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

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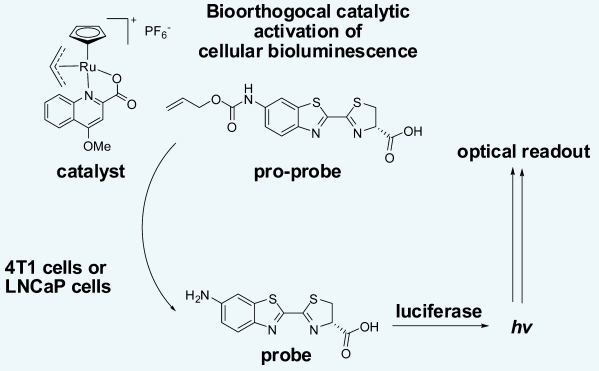
Supporting Information

ABSTRACT: The development of abiological catalysts that can function in biological systems is an emerging subject of importance with significant ramifications in synthetic chemistry and the life sciences. Herein we report a biocompatible ruthenium complex $[Cp(MQA)Ru(C_3H_5)]^+PF_6^-$ **2** (Cp = cyclopentadienyl, MQA = 4-methoxyquinoline-2-carboxylate) and a general analytical method for evaluating its performance in real time based on a luciferase reporter system amenable to high throughput screening in cells and by extension to evaluation in luciferase transgenic animals. Precatalyst **2** activates alloc-protected aminoluciferin **4b**, a bioluminescence pro-probe, and releases the active luminophore, aminoluciferin (**4a**), in the presence of luciferase-transfected cells. The formation and enzymatic turnover of **4a**, an overall process selected because it emulates pro-drug activation and drug turnover by an intracellular target, is evaluated in real time by photon counting as **4a** is converted by intracellular luciferase to oxyaminoluciferin and light. Interestingly, while the catalytic conversion (activation) of **4b** to **4a** in water produces multiple products, the presence of biological nucleophiles such as thiols prevents byproduct formation and provides almost exclusively luminophore **4a**. Our studies show that precatalyst **2** activates **4b** extracellularly, exhibits low toxicity at concentrations relevant to catalysis, and is comparably effective in two different cell lines. This proof of concept study shows that precatalyst **2** is a promising lead for bioorthogonal catalytic activation of pro-probes and, by analogy, similarly activatable pro-drugs. More generally, this study provides an analytical method to measure abiological catalytic activation of pro-probes and, by analogy with our earlier studies on pro-Taxol, similarly activatable pro-drugs in real time using a coupled biological catalyst that mediates a bioluminescent readout, providing tools for the study of imaging signal amplification and of targeted therapy.



INTRODUCTION

Biological catalysts (enzymes) have figured prominently in synthesis, medicine, materials science, and basic research, and their use has been successfully extended from aqueous biological systems to organic solvents.[1−4] In contrast, the complementary use of abiological catalysts in biological systems has received only recent attention.[5−11] The translation of increasingly important stoichiometric bioorthogonal processes[12] into catalytic processes with the potential for an amplified and/or targeted readout opens up many new opportunities and applications in research, imaging, diagnostics, and therapy. Our ultimate goal is to attach bioorthogonal catalysts to monoclonal antibodies (mAb), one of the most important emerging tools for targeting disease treatment,[13−15] or to targeting ligands which would allow for targeted catalytic turnover of a subsequently administered pro-drug at the site of mAb- or ligand-catalyst localization. This strategy would avoid problems with loading levels of drugs on mAb or copy number of mAb antigens on a targeted cell. By controlling catalytic release at the targeted site, one would control both the dose and the site of drug release by simply varying the amount of pro-drug administered. A similar strategy using pro-imaging agents could be used to amplify an optical signal as needed for early stage diagnostics. Key first steps toward these ends are the identification of biocompatible, bioorthogonal catalysts that could activate pro-probes and drugs and development of a general method to measure their efficacy.

