Bioorthogonal Catalysis: A General Method to Evaluate Metal-catalyzed Reactions in Real Time in Living Systems Using a Cellular Luciferase Reporter System

Hsiao-Tieh Hsu,$^{a,b}$ Brian M. Trantow,$^{a,b}$ Robert M. Waymouth$^a$ and Paul A. Wender$^{a,b}$

$^a$ Department of Chemistry, Stanford University, Stanford, California 94305-5080
$^b$ Department of Chemical and Systems Biology, Stanford University, Stanford, California 94305-5080

Supporting Information

Table of Contents

1. General information on chemical experiments .................................................. S2
2. Experimental Procedures and Analyses .......................................................... S3
3. Background Hydrolysis of Alloc-protected Luciferin 7 ................................. S4
4. HPLC Analysis of Product Distribution ......................................................... S5
5. Figures with Error Bars ...................................................................................... S6
6. Low Bioluminescence Intensity of the Positive Control ................................. S7
7. NanoDrop Calibration of 2 and 4b ..................................................................... S8
8. ICP-MS Analysis on Cell Lysates ................................................................. S8
9. MTT Assays ..................................................................................................... S9
10. Catalytic Cleavage of 4b by 2 in LNCaP Cells ............................................. S10
11. $^1$H/$^{13}$C NMR Spectra ................................................................................ S11
12. References ...................................................................................................... S16
General information on chemical experiments

1. Chemical reactions

All air- and moisture-sensitive reactions were carried out in glassware that was oven-dried (>130 °C) and cooled under nitrogen. Reactants were sealed with rubber septa or Teflon®-coated caps and maintained in an inert environment under a positive pressure of anhydrous N2. Stirring was accomplished via magnetic, Teflon®-coated stir bars or mechanical stirrers that were oven-dried and cooled under a nitrogen atmosphere. Vessels containing light-sensitive compounds were covered with aluminum foil. Solid reagents were measured on a Mettler Toledo AB104-S balance. Air- and moisture-sensitive liquids were transferred via syringe or cannula under an atmosphere of N2. Reaction temperatures refer to the external or bath temperature in which the reaction vessel was partially immersed. Room temperature (r.t.) indicates an external temperature of 20-25 °C. Elevated temperatures were achieved by the use of a silicon oil bath heated by a nichrome wire under constant voltage. Temperatures of 0 °C and -78 °C were maintained with ice/water mixtures and dry ice/acetone mixtures, respectively. Temperatures in between were maintained by the periodic addition of dry ice to a bath of acetone or acetonitrile (CH3CN). The term in vacuo refers to the use of a rotary evaporator with an attached vacuum membrane pump. Residual solvents were removed using vacuum held at < 1.0 Torr.

2. Reagents and Solvents

Unless otherwise noted, all commercial solvents and reagents were used without further purification. Reagents were purchased from Sigma-Aldrich unless otherwise indicated. Dichloromethane (CH2Cl2) was passed through an alumina drying column (Solvtek Inc.) using nitrogen pressure. Petroleum ether, pentane, ethyl acetate (EtOAc), and methanol (MeOH) were obtained from Fisher Scientific. Anhydrous dimethylformamide (DMF) and acetonitrile (CH3CN) were obtained from Acros Organics. Deuterated solvents were purchased from Cambridge Isotope Laboratories.

3. Chromatography

Analytical TLC was performed using 0.25 mm glass-backed silica gel 60F254-coated plates from EMD Chemicals Inc. and monitored at 254 nm. Plates were visualized by treatment with solutions of p-anisaldehyde or potassium permanganate and gentle heating. Preparative column chromatography was performed by running solvent under a pressure of air through gel (230-400 mesh, 60 Å), purchased from EMD Chemical Inc. Reverse-phase high pressure liquid chromatography (RP-HPLC) was performed on a Shimadzu LC-20AP using a preparative column (Shimadzu C18, 250 x 21.2 mm) or a semi-preparative column (Restek C18, 250 x 10 mm). The products were eluted utilizing a solvent gradient (solvent A = 0.1% TFA / H2O; solvent B = 0.1% TFA /CH3CN). Retention times are reported for analytical runs with ramp times of 15 or 20 minutes. Water was removed via lyophilization using a Virtis FreezeMobile 25EL lyophilizer.

