Boronic Acid for the Traceless Delivery of Proteins into Cells

Kristen A. Andersen, Thomas P. Smith, Jo E. Lomax, and Ronald T. Raines*

Page 1

Table of Contents

S1 Table of Contents
S2 Materials
S2 Instrumentation
S2 Statistics
S2 Synthesis
S7 Cell Culture
S7 Labeling of GFP
S10 Analysis of B-TML–GFP Internalization
S11 Colocalization of Internalized GFP with Organelles
S12 Cellular Internalization over Time
S13 Fructose Competition
S13 Labeling of Ribonuclease A
S14 References
S16 NMR Spectra
1. General Information

Materials. All chemicals were from Sigma–Aldrich (Milwaukee, WI) and were used without further purification. All glassware was flame-dried, and all reactions were performed under an atmosphere of N₂(g). Reagent grade solvents, i.e., dichloromethane (DCM), tetrahydrofuran (THF), triethylamine (TEA), and dimethylformamide (DMF), were dried over a column of alumina and were accessed under an atmosphere of N₂(g). The removal of solvents “under reduced pressure” refers to the use of a rotary evaporator with water-aspirator pressure (<20 torr) and a water bath of <40 °C. Column chromatography was performed with Silicycle 40–63 Å silica (230–400 mesh); thin-layer chromatography (TLC) was performed with EMD 250-µm silica gel 60-F254 plates. PBS contained Na₂HPO₄ (10 mM), KH₂PO₄ (1.8 mM), NaCl (137 mM), and KCl (2.7 mM) at pH 7.3. All procedures were performed at room temperature (~22 °C) unless noted otherwise.

Instrumentation. ¹H and ¹³C NMR spectra were acquired at ambient temperature with a Bruker Avance III 500i spectrometer at the National Magnetic Resonance Facility at Madison (NMRFAM) and referenced to residual protic solvent. Electrospray ionization (ESI) mass spectrometry of small molecules was performed with a Micromass LCT in the Mass Spectrometry Facility in the Department of Chemistry at the University of Wisconsin–Madison. Matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry of proteins was performed with a Voyager DE-Pro instrument at the Biophysics Instrumentation Facility at the University of Wisconsin–Madison. Absorbance measurements were made with an infinite M1000 plate reader from Tecan (Männedorf, Switzerland). Confocal microscopy was performed with an Eclipse TE2000-U laser scanning confocal microscope from Nikon (Tokyo, Japan), equipped with an AxioCam digital camera from Zeiss (Jena, Germany). Flow cytometry was performed at the University of Wisconsin–Madison Carbone Cancer Center Flow Cytometry Facility with a FACS Calibur instrument from BD Biosciences (San Jose, CA). Cytometry data were analyzed with the program FlowJo 8.7 from Treestar (Ashland, Oregon).

Statistics. Calculations were performed with Prism version 6 software from GraphPad Software (La Jolla, CA).

2. Synthesis

![Chemical structure of Compound 1](image)

Compound 1. 2,5-Dimethylphenol (5.0 g, 40.9 mmol) was dissolved in methanesulfonic acid (10 mL). Methyl-3,3-dimethylacrylate (5.61 g, 49.1 mmol) was added, and the resulting solution was heated to 70 °C for 24 h. The reaction mixture was then allowed to cool to room temperature and poured into separating funnel with 250 mL of water. The mixture was then extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with saturated aqueous NaHCO₃ followed by brine. The organic layers were combined and dried over NaSO₄(s). The solvent was
removed under reduced pressure and the crude product was purified by chromatography on a column of silica gel (2:8 EtOAc/hexanes) to afford an off-white solid (7.6 g, 92%).

$^1$H NMR (500 MHz, CDCl$_3$, δ): 1.45 (s, 6H), 2.27 (s, 3H), 2.46 (s, 3H), 2.60 (s, 2H), 6.83 (d, $J = 6.86$ Hz, 1H), 6.99 (d, $J = 6.85$ Hz, 1H)

$^{13}$C NMR (125 MHz, CDCl$_3$, δ): 16.28, 23.10, 27.63, 35.35, 45.61, 124.56, 127.98, 129.10, 129.49, 133.54, 149.75, 168.43

HRMS (ESI) calculated for [C$_{13}$H$_{16}$O$_2$]$^+$ (M+NH$_4^+$) requires $m/z$ 222.1489; found $m/z$ 222.1486