At the outset of our current studies, relatively few abiological catalysts had been shown to perform efficiently in water and even fewer in the presence of cells.[5,8,16−21] Of special interest, Kitamura reported a ruthenium catalyst that effected efficient cleavage of allyl protecting groups in water/MeOH (1:1) mixture.[22−28] The Meggers group had recently reported that Kitamura's catalyst, derived from quinaldic acid and related

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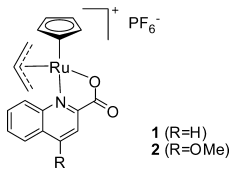
analogs, catalyze deallylation reactions and activate pro-fluorescent probes.[29] More recently, Rotello and co-workers have designed nanozymes loaded with Ru or Pd catalyst for fluorescence or pro-drug activation inside living cells,[30] showing the increasing interest and importance in bioorthogonal catalysis. As part of our own studies, we previously reported kinetic studies on the use of the Kitamura catalysts derived from quinaldic acid and from kynurenic acid for the cleavage of methyl allyl carbonate as an initial test substrate.[24,31] Both catalysts were reported to work in water as required for cellular work.[31] We have since investigated the use of the kynurenic acid catalyst in cleaving (activating) alloc-protected luciferin and aminoluciferin as well as other pro-probes, systems that upon release uniquely provide for "turn on" bioluminescence. Our interest in a bioluminescent readout is based on several factors. First, bioluminescence measurements provide better signal-to-noise ratios relative to fluorescence due to the avoidance of autofluorescence.[32] They are also amenable to high throughput screening.[33] Furthermore, they allow for real time study and evaluation of luciferin release in luciferase transfected cells, providing a signal only when the probe is released and is turned over by its intracellular target. Significantly and uniquely, such studies can be readily extended to luciferase transgenic animals for which a fluorescence readout is not possible due to deep tissue light scattering.[34−36] Herein we report a detailed analysis of the product distribution of this metal-catalyzed process in the absence of cells, the tolerability of the catalyst and process to cells, the successful demonstration of the first metal-catalyzed activation of a pro-bioluminescent probe, and, finally, its use in the real time evaluation of probe release into living cells based on its turnover by intracellular luciferase as measured by photon counting.

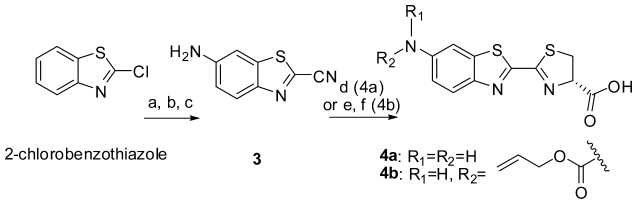
RESULTS AND DISCUSSION

Catalyst Selection. To be viable in living systems, a catalyst must satisfy a number of stringent chemical and biological requirements, such as being catalytically active at pH 7.4 and 37 °C in the presence of air, water, and biological functionalities (e.g., amines, thiols, alcohols, acids). The catalyst must also be nontoxic at concentrations relevant for catalysis. Based on these requirements, **1**, a precatalyst reported by Kitamura and co-workers, was initially identified as an attractive lead as it had been originally reported to cleave various allylic functionalities in the presence of air in water/MeOH (1:1) mixture.[23−27] However, precatalyst **1** proved to be insufficiently soluble in water for our studies. To address this problem and provide a potential handle for further tuning and modification of the catalyst, a more water-soluble analogue, precatalyst **2**, whose kinetic competence we reported previously, was selected for cellular studies.[31]



Our initial study of the release of luciferin from alloc-protected luciferin using precatalyst **2** was encouraging but complicated by uncatalyzed background hydrolysis of the alloc-protected carbonate substrate (SI Figure S1).[37] Therefore, the alloc-protected aminoluciferin **4b** (Scheme 1), incorporating a hydrolytically more robust carbamate linker, was next selected for study. As expected, in the absence of precatalyst **2**, no alloc cleavage of protected aminoluciferin **4b** was observed after 24 h (Table 1, Entry 1). The use of aminoluciferin (**4a**), rather than luciferin, offers further advantages as it can be modified and red-shifted allowing for more effective imaging in tissues.[38] **4a** was synthesized following the procedure reported by White,[39] and alloc-protected aminoluciferin **4b** was similarly prepared from 2-chlorobenzothiazole (Scheme 1).[40]

Scheme 1. Synthesis of Aminoluciferin 4a and Alloc-Protected Aminoluciferin 4b[a]



[a]Reagents and conditions: (a) KNO_3, H_2SO_4, 0 °C, 4 h, 67%; (b) Fe(0), $FeCl_3$, EtOH/CH_3COOH, 80 °C, 24 h, 88%; (c) KCN, DMSO, 120 °C, 24 h, 45%; (d) D-cysteine, K_2CO_3, MeOH/H_2O (1:1), r.t. 1 h, 58%; (e) allyl chloroformate, pyridine, CH_2Cl_2, r.t., 2 h, 90%; (f) D-cysteine, K_2CO_3, 1:1MeOH/H_2O, r.t., 1 h, 46%.

