## Bioluminescence

## A Selenium Analogue of Firefly D-Luciferin with Red-Shifted Bioluminescence Emission\*\*

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Bioluminescence imaging (BLI) makes use of light-generating luciferase enzymes, most commonly firefly luciferase (FLuc), *Renilla reniformis* luciferase, or *Gaussia* luciferase in combination with their appropriate substrates.<sup>[1]</sup> Noninvasive BLI of living subjects has become a routine technique in cancer biology research because it enables the monitoring of gene expression, gene delivery, tumor growth, enzyme activity, response to experimental drug therapies, and protein–protein interactions.<sup>[2]</sup>

The major limitation in invivo BLI experiments is absorption and scattering of light by tissue, which results in strong attenuation of BL signals that are emitted below 600 nm.<sup>[3]</sup> The yellow-green BL emission from the native FLuc (D-luciferin substrate,  $\lambda_{em,max} = 553-559$  nm),<sup>[4]</sup> which is the most red-shifted native bioluminescent system, decreases substantially with tissue depth. Consequently, the applications of this system are restricted mainly to small animals at superficial depths. To overcome this limitation, red-shifted variants of native FLuc have been selected by using random mutagenesis.<sup>[5]</sup> Considerable efforts have also been directed toward the development of analogues of D-luciferin<sup>[6]</sup> that produce a longer wavelength of light, which is an orthogonal approach to enzyme engineering. A recent report described the development of a D-luciferin analogue that emits in the near-infrared region.<sup>[6e]</sup> This analogue is an aminoluciferin-Cy5 conjugate and is based on bioluminescence resonance energy transfer (BRET). This type of modification, however, alters the cellular uptake properties of the substrate and likely changes its biodistribution in vivo. Herein, we describe a simple modification to D-luciferin, the production of a selenium analogue, which exhibits red-shifted BL emission. and we demonstrate its use for in vivo BLI.

We designed a D-luciferin analogue that contains a selenium atom in place of the native sulfur atom at position 1 (3c). We hypothesized that this replacement would red-shift

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the emission maximum because of the polar effect of the selenium atom, which has been previously reported in fluorophores.<sup>[7]</sup> Furthermore, it is known that several enzymes recognize selenium analogues equally as well as the natural sulfur-containing substrates,<sup>[8]</sup> which makes it likely that the derivative would be an efficient luciferase substrate. Finally, on account of the red shift induced by the 6'-amino substituent in **3b**, and the usefulness of the amino group in the preparation of bioluminogenic substrates,<sup>[2d, 9]</sup> we chose to preserve this functionality in our design.

The synthesis of native D-luciferin (3a)<sup>[10b-d]</sup> and its analogue 6'-amino-D-luciferin (3b)<sup>[6b]</sup> is straightforward and involves a condensation reaction<sup>[10]</sup> between 2-cyanobenzothiazole derivative 1a,b and cysteine (2a) (Scheme 1). Compound 3b is a competent substrate for FLuc and exhibits redshifted BL emission with  $\lambda_{em,max} = 578 \text{ nm.}^{[11]}$  The only known D-luciferin analogues that are tolerated by native FLuc contain 4'- or 6'-substitutions on the benzothiazole ring<sup>[6a,b]</sup> or naphthalene or quinoline in place of the benzothiazole,<sup>[12]</sup> although aliphatic<sup>[6c]</sup> and cyclic alkylaminoluciferin<sup>[6f]</sup> substrates can generate light with mutated variants of FLuc. We coupled seleno-D-cysteine (2b) with 2-cyano-6-aminobenzothiazole (1b) at room temperature in 0.5 M Tris-HCl buffer (pH 7.5) that contained 13% v/v DMSO to afford aminoseleno-D-luciferin (3c). The product was isolated by HPLC in 78% yield. Because the L enantiomer of luciferin generates no BL signal but inhibits FLuc,<sup>[13]</sup> it is desirable to use enantiomerically pure 2b in the synthesis, which can be prepared from elemental selenium.<sup>[14]</sup> In this manner, [<sup>75</sup>Se]3c and [<sup>77</sup>Se]3c for dual modality BLI/PET and BLI/MRI, respectively, are readily accessible. Finally, we note that other chalcogen-substituted cysteine derivatives, such as tellurocysteine (2c), which is known to react analogously to 2a, might also be successfully incorporated in the reaction shown in Scheme 1.