**Compound 2.** Compound 1 (3.5 g, 17.1 mmol) was dissolved in anhydrous THF (20 mL). The resulting solution was added dropwise to a suspension of LiAlH$_4$ (0.971 g, 25.6 mmol) in anhydrous THF (170 mL) that had been cooled to 0 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction was quenched by the slow addition of ethyl acetate (20 mL) followed by the slow addition of water (20 mL). The mixture was filtered to remove the aluminum salts, and the filtrate was dried over Na$_2$SO$_4$(s). The solvent was removed under reduced pressure, and the crude product was purified by chromatography on a column of silica gel (4:6 EtOAc/hexanes) to afford compound 2 as a colorless oil (2.8 g, 80%).

$^1$H NMR (500 MHz, CDCl$_3$, δ): 1.45 (s, 6H), 2.18 (s, 3H), 2.22 (t, $J = 7.12$ Hz, 2H), 2.48 (s, 3H), 3.59 (t, $J = 7.11$ Hz, 2H), 5.31 (s, 1H), 6.59 (d, $J = 7.60$ Hz, 1H), 6.86 (d, $J = 7.58$ Hz, 1H)

$^{13}$C NMR (125 MHz, CDCl$_3$, δ): 16.24, 25.69, 32.06, 39.90, 44.93, 61.36, 121.82, 125.57, 127.98, 131.55, 135.88, 153.54

HRMS (ESI) calculated for [C$_{13}$H$_{20}$O$_2$]$^+$ (M+•) requires $m/z$ 208.1458; found $m/z$ 208.1453

**Compound 3.** Compound 2 (2.5 g, 12 mmol) and tert-butyl dimethylchlorosilane (3.6 g, 24 mmol) were dissolved in DCM (120 mL). Triethylamine (5 mL, 36 mmol) was then added, and the reaction mixture was allowed to stir overnight. The solvent was removed under reduced pressure, and the crude product was purified by chromatography on a column of silica gel (2:8 EtOAc/hexanes) to afford compound 3 as a white solid (2.8 g, 73%).

$^1$H NMR (500 MHz, CDCl$_3$, δ): 0.03 (s, 6H), 0.89 (s, 9H), 1.59 (s, 6H), 2.15 (t, $J = 6.76$ Hz, 2H), 2.21 (s, 3H), 2.48 (s, 3H), 3.61 (t, $J = 6.77$ Hz, 2H), 5.82 (s, 1H), 6.60 (d, $J = 7.63$ Hz, 1H), 6.88 (d, $J = 7.57$ Hz, 1H)

$^{13}$C NMR (125 MHz, CDCl$_3$, δ): 5.41, 16.43, 18.30, 25.62, 25.92, 32.27, 39.68, 44.91, 61.75, 122.88, 125.17, 127.79, 132.07, 135.53, 153.87

HRMS (ESI) calculated for [C$_{19}$H$_{34}$O$_2$Si]$^+$ (M+H$^+$) requires $m/z$ 323.2401; found $m/z$ 323.2394
Compound 4. Boc-protected β-alanine (1.75 g, 9.3 mmol), EDC (2.3 g, 18.6 mmol), and DMAP (2.28 g, 18.6 mmol) were added to a flame-dried 250-mL round-bottom flask. The flask was evacuated and flushed with N₂(g), and DCM (42 mL) was added. Compound 3 (2.0 g, 6.2 mmol) was dissolved in dry DCM (20 mL), and the resulting solution was added drop-wise. The reaction mixture was allowed to stir overnight. The reaction mixture was diluted with 10% w/v aqueous NaHCO₃ and then washed with 1 N HCl. The solution was dried over NaSO₄(s), and solvent was removed under reduced pressure. The crude product was purified by chromatography on a column of silica gel (1:9 EtOAc/hexanes) to afford compound 4 as a white solid (1.98 g, 65%).

\[ \text{H NMR (500 MHz, CDCl}_3, \delta): 0.03 \text{ (s, 6H), 0.84 (s, 9H), 1.49–1.44 (m, 18H), 2.02 (s, 3H), 2.09–2.08 (m, 2H), 2.53 (s, 3H); 3.48–3.44 (m, 4H), 5.15–5.12 (bs, 1H), 6.92 (d, J = 7.71 Hz, 1H), 6.96 (d, J = 7.64 Hz, 1H)} \]

\[ \text{13C NMR (125 MHz, CDCl}_3, \delta): 5.36, 17.22, 18.24, 25.26, 25.92, 28.38, 31.88, 34.87, 35.94, 39.40, 46.06, 60.72, 79.39, 128.46, 128.95, 131.26, 136.34, 137.29, 148.57, 155.85, 171.28; HRMS (ESI) calculated for [C\text{27H47NO5Si}^+ (M+H^+) requires m/z = 494.3297; found m/z = 494.3294} \]