Product Distribution of the Catalytic Cleavage of 4b by 2. Cleavage of the alloc group of **4b** by precatalyst **2** to release aminoluciferin **4a** was initially studied in deionized (DI) water at 25 °C ($[\mathbf{4b}]_0$ = 30 mM, $[\mathbf{2}]_0$ = 1.5 mM). HPLC analysis of the reaction showed that three products were formed: the desired aminoluciferin (**4a**) (37%), mono- and diallylated derivatives **5** (41%) and **6** (22%) (Scheme 2). Similar decarboxylative allylation reactions of amines were observed using a related catalyst in water−organic solvent mixtures.[24] Since alkylated aminoluciferins such as **5** and **6** would complicate analysis of the luciferase readout,[39,41] efforts were next focused on conditions that would suppress their formation.

Initiation of the catalytic cycle involves the displacement of the Ru allyl by water to generate a Ru(II) solvate which subsequently reacts with **4b** to regenerate **2** with the liberation of **4a** (Scheme 3).[24] As a consequence, once **4a** starts to form, its more nucleophilic amine group competes with water, and upon entry into the catalytic cycle forms **5** which through a similar process leads to a second allylation and the formation of **6**.[24,42]

It follows from the above analysis that allylated products would be disfavored at lower substrate concentrations used in cellular studies. In agreement with this analysis, when the substrate concentration was lowered from 30 mM to the more biologically relevant level of 390 μM, the percentage of **4a** increased, but the overall conversion decreased as expected (Table 1, Entries 1−4). When the solvent was changed from DI water (pH = 5) to phosphate buffered saline (pH = 7.4) and the temperature was increased from 25 °C to physiologically relevant 37 °C, the formation of **4a** was favored over the side products and the total conversion favorably increased (Table 1, Entry 5). To explore whether the presence of nucleophilic species, as would be encountered in cellular studies, would be compatible with the precatalyst **2** and whether it would influence the product distribution, L-cysteine (1 equiv) was added to the reaction. Significantly, precatalyst **2** proved to be remarkably effective and under these conditions gave almost

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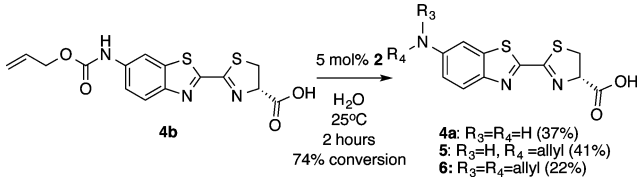
Table 1. Product Distribution from the Catalytic Cleavage of 4b by 2

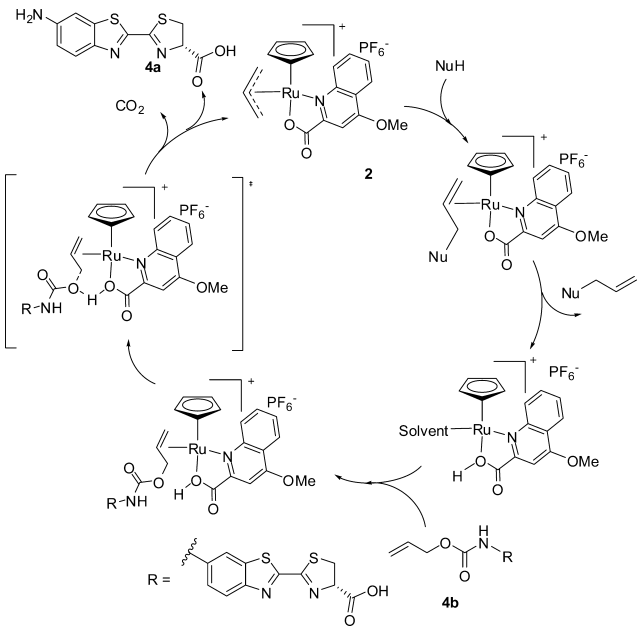
entry	$[4b]_0$ (μM)	2 (mol %)	T (°C)	pH	solvent	additives	% conversion[a]	% 4a[a]
1	390	0	25	5	H_2O	-	0	-
2	390	1	25	5	H_2O	-	19	63
3	390	2.5	25	5	H_2O	-	43	55
4	390	5	25	5	H_2O	-	56	49
5	470	5	37	7.4	PBS	-	78	66
6	490	5	37	7.4	PBS	L-cysteine	49	99
7	440	5	37	7.4	RPMI media[b]	-	40	97

[a]HPLC yield after 24 h as monitored with 2,4,6-trimethylphenol as the internal standard. [b]HEPES, amino acids, vitamins, D-glucose, glutathione (reduced), inorganic salts).