The absorbance spectra of **3b** and its selenium analogue, **3c**, are nearly identical; both contain a local maximum at 350 nm in 50 mM Tris-HCl buffer (pH 7.5), and the molar absorptivities are  $15100 \text{ m}^{-1} \text{ cm}^{-1}$  and  $15500 \text{ m}^{-1} \text{ cm}^{-1}$ , respectively. The value for **3b** is in good agreement with a previous measurement in 95% ethanol ( $15500 \text{ m}^{-1} \text{ cm}^{-1}$ ).<sup>[6a]</sup>



**Scheme 1.** Structure and synthesis of D-luciferin (**3 a**), amino-D-luciferin (**3 b**), and designed aminoseleno-D-luciferin (**3 c**).

In the presence of adenosine triphosphate (ATP),  $Mg^{2+}$ ions, and oxygen, 3c is a competent substrate for purified native FLuc and emits a red BL signal that is readily distinguished from the yellow-green BL signal of D-luciferin 3a and the orange BL signal of 3b (Figure 1a). The BL



*Figure 1.* a) Bioluminescence images of **3 a–c**. b) In vitro bioluminescence spectra of **3** (green), **3 b** (orange), and **3 c** (red) at 23 °C.

emission maxima for **3a**, **3b**, and **3c** were 559 nm, 588 nm, and 600 nm, respectively (Figure 1 b). The red-shifted BL signal of **3c** also persists at 37 °C (data not shown). The percentage of integrated BL emission above 600 nm, which is an important criterion for transmission through tissue in in vivo BLI, for **3c** is 55 %, which is higher than the values for **3a** and **3b**, 23 % and 41 %, respectively.

To determine the apparent Michaelis constant  $K_{\rm m}$  of 3c, which is defined as the substrate concentration at half of the maximum reaction velocity, the initial rate of luminescence was measured as a function of substrate concentration and is plotted in Figure 2a. The reaction of FLuc with 3c exhibits typical Michaelis–Menten kinetics, as shown by the linearity of the Lineweaver–Burk plot (Figure 2b). Nonlinear regression analysis of the Michaelis–Menten plot (Figure 2a) generated a  $K_{\rm m}$  value of  $(0.70 \pm 0.04) \,\mu$ M for 3c, which is not statistically different by *t*-test from the value for 3b,  $(0.62 \pm 0.05) \,\mu$ M (see the Supporting Information).<sup>[11]</sup> Thus, substitution of the sulfur atom for a selenium atom does not appreciably perturb the affinity of the substrate for FLuc.

To compare the emission rates of **3b** and **3c** under biologically relevant conditions, both substrates were incubated with a human breast adenocarcinoma cell line (MDA-MB-468) that stably expressed FLuc. The time course of BL emission in cell culture for each substrate (Figure 3) qualitatively resembles the invitro time course obtained with purified FLuc (see the Supporting Information). After an



*Figure 2.* a) Michaelis–Menten plot for 3c with light output from luminescence as a readout of the reaction rate. b) The same data represented in a Lineweaver–Burk plot. RLU = relative light units.

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**Figure 3.** a) Time course of baseline-corrected BL signals from equimolar solutions of **3b** (orange) and **3c** (red) in cell suspensions of MDA-MB-468 expressing FLuc. Lines represent the least squares best fits for the quasilinear basal signals from **3b** and **3c** after 10 min. b) False-color BL intensity images superimposed on the corresponding white-light images of the wells at various times.

initial period that is characterized by flash kinetics, 3c has a lower and more stable basal emission rate relative to **3b**; after 10 min, the signal from 3b decayed in a quasilinear manner with an emission rate decrease of approximately  $2.5 \times$  $10^4$  (photons s<sup>-1</sup>) min<sup>-1</sup> (R<sup>2</sup> = 0.994), whereas the emission from 3c decayed considerably slower. Extrapolation of each line to y=0 and subsequent integration of the area under each curve indicates that 3c emitted approximately 74% of the number of photons that were emitted by 3b (see the Supporting Information), which suggests a lower quantum yield for 3c if equal numbers of molecules reacted. Indeed, more efficient nonradiative relaxation would be expected for **3c** on account of the well-known heavy-atom effect.<sup>[15]</sup> However, a reduced quantum yield is not sufficient to explain the differences in the kinetic profiles of 3b and 3c. Recent work by Ribiero and Esteves da Silva<sup>[16a]</sup> indicates that dehydroluciferyl adenylate (L-AMP), a nonbioluminescent byproduct of the reaction,<sup>[17]</sup> behaves as a potent competitive inhibitor of FLuc ( $K_i = 3.8 \text{ nm}$ ),<sup>[16,18]</sup> and is responsible for much of the early decrease in BL emission. The faster initial decay of 3c and its lower basal level could be explained by the dehydroaminoselenoluciferyl-AMP byproduct having a lower  $K_{\rm i}$  value than the corresponding sulfur analogue.