OH

O

Si

O

O

Boc-beta-Ala, EDC

DMAP, DCM

65%

3 4

OH

O

Si

O

O

N

H

O

O

N

H

PCC, DCM

85%

5 6

OH

O

N

H

O

PCC, DCM

85%

5 6

OH

O

N

H

O

AcOH, THF:H₂O quant.

quant.

Compound 5. Compound 4 (1.5 g, 3.0 mmol) was dissolved in a mixture of THF (6 mL), H₂O (6 mL), and concentrated glacial acetic acid (18 mL). The reaction mixture was stirred and reaction progress was monitored with TLC. After 2 h, solvent was removed under reduced pressure, and the crude product was purified by passage through a short (2-in) plug of silica gel (2:8 EtOAc/hexanes) to afford compound 5 as a colorless oil (1.34 g, quant).

\[ \text{H NMR (500 MHz, CDCl}_3, \delta): 1.43 \text{ (s, 9H), 1.46 (s, 3H), 1.51 (s, 3H), 1.93–1.89 (m, 2H), 2.02 (s, 3H), 2.53 (s, 3H), 2.84–2.82 (m, 2H) 3.57–3.45 (m, 4H), 5.20 (bs, 1H), 6.92–6.91 (d, J = 7.70 Hz, 1H), 6.97–6.95 (d, J = 7.73 Hz, 1H)} \]

\[ \text{13C NMR (125 MHz, CDCl}_3, \delta): 17.22, 25.39, 28.41, 32.20, 34.94, 35.93, 39.50, 45.77, 60.38, 79.54, 128.65, 129.14, 131.43, 136.47, 137.09, 148.58, 155.86, 171.78; HRMS (ESI) calculated for [C\text{27H47NO5Si}^+ (M+H^+) requires m/z = 494.3297; found m/z = 494.3294} \]
Compound 6. A solution of compound 5 (1.0 g, 2.6 mmol) in anhydrous DCM (5 mL) was added slowly to a suspension of PCC (2.2 g, 10.4 mmol) in DCM (21 mL), and the reaction mixture was allowed to stir overnight. The reaction mixture was then filtered, and solvent was removed under reduced pressure. The crude product was purified by chromatography on a column of silica gel (4:6 EtOAc/hexanes) to afford compound 6 as a clear oil (0.83 g, 85%).

\[ \text{1H NMR (500 MHz, CDCl}_3, \delta): 1.52–1.44 \text{ (s, 16H), 2.04 (s, 3H), 2.55 (s, 3H), 2.93–2.55 (m, 4H), 3.51–3.48 (s, 2H), 5.08 (t, } J = 6.40 \text{ Hz, 1H), 6.97 (d, } J = 7.72 \text{ Hz, 1H), 7.02 (d, } J = 7.73 \text{ Hz, 1H), 9.51 (t, } J = 2.63 \text{ Hz, 1H)} \]

\[ \text{13C NMR (125 MHz, CDCl}_3, \delta): 17.18, 25.35, 28.37, 34.88, 35.90, 38.42, 56.70, 79.50, 129.23, 129.42, 131.70, 135.78, 135.91, 148.21, 155.80, 171.20, 202.81 \]

HRMS (ESI) calculated for \([\text{C}_{21}\text{H}_{31}\text{NO}_5]^+\) (M+H\(^+\)) requires \( m/z \) 378.2275; found \( m/z \) 378.2280