Scheme 2. Catalytic Cleavage of 4b by 2 in Water Generated 4a (37%), 5 (41%), and 6 (22%)



Scheme 3. Mechanism of the Catalytic Cleavage of 4b by 2



exclusively (99% selectivity) the desired **4a** relative to other allylated products (Table 1, Entry 6) after 24 h. When the reaction was run at 37 °C in the RPMI-1640 cell culture media, the reaction again proceeded readily (Table 1, Entry 7) and with high selectivity (97%) for the formation of **4a** over allylated products. Thus, while three products were observed for the reaction in water, the reaction in the more biologically relevant cell culture media favored almost exclusive formation of the desired product **4a**.

Catalytic Turnover of 4b and Bioluminescence Detection in Cellular Environment. To investigate in real time whether **2** can release **4a** from **4b** in the presence of cells, 4T1 cells (a mouse mammary carcinoma cell line) transfected with the gene of click beetle luciferase[43] were treated with alloc-protected substrate **4b** and precatalyst **2**. To monitor the release of **4a** and its interaction with its target enzyme luciferase, a charge-coupled device (CCD) camera (IVIS 100) was used to detect photons emitted from the enzyme-mediated conversion of **4a** to oxyaminoluciferin and light.[44] Significantly, robust and concentration dependent emission was observed (Figure 1a). When the cells were treated with precatalyst **2** at a constant concentration and **4b** at varied concentrations, the intensity of bioluminescence increased with increasing concentration of **4b** (Figure 1b), indicating that the reaction is catalytic in the presence of cells. If the reaction had been stoichiometric with respect to **2**, the intensity of bioluminescence would be expected to be constant since the concentration of **2** was constant. (For the purpose of clarity, all error bars in the bioluminescence figures are omitted. See SI for figures with error bars.)

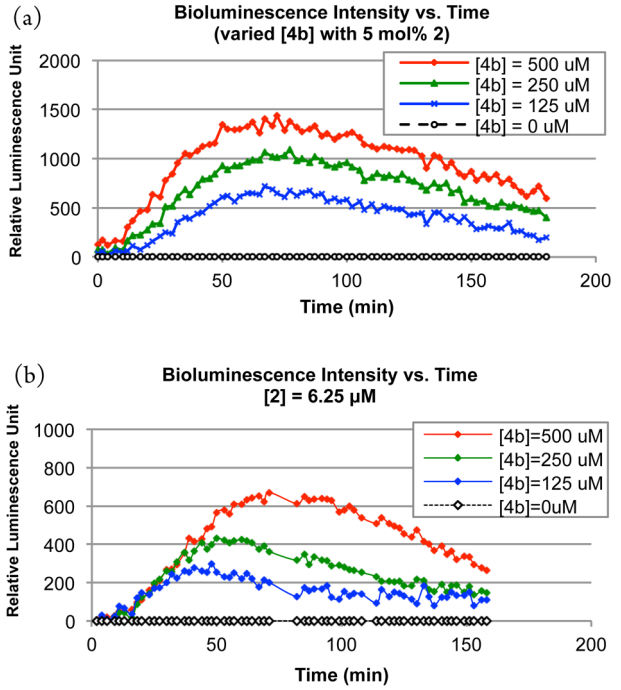


Figure 1. Bioluminescence intensity over time when 4T1 cells were (a) treated with varied concentrations of **4b** immediately followed by **2** (5 mol % relative to [**4b**]); (b) treated with varied concentrations of **4b** immediately followed by **2** at a fixed concentration ([**2**] = 6.25 μM).

To study whether the catalytic reaction took place intracellularly or extracellularly, the cells were treated with **2** for 15 min, followed by a PBS wash to remove extracellular catalyst, and then treated with **4b**. Under these conditions, no bioluminescence was observed, indicating that **2** was removed

