

Fluorogenic Label for Biomolecular Imaging

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Page	Contents
S1	Table of Contents
S2–S6	Procedures for Chemical Synthesis
S6	Reference
S7	Supplementary Figure 1 (Effect of pH on Spectroscopic Properties)
S8	Supplementary Figure 2 (HeLa Cells with Diurea–rhodamine 3) Supplementary Figure 3 (Immunostain of Cells with RNase A Conjugate)

General Experimental. Rh₁₁₀ (sold as Rhodamine 560) was obtained from Exciton (Dayton, OH). Dimethylformamide, tetrahydrofuran, and dichloromethane were drawn from a Baker CYCLE-TAINER solvent delivery system. All other reagents were obtained from Aldrich Chemical (Milwaukee, WI) or Fisher Scientific (Hanover Park, IL) and used without further purification. Thin-layer chromatography was performed using aluminum-backed plates coated with silica gel containing F₂₅₄ phosphor and visualized by UV illumination or developed with I₂, ceric ammonium molybdate, or phosphomolybdic acid stain. Flash chromatography was performed using open columns 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).

NMR spectra were obtained with a Bruker DMX-400 Avance spectrometer at the National Magnetic Resonance Facility at Madison (NMRFAM). Mass spectrometry was performed with a Micromass LCT (electrospray ionization, ESI) mass spectrometer at the Mass Spectrometry Facility in the Department of Chemistry or with a Perkin–Elmer Voyager (matrix assisted laser desorption ionization, MALDI) mass spectrometer in the Biophysics Instrumentation Facility.

Dimethylurea-Rh₁₁₀ (1). Rh₁₁₀ (500 mg, 1.363 mmol) was dissolved in anhydrous DMF (50 mL) under Ar(g). NaH (98 mg, 4.089 mmol) was added slowly, and the resulting purple solution was stirred at ambient temperature for 1 h. Dimethylcarbonyl chloride (138 μ L, 1.499 mmol) was added dropwise, and the reaction mixture was stirred at ambient temperature for 24 h. The reaction was quenched with a few drops of glacial acetic acid and solvent was removed under reduced pressure. The residue was partially purified by flash chromatography (silica gel, 0–6% v/v gradient of MeOH in CH₂Cl₂). The partially pure compound was recrystallized from MeOH to yield compound **1** as a red powder (93 mg, 17%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 8.54 (bs, 1H), 7.97 (d, *J* = 7.5 Hz, 1H), 7.78 (td, *J* = 7.5, 1.1 Hz, 1H), 7.70 (td, *J* = 7.5, 0.7 Hz, 1H), 7.63 (d, *J* = 2.1 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 1H), 7.12 (dd, *J* = 8.9, 2.1 Hz, 1H), 6.56 (d, *J* = 8.9 Hz, 1H), 6.44 (d, *J* = 1.8 Hz, 1H), 6.36 (d, *J* = 8.5 Hz, 1H), 6.32 (dd, *J* = 8.5, 1.9 Hz, 1H), 5.64 (bs, 2H), 2.93 (s, 6H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 168.85, 155.35, 152.53, 152.08, 151.32, 151.18, 142.92, 135.45, 129.89, 128.49, 127.63, 126.44, 124.49, 124.01, 115.12, 111.82, 111.10, 105.96, 105.35, 99.14, 83.83, 36.25 (2C). HRMS (ESI): *m/z* 402.1440 (MH⁺ [C₂₃H₂₀N₃O₄] = 402.1454).