Compound 7. Compound 6 (0.8 g, 2.1 mmol) and NaH2PO4 (0.26 g, 1.59 mmol) were dissolved in CH3CN (10 mL), and the resulting solution was cooled to -10 °C. A solution of 80% w/v sodium chlorite (0.6 g, 6.3 mmol) in water (10 mL) was added dropwise. The reaction mixture was stirred for 1 h at -10 °C and then allowed to warm to room temperature. Saturated sodium sulfite solution (2 mL) was added, and the reaction mixture was acidified to pH 2.0 with 1 N HCl followed by extraction with ethyl acetate (3 × 15 mL). The organic layer was washed with brine and then dried over NaSO4(s). The solvent was removed under reduced pressure, and the crude product was purified by chromatography on a column of silica gel (5–10% v/v MeOH/DCM) to afford compound 7 as a clear oil (0.61 g, 73%)

\[ \text{1H NMR (500 MHz, CDCl}_3, \delta): 1.43 (s, 9H), 1.54 (s, 3H), 1.63 (s, 3H), 2.02 (s, 3H), 2.55 (s, 3H), 2.85–2.74 (m, 4H), 3.48 (s, 2H), 5.16 (t, } J = 6.14 \text{ Hz, 1H), 6.93 (d, } J = 7.76 \text{ Hz, 1H), 6.98, (d, } J = 7.78 \text{ Hz, 1H) } \]

\[ \text{13C NMR (125 MHz, CDCl}_3, \delta): 17.18, 25.23, 28.37, 31.23, 31.55, 34.87, 35.90, 38.42, 47.48, 59.50, 128.82, 131.44, 136.04, 136.69, 148.26, 155.89, 171.27, 175.98 \]

HRMS (ESI) calculated for \([\text{C}_{21}\text{H}_{31}\text{NO}_6]^+\) (M+H\(^+\)) requires \( m/z \) 394.2225; found \( m/z \) 394.2219

Compound 8. Compound 7 (0.5 g, 1.2 mmol) was dissolved in a solution of 4 M HCl in dioxane (8.4 mL) and allowed to stir for 1 h. N\(_2\)(g) was then bubbled though the solution for 15 min to remove excess HCl, and solvent was removed under reduced pressure. Diethyl ether was added to precipitate compound 8 as its HCl salt (0.4 g; quant).

\[ \text{1H NMR (500 MHz, CD}_3\text{OD, } \delta): 1.54 (s, 6H), 2.18 (s, 3H), 2.53 (s, 3H), 2.77 (s, 2H), 2.99–3.01 (t, } J = 6.52 \text{ Hz, 2H), 3.25–3.28 (m, 6H), 6.65 (s, 1H), 6.83 (s, 1H) } \]
13C NMR (125 MHz, CD3OD, δ): 20.22, 25.50, 32.09, 33.08, 36.21, 39.80, 124.01, 133.41, 134.86, 137.33, 139.54, 150.55, 171.46, 175.54
HRMS (ESI) calculated for [C16H24NO4]+ (M–Cl)+ requires m/z 294.1779; found m/z 294.1793

**Compound 9.** 5-Amino-2-hydroxymethylphenylboronic acid (0.200 g, 1.07 mmol) was dissolved in 10.4 mL of dry acetonitrile. N,N-Disuccinimidyl carbonate (0.303 g, 1.18 mmol) was then added to the solution, followed by the dropwise addition of DIEA (0.20 mL, 2.14 mmol). The reaction mixture was allowed to stir overnight. The reaction mixture was then filtered to remove the insoluble N-hydroxysuccinimide (NHS) byproduct. The solvent from the filtrate was removed under reduced pressure to give the crude product as a yellow solid. The crude solid was dissolved in chloroform and cooled to –20 °C in the freezer for 1 h. The solution was filtered, and solvent was removed under reduced pressure to give compound 9 as a light yellow solid. The compound was used in the next step without further purification.

**Compound 10.** Compound 8 (0.080 g, 0.244 mmol) was dissolved in dry THF (2.4 mL). Compound 9 (0.078 g, 0.268 mmol) was then added to the solution, followed by the dropwise addition of DIEA (0.06 mL, 0.73 mmol). The reaction mixture was allowed to stir overnight. The reaction mixture was then filtered, and solvent was removed under reduced pressure. The crude product was purified by chromatography on a column of silica gel (3–5% v/v MeOH:DCM) to afford compound 10 as a white solid (0.083 g, 73%).