The in vivo performance of 3c was also compared with 3b in nude mice with subcutaneous tumor xenografts of a MDA-MB-468 cell line that were constitutively expressing FLuc (FLuc +). Each mouse was sequentially injected with e substrate with a 5 h delay between each substrate injection to allow for enzyme recovery and substrate clearance. After tail vein injection of either substrate into mice (n=4), BL emission was imaged at several time points (Figure 4a). To account for differences in tumor sizes among the mice, the BL





**Figure 4.** a) Representative BLI of a nude mouse implanted with FLuc + MDA-MB-468 xenografts after intravenous injection of either **3b** or **3c**. b) Time course of tumor-localized BL emission after intravenous injection of either **3b** or **3c** into mice implanted with FLuc + MDA-MB-468 xenografts. Each data point is the average baseline-corrected BL signal from four mice. To account for variations in tumor size, BL signals for each tumor were normalized to the maximum signal obtained from that tumor before averaging; the maximum signal was always obtained with substrate **3b** at t = 1 min. y error bars show the standard deviation of the BL signals.

signal at each time point and for each substrate was normalized to the maximum BL signal. This always occurred with substrate **3b** at the first time point (t=1 min). The normalized BL values for each substrate were averaged at each time point and plotted with their standard deviations (Figure 4b).

In contrast to in vitro studies, the time courses of in vivo BL emission for **3b** and **3c** are comparable; with the exception of the 5 min time point in Figure 4b, there is no statistically significant difference between the substrates (P > 0.05). The discrepancy between in vitro and in vivo experiments cannot be fully explained by the theoretically higher penetration of the red-shifted BL signal of **3c** through tissue (ca. 34%) because this effect is more than offset by its slower emission rate (see Figure 3). The differentially improved performance of **3c** in in vivo imaging may be attributed to some organism-level process, such as temperature regulation, perfusion, biodistribution, or elimination.<sup>[19]</sup> We note that such a process need only induce a small change in the steady-state concentration of a potent inhibitor species (for example,

L-AMP) relative to the in vitro conditions to induce a large change in the BL emission kinetics.

In conclusion, we have described a selenium-containing D-luciferin derivative **3c** and demonstrated that it is a competent substrate for native FLuc, exhibiting a red-shifted BL signal maximum relative to its sulfur-containing analogue **3b**. Although the substitution does not decrease the substrate's affinity for FLuc, it results in less light output in vitro, partly as a result of a lower quantum yield. For in vivo BLI applications, the substitution is benign; it has no net effect on the emission kinetics, likely because of a trade-off between greater tissue penetration of the BL emission and a lower quantum yield.

Our demonstration of the feasibility of chalcogen substitution without a detrimental effect on in vivo BLI enables the prospect of luciferin-based probes for multimodal imaging. One implementation for BLI/MRI relies upon the ability of FLuc to catalyze the conversion of D-luciferin into oxyluciferin, for which the enol tautomer is the predominant ground-state species.<sup>[4]</sup> Thus, the chalcogen at position 1, which is singly allylic in the substrate, becomes doubly allylic in the product. As both <sup>77</sup>Se and <sup>125</sup>Te are stable  $S = \frac{1}{2}$  nuclei with narrow lines and a wide range of chemical shifts, this chemical shift change is, in principle, detectable by MRI; we suspect that hyperpolarization might be required for the lowsensitivity <sup>77</sup>Se nucleus.<sup>[20]</sup> If successful, this method could be generally applied to BL/MR imaging of any luciferaseexpressing tumor model in mice.

