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Design and Synthesis of an Alkynyl Luciferin Analogue for Bioluminescence Imaging

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Abstract: Herein, the synthesis and characterization of an alkyne-modified luciferin is reported. This bioluminescent probe was accessed using C–H activation methodology and was found to be stable in solution and capable of light production with firefly luciferase. The luciferin analogue was also cell permeant and emitted more redshifted light than D-luciferin, the native luciferase substrate. Based on these features, the alkynyl luciferin will be useful for a variety of imaging applications.

Bioluminescence is a versatile imaging platform with applications ranging from metabolite biosensing to whole animal imaging.[1] At the heart of this technology are enzymes (luciferases) that catalyze the oxidation of small molecule substrates (luciferins).[2] During each enzymatic transformation, an electronically excited oxyluciferin is generated that emits a photon of light upon relaxation to the ground state.[3] Since mammalian cells do not produce large numbers of photons in the absence of incident light, bioluminescence can provide an exquisitely sensitive readout on biological processes in these environments.[4] Indeed, luciferase–luciferin pairs have been widely used to report on enzyme activities and gene expression patterns in live cells and tissue lysates.[1] Additionally, since bioluminescence does not require an excitation source, this technology is well suited for noninvasive imaging in whole animals, where delivery of excitation light is often inefficient or impractical.[1a,5]

The most widely used luciferases for cell and animal imaging originate from the insect family.[1b] These enzymes, including firefly luciferase (Fluc), catalyze the oxidation of D-luciferin (**1**) and release ≈500–600 nm light (Figure 1 a).[2b,3] Wavelengths of this sort can penetrate the skin of small rodents and be detected by sensitive cameras, making insect luciferases attractive for imaging in vivo.[6] Indeed, Fluc and related enzymes have been expressed in a variety of tissue and cell types, and when exposed to D-luciferin, light is produced.[1] D-Luciferin is also sufficiently bioavailable in rodents[7] and has been used extensively in preclinical models.[8]

Because of the sensitivity and user-friendly features of bioluminescence, there has been much interest in expanding the scope of the technology.[5d,9] Several efforts have been directed toward identifying other naturally occurring luciferase–luciferin pairs for multicomponent imaging.[1a,10] The instability and poor tissue penetrance of many luciferins have been prohibitive in many cases. Other attempts have focused on generating luciferases that provide altered emission spectra. For example, several insect luciferases have been engineered to emit different colors of light (ranging from ≈500–650 nm) with D-luciferin.[11] While these wavelengths can be adequately resolved in vitro, they cannot be easily discriminated in vivo, where tissue absorption and scatter modulate the color of light that ultimately reaches the detector.[6]

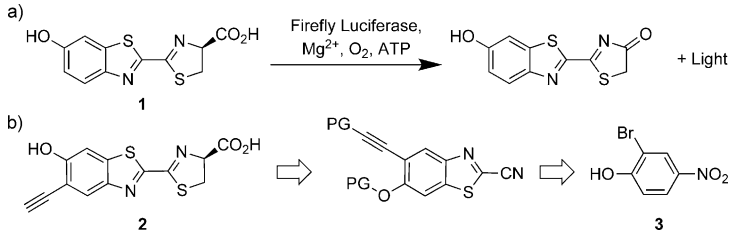

Figure 1. a) The luciferase-catalyzed oxidation of D-luciferin (**1**) produces visible light. b) Retrosynthetic analysis of alkynyl luciferin (PG = protecting group).

Compared to luciferase engineering efforts, there has been less work invested in crafting new luciferins. Substrate engineering is an obvious strategy to broaden the scope of bioluminescence technology, as the luciferin molecules can be modified to emit different colors of light or be selectively utilized by unique luciferases.[12,13] In some cases, the substrates have proven remarkably cell and tissue permeant and, thus, well suited for in vivo work.[14]

Continued efforts to develop unique bioluminescent tools would benefit from rapid access to diverse collections of light-

