), 2.26 (dt, *J* = 2.8, 6.8, 2H), 3.20 (s, 1H), 3.48 (q, *J* = 6.8, 2H), 6.33 (s, NH), 7.53 (d, *J* = 8.4, 2H), 7.72 (d, *J* = 8.4, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 18.29, 25.91, 28.80, 39.77, 69.02, 79.62, 82.92, 84.17, 125.42, 127.01, 132.43, 134.82, 166.93. HR-MS *m/z* calcd for $C_{15}H_{16}NO$ (M + H): 226.1226. Found: 226.1223.

(16) 1-ethynyl-2-naphthoic acid. **15** (66 mg, 0.23 mmol) was dissolved in ethanol/CH_2Cl_2 (3/2, 0.60 mL) and cooled to 0 °C. NaOH (1.0 M, 0.94 mL, 0.92 mmol) was slowly dripped in over 10 min. The reaction was warmed to rt over 2 h, and stirred for an additional 2 h. The reaction solution was washed with ether (2 × 4 mL), and the product was precipitated from the aqueous layer upon dropwise addition of HCl (1.0 M). The product was collected by filtration, dissolved in ether (3 mL), washed with H_2O (2 × 4 mL), and dried with Na_2SO_4. The organics were removed and the product was further purified over silica (CH_2Cl_2/CH_3OH (99:1)) yielding **5** (44 mg, 0.22 mmol, 96%) as an off-white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 5.30 (s, 1H), 7.74 (m, 2H), 7.86 (d, *J* = 8.4, 1H), 8.03 (m, 2H), 8.35 (d, *J* = 9.6, 1H). ^{13}C NMR (400 MHz, CD_3OD): δ 78.01, 88.10, 118.86, 119.13, 124.16, 125.82, 126.50, 126.89, 127.09, 127.54, 132.56, 133.28, 193.19. HR-MS *m/z* calcd for $C_{13}H_9O_2$ (M + H): 197.0597. Found: 197.0592.

Probe 3. **16** (15 mg, 0.08 mmol), HOBt (12 mg, 0.09 mmol), EDCI (14 mg, 0.09 mmol), and NMM (0.02 mL, 0.15 mmol) were dissolved in CH_3CN/DMF (2/1, 1.0 mL) at rt. After 15 min hex-5-yn-1-amine (11 mg, 0.08 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (3 mL), washed with 10% citric acid (2 × 4 mL), $NaHCO_3$ (sat) (2 × 4 mL), and brine (2 × 4 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate (70/30)) yielding **3** (13 mg, 0.042 mmol, 53%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.63 (m, 2H), 1.74 (m, 2H), 1.98 (t, *J* = 3.6, 1H), 2.26 (dt, *J* = 2.8, 6.9, 2H), 3.49 (m, 2H), 3.84 (s, 1H), 7.10 (s, NH), 7.53 (m, 2H), 7.84 (m, 3H), 8.36 (d, *J* = 9.7, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 18.30, 26.03, 28.60, 39.84, 68.93, 80.17, 84.20, 89.23, 116.27, 125.72, 127.05, 127.86, 127.88, 128.42,

129.74, 133.48, 133.83, 136.56, 167.26. HR-MS *m/z* calcd for $C_{19}H_{18}NO$ (M + H): 276.1383. Found: 276.1384.

(20) 4′-ethynylbiphenyl-4-carboxylic acid. 19 (22 mg, 0.068 mmol) was dissolved in ethanol/CH_2Cl_2 (3/2, 0.75 mL) and cooled to 0 °C. NaOH (1.0 M, 0.27 mL, 0.27 mmol) was slowly dripped in over 10 min. The reaction was warmed to rt in 2 h, and stirred for an additional 2 h. The reaction solution was washed with ether (2 × 3 mL), and the product was precipitated from the aqueous layer upon dropwise addition of HCl (1.0 M). The product was collected by filtration, dissolved in ether (2.6 mL), washed with H_2O (2 × 3 mL), and dried with Na_2SO_4. The organics were removed yielding **16** (10 mg, 0.045 mmol, 66%) as an off-white solid. ^{1}H NMR (400 MHz): δ 2.57 (s, 1H), 7.35 (dd, *J* = 2.4, 2H), 7.76 (br, 2H), 7.86 (m, 2H), 8.48 (m, 2H), 9.89 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 97.02, 105.67, 124.21, 129.85, 130.37, 130.76, 132.62, 133.26, 134.80, 136.03, 136.79, 192.79. HR-MS *m/z* calcd for $C_{15}H_{11}O_2$ (M + H): 223.0754. Found: 223.0746.

