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Expedient Synthesis of Electronically Modified Luciferins for Bioluminescence Imaging

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Supporting Information

ABSTRACT: Bioluminescence imaging with luciferase enzymes requires access to light-emitting, small-molecule luciferins. Here, we describe a rapid method to synthesize D-luciferin, the substrate for firefly luciferase (Fluc), along with a novel set of electronically modified analogues. Our procedure utilizes a relatively rare, but synthetically useful dithiazolium reagent to generate heteroaromatic scaffolds in a divergent fashion. Two of the luciferin analogues produced with this approach emit light with Fluc *in vitro* and in live cells. Collectively, our work increases the number of substrates that can be used for bioluminescence imaging and provides a general strategy for synthesizing new collections of luciferins.

Bioluminescence imaging is among the most popular methods for visualizing biological processes *in vitro*, in live cells, and even in whole organisms.[1,2] At the core of this technology are enzymes (luciferases) that catalyze the oxidation of small-molecule substrates (luciferins) to release visible light. Since cells and tissues do not normally emit significant numbers of visible photons, bioluminescence provides extremely high signal-to-noise ratios, making it well-suited for sensitive imaging applications.[3] Indeed, this technology is routinely used to monitor cell trafficking networks, gene expression patterns, and drug delivery mechanisms *in vivo*.[4,5] Despite its remarkable versatility, bioluminescence has been largely limited to monitoring one cell type or biological feature at a time. This is because only a handful of luciferases are suitable for biological work and, of these, nearly all utilize the same substrate (D-luciferin). Retooling bioluminescence technology for multicomponent imaging requires access to larger collections of light-emitting luciferins. Such molecules could potentially provide different colors of bioluminescent light or be utilized by novel luciferase variants. Unfortunately, luciferins have been notoriously difficult to produce, owing to a lack of rapid and reliable syntheses for these richly functionalized molecules. We report here an expedient method to prepare D-luciferin, along with a new class of light-emitting analogues. This chemistry is both efficient and scalable and will bolster ongoing efforts to expand the bioluminescence toolkit.

The vast majority of efforts to develop new bioluminescent tools have focused on mutating luciferase enzymes from the firefly (Fluc) and related organisms.[6,7] By contrast, only a handful of studies have focused on modifying the structure of D-luciferin (**1**), the substrate common to all insect luciferases. This disparity is surprising, given the prominent role of the small molecule in the light-emitting reaction. During the Fluc-catalyzed oxidation of D-luciferin, an excited-state version of the product (oxyluciferin) is generated; relaxation of this molecule to the ground state releases a photon of yellow-green light (Figure 1).[8] Since the chemical makeup of the excited-state

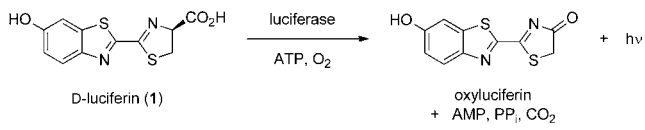

Figure 1. Luciferase-catalyzed oxidation of D-luciferin releases visible light.

emitter influences light production, modifications to the aromatic core can alter the wavelength and intensity of photons released. Miller and others have shown that luciferin variants containing a nitrogen atom in place of the exocyclic oxygen are efficiently processed by Fluc and emit red light.[9−11] In related work, Branchini and others replaced the entire benzothiazole core of D-luciferin with quinoline, naphthalene, and coumarin units. These analogues emitted different colors of light with Fluc, but elevated pH values were required to achieve robust emission in most cases.[12,13] Although these luciferins have somewhat limited utility in biological assays, they remain the only examples of Fluc substrates that do not contain a benzothiazole moiety.

We aimed to expand the repertoire of modified heteroaromatic luciferins suitable for biological studies. In particular, we were attracted to luciferins with benzimidazole and imidazoline rings (the nitrogenous counterparts to the benzothiazole and thiazoline units in D-luciferin, **2**−**4**). Heterocycles of this sort are capable of absorbing and emitting light, an important criterion for bioluminescent substrates.[14,15] White and McElroy have also shown that benzimidazole and other heterocycles are competitive inhibitors of Fluc, suggesting that **2**−**4** would be able to access the substrate binding pocket.[16] Last, since benzimidazole and imidazoline motifs are present in numerous pharmaceutical agents, we felt that the electronically modified analogues would possess reasonable bioavailability and metabolic stability for use in cells and animal models.[17,18]