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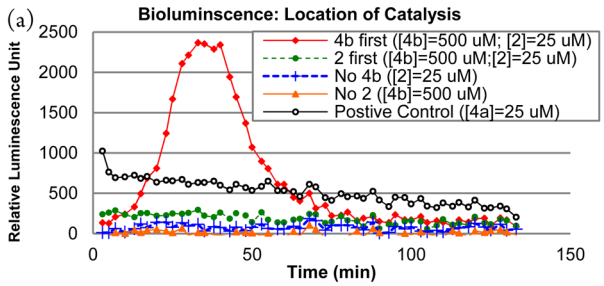
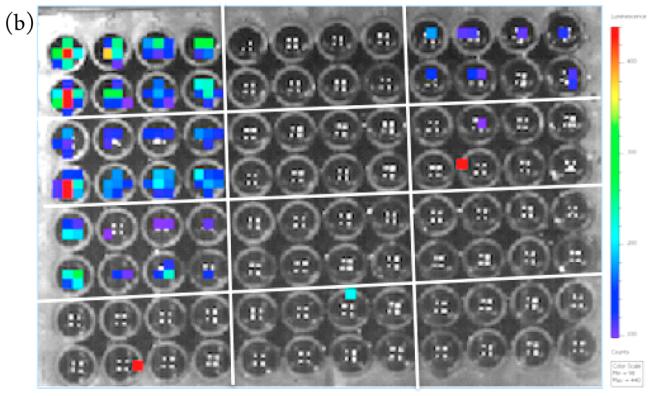
with the wash. However, when the cells were treated first with **4b** for 15 min, washed with PBS, and then treated with **2**, bioluminescence was observed and peaked at about 33 min after treatment of **2**. When either **2** or **4b** was absent, no bioluminescence was observed, indicating that **4b** itself was not turned over by luciferase in the cytosol (Figure 2 (a) and 2(b)).





Corresponding treatment conditions:

500μM **4b** (T=-15 min)→ PBS wash → 25μM **2** (T_0)	25μM **2** (T=-15 min)→ PBS wash → 500μM **4b** (T_0)	25μM **4a** (T_0)
250μM **4b** (T=-15 min)→ PBS wash → 12.5μM **2**(T_0)	12.5μM **2** (T=-15 min)→ PBS wash → 250μM **4b** (T_0)	12.5μM **4a** (T_0)
125μM **4b** (T=-15 min)→ PBS wash → 6.25μM **2**(T_0)	6.25μM **2** (T=-15 min)→ PBS wash → 125μM **4b** (T_0)	6.25μM **4a** (T_0)
0μM **4b** +25μM **2** (T_0)	0μM **2** + 500μM **4b** (T_0)	0μM **4a** (T_0)

Figure 2. (a) Bioluminescence intensity over time when 4T1 cells were treated with **4b** and **2** in different orders; (b) Bioluminescence image at T = 38 min in Figure 2(a) and corresponding treatment conditions.

Further, when cells treated with **4b** were washed repeatedly with PBS prior to treatment with **2**, the bioluminescence intensity decreased as the number of washes increased (Figure 3). This is consistent with **4b** readily partitioning between intracellular and extracellular space and being slowly removed from the latter with each wash. In contrast, **2** is either extracellular or rapidly and completely becomes extracellular as it is removed in a single wash.

To further explore the locations of **2** and **4b**, 4T1 cells were treated separately with **2** or **4b** for 15 min, and the extracellular treatment solutions were collected. Following treatment, the cells were washed three times with PBS with a 15 min interval

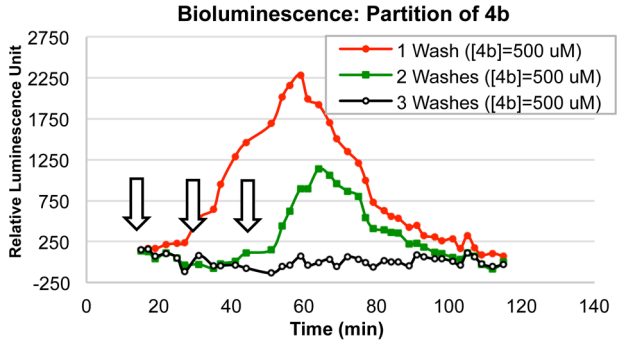


Figure 3. Bioluminescence intensity over time when 4T1 cells were treated with **4b** and washed with PBS before treatment with precatalyst **2**. (↓: PBS wash administered.).

in between each wash, and the PBS washes were collected. The concentrations of **2** or **4b** in the treatment solutions and PBS washes were measured with a NanoDrop 1000 spectrophotometer. After 15 min of treatment, all **2** was accounted for in the original treatment solution, which was made further evident from the absence of **2** in the subsequent PBS washes. This indicates that **2** did not significantly partition into the cell. On the other hand, only about 90% of **4b** was recovered in the treatment solution after 15 min, and **4b** was detected in the following PBS washes, showing that **4b** was able to enter and exit cells freely and was partitioned out with PBS washes (SI Figure S11).