1H NMR (500 MHz, CD3OD, δ): 1.56 (s, 3H), 2.20 (s, 3H), 2.55 (s, 3H), 2.80 (s, 2H), 2.87–2.85 (t, J = 6.16 Hz, 2H), 3.60–3.67 (t, J = 6.18 Hz, 2H), 5.03 (s, 2H), 6.63 (s, 2H), 6.68 (s, 1H), 7.28–7.29 (d, J = 8.22 Hz, 1H), 7.50–7.52 (d, J = 8.24 Hz, 1H), 7.62 (s, 1H)

13C NMR (125 MHz, CD3OD, δ): 18.66, 24.00, 24.73, 30.45, 35.16, 38.22, 70.56, 119.96, 120.95, 122.28, 122.62, 131.62, 133.49, 135.96, 137.84, 138.42, 147.83, 149.29, 156.70, 171.60, 173.36, 174.05; note: the signal for the carbon attached to boron was not observable due to quadrupolar relaxation

HRMS (ESI) calculated for the methyl boronic ester [C25H3111BN2O7]+ (M+H)+ requires m/z 483.2298; found m/z 483.2308
**Compound 11 (B-TML–NHS ester)**. Compound 10 (0.068 g, 0.129 mmol) was dissolved in dry DCM (1.3 mL). The reaction mixture was then cooled to 0 °C and DCC (0.032 g, 0.155 mmol) followed by N-hydroxysuccinimide (0.016 g, 0.142 mmol) were added. The reaction mixture was allowed to warm to room temperature and stir over night. The reaction mixture was then cooled to 0 °C and the solid was removed by filtration. The solvent was removed under reduced pressure, and the resulting residue and dissolved in ethyl acetate and chilled to –20 °C in a freezer for 3 h. The solids were removed by filtration, and the filtrate was extracted with water to remove any residual urea byproduct. The organic layers were combined, and the solvent was removed under reduced pressure to afford the compound 11 as a white solid (0.012 g, 20%).

$^1$H NMR (500 MHz, CD$_3$OD, δ): 1.61 (s, 6H), 2.20 (s, 3H), 2.55 (s, 3H), 2.75 (s, 4H), 2.93–2.90 (t, $J = 5.76$ Hz, 2H), 3.15 (s, 2H), 3.61–3.58 (t, $J = 6.74$ Hz, 2H), 5.03 (s, 2H), 6.66 (s, 1H), 6.84 (s, 1H), 7.28–7.29 (d, $J = 8.21$ Hz, 1H), 7.51–7.49 (d, $J = 8.19$ Hz, 1H), 7.61 (s, 1H)

$^{13}$C NMR (125 MHz, CD$_3$OD, δ): 20.24, 25.50, 26.45, 31.56, 36.54, 36.72, 40.04, 45.15, 72.11, 121.51, 122.51, 123.84, 124.29, 133.32m 133.95, 137.71, 139.17, 139.99, 149.38, 150.86, 158.22, 168.16, 171.79, 173.13; note: the signal for the carbon attached to boron was not observable due to quadrupolar relaxation

HRMS (ESI) calculated for the methyl boronic ester [C$_{29}$H$_{34}$^{10}BN$_3$O$_9$]$^+$ (M+Na)$^+$ requires $m$/$z$ 602.2286; found $m$/$z$ 602.2290

**Cell Culture**. Cell lines were obtained from American Type Culture Collection (Manassas, VA) and were maintained according to the recommended procedures. Medium and added components, trypsin (0.25% w/v), and Dulbecco’s PBS (DPBS) were the Gibco® brand from Thermo Fisher Scientific (Waltham, MA). Cells were grown in flat-bottomed culture flasks in a cell-culture incubator at 37 °C under CO$_2$(g) (5% v/v). Chinese hamster ovary (CHO K1) cells were grown in F12K nutrient medium and K562 cells were grown in RPMI 1640 medium, both supplemented with fetal bovine serum (FBS) (10% v/v), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were counted with a hemocytometer to determine the seeding density in 12-well plates from Corning Costar (Lowell, MA) or microscopy dishes from Ibidi (Madison, WI). All flow cytometry and confocal microscopy was performed with live cells, incubated on ice at the time of analysis.

**Labeling of GFP**

*Preparation of Green Fluorescent Protein (GFP)*

The gene encoding the eGFP variant GFP was inserted into the pET22b vector (Novagen), along with an N-terminal His$_6$ tag followed by a spacer region and a TEV protease recognition sequence. The GFP gene was modified with the following 17 substitutions to generate a “superfolding” variant that folds readily when produced in *Escherichia coli*: F64L, S65T, F99S, M153T, V163A, S30R, Y145F, I171V, A106V, Y39I, N105K, E111V, I128T, K166T, I167V, S205T, L221H, F223Y, T225N.$^{1-5}$ The vector also contained a T7 promoter and ampicillin resistance gene.