## **Experimental Section**

Synthesis of **3c**: Seleno-D-cystine (5.7 mg, 0.017 mmol), synthesized according to ref. [14], was reduced to **2b** by incubation in a solution of tris(2-carboxyethyl)phosphine hydrochloride (14.7 mg, 0.0513 mmol) in Tris-HCl (2.1 mL, 0.5 M, pH 7.5,) for 5 min. The aqueous solution of **2b** (0.034 mmol, 2 equiv) was added to a solution of **1b** (3.0 mg, 0.017 mmol, 1 equiv) in DMSO (300  $\mu$ L), mixed thoroughly, and allowed to react for 1.5 h at room temperature in an atmosphere of nitrogen. Aliquots of the reaction mixture were separated by reverse-phase HPLC on an Acclaim 120 C18 column with gradient elution (0% to 85% acetonitrile with water balance over 30 min; both elution solvents contained trifluoroacetic acid (0.1%); 1 mLmin<sup>-1</sup> flow-rate). Upon elution, the fractions that contained **3c** were combined and subjected to rotary evaporation to yield **3c** as a light-yellow solid (78%).

Bioluminescence images: The images in Figure 1 a were taken in the dark with a Nikon D70 digital camera equipped with an AF-S Nikkor 18–70 mm DX lens. Bioluminescent solutions were prepared as described for the BL spectra (see below), but on a larger scale and with ten times the concentration of FLuc. To collect the maximum amount of light, the largest available aperture size was used (f3.5). Integration times were 10 s for **3a** and 30 s for **3b** and **3c**.

Bioluminescence spectra: FLuc ( $5.0 \,\mu$ L of a solution of 130  $\mu$ g mL<sup>-1</sup> FLuc in phosphate-buffered saline (PBS) buffer, pH 7.4, containing 1% bovine serum albumin (BSA)) was added to a solution of PBS buffer (Dulbecco's 1 ×, 40  $\mu$ L, pH 7.4), ATP buffer (20  $\mu$ L, Stratagene; ATP (0.5 mM), CoA (0.5 mM), MgSO<sub>4</sub>, (10 mM), pH 7.8), and **3** (1  $\mu$ L, 1.5 mM in PBS buffer, pH 7.4). The combined solutions were mixed and incubated in a small-volume cuvette (Starna, 16.40F-Q-10/Z15) for 2 min to allow the BL signal to stabilize. BL spectra were collected on a wavelength-calibrated FluoroMax-3 fluorimeter (Horiba Jobin Yvon) with a blocked

excitation path (0.5 s integration time; 1 nm increments; 10 nm emission slit).

Determination of the  $K_m$  value: Two-fold serial dilutions of **3c** were performed with PBS (1 × , pH 7.4) to give eight 50 µL solutions ranging in concentration from 32.3 µM to 0.252 µM, each in a 1.5 mL microcentrifuge tube. Luciferase Assay Buffer II (100 µL, Promega) supplemented with ATP (3.31 mg mL<sup>-1</sup>, 6 mM) was added to each tube. Immediately before measuring the luminescence of a sample, a freshly prepared solution of QuantiLum Recombinant Luciferase (Promega, 50 µL, 1.1 µg mL<sup>-1</sup>) in PBS (1 × , pH 7.4) was added to the tube. The solution was briefly vortexed, and the resulting luminescence emission was measured over 10 s on a 20/20n luminometer (Turner Biosystems). The  $K_m$  value was determined by nonlinear regression analysis of the Michaelis–Menten plot by using the Enzyme Kinetics Wizard in the SigmaPlot 12.0 software package.

Cell culture kinetics: **3b** or **3c** (20  $\mu$ L of a 100  $\mu$ M solution in pH 7.4 PBS buffer) was added to a suspension of FLuc + cells (60  $\mu$ L, ca.  $6 \times 10^4$  MDA-MB-468 cells in pH 7.4 PBS buffer) with gentle mixing. The time course of BL emission was measured on by using an IVIS 200 optical imaging system (Caliper) with 10 s integration at each time point (no emission filter).

In vivo kinetics: All animal handling was performed in accordance with Stanford University's Animal Research Committee guidelines. Nude, athymic mice were subcutaneously implanted with  $10^7$ MDA-MB-468 FLuc + cells at a single site. Once tumors grew to approximately 5 mm in diameter (ca. 10 days), the mice were injected with **3b** or **3c** (100 µL of a 2.5 mM solution) into the tail vein. The mice were anesthetized with isoflurane, and the time course of BL emission from the tumor was measured by using an IVIS 200 optical imaging system with 30 s integration at each time point (no emission filter). Each substrate was tested in four different mice, and a 5 h delay between same-mouse experiments was employed to allow for clearance and FLuc recovery. In the absence of substrate, no BL signals were detected in the mice. No toxic effects were detected with **3c**.

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