

Ribonuclease-Activated Cancer Prodrug

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Materials

Uridine phosphoramidite and iodine oxidizing solution were from Glen Research (Sterling, VA). Minimum 70% *Z* isomer of 4-hydroxytamoxifen (remainder, primarily *E* isomer), 3-Å molecular sieves, tetrabutylammonium fluoride (TBAF), methylbenzimidazole, and all other commercial reagents were from Sigma–Aldrich (St. Louis, MO). Methylbenzimidazole triflate was made according to literature precedent from methylbenzimidazole.¹ Spectra/Por[®] dialysis bags (3500 MWCO) were from Fisher Scientific (Thermo Fisher Scientific, Walham, MA). *Escherichia coli* BL21(DE3) cells were from Novagen (Madison, WI). Cell-culture medium and supplements, as well as Dulbecco's phosphate-buffered saline (DPBS) were from Invitrogen (Carlsbad, CA). [*methyl*-³H]Thymidine (6.7 Ci/mmol) was from Perkin–Elmer (Boston, MA). HiTrap columns were from GE Biosciences (Piscataway, NJ). MES buffer was from Sigma–Aldrich (St. Louis, MO) and purified by anion-exchange chromatography to remove trace amounts of oligomeric vinylsulfonic acid.² Ribonuclease substrate 6-FAM–dArUdAdA–6-TAMRA was from Integrated DNA Technologies (Coralville, IA). Terrific Broth (TB) was from Research Products International Corp (Mt. Prospect, IL). Gels for SDS–PAGE were from Bio-Rad Laboratories (Hercules, CA). Ribonuclease-free 0.10 M sodium phosphate buffer, pH 7.4, containing NaCl (0.10 M) was prepared by making an aqueous solution of NaH₂PO₄ and NaCl (final concentrations: 0.10 M), adjusting the pH to 7.4 with aliquots of 10 M NaOH, adding diethylpyrocarbonate to 0.1% v/v, incubating for 1 h at 37 °C, and then autoclaving the resulting solution.

Instrumentation and Statistical Calculations:

¹H NMR spectra were acquired at the National Magnetic Resonance Facility at Madison (NMRFAM) at 298 K with a Bruker DMX-400 Avance spectrometer (Bruker AXS, Madison, WI, ¹H, 400 MHz; ¹³C, 101 MHz; ³¹P, 162 MHz). ¹³C and ³¹P spectra were proton decoupled. All ¹H and ¹³C NMR spectra were referenced to TMS. All ³¹P NMR spectra were referenced to an internal insert standard of H₃PO₄. Preparatory HPLC was performed on an instrument from Shimadzu Prominence (Kyoto, Japan) equipped with two LC-20AP pumps, a SPD-M20A photodiode array detector, and a CTO-20A column oven. Analytical HPLC was performed on an instrument from Waters (Milford, MA) equipped with two 515 pumps, a 717 plus autosampler, and a 996 photodiode array detector. Protein absorbance values were measured with a Varian Cary 50 UV–Vis Spectrometer from Agilent Technologies (Santa Clara, CA). [*methyl*-³H]Thymidine incorporation into MCF-7 genomic DNA was quantified by scintillation counting using a Microbeta TriLux liquid scintillation and luminescence counter (Perkin–Elmer, Wellesley, MA). Fluorescence measurements were made with a QuantaMaster1 photon-counting cuvette fluorometer from Photon Technology International (South Brunswick, NJ). Calculations for cell proliferation assays were performed using Prism version 5.02 from GraphPad Software (La Jolla, CA).

Determination of Partition and Distribution Coefficients

Values of log*P*, log*D*, and *pI* were calculated with calculator plugins in the program MartinView 5.4.1.1, 2011 from ChemAxon (Budapest, Hungary). Log*P* and log*D* values were set at default: calculations used equal weights of VG, KLOP, and PHYS methods and electrolyte concentrations (Na⁺, K⁺) and Cl[−] set to 0.1 mol/dm³. We did not consider tautomerization in our calculations.