The expression vector was transformed into electrocompetent BL21(DE3) *E. coli* cells from New England Biolabs (Ipswich, MA), then plated on LB agar containing ampicillin (200 µg/mL). On the following day, a single colony was used to inoculate 50 mL of LB medium, and the resulting culture was grown overnight at 37 °C in a shaking incubator. On the following day, 10 mL of starter culture was used to inoculate 1.00 L of Terrific Broth medium from Research Products International (Mt. Prospect, IL) in a 3.8-L glass flask with ampicillin at a final
concentration of 200 µg/mL. Flasks were shaken at 200 rpm at 37 °C in a shaking incubator until cells reached log phase (OD 0.6–0.8 at 600 nm). The production of GFP was induced by adding IPTG to a final concentration of 1 mM, and cells were grown overnight at 37 °C in a shaking incubator.

Cells were harvested by centrifugation for 20 min at 5,000 rpm at 4 °C. Cell pellets, which were bright yellow in color, were collected and resuspended in 15 mL of lysis buffer per 2 L of liquid growth, which was 50 mM Tris–HCl buffer, pH 7.0, containing NaCl (100 mM) and imidazole (30 mM). Cell pellets were vortexed and stored frozen at –20 °C overnight.

Cells were lysed with a TS Series cell disrupter from Constant Systems (Kennesaw, GA), and the lysate was subjected to centrifugation immediately for 1 h at 11,000 rpm at 4 °C. The supernatant was filtered through 5-µM syringe filters from EMD Millipore (Billerica, MA) and solid, pelleted material was discarded. Filtered cell lysates were purified by chromatography on Ni–NTA resin from GE Healthcare (Little Chalfont, UK) and eluted using a linear gradient of imidazole. The binding and wash buffer was 30 mM sodium phosphate buffer, pH 7.4, containing NaCl (0.50 M) and imidazole (20 mM). The elution buffer was 30 mM sodium phosphate buffer, pH 7.4, containing NaCl (0.50 M) and imidazole (0.50 M). Eluted fractions were collected, pooled, and dialyzed against 4 L of 20 mM Tris–HCl buffer, pH 7.0, containing EDTA (1 mM).

The dialyzed solution was purified again using anion-exchange chromatography on a hiTrap Q column from GE Healthcare. The protein was eluted by using a linear gradient of NaCl (0–1.00 M) in 20 mM Tris–HCl buffer, pH 7.0, containing EDTA (1.0 mM). Upon elution, colored fractions were pooled and concentrated (if necessary). The overall yield of GFP was ~120 mg per L of culture.

**Labeling of GFP with B-TML–NHS Ester**

B-TML–NHS ester (2.2 mg, 4 µmol, 100 equiv) was added to a 150-µL solution of GFP (300 µM, 0.04 µmol) in PBS. The vial was placed on a nutator in foil overnight at room temperature. The solution was transferred to 10,000 MWCO dialysis tubing and dialyzed twice against 4 L of PBS for 4 h at 4 °C. The extent of labeling was characterized by MALDI–TOF mass spectrometry. To determine the extent of labeling, the average mass of labeled protein was taken as a weighted average for all events within the defined peak range on the background-corrected MALDI–TOF spectra. The molecular weight of non-labeled protein was then subtracted from the given value to give the average total mass of all labels. This value was then divided by the molecular weight of the modifying small molecule minus the mass of N-hydroxysuccinimide (565.38 Da – 115.09 Da = 450.29 Da), which was lost during
conjugation, to give the average number of labels per molecule of protein. On average we
determined there to be approximately $3 \pm 1$ labels per GFP. The labeled protein is referred to as
B-TML–GFP. An identical procedure was used with non-boronated TML (Ac-TML) from Sigma–Aldrich in which the phenolic oxygen is protected as acetate. The Ac-TML–GFP was
also decorated with $3 \pm 1$ labels. The amount of labels was not statistically significant between
Day 1 and Day 33.

Figure S1. Labeling of GFP. MALDI–TOF spectra of (A) unmodified GFP, (B) B-TML–GFP,
and (C) Ac-TML–GFP. Data were fitted to a Gaussian curve (red line). Expected $m/z$: GFP,
29361; each B-TML moiety, 450; each Ac-TML moiety, 246.
Figure S2. Stability of B-TML–GFP. MALDI–TOF spectra of B-TML–GFP on Day 1 (which was the day of labeling and purification) and Day 33 after storage in PBS at 4 °C in the dark. Data were fitted to a Gaussian curve (red line). Expected \( m/z \): GFP, 29361; each B-TML moiety, 450.