Probe 4. 20 (10 mg, 0.045 mmol), HOBt (7.0 mg, 0.054 mmol), EDCI (10 mg, 0.054 mmol), and NMM (0.01 mL, 0.090 mmol) were dissolved in CH_3CN/DMF (2/1, 0.5 mL) at rt. After 15 min hex-5-yn-1-amine (7.0 mg, 0.050 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (2 mL), washed with 10% citric acid (2 × 3 mL), $NaHCO_3$ (sat) (2 × 3 mL), and brine (2 × 3 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/CH_2Cl_2 (75/25)) yielding **4** (11 mg, 0.033 mmol, 72%) as a white solid. ^{1}H NMR (400 MHz, Acetone-d_6): δ 1.21 (m, 2H), 1.63 (m, 2H), 2.26 (dt, *J* = 2.8, 6.9, 2H), 2.67 (s, 1H), 3.41 (m, 2H), 4.79 (s, 1H), 7.40 (m, 2H), 7.44 (m, 2H), 7.72 (m,

2H), 7.97 (d, J = 2, 2H). HR-MS *m/z* calcd for $C_{21}H_{20}NO$ (M + H): 302.1539. Found: 302.1544.

Probe 5. **21** (25 mg, 0.14 mmol), HOBt (22 mg, 0.16 mmol), EDCI (32 mg, 0.16 mmol), and NMM (0.03 mL, 0.27 mmol) were dissolved in CH_3CN/DMF (2/1, 2.0 mL) at rt. After 15 min hex-5-yn-1-amine (20 mg, 0.15 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (4 mL), washed with 10% citric acid (2 × 5 mL), $NaHCO_3$ (sat) (2 × 5 mL), and brine (2 × 5 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate (50/50)) yielding **5** (24 mg, 0.081 mmol, 58%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (m, 8H), 1.57 (m, 8H), 1.95 (dt, J = 2.8, 6.8, 2H), 2.20 (br, 6H), 3.25 (q, J = 9.2, 2H), 5.48 (br, NH). ^{13}C NMR (400 MHz, $CDCl_3$): δ 25.88, 25.93, 28.61, 28.84, 28.91, 29.10, 29.37, 29.41, 68.28, 68.88, 84.21, 84.91, 173.29. HR-MS *m/z* calcd for $C_{17}H_{28}NO$ (M + H): 262.2165. Found: 262.2161.

Probe 6. **25** (17 mg, 0.14 mmol), HOBt (22 mg, 0.16 mmol), EDCI (31 mg, 0.16 mmol), and NMM (0.03 mL, 0.27 mmol) were dissolved in CH_3CN/DMF (2/1, 2.5 mL) at rt. After 15 min hex-5-yn-1-amine (20 mg, 0.15 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (4 mL), washed with 10% citric acid (2 × 5 mL), $NaHCO_3$ (sat) (2 × 5 mL), and brine (2 × 5 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate (60/40)) yielding **6** (9.0 mg, 0.044 mmol, 32%) as a white solid. ^{1}H NMR (400 MHz): δ . ^{13}C NMR (400 MHz): δ . HR-MS *m/z* calcd for $C_{13}H_{18}NO$ (M + H): 204.1383. Found: 204.1385.