4. Physical and Spectroscopic Measurements

Nuclear magnetic resonance spectra were taken on a Varian Inova 500 (1H at 500 MHz, 13C at 125 MHz) or Varian Mercury 400 (1H at 400 MHz, 13C at 100 MHz) magnetic resonance spectrometer. 1H NMR referenced to residual solvent peak (CDCl3 = 7.26 ppm, CD3OD = 3.31 ppm, or D2O = 4.79 ppm). 13C chemical shifts are reported relative to the solvent (CDCl3 = 77.1 ppm, CD3OD = 49.0 ppm). Infrared spectra were measured on a Perkin-Elmer 1600 Series Fourier transform spectrometer (FTIR), and are reported in wavenumbers (cm⁻¹). High-resolution mass spectra (HRMS) were obtained from the Vincent Coates Foundation.
laboratory at Stanford University.

5. HPLC Analysis of Product Distribution of the Catalytic Cleavage of 4b

Unless otherwise noted, 4b (1.5-1.8mg, 0.004-0.005 mmol) was dissolved in 8.487 mL of solvent (DI water, PBS, or RPMI-1640 media) in a vial with stirring at 37°C. 1.513 mL of 130-160 µM 2 stock solution in respective solvents (DI water, PBS, or RPMI-1640 media) was added to the stirring mixture. The initial total volume of all reactions was 10 mL. The reaction was stirred at the indicated temperature, and the reaction was monitored with HPLC (Shimadzu LC-20AP, semi-preparative column) by taking a 1.5-mL aliquot from the reaction approximately every 80 minutes with 200 µL of 5 mM 2,3,5-trimethylphenol (TMP) stock solution in MeOH as the internal standard. The products were eluted utilizing a solvent gradient (solvent A = 0.1% TFA / H₂O; solvent B = 0.1% TFA / CH₃CN, 5% - 70% B/A in 30 minutes.)

6. Caged Luciferin Stability and Release Assays

7 was dissolved in 750 µL D₂O at 55 mM. Acetone (1 equivalent) was then added to the solution and it was transferred to a dry NMR tube. A Varian Inova 500 MHz NMR spectrometer was brought to 37°C and the prepared sample was used to lock and shim the instrument. Catalyst 2 (5 mol%) was subsequently dissolved in an additional 750 µL D₂O and added to the NMR tube and ¹H-NMR scans begun at once and continued at periodic time points. The reaction progress was monitored by shifts of both allylic and aromatic protons relative to the internal standard.

Experimental Procedures and Analyses

1. Catalytic cleavage of 4b by 2: characterization of side products 5 and 6

2 (0.5 mg, 0.00088 mmol) was dissolved in 1.5 mL of DI water in a vial with stirring at room temperature. 4b (15 mg, 0.0413 mmol) was added to the stirring solution, and the reaction was stirred at room temperature for 5 hours. The reaction mixture was separated with RP-HPLC to yield 4a (2.2 mg, 0.0079 mmol), 5 (2.8 mg, 0.0088 mmol), and 6 (1.7 mg, 0.0047 mmol).

Characterization of 5:

¹H NMR (500 MHz, CD₃OD): δ 7.79 (d, J = 10Hz, 1H), 7.05 (d, J = 5Hz, 1H), 6.95 (dd, J = 5, 10Hz, 1H), 6.03-5.96 (m, 1H), 5.35 (t, J = 10 Hz, 1H), 5.32 (dd, J = 5, 10 Hz, 1H), 5.19 (dd, J = 5, 10Hz, 1H), 3.86 (d, J = 5Hz, 2H), 3.75 (dd, J = 2.5, 5 Hz, 2H) ppm. ¹³C NMR (500 MHz, CD₃OD): δ 173.38, 167.85, 156.08, 149.00, 146.95, 139.96, 135.57, 125.51, 117.63, 117.15, 103.24, 79.15, 47.67, 35.77 ppm. FT-IR (thin film): ν = 3354 (s), 2356 (w), 2339 (w), 1651 (m), 1190 (m), 1132 (w), 881 (w), 800 (w), 721 (m), 667 cm⁻¹. HRMS-ESI (m/z): [M + H]+ calc’d for C₁₄H₁₃N₃O₂S₂, 320.0527; found, 320.0514. Tᵣ (RP-HPLC, 5% MeCN/H₂O → 70% MeCN/H₂O in 30 mins): 25 min.