Recombinant Protein Production

Human pancreatic ribonuclease (RNase 1) was produced as described previously,³ except that after purification by the two chromatographic steps, the protein was re-purified by both steps to ensure purity. Following purification, purity and apparent molecular mass of RNase 1 was verified with SDS-PAGE.

Fluorescence Assay for Ribonucleolytic Activity

The ribonucleolytic activity of RNase 1 was determined by quantitating its ability to cleave 6-FAM-dArUdAdA-6-TAMRA, as described previously.^{3,4} Assays were carried out at ambient temperature in 2.0 mL of 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M). Fluorescence data were fitted to eq 1, in which $\Delta I/\Delta t$ is the initial reaction velocity, I_0 is the fluorescence intensity before addition of ribonuclease, I_f is the fluorescence intensity after complete substrate hydrolysis, and $[E]$ is the total ribonuclease concentration. Data were the average of three experiments. The activity of RNase 1 was $(6.0 \pm 0.7) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$. This value is similar to previous values (29% of that in ref. 3, though that enzyme lacked residue 128; 21% of that in ref. 5, though those assays were performed in a DPBS buffer containing 0.1 mg/mL BSA).

$$k_{\text{cat}}/K_M = (\Delta I/\Delta t) / (I_f - I_0)[E] \quad (1)$$

HPLC Assay for Cleavage of Uridine 3'-(4-Hydroxytamoxifen phosphate) by RNase 1

Uridine 3'-(4-hydroxytamoxifen phosphate) (UpHT) was dissolved to a final concentration of 0.090 mM in 0.10 M MES-NaOH buffer, pH 6.0, containing NaCl (0.10 M NaCl). One aliquot (900 μL) of this solution was placed in HPLC vial 1, and another aliquot (300 μL) was placed in HPLC vial 2. Vials with UpHT were pre-incubated at 37 °C in an HPLC autosampler. Buffer A was 0.10 M triethylammonium acetate (TEAA) in H_2O ; Buffer B was 0.10 M TEAA in acetonitrile/ H_2O 95:5. RNase 1 was diluted in 0.10 M MES-NaOH buffer, pH 6.0, to a concentration of 8.5 μM (~125 mg/L). A Varian 150/4.6 Microsorb-MV 100-5 C18 reverse-phase column was equilibrated with Buffer A/Buffer B 3:7. All samples were eluted isocratically at 1 mL/min for 5 min with the same solution. First, 50 μL from vial 1 was analyzed to obtain a baseline reading. Then, an aliquot (1 μL) of the RNase 1 solution was added to the remaining 850 μL of vial 1 (final RNase 1 concentration: 10 nM, ~0.15 mg/L). Twelve assays were thus performed at known times. An aliquot (50 μL) of vial 2 was analyzed to assess the amount of UpHT activated in the absence of RNase 1. An aliquot (9 μL) of a concentrated solution of RNase 1 (144 μM) was then added to the remaining 250 μL in vial 1 to cleave >95% of the UpHT. After ~1.5 h, an aliquot (50 μL) of this solution was analyzed to quantify the total amount of attainable HT (after adjusting for dilution by RNase 1). The entire experiment was run in triplicate (for overlaid HPLC traces from one experiment, see: Figure S1). Baseline readings were subtracted from the two HT peaks to obtain HT concentrations at each timepoint. Values are the mean \pm SE for each timepoint. All data accounted for the dilution that accompanies the addition of RNase 1. These experiments were repeated to analyze the stability of UpHT (40 μM) in ribonuclease-free 0.10 M sodium phosphate buffer, pH 7.4, containing NaCl (0.10 M); cell medium in the absence of RNase 1; and cell medium in the presence of RNase 1 (0.4 mg/L).