(27) Ethyl 2-oxo-7-(trifluoromethylsulfonyloxy)-2*H*-chromene-3-carboxylate. 26 (100 mg, 0.43 mmol) was dissolved in dry CH_2Cl_2 (2 mL). Pyridine (0.1 mL, 1.3 mmol) was added and the solution stirred at rt for 10 min. Trifluoromethanesulfonic anhydride (0.08 mL, 0.47 mmol) was slowly dripped into the solution causing a rapid color change to bright orange. After 1 h the reaction was stopped and H_2O (4 mL) was added and the reaction was extracted with CH_2Cl_2 (3 × 5 mL). The combined organics were washed with 10% citric acid (2 × 5 mL), H_2O (2 × 5 mL), and brine (2 × 5 mL), and dried with $MgSO_4$. The volatiles were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate 70/30)) yielding **27** as a yellow solid (115 mg, 0.31 mmol, 73%). ^{1}H NMR (400 MHz, CD_2Cl_2): δ 1.39 (t, *J* = 7.2, 3H), 4.39 (q, *J* = 7.2, 2H), 7.30 (m, 2H), 7.74 (d, *J* = 8.4, 1H), 8.51 (s, 1H). ESI *m/z* calcd for $C_{13}H_{10}F_3O_7S$ (M + H): 367. Found: 367.

(28) Ethyl 2-oxo-7-((trimethylsilyl)ethynyl)-2*H*-chromene-3-carboxylate. 27 (115 mg, 0.31 mmol) was dissolved in a triethylamine/pyridine (7/1, 3 mL) solution. To the solution was added triphenylphosphine (109 mg, 0.42 mmol) and palladium (II) acetate (catalytic). The solution was heated to 90 °C, and the trimethylsilylacetylene (0.1 mL, 0.66 mmol) was added portionwise. After 4 h the volatiles were removed *in vacuo*. The product was purified over silica (hexanes/ethyl acetate (80/20)) yielding **28** as a yellow solid (54 mg, 0.17 mmol, 55%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 9H), 1.13 (t, *J* = 7.2, 2H), 4.13 (q, *J* = 7.2, 3H), 7.09 (m, 2H), 7.25 (d, *J* = 8.2, 1H), 8.20 (s, 1H). ^{13}C NMR (300 MHz, $CDCl_3$): δ 0.03, 14.52, 62.35, 100.86, 103.25, 118.04, 118.58, 119.95, 128.60, 129.48, 129.57, 148.04, 155.06, 156.69, 163.18. HR-MS *m/z* calcd for $C_{17}H_{19}O_4Si$ (M + H): 315.1047. Found: 315.1050.

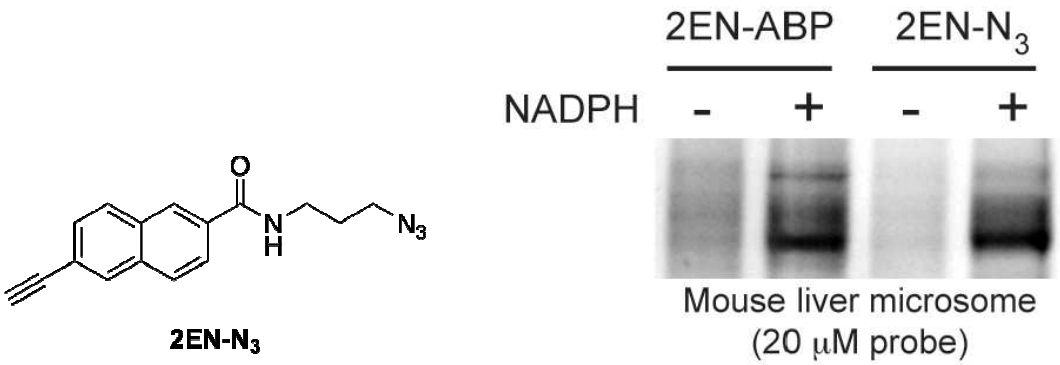
(29) 7-ethynyl-2-oxo-2*H*-chromene-3-carboxylic acid. **28** (41 mg, 0.13 mmol) was dissolved in ethanol/CH_2Cl_2 (3/2, 1.5 mL) and cooled to 0 °C. NaOH (1.0 M, 0.52 mL, 0.52 mmol) was slowly dripped in over 10 min. The reaction was warmed to rt in 2 h, and stirred for an additional 2 h. The reaction solution was washed with ether (2 × 5 mL), and the product was precipitated from the aqueous layer with HCl (1.0 M). The product was collected by filtration, dissolved in ether (3.0 mL), washed with H_2O (2 × 5 mL), and dried with Na_2SO_4. The organics were removed yielding **29** (27 mg, 0.13 mmol, 97%) as a yellow solid. ^{1}H NMR (300 MHz, CD_2Cl_2): δ 3.53 (s, 1H), 7.55 (m, 2H), 7.75 (d, *J* = 8.1, 1H), 8.91 (s, 1H). HR-MS *m/z* calcd for $C_{15}H_{11}O_2$ (M + Na): 237.0158. Found: 237.0158.