Characterization of 6:

¹H NMR (500 MHz, CD₃OD): δ 7.86 (d, J = 5Hz, 1H), 7.21 (d, J = 2.5Hz, 1H), 7.07 (dd, J = 2.5, 5Hz, 1H), 5.98-5.92 (m, 2H), 5.39 (t, J = 10 Hz, 1H), 5.22 (d, J = 10 Hz, 2H), 5.21 (dd, J
= 5, 10Hz, 2H), 4.10 (d, J = 5Hz, 4H), 3.76 (dd, J = 2.5, 5 Hz, 2H) ppm. $^{13}$C NMR (500 MHz, CD$_3$OD): δ = 176.71, 173.42, 167.75, 156.02, 149.89, 145.99, 139.92, 134.56, 125.27, 116.61, 115.39, 108.95, 103.27, 79.27, 54.21, 35.76, 35.66 ppm. FT-IR (thin film): ν = 3572 (s), 2932 (w), 2829 (w), 1673 (m), 1582 (m), 1489 (w), 1381 (w), 1193 (m), 1134 (w), 988 (w), 883 (w), 800 (w), 722 cm$^{-1}$.

HRMS-ESI (m/z): [M + H]$^+$ calc’d for C$_{17}$H$_{17}$N$_2$O$_2$S$_2$, 360.0840; found, 360.0824. T$_r$ (RP-HPLC, 5% MeCN/H$_2$O → 70% MeCN/H$_2$O in 30 mins): 31 min.

2. Synthesis of alloc-protected luciferin 7

To a 10 mL round bottom flask with stir bar was added a solution of luciferin (20 mg, 0.07 mmol) in H$_2$O (3 mL). 340µL 0.5M NaOH solution was added to the flask at 0 °C, leading to the solubilization of the luciferin. Allyl chloroformate (30µL, 0.282 mmol) was added to the mixture and the reaction stirred for 45 min, during which time the solution turned from fluorescent yellow to opaque white. The reaction mixture was carefully acidified to pH = 3 and the product was washed with H$_2$O and centrifuged twice to collect the solids. The product was dried on a high vacuum to give 7 (10.6 mg, 41%) as a white solid, which was chromatographically homogenous by RP-HPLC.

$^1$H NMR (400 MHz, CD$_3$OD): δ = 8.11 (d, J = 9.2 Hz, 1 H), 7.95 (d, J = 2.0 Hz, 1 H), 7.43 (dd, J = 2.0, 9.2 Hz, 1 H), 6.09-5.99 (m, 1 H), 5.48-5.40 (m, 2 H), 5.33 (dd, J = 1.2, 10.4 Hz, 1 H), 4.77 (d, J = 5.6 Hz, 2 H), 3.79 (dd, J = 2.4, 8.8 Hz, 2 H) ppm. $^{13}$C NMR (100 MHz, acetone-d$_6$): δ = 170.3, 165.2, 161.6, 153.1, 151.1, 150.2, 136.6, 131.7, 124.9, 121.4, 114.9, 78.4, 69.1, 34.7 ppm. FT-IR (thin film): ν = 3054 (w), 2924 (s), 2854 (m), 1753 (m), 1732 (m), 1490 (w), 1446 (w), 1265(s), 1073 (w), 1031 (w), 738 (s), 705 (w) cm$^{-1}$. HRMS-ESI (m/z): [M + H]$^+$ calc’d for C$_{15}$H$_{13}$N$_2$O$_2$S$_2$, 365.0266; found, 365.0245. T$_r$ (RP-HPLC, 5% MeCN/H$_2$O → 70% MeCN/H$_2$O in 30 mins): 12.3 min.