Monoacetamide-Rh₁₁₀ (2). Rh₁₁₀ (500 mg, 1.363 mmol) was dissolved in anhydrous DMF (15 mL) under Ar(g). NaH (65 mg, 2.73 mmol) was added slowly, and the resulting brown solution was stirred at ambient temperature for 1 h. Acetic anhydride (107 μ L, 1.136 mmol) was added dropwise in anhydrous DMF (1.0 mL), and the reaction mixture was stirred for 24 h. The reaction was quenched with a few drops of glacial acetic acid and the solvent was removed under reduced pressure. The residue was partially purified by flash chromatography (silica gel, 0–5% v/v gradient of MeOH in CH₂Cl₂). This crude compound was further purified by flash chromatography (silica gel, 5% v/v MeOH in CH₂Cl₂). Compound **2** was isolated as an orange solid (188 mg, 44%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 10.20 (bs, 1H), 7.98 (d, *J* = 7.7 Hz, 1H), 7.79 (m, 2H), 7.70 (td, *J* = 7.4, 0.8 Hz, 1H), 7.26 (d, *J* = 7.6 Hz, 1H), 7.07 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.64 (d, *J* = 8.6 Hz, 1H), 6.45 (d, *J* = 1.8 Hz, 1H), 6.37 (d, *J* = 8.5 Hz, 1H), 6.33 (dd, *J* = 8.6, 2.0 Hz, 1H), 5.67 (bs, 2H), 2.06 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 168.87, 168.78, 152.47, 151.95, 151.36, 151.16, 141.15, 135.50, 129.99, 128.50, 128.25, 126.33, 124.55, 123.99, 114.64, 113.41, 111.25, 106.00, 105.18, 99.11, 83.49, 24.10. HRMS (ESI): *m/z* 373.1177 (MH⁺ [C₂₂H₁₇N₂O₄] = 373.1188).

Bis(dimethylurea)-Rh₁₁₀ (3). Rh₁₁₀ (500 mg, 1.363 mmol) was dissolved in anhydrous DMF (50 mL) under Ar(g). NaH (134 mg, 5.589 mmol) was added slowly, and the resulting brown solution was stirred at ambient temperature for 1 h. Dimethylcarbonyl chloride (250 μ L, 2.73 mmol) was added dropwise and the mixture was stirred at ambient temperature for 24 h. The reaction was quenched with a few drops of glacial acetic acid and solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica gel, EtOAc). Compound **3** was isolated as an off-white solid (359 mg, 56%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 8.58 (bs, 2H), 8.01 (d, *J* = 7.4 Hz, 1H), 7.79 (td, *J* = 7.4, 1.3 Hz, 1H), 7.72 (td, *J* = 7.5, 0.9 Hz, 1H), 7.66 (d, *J* = 2.3 Hz, 2H), 7.28 (d, *J* = 7.6 Hz, 1H), 7.18 (dd, *J* = 8.8, 2.1 Hz, 2H), 6.62 (d, *J* = 8.8 Hz, 2H), 2.93 (s, 12H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 168.79, 155.33 (2C), 152.63, 150.89 (2C), 143.14 (2C), 135.65, 130.10, 127.75 (2C), 125.97, 124.69, 123.97, 115.44 (2C), 111.36 (2C), 105.95 (2C), 82.65, 36.24 (4C). HRMS (ESI): *m/z* 473.1831 (MH⁺ [C₂₆H₂₅N₄O₅] = 473.1825).

Monoacetamide-Monourea-Rh₁₁₀ (4). Dimethylurea-Rh₁₁₀ **1** (66 mg, 0.164 mmol) was dissolved in anhydrous DMF (1.0 mL) and anhydrous pyridine (1.0 mL) under Ar(g). Acetyl chloride (94 μ L, 1.32 mmol) was added, and the reaction mixture was stirred at ambient temperature for 5 h. Solvent was removed under reduced pressure, and the residue dissolved in CH₂Cl₂. This solution was washed with 5% HCl and saturated brine. The organic layer was then dried over anhydrous MgSO₄(s), and the solvent

was removed under reduced pressure. The residue was purified by flash chromatography (silica gel, EtOAc) to give the compound **4** as a pale yellow solid (43 mg, 59%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ (ppm): 8.59 (bs, 1H), 7.95 (d, $J = 6.7$ Hz, 1H), 7.58 (m, 2H), 7.31 (d, $J = 1.4$ Hz, 1H), 7.28 (d, $J = 1.9$ Hz, 1H), 7.19 (d, $J = 8.6$ Hz, 1H), 7.07 (bs, 1H), 7.03 (dd, $J = 8.8, 2.1$ Hz, 1H), 6.98 (d, $J = 7.2$ Hz, 1H), 6.61 (d, $J = 8.7$ Hz, 1H), 6.58 (d, $J = 8.8$ Hz, 1H), 3.00 (s, 6H), 2.08 (s, 3H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ (ppm): 170.11, 169.29, 155.72, 153.09, 151.55, 151.46, 141.68, 140.36, 135.35, 129.81, 128.12, 128.00, 126.15, 124.83, 124.13, 116.07, 115.58, 113.66, 112.49, 107.88, 107.60, 83.45, 36.51 (2C), 24.34. HRMS (ESI): m/z 466.1398 (MNa^+ [$\text{C}_{25}\text{H}_{21}\text{N}_3\text{O}_5\text{Na}$] = 466.1379).