Probe 7. **29** (27 mg, 0.12 mmol), HOBt (21 mg, 0.15 mmol), EDCI (29 mg, 0.15 mmol), and NMM (0.03 mL, 0.27 mmol) were dissolved in CH_3CN/DMF (2/1, 3.0 mL) at rt. After 15 min hex-5-yn-1-amine (19 mg, 0.14 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (4 mL), washed with 10% citric acid (2 × 5 mL), $NaHCO_3$ (sat) (2 × 5 mL), and brine (2 × 5 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate (50/50)) yielding **7** (24 mg, 0.082 mmol, 65%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.64 (m, 4H), 2.27 (dt, *J* = 2.8, 6.8, 2H), 3.36 (s, 1H), 3.49 (q, *J* = 6.8, 2H), 4.34 (s, 1H), 7.45 (m, 2H), 7.65 (d, *J* = 8.1, 1H), 8.88 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 18.29, 25.97, 28.65, 39.56, 62.30, 68.93, 82.33, 84.07, 119.02, 120.11, 120.28, 128.05, 128.35, 128.99, 129.77, 147.78, 154.91, 156.38. HR-MS *m/z* calcd for $C_{18}H_{16}NO_3$ (M + H): 294.1125. Found: 294.1116.

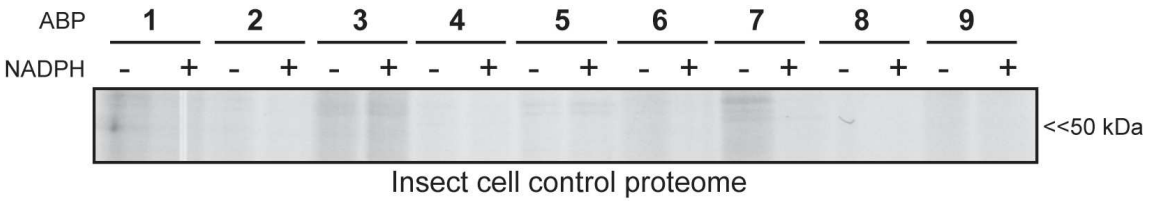
Probe 8. **30** (50 mg, 0.17 mmol) was dissolved in THF (3 mL), NaH (6.8 mg, 0.17 mmol) was added, and the solution was heated to 50 °C. After 1 h of heating hex-5-ynyl-4-methylbenzenesulfonate (1.0 M in THF, 0.14 mL, 0.14 mmol) was added and the reaction was stirred vigorously at 50 °C for 2 h. The reaction was quenched with H_2O, and the product was extracted with ethyl acetate (2 × 4 mL). The organic layer was washed with 10% citric acid (2 × 6 mL), $NaHCO_3$ (sat) (2 × 6 mL), and NaOH (1.0 M, 2 × 2 mL), dried with Na_2SO_4, and removed *in vacuo*. The product was further purified over silica (hexanes/ethyl acetate (90/10)) yielding **8** (29 mg, 0.077 mmol, 55%) as a white solid. ^{1}H NMR (400 MHz) δ ^{13}C NMR (400 MHz) δ HR-MS *m/z* calcd for $C_{26}H_{33}O_2$ (M + H): 377.2475. Found: 377.2471.