**Background Hydrolysis of Alloc-protected Luciferin 7**

![Figure S1](image_url)

Figure S1. [8]/[7] over time determined by $^1$H NMR. Formation of 8 was observed in the absence of 2.

Since the background hydrolysis of 7 will complicate the bioluminescence readout, alloc-protected aminoluciferin 4b, incorporating a hydrolytically more robust carbamate linker, was next selected for study.
HPLC Analysis of Product Distribution

Figure S2. HPLC chromatogram of compounds 4a, 4b, 5, and 6.

Table S1. HPLC calibration of compounds 4a, 4b, 5, and 6 (TMP: internal standard)

| Compound | $T_r$ (mins) | Amount/peak area (µmol) |
|----------|--------------|-------------------------|
| 4a       | 13.3         | 3.04E-07                |
| 5        | 22.3         | 2.87E-07                |
| 4b       | 25           | 1.91E-07                |
| TMP      | 28           | 5.87E-07                |
| 6        | 31           | 2.27E-07                |

Figure S3. Product distributions of the reaction between 2 and 4b after 24 hours with varied catalyst load. ([4b]₀ = 390 µM. A = 500 µM 4b stock solution in DI water; B = 100 µM 2 stock solution in DI water. 0 mol%: 3.5 mL A+0 mL B+1 mL DI water; 1 mol%: 3.5 mL A+0.175 mL B+0.825 mL DI water; 2.5 mol%: 3.5 mL A+0.4375 mL B+0.5625 mL DI water; 5 mol% = 2.5 mL A+0.875 mL B+0.125 mL DI water. Total volume of each reaction = 4.5 mL.)
Figure S4. Product distribution of the reaction between 2 and 4b over time with 5 mol% catalyst load. (a) 37 °C in PBS ([4b]₀ = 470 µM; [2]₀ = 23.5 µM); (b) 37 °C in PBS with 1 equivalent of L-cysteine ([4b]₀ = 490 µM; [2]₀ = 24.5 µM; [L-cys]₀ = 490 µM); (c) 37°C in RPMI-1640 media ([4b]₀ = 440 µM; [2]₀ = 22 µM).

Figures with Error Bars

Figure S5. Bioluminescence intensity over time when 4T1 cells were treated with varied concentrations immediately followed by 2 (5 mol% relative to [4b]). (b) Bioluminescence intensity over time when 4T1 cells were treated with varied concentrations of 4b immediately followed by 2 ([2] = 6.25 µM).

Figure S6. Treatment of 4T1 cells with 4b and 2 in different orders. ♦: treated with 4b at T= -15min ([4b] = 500 µM)→PBS wash and treated with 2 at T₀ ([2] = 25 µM); •: treated with 2 at T=-15 min ([2] = 25 µM)→PBS wash and treated with 4b at T₀ ([4] = 500 µM); ◊: treated with 2 at T₀ ([2] = 25 µM); Δ: treated with 4b at T₀ ([4b] = 500 µM). O: positive control – treated with 4a at T₀ ([4a] = 25 µM);
Figure S7. Bioluminescence over time when 4T1 cells were treated with 4b and washed with PBS before the treatment of 2. •: treated with 4b at T₀ ([4b] = 500 µM) → 1 PBS wash and treated with 2 ([2] = 25 µM); •: treated with 4b at T₀ ([4b] = 500 µM) → 2 PBS washes with a 15-minute interval and treated with 2 ([2] = 25 µM); •: treated with 4b at T₀ ([4b] = 500 µM) → 3 PBS washes with 2 15-minute intervals and treated with 2 ([2] = 25 µM). ə: PBS wash administered.