Diacetamide-Rh₁₁₀ (5). Rh₁₁₀ (200 mg, 0.545 mmol) was dissolved in anhydrous DMF (2.0 mL) and anhydrous pyridine (2.0 mL) under Ar(g). Acetyl chloride (0.311 mL, 4.36 mmol) was added dropwise, and the reaction mixture was stirred at ambient temperature for 24 h. The solution was poured into ice water containing 5% v/v HCl and the pale pink precipitate was collected by filtration. The crude product was purified by flash chromatography (silica gel, EtOAc). Compound **5** was isolated as an off-white solid (135 mg, 60%). $^1\text{H NMR}$ (400 MHz, $\text{DMSO}-d_6$) δ (ppm): 10.24 (bs, 2H), 8.02 (d, $J = 7.5$ Hz, 1H), 7.84 (d, $J = 1.9$ Hz, 2H), 7.79 (td, $J = 7.4, 1.3$ Hz, 1H), 7.73 (td, $J = 7.5, 0.9$ Hz, 1H), 7.28 (d, $J = 7.4$ Hz, 1H), 7.14 (dd, $J = 8.7, 2.2$ Hz, 2H), 6.72 (d, $J = 8.7$ Hz, 2H), 2.07 (s, 6H). $^{13}\text{C NMR}$ (100 MHz, $\text{DMSO}-d_6$) δ (ppm): 168.91 (2C), 168.66, 152.53, 150.82 (2C), 141.41 (2C), 135.76, 130.21, 128.36 (2C), 125.71, 124.75, 123.98, 115.13 (2C), 112.84 (2C), 106.04 (2C), 81.97, 24.09 (2C). HRMS (ESI): m/z 437.1127 (MNa^+ [$\text{C}_{24}\text{H}_{18}\text{N}_2\text{O}_5\text{Na}$] = 437.1113).

Morpholinourea-Rh₁₁₀ (6). Rh₁₁₀ (500 mg, 1.36 mmol) was dissolved in anhydrous DMF (50 mL) under Ar(g). NaH (67 mg, 2.86 mmol) was added portion-wise, and the resulting purple-brown solution was stirred at ambient temperature for 1 h. 4-Morpholinecarbonyl chloride (0.156 mL, 1.36 mmol) was added dropwise and the mixture was stirred for 24 h. Solvent was removed under reduced pressure and the residue was purified by flash chromatography (silica gel, 100:7:1 v/v/v CHCl_3 :MeOH:AcOH). The purification gave compound **6** as a red-orange crystalline solid (176 mg, 29%). $^1\text{H NMR}$ (400 MHz, $\text{DMSO}-d_6$) δ (ppm): 8.80 (bs, 1H), 7.97 (d, $J = 7.9$ Hz, 1H), 7.78 (td, $J = 7.4, 1.4$ Hz, 1H), 7.70 (td, $J = 7.5, 0.6$ Hz, 1H), 7.62 (d, $J = 2.6$ Hz, 1H), 7.25 (d, $J = 7.4$ Hz, 1H), 7.09 (dd, $J = 8.6, 1.8$ Hz, 1H), 6.58 (d, $J = 9.1$ Hz, 1H), 6.44 (d, $J = 2.0$ Hz, 1H), 6.36 (d, $J = 8.2$ Hz, 1H), 6.32 (dd, $J = 8.5, 2.2$ Hz, 1H), 5.65 (bs, 2H), 3.60 (m, 4H), 3.43 (m, 4H). $^{13}\text{C NMR}$ (100 MHz, $\text{DMSO}-d_6$) δ (ppm): 168.86, 154.78, 152.55, 152.06, 151.31, 151.12, 142.64, 135.46, 129.94, 128.50, 127.81, 126.42, 124.52, 124.02, 115.01, 112.08, 111.14, 105.97, 105.33, 99.15, 83.74, 65.95 (2C), 44.17 (2C). HRMS (ESI): m/z 444.1570 (MH^+ [$\text{C}_{25}\text{H}_{22}\text{N}_3\text{O}_5$] = 444.1559).