Probe 9. **33** (22 mg, 0.085 mmol), HOBt (14 mg, 0.10 mmol), EDCI (20 mg, 0.10 mmol), and NMM (0.019 mL, 0.17 mmol) were dissolved in CH_3CN/DMF (2/1, 2.0 mL) at rt. After 15 min hex-5-yn-1-amine (12 mg, 0.093 mmol) was added portionwise and the solution stirred for 4 h. The reaction was diluted with ethyl acetate (4 mL), washed with 10% citric acid (2 × 5 mL), $NaHCO_3$ (sat) (2 × 5 mL), and brine (2 × 5 mL), and dried with Na_2SO_4. The organic solvents were removed *in vacuo* and further purified over silica (hexanes/ethyl acetate (60/40)) yielding **9** (11 mg, 0.032 mmol, 38%) as a white solid. ^{1}H NMR (400 MHz, Acetone-d_6): δ 1.56 (m, 2H), 1.70 (m, 2H), 2.09 (s, 1H), 2.23 (dt, *J* = 2.4, 6.8, 2H), 3.36 (q, *J* = 6.4, 2H), 4.88 (s, 2H), 6.38 (d, *J* = 9.6, 1H), 7.05 (d, *J* = 2.4, 1H), 7.69 (s, 1H), 7.99 (d, *J* = 2.0, 1H), 8.07 (d, *J* = 10, 1H). ^{13}C NMR (400 MHz, Acetone-d_6): δ 17.73, 25.87, 28.91, 38.18, 69.36, 72.35, 84.09, 107.28, 114.70, 114.97, 116.97, 126.44, 131.00, 143.35, 144.88, 147.60, 147.72, 159.41, 167.53. HR-MS *m/z* calcd for $C_{13}H_{18}NO$ (M + H): 340.1179. Found: 340.1176.

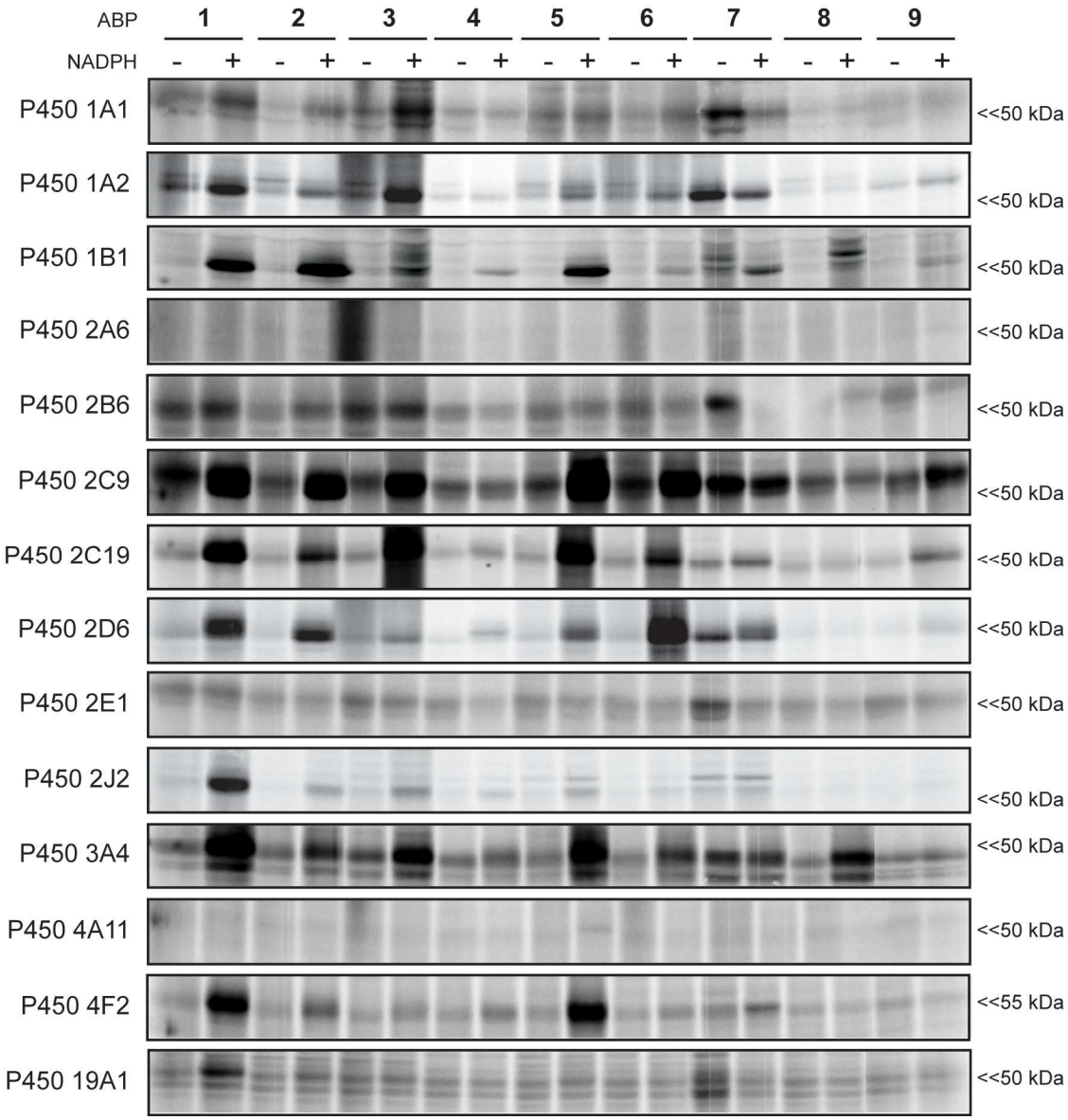
Supporting Figures.