To probe whether the absence of bioluminescence observed when **2** is administered first followed by a wash is caused by catalyst inactivation, the cells were treated with **2** for 15 min and **4b** was administered without a PBS wash. Bioluminescence was observed in this set of treatments, indicating that the absence of bioluminescence was not caused by catalyst inactivation, but rather by **2** residing preferentially on the 4T1 cell membrane or in the extracellular space where it is readily removed by washing (Figure 4). Finally, we sought to

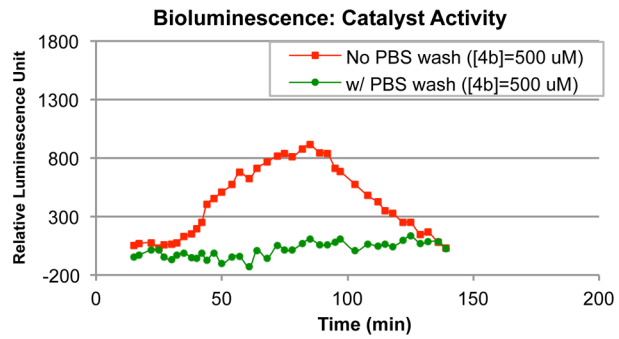


Figure 4. Treatment of 4T1 cells with **2** ([**2**] = 25 μM) at T_0 followed by **4b** ([**4b**] = 500 μM) at T = 15 min.

cross check the washing studies by ICP-MS analysis of the cell lysate. No trace ruthenium was detected in the cytosol component. This result is further evidence indicating that the catalytic cleavage of **4b** by **2** occurs extracellularly. (SI Figure S12.)

An important requirement for a bioorthogonal catalyst is low toxicity at concentrations relevant to catalysis. MTT cell viability assays were performed using the treatment conditions of **2** and **4b** used for bioluminescence experiments. The treatment conditions of 500 μM and 375 μM **4b** with 5 mol %

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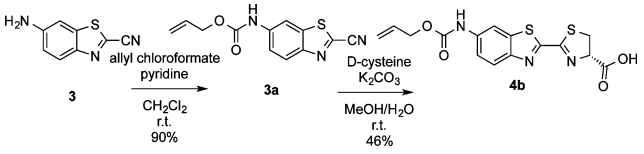
2 caused little cytotoxicity, while 250 μM **4b** with 5 mol % **2** and lower concentrations showed no statistically significant effect on cell proliferation. With varying concentrations of **2**, MTT assays showed that cell viability was >70% at the solubility limit of 100 μM for **2** (SI Figure S13). Finally, similar treatments of **2** and **4b** were performed on luciferase-transfected LNCaP cells (a human prostate adenocarcinoma cell line) and comparable bioluminescence results were observed, showing that our system is robust across different cell lines (SI Figure S14).

CONCLUSIONS

In conclusion, here we report a bioorthogonal ruthenium precatalyst **2** that can cleave novel caged bioluminescence probe **4b** in a cellular environment and an analytical method that allows for real time evaluation of probe release and enzymatic turnover in living cells using a luciferase reporter system. This method measures catalyst cleavage of a pro-probe and intracellular enzyme mediated turnover of the released probe, thus providing metrics of critical value in assessing the performance of new abiological catalysts and caging strategies as required for analogously cleavable pro-drugs. Catalytic cleavage of **4b** in cell culture media at 37 °C proceeded with exceptionally high selectivity, giving only the desired amino-luciferin **4a** and dramatically contrasting the process in pure water or PBS where side products were observed. In this case, the low product selectivity observed in the "model" cell-free studies proved not to be predictive of the more complex but more selective cellular studies. Bioluminescence studies showed that **2** can catalytically activate **4b** in cellular environments to produce real time bioluminescence readout. The results from a series of treatments varying the order of incubation with **2** and **4b** and ICP-MS experiments are consistent with catalysis occurring extracellularly in this study as would be desired for the release of the drug from a prodrug by a catalyst attached to a cell surface bound antibody and targeting ligands. It is anticipated from our earlier work that cell-penetrating guanidinium-rich transporters and monoclonal catalyst conjugates could target the catalyst as well as deliver it intracellularly for applications requiring intracellular drug release. MTT assays showed that this catalyst–substrate system has little to no toxicity at concentrations relevant to catalysis. Finally, comparable catalytic performance was observed in a second luciferase-transfected cell line, indicating that this system can be extended to other cell types. This proof of concept study shows that precatalyst **2** is a promising lead for further exploration of pro-drug activation, imaging signal amplification and targeted therapy. In addition, this study provides a general analytical procedure to evaluate new bioorthogonal catalysts in cells using a real time bioluminescence reporter system. Work toward further biological applications of precatalyst **2** and related bioorthogonal catalysts using transporters and targeted delivery systems are currently in progress in our lab.