Human Cell Culture

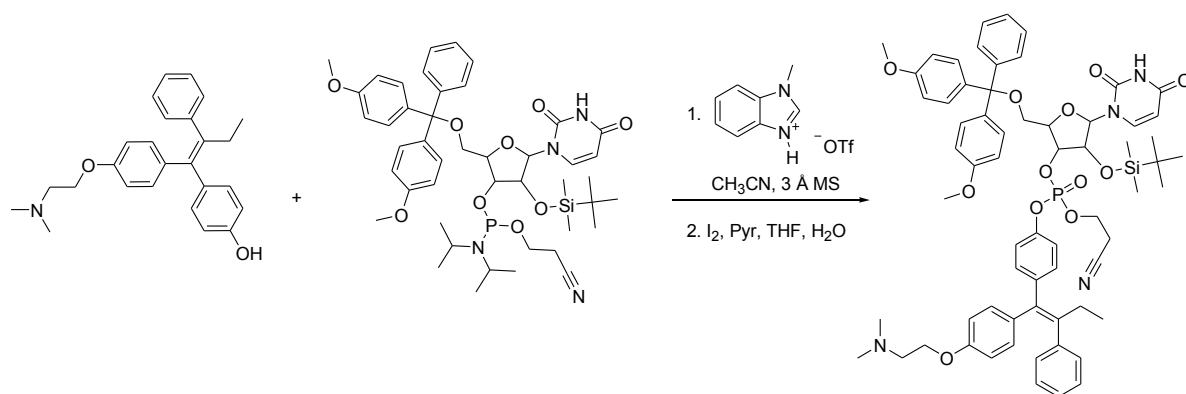
The MCF-7 cell line was a generous gift from Professor J. Wesley Pike (University of Wisconsin-Madison). Cells were grown at 37 °C in a cell-culture incubator in flat-bottomed

culture flasks under CO₂ (5% v/v). “Medium A” for the growth and initial plating of cells was Dulbecco’s modified Eagle’s medium (DMEM) containing phenol red and supplemented with fetal bovine serum (FBS; GIBCO; 10% v/v), penicillin (100 units/mL), streptomycin (100 µg/mL), human recombinant insulin (10 µg/mL), and MEM non-essential amino acids solution (100 µM). “Medium B” for assays of UpHT stability and effect on cell-proliferation was DMEM without phenol red and without FBS, supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL), human recombinant insulin (10 µg/mL), MEM non-essential amino acids solution (100 µM), and sodium pyruvate (1 mM).

Cell-Proliferation Assays

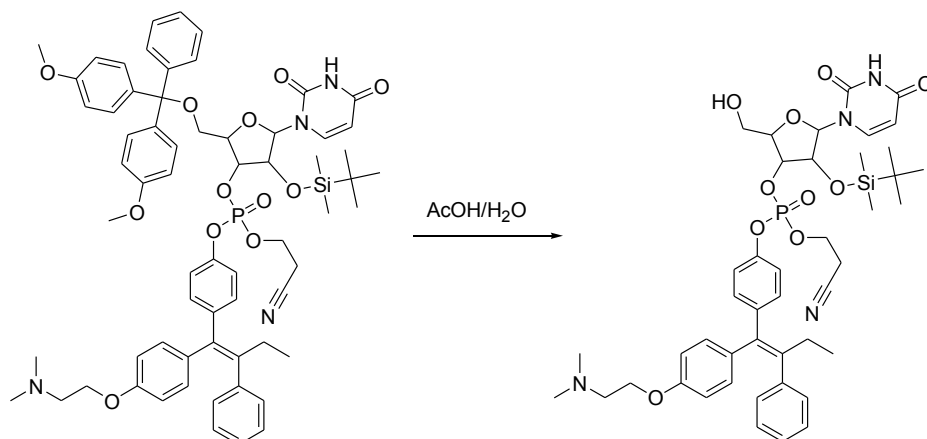
The effect of UpHT and HT on the proliferation of MCF-7 cells was assayed by monitoring the incorporation of [*methyl*-³H]thymidine into cellular DNA, as described previously.⁶ The assay was also similar to that used in a previous study on the toxicity of tamoxifen for MCF-7 cells.⁷ For assays, MCF-7 cells (100 µL of a solution of 5.0×10^4 cells/mL) in medium A were plated in each well of 96-well plates and incubated for 22 h to allow for cell adherence. Medium was removed, cells were washed with DPBS, and medium B (50 µL) was added. UpHT and HT were first dissolved in DMSO, and then diluted 1000-fold with medium B, resulting in a final DMSO concentration of 0.1% v/v. This stock solution was serially diluted with medium B containing DMSO (0.1% v/v). The highest concentration HT dilutions appeared to be cloudy, indicating that these concentrations might be reaching the solubility limit of HT in medium. Aliquots (50 µL) of these serial dilutions were added to wells (final DMSO concentration: 0.05% v/v). RNase 1 was diluted in medium B, and wells received either 1 µL of stock RNase 1 for a final concentration of 0.42 µM (~6.2 mg/L, ~15× plasma concentration of ~0.4 mg/L) or 1 µL of medium B, and the analyzed drug concentrations take into account this dilution. RNase 1 control wells received no drug, but did receive RNase 1. After a 2-h incubation, MCF-7 cells were treated with [*methyl*-³H]thymidine for 4 h, and the incorporation of radioactive thymidine into cellular DNA was quantified by liquid scintillation counting. The results are shown as the percentage of [*methyl*-³H]thymidine incorporated relative to control cells treated with medium B containing DMSO (final DMSO concentration 0.05% v/v). Data are the average of three measurements for each concentration, excluding those measurements that were determined to be outliers by the Grubb’s test for outliers⁸ with $p = 0.05$. The entire experiment was repeated in triplicate. Values for IC₅₀ were calculated by fitting the curves by nonlinear regression with eq 2, in which y is the total DNA synthesis following the [*methyl*-³H]thymidine pulse, and h is the slope of the curve.