Hydrolysis of labeled GFP by CHO K1 cell lysate

CHO K1 cells were grown to confluence in a 10-cm\(^2\) dish before their collection and lysis with M-PER (Thermo Fisher Scientific). The presence of esterase activity in the lysate was verified by a colorimetric assay using \( p \)-nitrophenyl acetate (data not shown). A solution of B-TML–GFP (10 \( \mu \)g) was added to 200 \( \mu \)L of CHO K1 cell lysate, and the reaction mixture was nutated at ambient temperature for 12 h. The GFP was subsequently purified with HisPur\textsuperscript{TM} Ni-NTA Magnetic Beads (Thermo Fisher Scientific). The regeneration of unlabeled GFP was confirmed with MALDI–TOF mass spectrometry (Figure 1B).

Analysis of B-TML–GFP Internalization
**Flow Cytometry.** CHO K1 cells were seeded at a density of 50,000 cells/well in 12-well plates. Cells were incubated with GFP (10 µM), B-TML–GFP (10 µM), or Ac-TML–GFP (10 µM) for 4 h. Cells were then rinsed twice with DPBS and released from the plate with 250 µL of trypsin/EDTA (0.25% w/v). Cells were resuspended in an additional 500 µL of medium and incubated on ice until analyzed with flow cytometry. The fluorescence intensity of at least 20,000 events was measured by flow cytometry. Alexa Fluor488® was excited with a 488-nm solid-state laser, and the emission was measured through a 530/30 bandpass filter (Figure 1C).

**Microscopy.** CHO K1 cells were seeded at a density of 50,000 cells/dish in 35-mm µ-dish microscopy imaging dishes from Ibidi. Cells were incubated with GFP (10 µM), B-TML–GFP (10 µM), or Ac-TML–GFP (10 µM) for 4 h. Cells were then rinsed twice with DPBS and cell nuclei were stained with Hoechst 33342 (2 µg/mL) for 5 min at 37 °C and cell membrane was stained with WGA-594 (5.0 µg/mL) (Invitrogen) for 15 min on ice. Cells were then washed twice with wash buffer, and examined live using a scanning confocal microscope (Figure 1D).

**Colocalization of Internalized GFP with Organelles**

*Microscopy.** CHO K1 cells were seeded at a density of 50,000 cells/dish in 35-mm µ-dish microscopy imaging dishes from Ibidi. Cells were incubated with 10 µM B-TML–GFP for 4 h, then rinsed twice with DPBS. Cell nuclei were stained with Hoechst 33342 (2 µg/mL) for 5 min at 37 °C, and other cellular components were stained with WGA-594 (5.0 µg/mL) for 15 min on ice, CellTracker™ Orange CMTMR dye (1 µM) for 15 min at 37 °C, Transferrin-594 (25 µg/mL) for 15 min at 37 °C, or LysoTracker Red (50 nM) for 30 min at 37 °C (all from Invitrogen). Cells were then washed twice with wash buffer, and examined live using a scanning confocal microscope.
Figure S3. Confocal microscopy images of live cells after a 4-h incubation of CHO K1 cells with B-TML–GFP, costained subsequently with various organelle markers. Scale bars: 10 μm.

Cellular Internalization over Time

Microscopy. CHO K1 cells were seeded at a density of 50,000 cells/dish in an 8-well μ-slide microscopy imaging dish from Ibidi. Cells were incubated with GFP (10 μM) or B-TML–GFP (10 μM) for 4 or 24 h. Cells were then rinsed twice with DPBS, and cell nuclei were stained with Hoechst 33342 (2 μg/mL) for 5 min at 37 °C, and cell membranes were stained with WGA-594 (5.0 μg/mL) for 15 min on ice. Cells were then washed twice with wash buffer, and examined live using a scanning confocal microscope.