Morpholinourea-Rh₁₁₀ Trimethyl Lock (8). Compound **6** (54 mg, 0.122 mmol) was dissolved in a mixture of anhydrous DMF (0.9 mL) and anhydrous pyridine (0.6 mL) under Ar(g). To this solution was added EDC (47 mg, 0.244 mmol) and 3-(2'-acetoxo-4',6'-dimethylphenyl)-3,3-dimethylpropanoic acid **7** (**1**) (64 mg, 0.244 mmol). The resulting solution was stirred for 48 h at ambient temperature. Solvent was removed under reduced pressure, and the residue was taken up in CH_2Cl_2 . This solution was washed with 5% HCl and saturated brine. The organic layer was dried over anhydrous MgSO_4 (s), and the solvent was removed under reduced pressure. The pale orange residue was purified by flash chromatography (silica gel, 2:1 EtOAc:hexanes) to give compound **8** as a pale yellow solid (68 mg, 81%). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ (ppm): 7.97 (m, 1H), 7.63 (td, $J = 7.4, 1.3$ Hz, 1H), 7.58 (td, $J = 7.4, 1.2$ Hz, 1H), 7.55 (bs, 1H), 7.39 (d, $J = 2.2$ Hz, 1H), 7.36 (d, $J = 2.0$ Hz, 1H), 7.08 (m, 2H), 6.94 (dd, $J = 8.6, 1.9$ Hz, 1H), 6.90 (bs, 1H), 6.79 (d, $J = 1.4$ Hz, 1H), 6.65 (dd, $J = 8.6, 2.1$ Hz, 1H), 6.63 (d, $J = 1.5$ Hz, 1H), 6.57 (d, $J = 8.7$ Hz, 1H), 6.55 (d, $J = 8.6$ Hz, 1H), 3.69 (m, 4H), 3.48 (m, 4H), 2.59 (ABq, $J = 13.4$ Hz, 2H), 2.42 (s, 3H), 2.37 (s, 3H), 2.23 (s, 3H), 1.68 (s, 3H), 1.67 (s, 3H). $^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ (ppm): 172.11, 169.96, 169.89, 154.74, 153.22, 151.66, 151.59, 150.05, 141.34, 140.08, 139.02, 137.28, 135.17, 133.19, 132.87, 129.71, 128.15, 128.09, 126.24, 124.87, 124.06, 123.45, 115.52, 115.16, 113.68, 112.76, 107.43 (2C), 83.25, 66.47 (2C), 51.03, 44.27 (2C), 40.35, 32.16, 32.11, 25.51, 21.94, 20.17. HRMS (ESI): m/z 712.2657 (MNa^+ [$\text{C}_{40}\text{H}_{39}\text{N}_3\text{O}_8\text{Na}$] = 712.2635).

t-Boc-Rh₁₁₀ (9). Rh₁₁₀ (403 mg, 1.10 mmol) was dissolved in anhydrous DMF (15 mL) under Ar(g). NaH (53 mg, 2.20 mmol) was added slowly, and the resulting brown solution was stirred for 1 h. Di-*tert*-butyl dicarbonate (200 mg, 0.916 mmol) was added, and the reaction mixture was stirred at ambient temperature for 24 h. The reaction was quenched with a few drops of glacial acetic acid and the solvent was removed under reduced pressure. The orange solid was taken up into 80 mL of CH_2Cl_2 :MeOH (1:1 v/v) and filtered to remove unreacted Rh₁₁₀ (98 mg). The mother liquor was concentrated under reduced pressure and the residue was purified by flash chromatography (silica gel, 5:3:2 v/v/v