Figure S8. Treatment of 4T1 cells with 2 at T₀ followed by 4b at T = 15 min. When a PBS wash was not performed between the treatments, bioluminescence was still observed. ■: treated with 2 ([2] = 25 µM) → treated with 4b at T = 15 min; •: treated with 2 at T₀ ([2] = 25 µM) → PBS wash and treated with 4b ([4b] = 500 µM) at T = 15 min.

Low Bioluminescence Intensity of the Positive Control

Low level of bioluminescence intensity was observed with the positive control in Figure 2. One major factor is the concentrations used for the control and the substrate: the positive control was conducted at a significantly (20-fold) lower concentration of 4a (25 µM) relative to 4b (500 µM with 5 mol% catalyst load). The relative signal intensity is further impacted by the timing of signal collection. We have previously reported that when luciferase-transfected PC3M cells are treated with luciferin only, a very strong initial bioluminescence signal was observed, but the signal faded away rapidly, and is close to baseline after ~3 to 4 minutes. Since the first bioluminescence image taken in Figure 2 was 2 minutes after the cells were treated with 2 due to the exposure time of the CCD camera, the first 2 images only captured the end of the initial burst of bioluminescence signal of the positive control, and the low level of bioluminescence in the remaining time was the fading “tail” approaching baseline that was
observed previously. An experiment was performed to confirm that aminoluciferin turnover in luciferase-transfected 4T1 cells also follows a similar pattern (Figure S9).

**Figure S9.** Bioluminescence intensity vs. time when 4T1 cells were treated with aminoluciferin 4a. Camera was shortened from 2 minutes (Figure 2) to 5 seconds to capture the initial strong bioluminescence of the positive control.

**NanoDrop Calibration of 2 and 4b**

(a) Calibration curve of 2 on NanoDrop 1000 spectrophotometer in PBS: $\lambda_{\text{max}} = 240$ nm, $\text{AU} = 0.004$ $[2] - 0.003$, $R^2 = 0.99984$.

(b) Calibration curve of 4b on NanoDrop 1000 spectrophotometer in PBS: $\lambda_{\text{max}} = 330$ nm, $\text{AU} = 0.0011$ $[4b] - 0.0073$, $R^2 = 0.99928$.

**Treatment Solutions (TS) and PBS Washes Analyses**

(a) Concentration of (a) 2 and (b) 4b in extracellular treatment solutions (TS) and PBS washes of 4T1 cells calculated from calibration curves in Figure S10.
ICP-MS Analysis of Cell Lysates

4T1 cells were treated with 2 at varied concentrations identical to previous bioluminescence experiments (50 µM, 25 µM, 12.5 µM, and 0 µM) for 15 minutes. The cells were then washed with PBS and lysed with concentrated HCl(aq). ICP-MS experiments were performed on the cell lysates by Stanford University Environmental Measurements Facility (Thermo Xseries II), and the result showed that no trace ruthenium was detected in the cell lysates (Figure S12). This observation further supported the hypothesis that 2 cleaves 4b extracellularly. In addition, it suggests that 2 might have low affinity for cell membrane because if 2 had bound to cell membrane tightly, ruthenium is likely to be released from the membrane into the aqueous phase when the cells were lysed with concentration HCl(aq).

Figure S12. ICP-MS experiments on cell lysates. (a) Calibration curve of ruthenium. The ruthenium concentration detected by ICP-MS is consistent with the calculated ruthenium concentration from the concentration of 2. (b) ICP-MS on cell lysates. QC = quality control ([Ru] = 1.40 ppm); CL = cell lysate. The concentration in the parenthesis is the concentration of 2 that the cells were treated with before they were lysed with concentrated HCl(aq).