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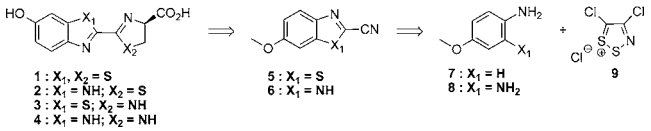
Analogues **2**–**4**, like D-luciferin, contain a unique 2–2′ linkage of heteroaromatic rings. This connectivity is scarcely observed in known natural products, and facile methods to prepare such linkages are rare.[19] In fact, the first synthesis of D-luciferin, reported by White in 1963, is basically the same route used to produce nearly all luciferins today.[20] This synthesis proceeds through a cyanobenzothiazole intermediate (**5**), which, upon protecting group removal, can be condensed with D-cysteine to provide the native luciferin. This condensation is both mild and high-yielding, making it an appealing method for late-stage introduction of the luciferin stereocenter. Unfortunately, the White synthesis of **5** requires seven steps and is not amenable to heteroatom substitutions.

Recognizing the utility of cyano heterocycles for luciferin production, we aimed to identify a more expedient route to **5**, along with the analogous cyanobenzimidazole **6**. Condensation of these scaffolds with either D-cysteine or diaminopropionic acid could provide the entire set of luciferin analogues (**2**–**4**). To access **5** and **6** in tandem, we were drawn to the dithiazolium chloride **9** (Scheme 1). This reagent, also known

Scheme 1. Retrosynthetic Analysis of Luciferin Analogues

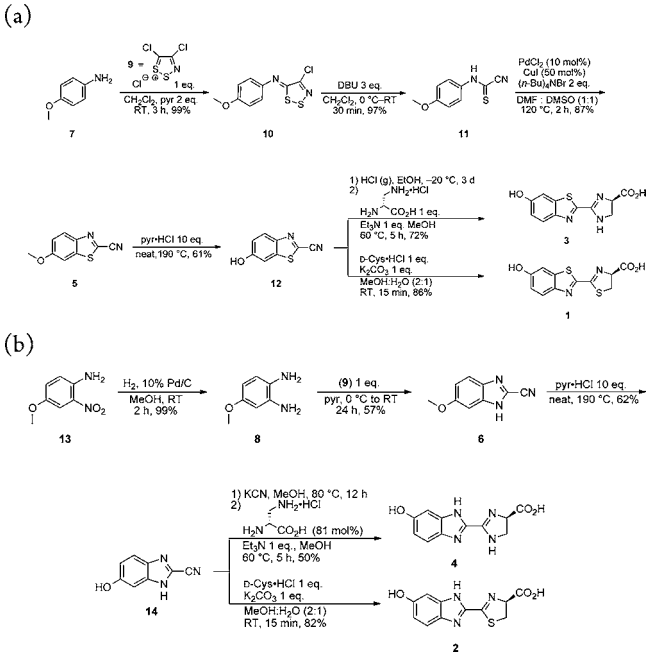


as Appel's salt, has previously been used to synthesize both benzothiazole and benzimidazole scaffolds from anilines.[21] Appel's salt condenses readily with arylamines, and the resulting iminodithiazoles can be easily opened with a variety of nucleophiles.[22] If the nucleophile is present on the aniline itself (as in the case of *o*-aminoanilines), cyanobenzimidazole structures can be isolated directly. In the absence of intramolecular nucleophiles, the dithiazole adduct can be fragmented with a variety of exogenously supplied reagents. When amidine bases are used, dithiazole cleavage provides thioformamides; molecules of this sort can be readily cyclized to cyanobenzothiazoles.[22]

To investigate the utility of Appel's salt for luciferin synthesis, we first used the reagent to prepare the cyanobenzothiazole **5** (en route to D-luciferin, Scheme 2a). *p*-Anisidine was treated with **9** to provide the expected dithiazole adduct **10**. This intermediate was isolable using standard chromatographic techniques and found to be remarkably shelf stable. Treatment of **10** with excess DBU produced cyanothioformamide **11** in excellent yield. Palladium- and copper-mediated cyclization of this compound generated the key cyanobenzothiazole **5**. Notably, this route to **5** is four steps shorter than the sequence employed by White and provides the compound in markedly better yield (84% versus 10% overall). The desired luciferin **1** was eventually isolated by removal of the methyl protecting group from **5**, followed by condensation with D-cysteine under mild conditions. The functional activity of the isolated luciferin was also confirmed in light emission assays with Fluc (Figure S1).