hexanes:CH₂Cl₂:EtOAc). *t*-Boc-Rh₁₁₀ was isolated as an orange powder (171 mg, 43%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 9.65 (bs, 1H), 7.97 (d, *J* = 8.1 Hz, 1H), 7.78 (td, *J* = 7.4, 1.5 Hz, 1H), 7.70 (td, *J* = 7.5, 0.7 Hz, 1H), 7.52 (d, *J* = 1.8 Hz, 1H), 7.24 (d, *J* = 7.9 Hz, 1H), 7.09 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.59 (d, *J* = 8.8 Hz, 1H), 6.43 (d, *J* = 1.9 Hz, 1H), 6.36 (d, *J* = 8.5 Hz, 1H), 6.32 (dd, *J* = 8.5, 2.4 Hz, 1H), 5.66 (bs, 2H), 1.48 (s, 9H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 168.84, 152.64, 152.52, 151.98, 151.36, 151.30, 141.61, 135.46, 129.93, 128.49, 128.22, 126.34, 124.54, 124.00, 113.93, 112.55, 111.21, 105.26, 104.93, 99.08, 83.55, 79.64, 28.03 (3C). HRMS (ESI): *m/z* 431.1593 (MH⁺ [C₂₅H₂₃N₂O₅] = 431.1607).

4-Maleimidobutyric Acid (10). 4-Aminobutyric acid (20.0 g, 194 mmol) was dissolved in glacial acetic acid (250 mL). Maleic anhydride (19.0 g, 194 mmol) was added, and the mixture was heated at reflux for 4 h with removal of the condenser for the final 20 min to allow escape of H₂O. The reaction mixture was concentrated under reduced pressure, and residual acetic acid was removed by its azeotrope with toluene. Purification by flash chromatography (silica gel, CH₂Cl₂) gave the compound **10** as a white crystalline solid (12.6 g, 35%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 7.00 (s, 2H), 3.42 (t, *J* = 6.8 Hz, 2H), 2.20 (t, *J* = 7.3 Hz, 2H), 1.71 (p, *J* = 7.0 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 173.82, 171.17 (2C), 134.50 (2C), 36.58, 30.84, 23.41. HRMS (ESI): *m/z* 182.0456 (M-H [C₈H₈NO₄] = 182.0453).

Maleimidourea-Rh₁₁₀-*t*-Boc (11). 4-Maleimidobutyric acid **10** (162 mg, 0.885 mmol) was dissolved in anhydrous THF (2.0 mL) under Ar(g). Hünig's Base (0.206 mL, 1.180 mmol) was added followed by diphenyl phosphoryl azide (244 mg, 0.885 mmol). The resulting solution was stirred at ambient temperature for 4 h, and the reaction mixture was then heated at reflux for 1 h. *t*-Boc-Rh₁₁₀ **9** (127 mg, 0.295 mmol) was then added in anhydrous THF (2.0 mL), and the reaction mixture was heated at reflux for 18 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 60% v/v EtOAc in hexanes). The purification gave compound **11** as a pale yellow crystalline solid (101 mg, 56%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.99 (d, *J* = 7.0 Hz, 1H), 7.68 (bs, 1H), 7.63 (td, *J* = 7.4, 1.3 Hz, 1H), 7.58 (td, *J* = 7.4, 1.1 Hz, 1H), 7.36 (d, *J* = 1.8 Hz, 1H), 7.17 (bs, 1H), 7.13 (d, *J* = 1.7 Hz, 1H), 7.05 (m, 2H), 6.94 (dd, *J* = 8.8, 2.0 Hz, 1H), 6.64 (d, *J* = 8.8 Hz, 1H), 6.63 (s, 2H), 6.53 (d, *J* = 8.5 Hz, 1H), 5.87 (t, *J* = 5.8 Hz, 1H), 3.52 (t, *J* = 6.6 Hz, 2H), 3.17 (m, 2H), 1.75 (p, *J* = 6.6 Hz, 2H), 1.51 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 170.91 (2C), 170.36, 155.44, 153.15, 152.66, 151.77, 151.54, 141.71, 140.83, 135.41, 134.04 (2C), 129.78, 128.47, 128.13, 126.16, 124.91, 124.08, 114.98, 114.18, 112.59, 111.69, 106.32, 106.19, 84.03, 80.95, 37.07, 35.07, 28.86, 28.30 (3C). HRMS (ESI): *m/z* 611.2147 (MH⁺ [C₃₃H₃₁N₄O₈] = 611.2142).