$$y = 100\% / (1 + 10^{(\log(\text{IC}_{50}) - \log[\text{ribonuclease}])h}) \quad (2)$$

Protected Uridine 3'-(4-Hydroxytamoxifen phosphate)

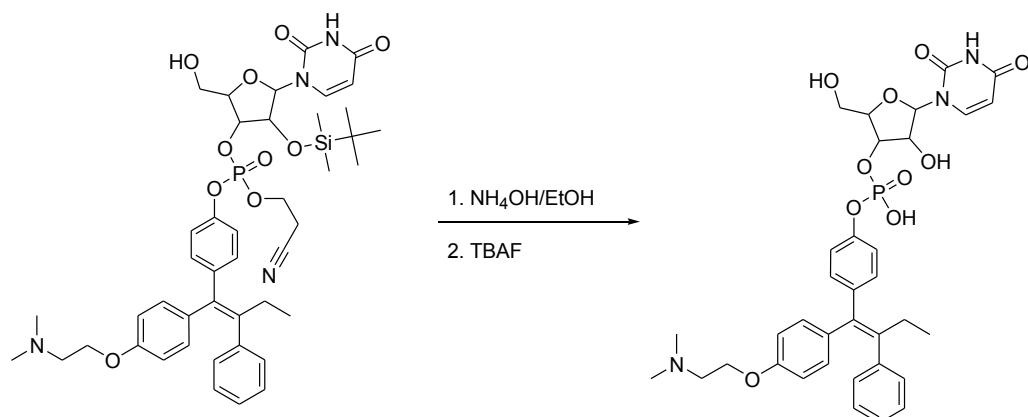
A 20-mL scintillation vial was flame-dried under vacuum. To this vial were added 3-Å molecular sieves (10 beads) followed by 4-hydroxytamoxifen (0.100 g, 0.258 mmol), phosphoramidite (0.222 g, 0.258 mmol), and anhydrous acetonitrile (2.58 mL). After stirring for 5 min, *N*-methylbenzimidazolium triflate (0.073 g, 0.258 mmol) was added and the reaction mixture was allowed to stir for an additional 2.5 h. The solution was then decanted from the sieves into a new scintillation vial, concentrated to remove acetonitrile, and treated with a 0.02 M solution of I₂ (12.6 mL, 2.52 mmol) in tetrahydrofuran, pyridine, and water (Glen Research Oxidizing Solution) for 1 h. The reaction mixture was then concentrated under vacuum and purified by flash silica gel chromatography (MeOH/DCM 1:9) to give protected uridine 3'-(4-hydroxytamoxifen phosphate) as a mixture of isomers (0.279 g, 93%).

