

## Robust Light Emission from Cyclic Alkylaminoluciferin Substrates for Firefly Luciferase

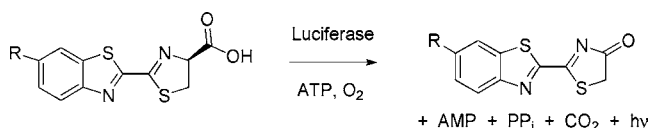
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**Abstract:** Firefly luciferase utilizes the chemical energy of ATP and oxygen to convert its substrate, D-luciferin, into an excited-state oxyluciferin molecule. Relaxation of this molecule to the ground state is responsible for the yellow-green light emission. Synthetic cyclic alkylaminoluciferins that allow robust red-shifted light emission with the modified luciferase Ultra-Glo are described. Overall light emission is higher than that of acyclic alkylaminoluciferins, aminoluciferin, and the native substrate D-luciferin.

Beetle luciferases chemically generate light by converting D-luciferin into an excited-state molecule of oxyluciferin (Figure 1).<sup>1,2</sup> The energy difference between the excited and ground states determines the wavelength of the bioluminescence.<sup>3</sup> Despite the fact that the light emission properties of beetle luciferases are inherently limited by the luciferin structure, there has been little work on the synthesis of modified luciferin substrates since the late 1960s.<sup>4–6</sup> Here we describe conformationally restricted cyclic alkylaminoluciferin substrates that result in red-shifted and more intense light emission with Ultra-Glo luciferase.<sup>7</sup>



**Figure 1.** Beetle luciferases catalyze the conversion of D-luciferin (R = OH) or 6'-aminoluciferin (R = NH<sub>2</sub>) into the corresponding oxyluciferin. Light emission occurs from the oxyluciferin excited state.

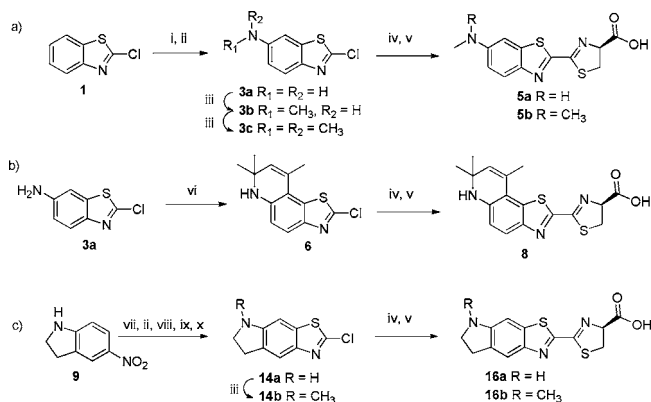
White and McElroy established that the 6'-hydroxyl of D-luciferin (LH<sub>2</sub>) can be replaced with an amino group;<sup>8</sup> 6'-aminoluciferin (6'-NH<sub>2</sub>LH<sub>2</sub>) emits light at a longer wavelength and has ~10-fold higher affinity for luciferase than LH<sub>2</sub>.<sup>9</sup> On the other hand, no light is emitted when the 6'-hydroxyl is removed or replaced with 6'-methoxy, ethoxy, or N-acetylamino groups. This is not due to a lack of binding; conversely, these and other bulky modifications are well-tolerated.<sup>10</sup> Rather, these modifications prevent light emission.

We hypothesized that replacement of the phenolic hydroxyl of luciferin with more strongly electron-donating alkylamino groups would red-shift the spectral properties of the luciferin chromophore while maintaining high affinity for the luciferase. Furthermore, we hypothesized that restriction of the conformational flexibility of the alkylaminoluciferin by cyclization of the arylamine would maintain a high quantum yield of bioluminescence. Conversely, free rotation around the aryl–nitrogen bond in acyclic alkylaminoluciferins was predicted to result in a reduced quantum yield as a result of poor overlap of the nitrogen lone pair with the conjugated system of the luciferin, particularly in the context of the luciferin binding pocket.

We first synthesized acyclic alkylaminoluciferins from **1** (Scheme 1a). Reductive alkylation of **3a** followed by nucleophilic substitution

with cyanide and condensation with D-cysteine afforded 6'-MeNHLH<sub>2</sub> (**5a**) and 6'-Me<sub>2</sub>NLH<sub>2</sub> (**5b**). However, attempts to construct cyclic alkylamino modifications from **3a** were stymied by the exclusive formation of the undesired 6,7 isomer rather than the 5,6 ring fusion (Scheme 1b).