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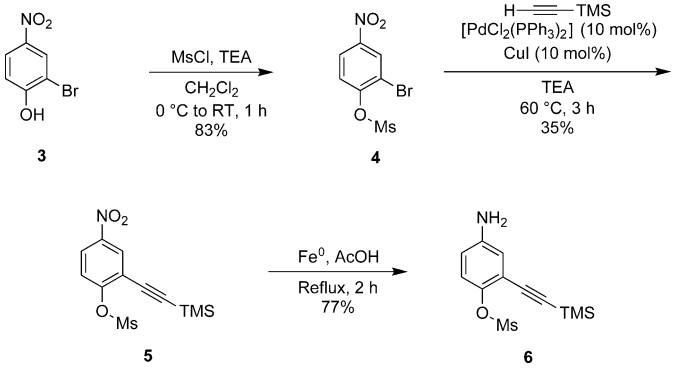
emitting luciferins. These scaffolds have been notoriously difficult to synthesize owing to their electron-rich and highly substituted cores. Late-stage modifications to luciferin molecules are also complicated. For example, most attempts to derivatize D-luciferin (**1**) have focused on altering the 6′-position by alkylation or acylation chemistries.[7a,15] While facile, these strategies have produced scaffolds that are somewhat limited in scope. Electron donation is required for robust emission and, thus, the 6′-position is particularly sensitive to modification.

We aimed to develop a bioluminescent probe modified at an alternative ring position. We were initially drawn to the 5′-alkyne derivative (**2**) shown in Figure 1b. Previous work established that 5′-fluoro and other small substituents were well tolerated by Fluc and minimally perturbing to the bioluminescent reaction.[12b] Modeling analyses suggested that the alkyne would be similarly accommodated in the luciferase active site (Figure 2a). Furthermore, computational data[16] indicated that **2** would be a viable light emitter (Figure 2b).

We were further attracted to alkyne **2** as its benzothiazole core could be accessed using C–H activation chemistry previously reported by our group.[17] However, the functionalized luciferin still presented some synthetic challenges. Electron-rich heterocycles like **2** are susceptible to nonspecific oxidation and are thus difficult to handle and prepare on scale. Methods to produce highly substituted benzothiazoles are also rare.



Scheme 1. Installation of the alkyne substituent. TEA = triethylamine.

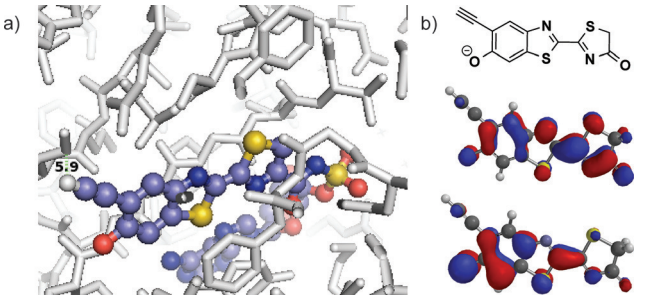
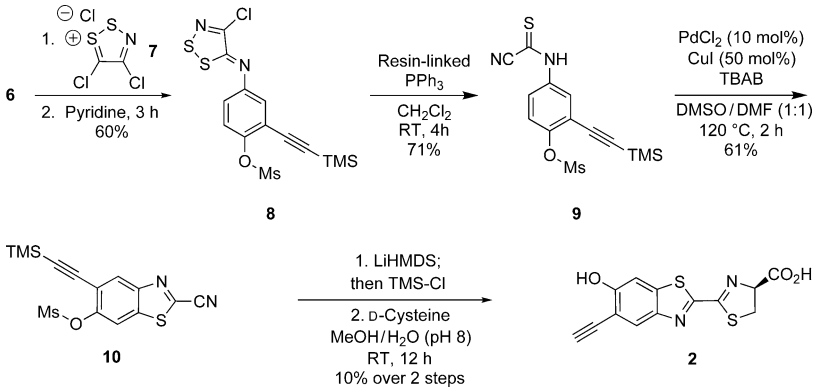


Figure 2. In silico analyses of D-luciferin. a) Overlay of **2** with firefly luciferase (PDB ID: 4G36) suggests that the alkyne motif will be tolerated. b) B3LYP/6-311** MO predictions[16] of the HOMO (middle) and LUMO (bottom) of the oxidized product (top).