EXPERIMENTAL SECTION

Synthesis of 2. Catalyst **2** was synthesized and characterized following literature procedures.[31] The spectral data matched that reported in the literature.[31] ^{1}H NMR (500 MHz, acetone-d_6): δ 8.44 (dd, J = 3, 6 Hz, 1H), 8.15–8.08 (m, 2H), 7.92 (dt, J = 3, 6 Hz, 1H), 7.59 (s, 1H), 6.53 (s, 1H), 4.93–4.86 (m, 2H), 4.72–4.70 (m, 1H), 4.41–4.39 (m, 1H), 4.35 (s, 1H) ppm.



Synthesis of 4a and 4b. **3** was synthesized from 2-chlorobenzothiazole following literature procedures.[39,40] The spectral data matched that in the literature.[39] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95 (d, J = 8 Hz, 1H), 7.09 (d, J = 4 Hz, 1H), 6.96 (dd, J = 4, 8 Hz, 1H), 4.14 (s, 2H) ppm. MS-ESI (m/z): $[M + H]^+$ calc'd for $C_8H_5N_3S$, 176.03, found 176.1. **4a** was synthesized following reported procedures.[39] The spectral data matches that in the literature.[39] ^{1}H NMR (300 MHz, D_2O): δ 8.04 (d, J = 9 Hz, 1H), 7.96 (d, J = 3 Hz, 1H), 7.47 (dd, J = 3, 9 Hz, 1H), 5.41 (t, J = 9 Hz, 1H), 3.86 (dd, J = 9, 15 Hz, 1H), 3.75 (dd, J = 9, 15 Hz, 1H) ppm. MS-ESI (m/z): $[M + H]^+$ calc'd for $C_{11}H_9N_3O_2S_2$, 280.3. Found 280.4. To synthesize **3a**, **3** (50.8 mg, 0.290 mmol) was dissolved in 10 mL of distilled CH_2Cl_2 in a flame-dried round-bottom flask with stirring. 120 μL of distilled pyridine was added to the stirring solution. 150 μL of allyl chloroformate was dissolved in 5 mL of distilled CH_2Cl_2 in a separated vial and was slowly added to the stirring solution, where a yellow precipitate started to form. The reaction was monitored by TLC and seemed completed after 2 h. The solution was then washed with 0.25 M $HCl_{(aq)}$ (3 × 30 mL), dried with brine (3 × 30 mL), and $MgSO_{4(aq)}$. The organic layer was filtered and the solvent was removed in vacuo to yield a pale yellow crude product. The crude product was purified with flash column chromatography to obtain a white solid (67.4 mg, 90%). TLC R_f: 0.29 (25% EtOAC/hexane). ^{1}H NMR (500 MHz, CD_3OD): δ 8.45 (d, J = 2.5 Hz 1H), 8.08 (d, J = 5 Hz, 1H), 7.60 (dd, J = 2.5, 5 Hz, 1H), 6.06–5.98 (m, 1H), 5.40 (ddd, J = 2.5, 5, 10 Hz, 2H), 5.25 (dd, J = 5, 10 Hz, 1H), 4.68 (d, J = 5 Hz, 2H) ppm. ^{13}C NMR (500 MHz, $CDCl_3$): δ 153.20, 148.56, 138.87, 137.47, 135.21, 132.20, 125.79, 120.04, 119.24, 113.41, 109.96, 66.75 ppm. FT-IR (thin film): ν = 3346 (m), 2227 (m), 1720 (s), 1568 (s), 1529 (s), 1480 (s), 1400 (m), 1283 (m), 1232 (s), 1216 (s), 1134 (m), 1066 (m), 1040 (m), 926 (w), 818 (w), 767 (w) cm^{-1}. HRMS-ESI (m/z): $[M + H]^+$ calc'd for $C_{12}H_{10}O_2N_3S$, 260.0488; found, 260.0492.