MTT Assays for Cell Viability

Figure S13. (a) MTT assay for cell viability under bioluminescence treatment conditions. (b) MTT assay for cell viability as a function of log([2]).
Catalytic Cleavage of 4b by 2 in LNCaP Cells

To explore whether the catalytic activated bioluminescence can be extended to a different cell type, luciferase-transfected LNCaP cells (a human prostate adenocarcinoma cell line) were treated with 2 and 4b. When the cells were treated with 4b immediately followed by 2, dose-dependent photon emission was observed. When the cells were treated with pre-catalyst 2 for 15 minutes, followed by a PBS wash to remove extracellular catalyst, and then treated with alloc-protected aminoluciferin 4b, no bioluminescence was observed. When the treatment order was reversed, bioluminescence was observed and peaked at about 25 minutes after treatment with 2. When either 2 or 4b was absent, no bioluminescence was observed (Figure S14). This is analogous to what was observed in the luciferase-transfected 4T1 cells, indicating that this system is robust across different cell lines.

Figure S14. (a) Bioluminescence intensity over time when LNCaP cells were treated with varied concentrations of 4b immediately followed by 2 (5 mol% with relative to [4b]). (b) Bioluminescence intensity over time when LNCaP cells were treated with 4b and 2 in different orders. ●: treated with 4b at T = -15 min ([4b] = 500 µM) → PBS wash and treated with 2 at T₀ ([2] = 25 µM); ○: treated with 2 at T = -15 min ([2] = 25 µM) → PBS wash and treated with 4b at T₀ ([4b] = 500 µM); ●: treated only with 2 at T₀ ([2] = 25 µM); ●: treated only with 4b at T₀ ([4b] = 500 µM); ○: positive control – treated with 4a at T₀ ([4a] = 5 µM).
$^{1}H/^{13}C$ NMR Spectra

**Parameter** | **Value**
--- | ---
1. Solvent | CDCl$_3$
2. Temperature | 29.0
3. Pulse Sequence | sdpul
4. Number of Scans | 1024
5. Relaxation Delay | 9.5000
6. Spectrometer Frequency | 125.67
7. Spectral Width | 33000.3
8. Lowest Frequency | 4463.37
9. Nucleus | 13C
10. Acquired Size | 49508
11. Spectral Size | 131072
| Parameter          | Value   |
|--------------------|---------|
| 1 Solvent          | CD3OD   |
| 2 Temperature      | 29.0    |
| 3 Pulse Sequence   | s2pol   |
| 4 Number of Scans  | 16      |
| 5 Relaxation Delay | 0.0000  |
| 6 Spectrometer Frequency | 400.75 |
| 7 Spectral Width   | 8000.0  |
| 8 Lowest Frequency | -1496.2 |
| 9 Nucleus          | 1H      |
| 10 Acquired Size   | 32000   |
| 11 Spectral Size   | 65536   |

![NMR Spectrum 1](image1)

| Parameter          | Value   |
|--------------------|---------|
| 1 Solvent          | CD3OD   |
| 2 Temperature      | 29.0    |
| 3 Pulse Sequence   | s2pol   |
| 4 Number of Scans  | 1724    |
| 5 Relaxation Delay | 0.5000  |
| 6 Spectrometer Frequency | 125.67 |
| 7 Spectral Width   | 33033.3 |
| 8 Lowest Frequency | -4316.9 |
| 9 Nucleus          | 13C     |
| 10 Acquired Size   | 49509   |
| 11 Spectral Size   | 131372  |

![NMR Spectrum 2](image2)
References

1. Kiesewetter, M. K., Waymouth, R. M. (2010) Kinetics of an Air- and Water-Stable Ruthenium(IV) Catalyst for the Deprotection of Allyl Alcohol in Water. Organometallics 29, 6051–6056.

2. Katz, L. (1951) Antituberculous Compounds. II. 2-Benzalhydrazinobenzothiazoles1a. J. Am. Chem. Soc. 73, 4007–4010.

3. White, E. H., Wörther, H., Seliger, H. H., McElroy, W. D. (1966) Amino Analogs of Firefly Luciferin and Biological Activity Thereof1d. J. Am. Chem. Soc. 88, 2015–2019.

4. Jones, L. R., Goun, E. A., Shinde, R., Rothbard, J. B., Contag, C. H. (2006) Releasable Luciferin–Transporter Conjugates: Tools for the Real-Time Analysis of Cellular Uptake and Release. J. Am. Chem. Soc. 128, 6526–6527.