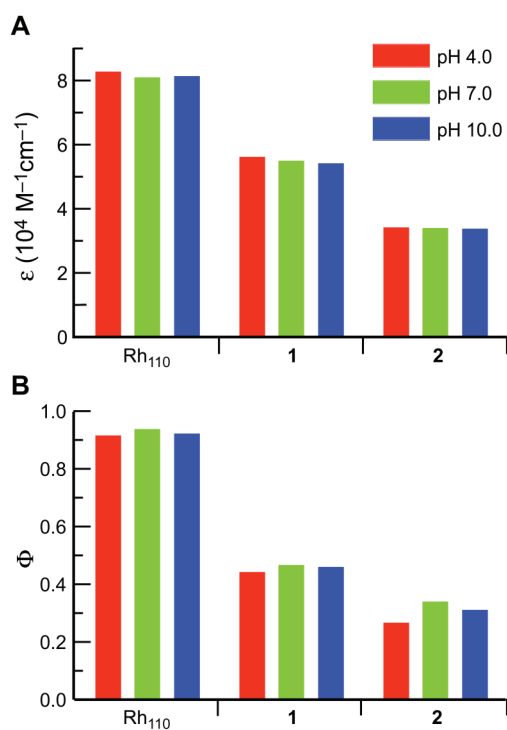
Maleimidourea-Rh₁₁₀ (12). Maleimidourea-Rh₁₁₀-*t*-Boc **11** (123 mg, 0.201 mmol) was dissolved in a mixture of CH₂Cl₂ (8 mL) and TFA (2 mL). The resulting solution was stirred at 0°C for 10 min and ambient temperature for 90 min. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 5% v/v MeOH in CH₂Cl₂). Compound **12** was isolated as an orange crystalline solid (94 mg, 91%). ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 8.89 (bs, 1H), 7.97 (d, *J* = 7.8 Hz, 1H), 7.78 (td, *J* = 7.5, 1.2 Hz, 1H), 7.70 (td, *J* = 7.5, 0.9 Hz, 1H), 7.63 (d, *J* = 1.9 Hz, 1H), 7.25 (d, *J* = 7.6 Hz, 1H), 7.02 (s, 2H), 6.86 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.54 (d, *J* = 8.7 Hz, 1H), 6.43 (d, *J* = 2.2 Hz, 1H), 6.36 (d, *J* = 8.3 Hz, 1H), 6.32 (dd, *J* = 8.6, 2.0 Hz, 1H), 6.26 (t, *J* = 5.9 Hz, 1H), 5.65 (bs, 2H), 3.44 (t, *J* = 7.0 Hz, 2H), 3.06 (q, *J* = 6.4 Hz, 2H), 1.66 (p, *J* = 6.9 Hz, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 171.18 (2C), 168.83, 154.92, 152.52, 152.09, 151.40, 151.31, 142.58, 135.42, 134.53 (2C), 129.90, 128.47, 128.10, 126.44, 124.46, 124.03, 113.56, 111.36, 111.10, 105.31, 104.23, 99.14, 83.86, 36.63, 34.92, 28.80. HRMS (ESI): *m/z* 511.1605 (MH⁺ [C₂₈H₂₃N₄O₆] = 511.1617).