Encouraged by these results, we next investigated whether Appel's salt could provide access to the benzimidazole intermediate **6** (Scheme 2b). Gratifyingly, this molecule was isolated in a single step upon incubation of bis-aniline **8** with **9**. In this reaction, the initial dithiazole adduct is likely trapped by the *o*-amino substituent of **8**, providing the cyclized product.

Scheme 2. Synthesis of Luciferin Scaffolds: (a) D-Luciferin (1) and the Imidazoline Analogue 3; (b) Benzimidazole Analogues 2 and 4



Cyanobenzimidazole **6** was ultimately demethylated and condensed with D-cysteine as above to isolate luciferin **2**. Multigram quantities of both luciferins **1** and **2** have been produced using the routes outlined in Scheme 2, highlighting the scalability of the approach.

The cyano heterocycles produced with Appel's salt can be condensed with a variety of other 1,2-disubstituted nucleophiles in addition to D-cysteine. We exploited this mode of reactivity to generate the imidazoline rings present in luciferins **3** and **4**. First, intermediates **12** and **14** were converted into the corresponding imidates using standard conditions. The imidates were not isolated but treated directly with diaminopropionic acid to afford the desired luciferins in reasonable yield.

Our initial efforts to characterize luciferins **2** and **4** were complicated by tautomerism. Benzimidazole scaffolds are known to undergo rapid N–H isomerization in solution (Figure S2), resulting in significantly broadened ^{1}H and ^{13}C NMR signals. We were also concerned that such rapid tautomerization would suppress bioluminescent light emission from the analogues. Such quenching behavior has been observed with other electronically excited benzimidazoles.[23,24] To mitigate against potential quenching effects and aid our structural characterization efforts, we prepared a methylated version of **2** (Scheme S1). Interestingly, only one *N*-methyl regioisomer (**15**, Scheme S1) was formed in reasonable yield from intermediate **6**.

With the nitrogenous analogues in hand, we assayed the compounds for light emission with Fluc. Luciferins **2**–**4** and **15** were incubated with the enzyme, ATP, and coenzyme A (to reduce product inhibition) at pH 7.4.[25] Light emission was measured using a cooled CCD camera, and representative images are shown in Figure 2. No photons were detected for analogue **3**, and only minimal light emission was observed with the related imidazoline **4** at low substrate concentrations. These reduced intensities may be attributed to poor binding to Fluc,

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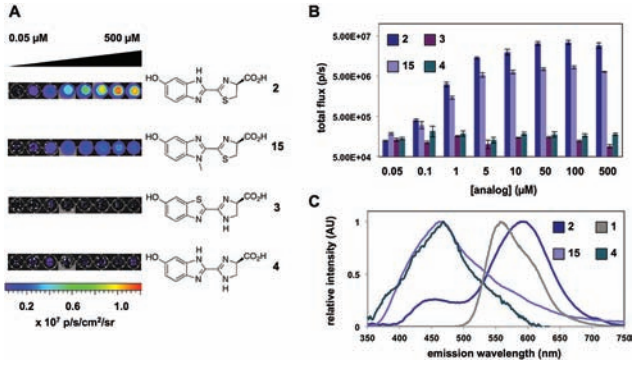

Figure 2. Light production from luciferin analogues. (A) Bioluminescence images from analogues **2**–**4** and **15** (0.05–500 μM) incubated with Fluc or no enzyme. (B) Quantification of the images from (A). (C) Bioluminescence emission spectra for luciferins **1**, **2**, **4**, and **15**.

lower efficiencies of light production, or a combination of factors (Figure S3). By contrast, robust emission was observed with the benzimidazole variants **2** and **15**, suggesting that these molecules can be converted to light-emitting species in the enzyme active site (Figure 2). Both analogues are weaker emitters than the native substrate (~100-fold reduced emission intensities in the low μM range, Figure S1B) but on par with other luciferin scaffolds used in biological assays.[10] Additional improvements in light output may also be obtained using the analogues in combination with mutant luciferases.[9,26] Importantly, the bioluminescence emissions from **2** and **15** are long-lived (Figure S4). Prolonged light release is necessary for numerous imaging applications *in vivo* and has been difficult to achieve with other luciferins.