To synthesize **4b**, **3a** (100.9 mg, 0.389 mmol) was dissolved in MeOH (2 mL) and H_2O (2 mL) in a round-bottom flask covered with aluminum foil. K_2CO_3 (108.0 mg, 0.781 mmol) was added to the solution. D-cysteine–HCl (122.4 mg, 0.697 mmol) was added to the solution and the solution was stirred under room temperature for 1 h. Afterward, MeOH in the reaction mixture was removed in vacuo, and the pH of the aqueous phase was brought down to 3 by adding 1 M $HCl_{(aq)}$ dropwise, wherein a light yellow precipitate formed. The solution and precipitate was transferred to a test tube and centrifuged, and the supernatant was removed and the solid was washed and suspended with H_2O (3 mL). This step was repeated three times. The supernatant was removed and residual water was removed under vacuum overnight. The crude product was purified by RP-HPLC to yield a light yellow solid (58.1 mg, 45.9%). T_r (RP-HPLC, 5% MeCN/H_2O → 90% MeCN/H_2O in 25 min): 9 min. ^{1}H NMR (500 MHz, D_2O): δ 7.79 (s, 1H), 7.62 (d, J = 10 Hz, 1H), 7.21 (d, J = 10 Hz, 1H), 6.02–5.95 (m, 1H), 5.37 (d, J = 15 Hz, 1H), 5.27 (d,

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J = 15 Hz, 1H), 5.19 (t, J = 5 Hz, 1H), 4.62 (d, J = 2.5 Hz, 2H), 3.78 (dd, J = 2.5, 10 Hz, 1H), 3.58 (dd, J = 2.5, 10 Hz, 1H) ppm. ^{13}C NMR (500 MHz, D_2O): δ = 177.50, 165.20, 159.03, 154.52, 147.03, 137.01, 135.81, 132.27, 123.03, 118.52, 117.81, 109.40, 80.34, 66.11, 36.30 ppm. FT-IR (thin film): ν = 3419 (s), 2927 (w), 2359 (w), 1731 (m), 1582 (m), 1526 (w), 1490 (w), 1386 (w), 1219 (m), 1072 (w), 1051 (w), 870 (w), 822 (w), 762 (w) cm^{-1}. HRMS-ESI (m/z): [M + H]$^+$ calc'd for $C_{15}H_{14}N_3O_4S_2$, 364.0425; found, 364.0416.

HPLC Analysis of Product Distribution of the Catalytic Cleavage of 4b. Unless otherwise noted, **4b** (1.5−1.8 mg, 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, semipreparative column) by taking a 1.5 mL aliquot from the reaction approximately every 80 min 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/H_2O; solvent B = 0.1% TFA/CH_3CN, 5% − 70% B/A in 30 min).

Catalytic Cleavage of 4b and Bioluminescence Studies in Live Cells. A 4T1 cell line expressing click beetle luciferase (CBL) was obtained and grown under selection with RPMI-1640 media supplemented with 10% FBS and penicillin/streptomycin. Cells were plated at 10 000 cells/well in a 96-well, black flat bottom plate and incubated at 37 °C with 5% CO_2 24 h prior to the assay. The cell media was removed and washed with phosphate buffer saline (PBS) prior to treatment with **2**, **4a**, and **4b**. The treatment order of each experiment is detailed in the corresponding figure. After treatment with compounds, bioluminescence of the wells was measured using a charge-coupled (CCD) device camera (exposure time = 2 min unless otherwise noted) and analyzed with Living Image Software (IVIS 100, Xenogen Corp., Alameda, CA). Relative luminescence unit was obtained by subtracting background signals when cells were only treated with PBS from the bioluminescence readout. The same procedure was used for luciferase-transfected LNCaP cells.

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 min. 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).

MTT Assays. 4T1 cells were plated in 96-well plates and incubated at 37 °C with 5% CO_2 24 h prior to the assay. The cells were treated with either a serial dilution of **2** or the identical conditions for bioluminescence studies (various concentrations of **2** and **4b**) for 2 h at 37 °C. The media was then removed and the cells were resubjected to fresh media and incubated for a further 24 h (48 total). Viability was assayed by adding 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. After 2 h of incubation at 37 °C, 100 μL of solubilizing solution (10% Triton X-100, 90% 0.1 N HCl in isopropanol) was added to each well, and colorimetry data was obtained on a plate reader. Numerical data from the plate reader was normalized against the positive control. Cytotoxicity of **2** and the treatment conditions for bioluminescence study was determined by the degree of cell viability relative to untreated and cell-free control wells (100% and 0% viability, respectively).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00469.

Data on synthesis, characterization, HPLC calibration of product distribution, figures with error bars, additional bioluminescence experiments, PBS wash experiments, and MTT assays for cytotoxicity study; selected NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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