To access the desired heterocycle, we began with trisubstituted phenol **3**. The hydroxy substituent was first protected with a mesyl group (Scheme 1).[18] Other classic phenol protecting groups (e.g., silyl and methyl) were explored, but most proved either incompatible with subsequent transformations (in the case of bulky silyl groups) or difficult to remove later on in the synthesis (in the case of methyl groups). Mesylate **4** was ultimately subjected to Sonogashira conditions for alkyne installation. Notably, this reaction was readily scalable and provided decagram quantities of **5** (Scheme S1, Supporting Information). The nitro group of **5** was reduced using iron filings and glacial acetic acid[19] to reveal aniline **6** in good yield and purity. Compound **6** was then treated with Appel's salt **7**, and the resulting adduct was fragmented with resin-linked PPh_3 to yield thioamide **9** (Scheme 2).[20] It should be noted that while other bulky nucleophiles (e.g., DBU and DBA)[21] can be used for such fragmentations, they resulted in premature deprotection of the mesyl group and reduced overall yields in this case. Subsequent cyclization of thioamide **9** by palladium- and copper-catalyzed C–H activation[22] provided **10** in 61% yield. Attempts to isolate **10** directly from **8** by thermal cyclization resulted in product decomposition and were not further pursued. The desired alkyne luciferin **2** was ultimately isolated following mesyl-group removal[23] and cysteine condensation. Importantly, luciferin **2** was stable for weeks as a solid material and in aqueous solution.



Scheme 2. Synthesis of alkyne luciferin **2** by using C–H activation chemistry. LiHMDS = lithium hexamethyl disilazide; TBAB = tetrabutylammonium bromide.

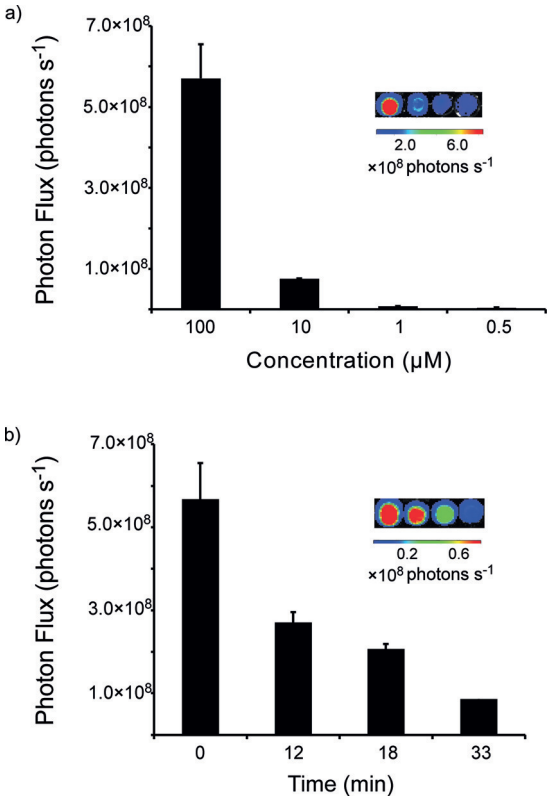


Figure 3. a) Alkynyl luciferin **2** produces light upon incubation with Fluc. Solutions of **2** (0.5–100 μM) were mixed with Fluc, ATP, and CoA in pH 8 buffer in 96-well plates. Light emission was measured using a cooled CCD camera. Sample images are shown in the inset. b) Analogue **2** exhibits sustained light emission. Compound **2** (100 μM) was incubated with Fluc, ATP, and CoA. Light emission was measured over time, and sample images are shown. For a–b, error bars represent the standard deviation of the mean for three replicate experiments.

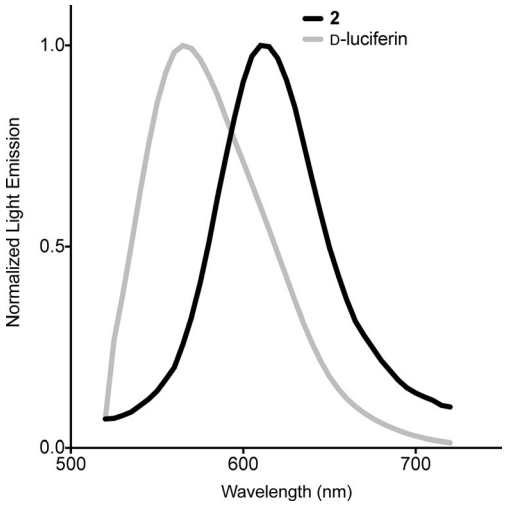


Figure 4. Normalized bioluminescence emission spectra for alkynyl luciferin **2** (λ_{max} 610 nm) and D-luciferin **1** (λ_{max} 565 nm). Samples (100 μM) were combined with Fluc (10 μg) and monitored at 25 °C.