**Scheme 1.** Synthesis of Alkylaminoluciferins: (a) Reductive Alkylation of Intermediate **3a** Afforded Acyclic Alkylaminoluciferins; (b) Attempts To Form Cyclic Alkylaminoluciferins from **3a** Gave Exclusive Formation of the Undesired 6',7'-Fused Isomer (e.g., **8**); (c) Synthesis of 5',6'-Fused Cyclic Alkylaminoluciferins **16** Required Reversal of the Order of Ring Formation<sup>a</sup>



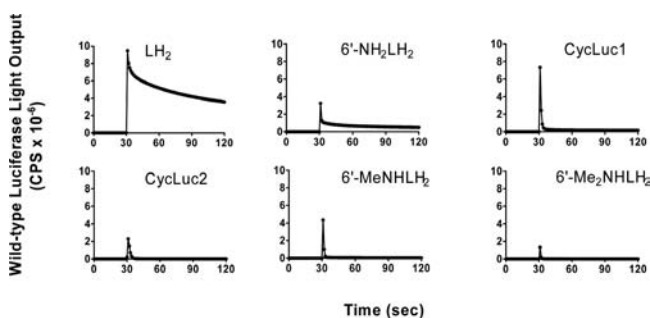
<sup>a</sup> Conditions: (i) HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>; (ii) SnCl<sub>2</sub>·2H<sub>2</sub>O, EtOH, reflux; (iii) NaBH(OAc)<sub>3</sub>, 37% HCHO, DCE, HOAc; (iv) KCN, DMSO, 130 °C; (v) D-Cys, aq. MeOH, pH 8; (vi) 5 mol % In(OTf)<sub>3</sub>, acetone, rt; (vii) TFAA, TEA, rt; (viii) KSCN, Br<sub>2</sub>, AcOH, rt; (ix) *t*-BuONO<sub>2</sub>, CuCl, CH<sub>3</sub>CN; (x) NaBH<sub>4</sub>, EtOH.

Thwarted by the positional reactivity preferences of **3a**, we examined approaches for constructing the benzothiazole ring as the final step (Scheme 1c). Protection of **9** as a trifluoroacetamide followed by reduction of the nitro group and oxidative ring formation yielded the desired 2-aminobenzothiazole. Diazotization of the 2-amino group and concomitant displacement by chlorine followed by reductive removal of the trifluoroacetamide protecting group afforded **14a**. Introduction of the 2-cyano group and subsequent treatment with D-cysteine afforded the respective luciferin analogue **16a** (CycLuc1). The N-methylated derivative **16b** (CycLuc2) was similarly constructed from **14b**.

We next measured the emission wavelength of bioluminescence for our synthetic aminoluciferin substrates. Relative to LH<sub>2</sub>, 6'-NH<sub>2</sub>LH<sub>2</sub> exhibited red-shifted light emission from wild-type firefly luciferase (593 vs 557 nm). We found that 6'-MeNHLH<sub>2</sub> extended this emission shift to 609 nm, while 6'-Me<sub>2</sub>NLH<sub>2</sub> was even more red-shifted to 623 nm. This emission wavelength is identical to that of the railroad worm beetle luciferase *PxhRE*, the most red-shifted luciferase reported to date.<sup>11</sup> Surprisingly, luciferase emission with the cyclic aminoluciferins was less red-shifted than with

their acyclic counterparts (599 nm for CycLuc1 and 607 nm for CycLuc2; see Table S1 in the Supporting Information).

Measurement of the emission intensity as a function of wavelength revealed a significant reduction in light output for all of the aminoluciferins relative to D-luciferin. The lower intensity of light emission could arise from either kinetic factors (lower rate of enzymatic turnover or product inhibition) or an inherently lower efficiency of light production (lower quantum yield or failure to produce oxyluciferin), or both. We therefore performed a rapid-injection experiment in which the light output was measured during and immediately after mixing of the luciferin and luciferase.<sup>12</sup> All of the substrates gave a robust initial burst of light and are thus capable of rapid conversion to a light-emitting oxyluciferin (Figure 2). However, sustained light output was low after the initial burst, consistent with product inhibition.<sup>13</sup> Addition of coenzyme A, which has been shown to reduce product inhibition by L-AMP,<sup>1</sup> failed to prevent this effect (Figure S1 in the Supporting Information). Encouragingly, the initial flash of light from cyclic substrate CycLuc1 substantially exceeded that from all of the other aminoluciferins (Figure 2).