We next analyzed the bioluminescence emission profiles for **2**, **4**, and **15**. The spectra for these analogues, like most luciferins, are quite broad and indicate the presence of tautomers in aqueous solution (Figure 2C). Benzimidazole analogue **2** was found to emit maximally at 578 nm, slightly red-shifted from D-luciferin (λ_{max} = 557 nm) at room temperature. The bioluminescence spectrum of **2** is also substantially different from the analogue's fluorescence profile, indicating a potential role for Fluc in modulating the color of light released (Table S1). Interestingly, the bioluminescence spectrum for benzimidazole analogue **15** is substantially blue-shifted from those of luciferins **1** and **2**. With peak emission near 460 nm, analogue **15** emits the largest percentage of blue light among the known Fluc substrates. This result also implies that **15** may be useful for multicomponent imaging applications, as its emission can be readily resolved from other luciferins using appropriate filter sets.

To probe whether the light-emitting luciferins would also be useful for cell studies, we incubated **2**, **4**, and **15** with Fluc-expressing HEK 293 cells. Photon emission was measured using a cooled CCD camera, and sample images are shown in Figure 3. Dose-dependent light emission was observed for both **2** and **15**, with photon intensities peaking around 10–20 min after substrate addition (Figure S5). No emission was observed from the nitrogenous analogue **4** in this assay, even at high substrate concentrations. It should also be noted that no light was observed in the absence of the analogues, or when the compounds were incubated with non-luciferase-expressing cells (Figure 3). These results are consistent with the light-emitting behavior of D-luciferin in whole cells, and suggest that the

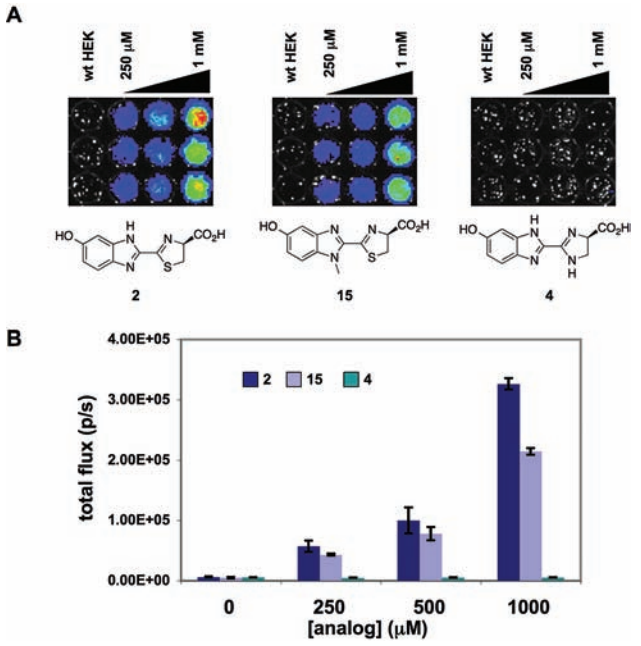

Figure 3. Cellular imaging with luciferin analogues. (A) Bioluminescence images from **2**, **4**, and **15** (250 μM–1 mM) incubated with luciferase-expressing HEK 293 cells or wild-type cells (wt HEK). (B) Quantification of the images from (A).

benzimidazole scaffolds are sufficiently biocompatible for use in cellular imaging studies.[2,10]

In summary, we have developed a facile method to prepare luciferins from aniline starting materials and Appel's salt. This procedure was used to synthesize D-luciferin, the native substrate for Fluc, along with a series of nitrogenous analogues. Two of the analogues were found to emit light with purified Fluc and in live cells, and these scaffolds will be generally useful for imaging studies. More broadly, the chemistry reported here provides a gateway to access additional luciferin architectures. For example, the adducts formed upon aniline condensation with Appel's salt can be selectively fragmented to access quinazolines, benzoxazoles, and a variety of other heterocycles in addition to the benzothiazole and benzimidazole scaffolds examined here.[22] This diverse manifold of reactivity will likely be exploited for synthesizing new classes of heteroaromatic luciferins in the near future. The ability to rapidly access novel luciferin substrates will expand the imaging toolkit and inspire new applications of bioluminescence technology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, full spectroscopic data for all new compounds, and additional images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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