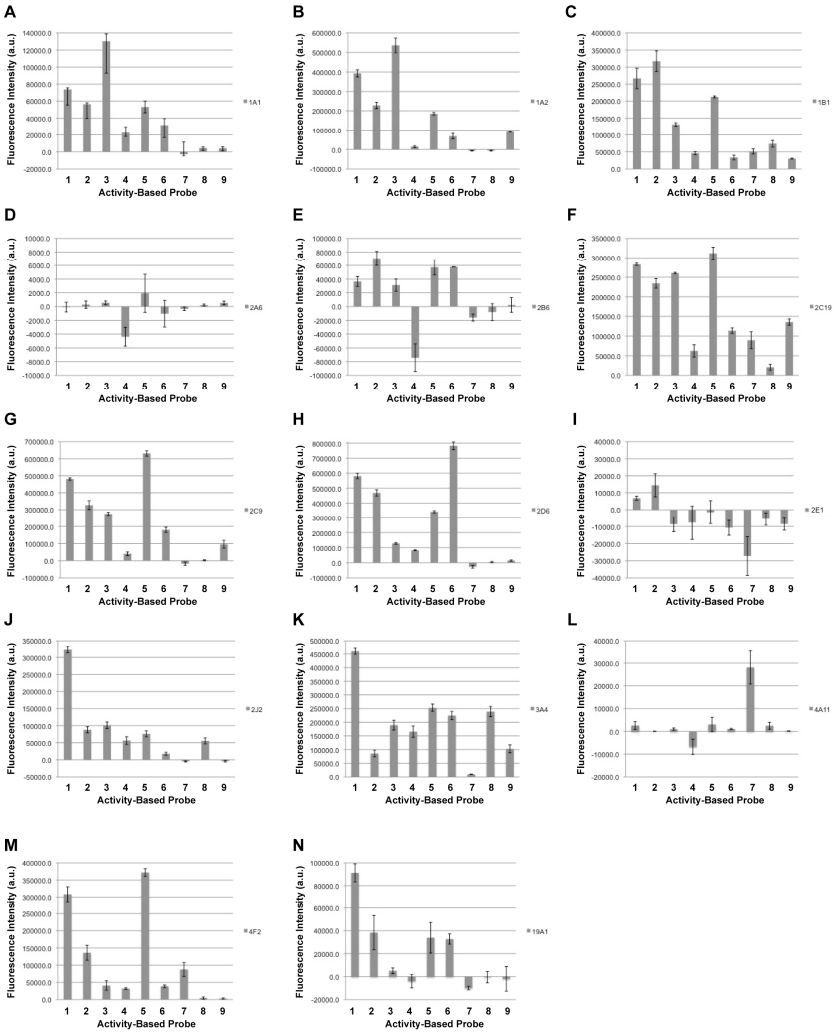
Supporting Figure 1. Azide variant of 2EN-ABP. We synthesized **SI-1** to confirm that the majority of P450 labeling by aryl acetylene probes was due to the aryl acetylene, rather than the terminal alkyne on the aliphatic linker group. Mouse liver microsomes (1 mg/mL) were treated with either 2EN-ABP or **SI-1** (20 μM), NADPH (1 mM; not added to the control samples), and incubated at 37 ^{o}C for 1 h. Following incubation, probe-labeled proteomes were treated with rhodamine-azide (100 μM; 6 mM stock solution in DMSO) followed by TCEP (0.5 mM; 25 mM stock in water) and ligand (100 μM; 1.7 mM stock in DMSO:t-butanol (1:4)). The samples were vortexed, and cycloaddition was initiated by the addition of $CuSO_4$ (1 mM; 50 mM stock in water). Samples were vortexed and left at rt in the dark for 1 h at which time 2× SDS-PAGE loading buffer (50 μL) was added. The samples were heated at 90 °C for 8 min, loaded onto SDS-PAGE gels (30 μL per well), and visualized by in-gel fluorescent scanning using a Hitachi FMBio Ile flatbed scanner (MiraiBio, Alameda, CA).