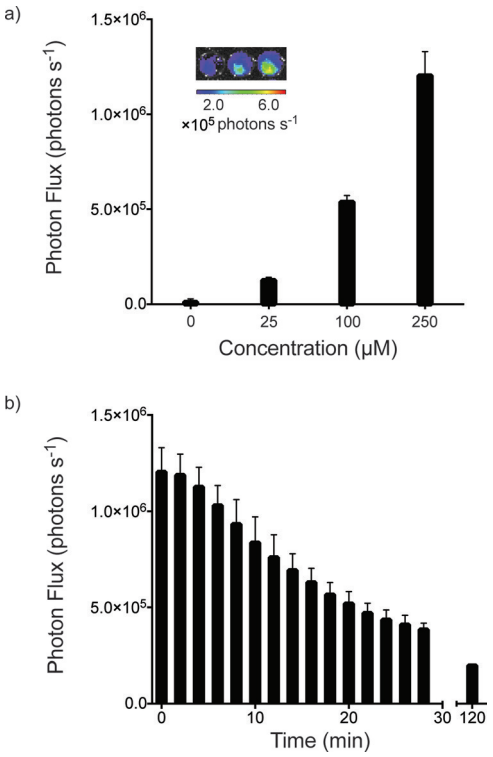


Figure 5. a) Alkynyl luciferin **2** produces light when incubated with HEK293 cells. Analogue **2** (25–250 μM in PBS) was added to cells (100,000 cells per well). Sample images are shown (inset). b) Analogue **2** exhibits sustained light emission with HEK293 cells. Analogue **2** (250 μM) was incubated with HEK293 cells (100,00 cells per well) and photon production was monitored over time. For a, error bars represent the standard deviation of the mean for 6 replicate experiments. For b, error bars represent the standard deviation of the mean for 3 replicate experiments.

Luciferin **2** was also found to be a viable substrate for firefly luciferase (Fluc). When **2** was incubated with Fluc in the presence of ATP, bioluminescent light was observed. As shown in Figure 3, light emission was both concentration-dependent and sustained. The overall photon output from **2** is weaker than that observed with D-luciferin (the native substrate), but on par with other luciferin analogues used in biological assays (Figure S1, Supporting Information).[12b] The measured K_m value was 8.5 ± 1 μM, and the apparent V_{max} was 130 ± 5 × 10^6 photons s^{-1} (Figure S5, Supporting Information). Interestingly, the bioluminescence emission spectrum of **2** was substantially redshifted compared to D-luciferin (λ_{max} = 610 nm at 25 °C, Figure 4). In fact, the alkynyl luciferin spectrum is similar to those of aminoluciferins used in bioluminescence imaging.[14,15b,12e]

We further evaluated the luciferin analogue in live cells. Fluc-expressing HEK293 cells were incubated with **2**, and bioluminescent images were acquired. As shown in Figure 5a, dose-dependent light emission was observed, indicating that the alkynyl luciferin is cell permeable. The photon outputs from cultures treated with **2** were weaker than cultures treated with D-luciferin (Figure S7, Supporting Information). However, the intensities observed are similar to other luciferin analogues and sufficient for some cellular imaging applications.[12b–d] Importantly, the light emission from cells treated with **2** was also sus-

tained (Figure 5b). Prolonged emission is desirable for routine imaging experiments.

We also recognized that **2** could be further "clicked"[24] with azido appendages by copper-catalyzed azide–alkyne cycloaddition (CuAAC). This transformation could potentially expedite the production of new luciferin analogues by using **2** as a platform for late-stage modification. Model reactions with **2** and various azido compounds suggested that the CuAAC diversification strategy is feasible (Figures S2–S3 and S5, Supporting Information). Notably, the cycloaddition can proceed in aqueous solvents and in the absence of copper chelators (Figure S6, Supporting Information). We envision using CuAAC to produce different classes of luciferins that can be screened for selective processing by mutant luciferases. Recent crystallographic analyses have revealed Fluc amino acids in close proximity to the 5′ carbon of a bound luciferin intermediate.[11h,25] These amino acids could potentially be mutated to complement more bulky, steric appendages on the luciferin ring, thereby facilitating the development of substrate-specific (i.e., orthogonal) bioluminescent tools.

Conclusion

In conclusion, we identified an alkyne-modified luciferin **2** for use in bioluminescence assays. This scaffold is isolable in reasonable quantities and is a functional light emitter with luciferase. The alkynyl probe can also be selectively modified with azido appendages by CuAAC. Such designer luciferins are applicable to multicomponent imaging or biosensing in cells and live organisms.[26] Based on the accessibility and unique features of **2**, we anticipate that the alkynyl probe will find use in various imaging assays and further expand the scope of bioluminescence technology.

Experimental Section

Experimental details are available in the Supplementary Information.

Acknowledgements

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