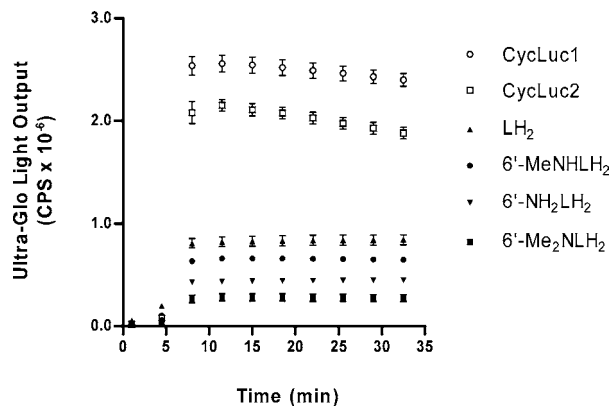


**Figure 2.** Light emission from 10  $\mu\text{M}$  luciferin substrates after rapid injection of wild-type firefly luciferase at the 30 s time point.

The burst kinetics of firefly luciferase, even with its native substrate D-luciferin, is well-known (Figure 2).<sup>1</sup> To provide a steady level of light emission, Promega has developed Ultra-Glo, a highly mutated form of luciferase designed to be more stable and resistant to inhibition during high-throughput screening assays.<sup>7,14</sup> It has also been shown that when this luciferase is used with P450-Glo buffer, it allows sustained light emission from substrates that lead to product inhibition of wild-type firefly luciferase.<sup>5,15</sup>

When tested with Ultra-Glo luciferase in P450-Glo buffer at a concentration of 1  $\mu\text{M}$ , CycLuc1 and CycLuc2 exhibited dramatically higher light output than any of the other substrates (Figure 3). CycLuc1 emitted 5.7-fold more light than aminoluciferin and 3.2-fold more light than D-luciferin. Similarly, CycLuc2 emitted 4.7-fold more light than aminoluciferin and 2.6-fold more light than D-luciferin. The superior light output of CycLuc1 was maintained across a wide concentration range (1–100  $\mu\text{M}$ ; Figure S2), indicating that this behavior is not simply due to higher affinity for the luciferase. Even in tests at exorbitantly high substrate concentrations (750  $\mu\text{M}$ ), the light emission from CycLuc1 greatly exceeded that from any of the other aminoluciferins and was equivalent to that of D-luciferin (Figure S2). In contrast, the acyclic substrate 6'-MeNHLH<sub>2</sub> emitted ~50% more light than aminoluciferin at 1  $\mu\text{M}$  but was no better at concentrations over 100  $\mu\text{M}$ , and 6'-Me<sub>2</sub>NLH<sub>2</sub> had uniformly lower light emission at all of the tested concentrations (Figure S2).

The superior light output of CycLuc1 and CycLuc2 demonstrates that they are more efficient light emitters than their respective acyclic counterparts and that both monoalkyl and dialkylaminoluciferins can be efficient substrates (Figure 3). We hypothesize that this is due to the increased rigidity and restricted bond rotation of the cyclic



**Figure 3.** Ultra-Glo light output with 1  $\mu\text{M}$  luciferin substrate.

alkylaminoluciferins, which lead to an increase in the relative quantum yield.<sup>16</sup> In the context of wild-type firefly luciferase, product inhibition overshadowed this effect and limited the light output (Figure 2).

The use of cyclic aminoluciferin substrates or pro-substrates for in vitro assays with Ultra-Glo is expected to improve the sensitivity of detection.<sup>17</sup> However, despite its utility for high-throughput screening applications, Ultra-Glo is not available as a genetic construct that can be introduced into living organisms for bioluminescence imaging.<sup>9,18</sup> Future work will be directed toward mutation of luciferase in order to relieve product inhibition and allow sustained light emission from cyclic aminoluciferins under physiological conditions using a luciferase that can be readily expressed within cells and whole organisms.

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**Supporting Information Available:** Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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