Supporting Figure 2. Probe labeling of insect cell control proteomes. BD insect cell control microsomal proteomes (50 µL of 1.0 mg/mL protein in PBS) were treated with individual probes (**1**-**9**) (20 µM; 0.5 µL of a stock solution in DMSO) in the presence or absence of NADPH (1 mM; 0.5 µL of a stock solution in PBS). Samples were incubated at 37 °C for 1 h. Following incubation, probe-labeled proteomes were treated with rhodamine-azide (100 µM; 6 mM stock solution in DMSO) followed by TCEP (0.5 mM; 25 mM stock in water) and ligand (100 µM; 1.7 mM stock in DMSO:t-butanol (1:4)). The samples were vortexed, and cycloaddition was initiated by the addition of $CuSO_4$ (1 mM; 50 mM stock in water). Samples were vortexed and left at rt in the dark for 1 h at which time 2× SDS-PAGE loading buffer (50 µL) was added. The samples were heated at 90 °C for 8 min, loaded onto SDS-PAGE gels (30 µL per well), and visualized by in-gel fluorescent scanning using a Hitachi FMBio Ile flatbed scanner. As seen in the gel image, no NADPH-dependent probe labeling of any endogenous insect proteins was observed in the molecular mass region that contains recombinantly expressed human P450s.