Figure S4. Internalization of B-TML–GFP at 4 and 24 h. (A) Confocal microscopy of CHO K1 cells incubated with unlabeled GFP or B-TML–GFP (10 μM) for 4 or 24 h. Cells were stained with WGA-594 (red) and Hoechst 33342 (blue). Scale bars: 10 μm. (B) Flow cytometry analysis of CHO K1 cells incubated with either unlabeled GFP or B-TML–GFP (10 μM) for 4 or 24 h.
**Fructose Competition**

10 µM B-TML–GFP was preincubated in an aqueous solution of fructose (175 mM) for 30 min, then used to treat CHO K1 cells for 4 h before analysis by either confocal microscopy or flow cytometry as described above and shown in Figure 2.

**Labeling of Ribonuclease A**

*Preparation of FLAG–Ribonuclease A (RNase A) and G88R RNase A*

FLAG–RNase A and G88R RNase A were produced and purified by methods described previously.6,7

*Labeling of FLAG–RNase A and G88R RNase A*

The labeling of FLAG–RNase A and G88R RNase A with B-TML–NHS ester, and characterization of the extent of labeling was carried out as described for GFP. On average, there were 1.6 ± 0.7 labels per FLAG–RNase A, and 2 ± 1 labels per G88R RNase A.

![MALDI–TOF mass spectra](image)

**Figure S5.** MALDI–TOF mass spectra of (A) unmodified FLAG–RNase A, (B) B-TML–labeled FLAG–RNase A, (C) G88R RNase A, and (D) B-TML–labeled G88R RNase A. Data were fitted to a Gaussian curve (red line). Expected \( m/z \): FLAG–RNase A, 14815; G88R RNase A, 13790; each B-TML moiety, 450.

*Hydrolysis of labeled FLAG–RNase A by K562 cell lysate*

K-562 cells were grown to confluence in a T75 flask before their collection and lysis with M-PER from Thermo Fisher Scientific. The presence of esterase activity in the lysate was verified...
by a colorimetric assay using \( p \)-nitrophenyl acetate (data not shown). A solution of esterified FLAG–RNase A (10 µg) was added to 200 µL of K-562 cell lysate, and the reaction mixture was nutated at ambient temperature for 12 h. FLAG–RNase A was purified with anti-FLAG\textsuperscript{®} M2 Magnetic Beads from Sigma–Aldrich. The regeneration of unlabeled FLAG–RNase A was confirmed with MALDI–TOF mass spectrometry.

**Figure S6.** K562 cell lysate-treated FLAG–RNase A that had been conjugated to B-TML. Data were fitted to a Gaussian curve (red line). Expected \( m/z \): FLAG–RNase A, 14815; each B-TML moiety, 450.

**Cell-Proliferation Assays**
The effect of B-TML–labeled G88R RNase A and unlabeled G88R RNase A on the proliferation of K-562 cells was assayed using a CyQUANT\textsuperscript{®} NF Cell Proliferation Assay Kit (Invitrogen). Briefly, \( 5 \times 10^4 \) cells/mL were added to each well of a 96-well plate in 100 µL of serum-free RPMI 1640 medium. Cells were incubated with G88R RNase A or B-TML–G88R RNase A for 48 h, with PBS and \( \text{H}_2\text{O}_2 \) serving as negative and positive controls, respectively. Cells were then washed and incubated in CyQUANT\textsuperscript{®} reagent for 30 min and fluorescence intensity was measured with excitation at \( \sim 485 \) nm and emission detection at \( \sim 530 \) nm. Data are the average of two measurements for each concentration, and the entire experiment was repeated in triplicate. The results are shown as the percentage of dye incorporated relative to control cells treated with PBS. Values for \( \text{IC}_{50} \) were calculated by fitting the curves by nonlinear regression to the equation: \[ y = \frac{100\%}{1 + 10^{(\log(\text{IC}_{50}) - \log[\text{ribonuclease}]R)}} \], where \( y \) is the total DNA synthesis following the CyQUANT dye pulse and \( R \) is the slope of the curve (Figure 3).

**References**


NMR Spectra

$^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 1
$^{1}$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 2
$^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 3
$^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 4
$^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 5
$^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 6
$^1$H NMR (CDCl$_3$) and $^{13}$C NMR (CDCl$_3$) of Compound 7
$^1$H NMR (CD$_3$OD) and $^{13}$C NMR (CD$_3$OD) of Compound 8
$^{1}$H NMR (CD$_3$OD) and $^{13}$C NMR (CD$_3$OD) of Compound 10
$^1$H NMR (CD$_3$OD) and $^{13}$C NMR (CD$_3$OD) of Compound 11