¹H NMR (400 MHz, CD₃OD) (Mixture of isomers) δ = 7.91–7.83 (m, 1H), 7.43–7.35 (m, 2H), 7.34–7.20 (m, 8H), 7.19–7.01 (m, 8H), 6.91–6.82 (m, 4H), 6.80–6.73 (m, 2H), 6.65–6.56 (m, 2H), 6.00–5.85 (m, 1H), 5.43–5.31 (m, 1H), 5.08–4.96 (m, 1H), 4.64–4.50 (m, 1H), 4.48–4.36 (m, 2H), 4.34–4.21 (m, 1H), 4.15–4.01 (m, 2H), 3.76 (as, 6H), 3.65–3.45 (m, 2H), 3.40–3.10 (m, 2H), 2.95–2.85 (m, 2H), 2.74 (as, 6H), 2.51–2.30 (m, 2H), 0.95–0.82 (m, 12H), 0.20–0.06 (m, 6H). **¹³C NMR (101 MHz, CD₃OD)** (Major Isomer) δ = 165.6, 160.3, 157.2, 152.1, 150.0, 145.8, 143.8, 143.3, 142.7, 141.7, 138.2, 137.6, 136.3, 133.2, 132.2, 131.5, 130.8, 129.3, 129.2, 129.0, 128.3, 127.4, 121.2, 118.2, 115.6, 114.7, 114.5, 103.3, 88.8, 83.4, 79.4, 75.7, 65.3, 63.7, 63.3, 57.9, 55.9, 44.3, 30.0, 26.2, 20.2, 19.0, 13.8, –4.7. **³¹P NMR (162 MHz, CD₃OD)** (Mixture of isomers) δ = –8.57, –8.82, –9.02. **HRMS** (ESI) m/z 1163.4998 [calc'd for C₆₅H₇₆N₄O₁₂PSi (M+H) 1163.4962].

Semi-protected Uridine 3'-(4-Hydroxytamoxifen phosphate)

Acetic acid/H₂O 60:40 (4.6 mL) was added to a 20-mL scintillation vial containing 4-hydroxytamoxifen phosphate (0.270 g, 0.232 mmol), and the reaction mixture was allowed to stir at room temperature for 5 h until the starting material was consumed as determined by TLC (10% MeOH, 90% DCM). The reaction was then quenched by adding saturated sodium bicarbonate (5 mL), extracted with ethyl acetate (4 × 5 mL), dried over sodium sulfate, and concentrated under vacuum. The resulting residue was purified using flash silica gel chromatography (10% MeOH, 90% DCM) to give semi-protected uridine 3'-(4-hydroxytamoxifen phosphate) (0.180 g, 90%).

¹H NMR (400 MHz, CD₃OD) (Mixture of isomers) δ = 8.17–7.95 (m, 1H), 7.35–7.21 (m, 3H), 7.20–6.90 (m, 6H), 6.81 (d, J = 8.6, 2H), 6.64 (d, J = 8.6, 2H), 6.09–5.89 (m, 1H), 5.82–5.66 (m, 1H), 4.63–4.25 (m, 4H), 4.20–4.07 (m, 2H), 3.87–3.53 (m, 2H), 3.43–3.26 (m, 3H), 2.97–2.90 (m, 2H), 2.87–2.74 (m, 6H), 2.50–2.40 (m, 2H), 0.97–0.79 (m, 12H), 0.15–0.01 (m, 6H). **¹³C NMR (101 MHz, CD₃OD)** (Major Isomer) δ = 164.4, 156.1, 151.0, 148.7, 141.9, 140.7, 137.0, 136.1, 130.8, 130.3, 129.4, 127.6, 125.9, 119.9, 118.8, 116.8, 114.1, 113.3, 102.1, 87.2, 83.7, 78.9, 74.3, 63.9, 62.3, 60.7, 56.5, 42.9, 28.5, 24.8, 18.8, 17.6, 12.4, –6.02. **³¹P NMR (162 MHz, CD₃OD)** (Mixture of Isomers) δ = –8.53, –8.64, –8.71, –8.81. **HRMS (ESI)** m/z 861.3652 [calc'd for C₄₄H₅₈N₄O₁₀PSi (M+H) 861.3655].