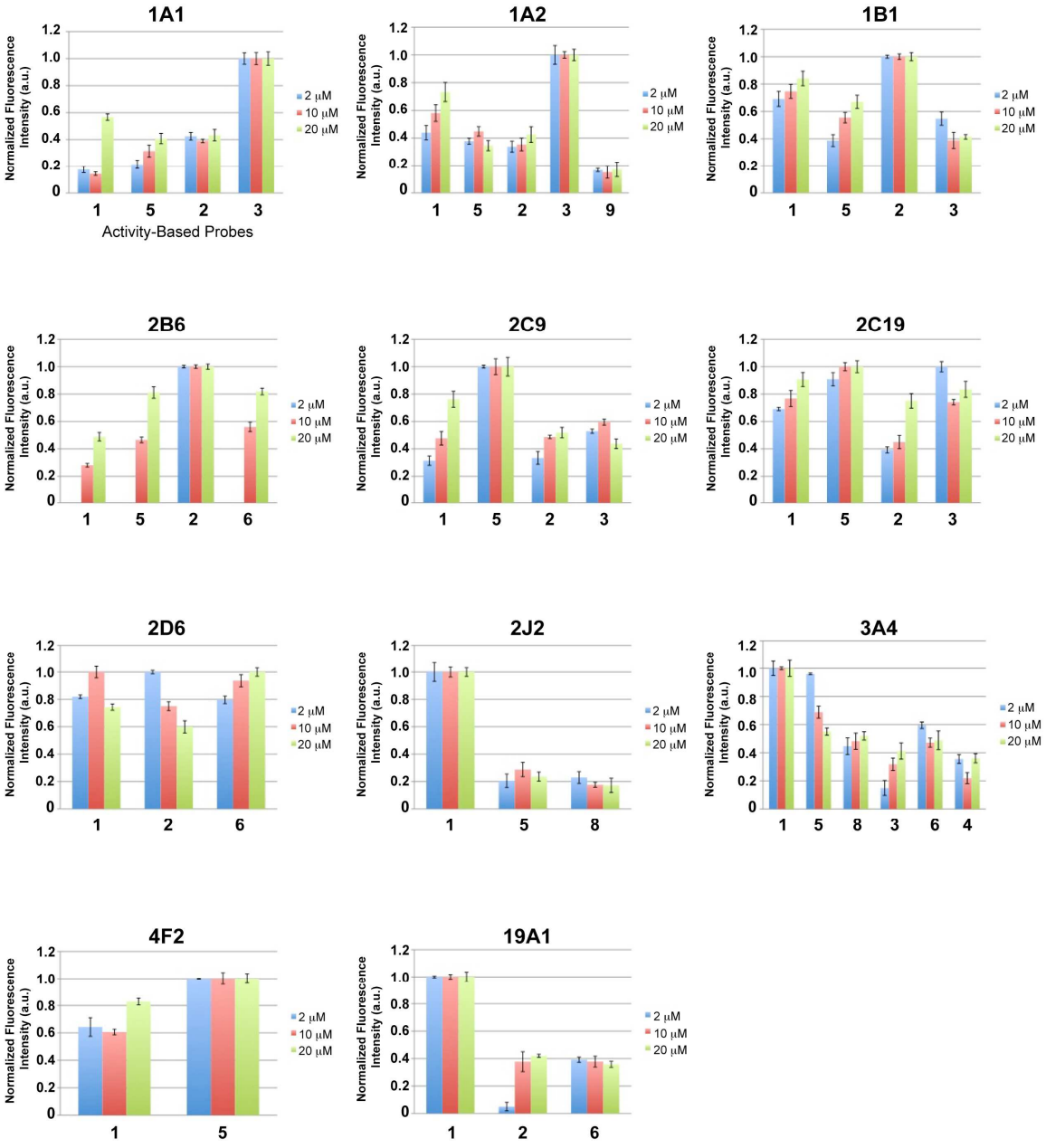


Supporting Figure 3. Probe labeling of the 14-member P450 panel. Full SDS-PAGE separation was performed, but only the 45-55 kDa region is shown, where NADPH-dependent probe-labeled proteins were detected (consistent with the molecular masses of P450 enzymes).



Supporting Figure 4. Quantitative analysis of P450 probe labeling. This is an expanded analysis of the one reported in Figure 4 of the manuscript. This image shows the quantitative analysis of probe labeling for all P450s. (A) 1A1; (B) 1A2; (C) 1B1; (D) 2A6; (E) 2B6; (F) 2C19; (G) 2C9; (H) 2D6; (I) 2E1; (J) 2J2; (K) 3A4; (L) 4A11; (M) 4F2; (N) 4A11. A negative value reflects that labeling without NADPH was greater than labeling in the presence of NADPH. These occurrences mostly reflect variable

differences in low signal intensity values and are therefore not likely of relevance for specific probe-P450 interactions.



Supporting Figure 5. Concentration dependence of probe labeling. This image shows the concentration dependence of probe labeling for all P450s, except 2A6, 2E1, and 4A11 because probe labeling at 20 μM was too weak.