Maleimidourea-Rh₁₁₀ Trimethyl Lock (13). Maleimidourea-Rh₁₁₀ **12** (24 mg, 0.047 mmol) was dissolved in a mixture of anhydrous DMF (1.0 mL) and anhydrous pyridine (1.0 mL) under Ar(g). EDC (18 mg, 0.094 mmol) and 3-(2'-acetoxo-4',6'-dimethylphenyl)-3,3-dimethylpropanoic acid **7** (**1**) (15 mg, 0.094 mmol) were added, and the reaction mixture was stirred at ambient temperature for 24 h. Solvent was removed under reduced pressure, and the pale orange residue was purified by flash chromatography (silica gel, 0–30% v/v gradient of EtOAc in hexanes). Compound **13** was isolated as an off-white crystalline solid (28 mg, 79%). ¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.00 (d, *J* = 6.9 Hz, 1H), 7.68 (bs, 1H), 7.64 (td, *J* = 7.4, 1.3 Hz, 1H), 7.60 (td, *J* = 7.5, 1.0 Hz, 1H), 7.38 (d, *J* = 1.9 Hz, 1H), 7.29 (bs, 1H), 7.12 (d, *J* = 2.2 Hz, 1H), 7.07 (d, *J* = 7.2 Hz, 1H), 7.04 (dd, *J* = 8.7, 2.2 Hz, 1H), 6.81 (d, *J* = 1.8 Hz, 1H), 6.69 (s, 2H), 6.69 (dd, *J* = 8.6, 2.2 Hz, 1H), 6.64 (d, *J* = 1.6 Hz, 1H), 6.58 (d, *J* = 8.6 Hz, 1H), 6.57 (d, *J* = 8.7 Hz, 1H), 5.66 (t, *J* = 5.9 Hz, 1H), 3.60 (t, *J* = 6.5 Hz, 2H), 3.21 (q, *J* = 6.3 Hz, 2H), 2.64 (ABq, *J* = 13.5 Hz, 2H), 2.45 (s, 3H), 2.39 (s, 3H), 2.24 (s, 3H), 1.81 (p, *J* = 6.5 Hz, 2H), 1.70 (s, 3H), 1.69 (s, 3H). ¹³C NMR

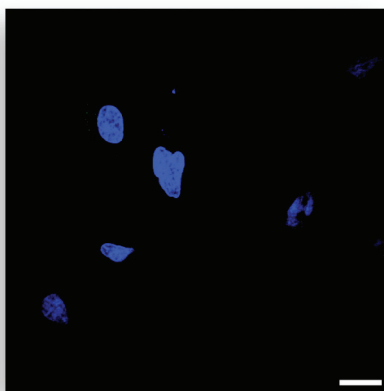
(100 MHz, CDCl₃) δ (ppm): 172.02, 170.98 (2C), 170.20, 170.12, 155.02, 153.18, 151.56, 151.51, 149.99, 141.63, 139.97, 138.94, 137.25, 135.22, 134.14 (2C), 133.16, 132.90, 129.72, 128.23 (2C), 126.24, 124.93, 124.08, 123.43, 115.20, 114.98, 113.72, 111.94, 107.55, 106.39, 83.58, 50.95, 40.31, 36.93, 35.03, 32.09, 32.06, 28.85, 25.55, 21.92, 20.16. HRMS (ESI): m/z 779.2715 (MNa⁺ [C₄₃H₄₀N₄O₉Na] = 779.2693).

Reference

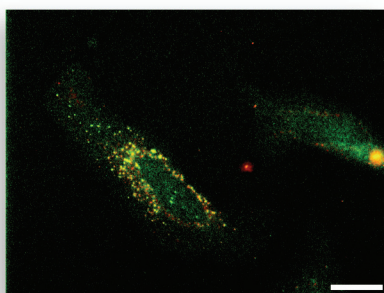
1. Amsberry, K. L., Gerstenberger, A. E., and Borchardt, R. T. (1991) Amine prodrugs which utilize hydroxy amide lactonization. II. A potential esterase-sensitive amide prodrug. *Pharm. Res.* 8, 455–461.



Supplementary Figure 1. (A) Effect of pH on the extinction coefficient of Rh₁₁₀, urea **1**, and amide **2**. (B) Effect of pH on the quantum yield of Rh₁₁₀, urea **1**, and amide **2**.



Supplementary Figure 2. Unwashed HeLa cells incubated for 1 h with diurea **3** (10 μ M) at 37 °C in DMEM (5% v/v CO₂(g), 100% humidity) and counter-stained with Hoechst 33342. Scale bar: 20 μ m.



Supplementary Figure 3. HeLa cells incubated for 1 h with the fluorogenic label **13**-RNase A conjugate (10 μ M) at 37 °C in DMEM (5% v/v CO₂(g), 100% humidity). Cells were fixed with 4% paraformaldehyde, washed extensively, and counter-stained with a primary antibody to RNase A and secondary antibody labeled with AlexaFluor 594. Cells were imaged on a Nikon Eclipse E800 fluorescence microscope (Melville, NY) equipped with a Photometrics CoolSnap HQ cooled CCD camera (Roper Scientific, Tucson, AZ). Excitation light was provided by a mercury lamp. Scale bar: 20 μ m.