Uridine 3'-(4-Hydroxytamoxifen phosphate)

Anhydrous ethanol (1.2 mL) was added to a 20-mL scintillation vial containing semi-protected uridine 3'-(4-hydroxytamoxifen phosphate) (0.051 g, 0.059 mmol) at room temperature followed by ammonium hydroxide (0.04 mL, 1.185 mmol) and the reaction was allowed to stir for 3 h before concentrating under vacuum to give a crude residue that was used directly in the next step.

A portion of the resulting crude residue (0.030 g, 0.037 mmol) was dissolved in anhydrous acetonitrile (4.3 mL) at 0 °C, and a solution of tetrabutylammonium fluoride (0.04 mL, 0.040 mmol) was added in one portion. The reaction mixture was allowed to warm to room temperature while stirring for 5 h. The reaction mixture was then diluted with 4.3 mL of H_2O and loaded onto a column of Macherey–Nagel VP 250/21 Nucleosil 100-5 C18 reverse-phase HPLC resin that had been pre-equilibrated in a solution of acetonitrile (25% v/v) in H_2O , and maintained continually at 35 °C. Sample was washed with the same solution for 13 min. Product was eluted with a linear gradient (25–100% v/v acetonitrile in H_2O) for 68 min, providing uridine 3'-(4-hydroxytamoxifen phosphate) (0.020 g, 69%, for Bu_4N^+ salt).

^1H NMR (400 MHz, CD_3OD) (Both Olefin Isomers) δ = 8.03 (d, J = 8.1, 0.77H), 7.99 (d, J = 8.2, 0.23H), 7.23 (d, J = 8.2, 1.54H), 7.17–7.03 (m, 7.00H), 6.97 (d, J = 8.5, 0.46H), 6.87 (d, J = 8.5, 0.46H), 6.77 (at, J = 8.6, 2.00H), 6.61 (d, J = 8.6, 1.54H), 5.96 (d, J = 5.5, 0.77H), 5.90 (d, J = 5.5, 0.23H), 5.69 (at, J = 6.8, 1H), 4.77–4.66 (m, 0.77H), 4.64–4.56 (m, 0.23H), 4.35–4.0 (m, 4.00H), 3.80–3.66 (m, 2.00H), 3.63–3.48 (m, 0.46H), 3.31–3.17 (m, 2.00H), 2.80 (s, 1.38H), 2.74 (s, 4.62H), 2.46 (q, J = 7.3, 2.00H), 0.90 (t, J = 7.3, 3.00H). **^{13}C NMR (101 MHz, CD_3OD)** (Major Isomer Peaks + 4 Bu_4N^+ Salt Signals) δ = 164.7, 155.9, 151.4, 151.0, 142.3, 141.6, 141.1, 138.8, 137.7, 136.5, 131.7, 130.0, 129.5, 127.5, 125.8, 119.8, 113.1, 101.4, 88.5, 84.0, 74.6, 73.9, 62.4, 60.8, 58.1, 56.6, 42.9, 28.5, 23.4, 19.3, 12.5, 12.4. **^{31}P NMR (162 MHz, CD_3OD)** (Both Olefin Isomers) δ = –5.90, –6.09. **HRMS (ESI)** m/z 716.2324 [calc'd for $\text{C}_{35}\text{H}_{40}\text{N}_3\text{O}_{10}\text{PNa}$ ($\text{M}+\text{Na}$) 716.2344].

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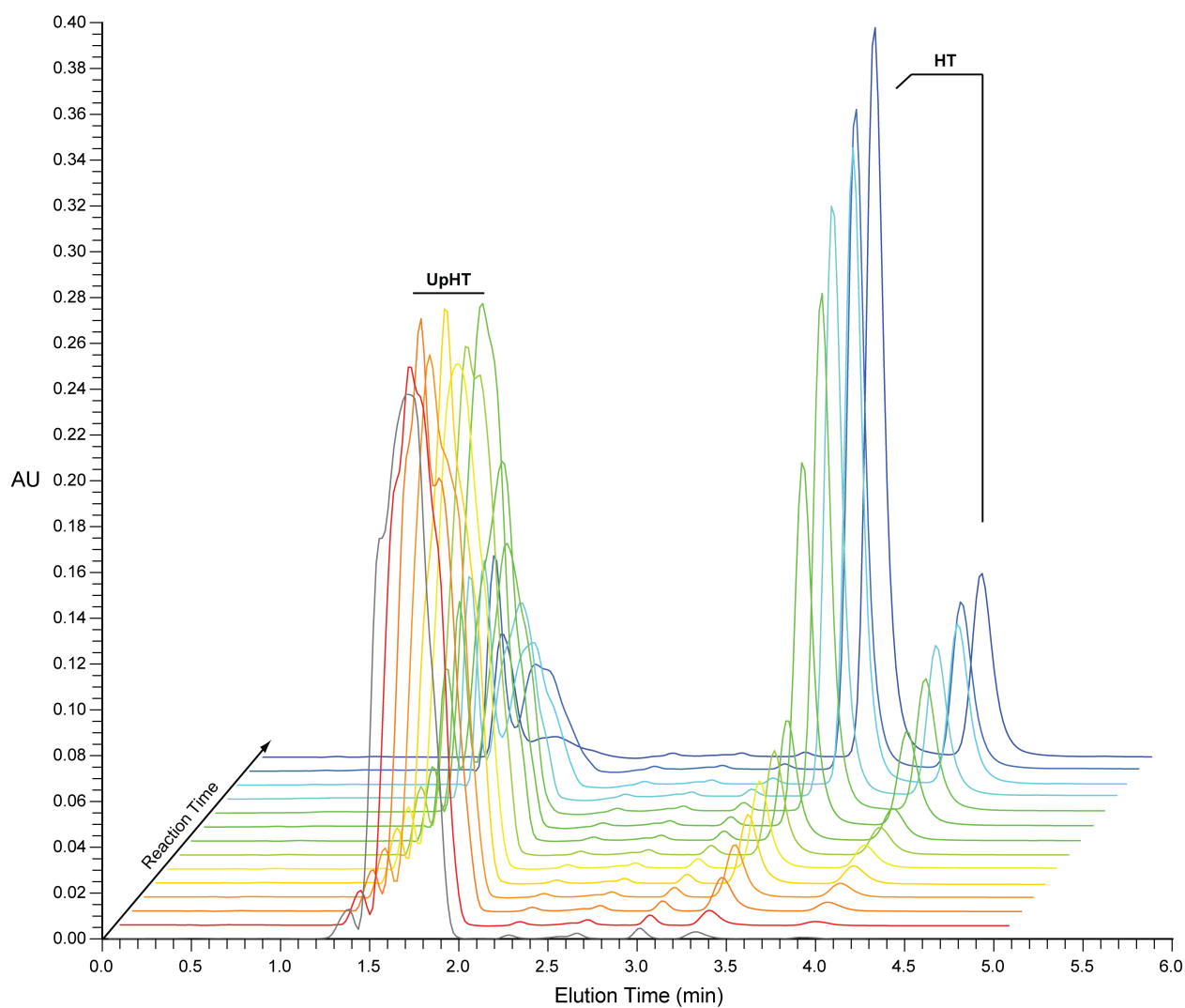


Figure S1. Compilation of HPLC traces from one UpHT-activation experiment. The release of free HT by RNase 1 at 37 °C was monitored over time at 245 nm by the appearance of peaks for the HT isomers at ~3.3 and ~3.9 min. The initial trace (gray) is prior to the addition of RNase 1; the final trace (blue) is after the addition of excess RNase 1.

