Bioluminescence

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D-Luciferin Analogues: a Multicolor Toolbox for Bioluminescence Imaging**

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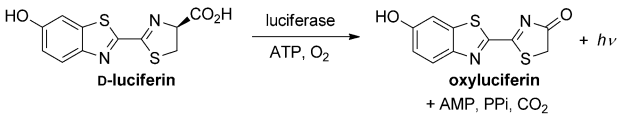
luciferases · luciferins · luminescence · multicolor imaging

Bioluminescence, the conversion of chemical energy into light by a living organism, is beautifully illustrated by the flashing light produced by fireflies. Firefly luciferase (Fluc) 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 releases a photon of yellow-green light (Scheme 1).[1] Because cells



Scheme 1. Luciferase-catalyzed oxidation of D-luciferin releases visible light. Light emission occurs from the excited state of oxyluciferin.

and tissues do not normally emit a significant number of visible photons, bioluminescence provides extremely high signal-to-noise ratios, thus making it well-suited for sensitive imaging applications.[2] Indeed, bioluminescence imaging is among the most popular methods for visualizing biological processes in vitro, in live cells, and even in whole organisms.[3] For example, noninvasive bioluminescence imaging of living subjects has become a routine technique in cancer biology research for the monitoring of gene expression, gene delivery, tumor growth, enzyme activity, response to experimental drug therapies, and protein–protein interactions.[2,4]

Despite its remarkable versatility, bioluminescence has been largely limited to monitoring one cell type or biological feature at a time. This limitation is a result of the existence of only a handful of luciferases that are suitable for biological work and, of these, nearly all are based on the same substrate (D-luciferin). Retooling bioluminescence technology for multicomponent imaging requires access to a large collection of light-emitting luciferins, which need to provide bioluminescent light in different colors. On the other hand, to avoid the absorption and scattering of light by tissue and to get deep tissue penetration, analogues of luciferins that could effectively emit in the far-red to near-infrared region (>600 nm) are also highly desired. Recently, the research groups of Miller,[5] Moerner,[6] and Prescher[7] independently disclosed their studies on alkylaminoluciferin, aminoselenoluciferin, and luciferins with a benzimidazole scaffold, respectively. The designs of the three types of luciferin analogues are based on simple alteration of the hydroxy moiety or sulfur atom of original D-luciferin (Figure 1), by taking advantage of the experiences accumulated in the development of fluorescent

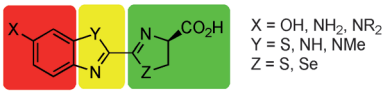
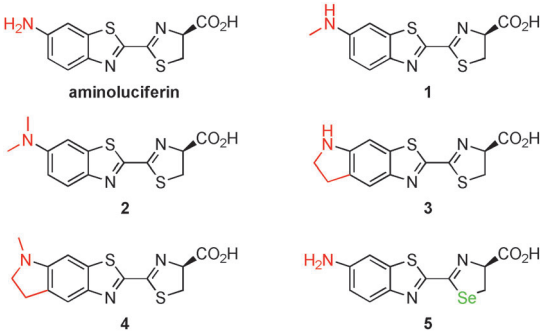


Figure 1. Modules of luciferin analogues.

dyes.[8] The three analogues show excellent bioluminescence properties and great potential in bioluminescence imaging. Herein, these important discoveries are summarized.

Even though aminoluciferins were actively studied only in recent years, it was back in 1966 when White, McElroy, et al. established that the 6′-hydroxy group of D-luciferin can be replaced with an amino group.[9] Relative to D-luciferin, aminoluciferin emits light at a longer wavelength with wild-type firefly luciferase (593 vs. 557 nm).[10] Recently, Miller et al. extended this concept to the design of four alkylaminoluciferins (**1**–**4**, Scheme 2).[5] Compared with D-luciferin and aminoluciferin, **1**–**4** exhibited red-shifted emission (Table 1)



Scheme 2. Structures of aminoluciferin and luciferin analogues **2**–**5**.

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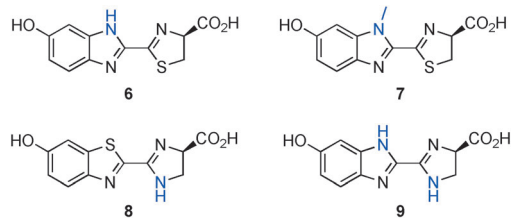
Table 1: Emission maxima for wild-type luciferase and Ultra-Glo with each luciferin substrate, as well as their applications in bioluminescence imaging.

Substrate	λ_{max} [nm]		Detection in vivo
	wild-type	Ultra-Glo	
d-luciferin	557	551	yes
aminoluciferin	594	576	yes
1	609	590	no
2	623	601	no
3	599	583	no
4	607	600	no
5	600	not given	yes
6	578	not given	yes
7	460	not given	yes

with wild-type firefly luciferase, but with a significant reduction in light output. The authors indicated that the low intensity of light emission is due to 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. However, when tested with Ultra-Glo luciferase (a highly mutated form of luciferase designed to be more stable and resistant to inhibition during high-throughput screening assays) in P450-Glo buffer, **3** and **4** exhibited dramatically higher output across a wide concentration range (1–100 μM).[5] For example, **3** emitted 5.7 times more light than aminoluciferin and 3.2 times more light than D-luciferin at a concentration of 1 μM. Unfortunately, Ultra-Glo is not available as a genetic construct that can be introduced into living organisms for bioluminescence imaging.

Through combination of the electron-donating effect of an amino group and the polar effect of a selenium atom,[11] Moerner and co-workers developed a selenium analogue of D-luciferin (**5**, Scheme 2) that shows red-shifted bioluminescence emission relative to its sulfur-containing analogue.[6] In the presence of ATP, Mg^{2+}, and oxygen, **5** emits a red bioluminscence signal with purified native Fluc. This signal is readily distinguished from the yellow-green bioluminscence signal of D-luciferin and the orange bioluminscence signal of aminoluciferin. The percentage of integrated bioluminscence emission above 600 nm (an important criterion for transmission through tissue in in vivo bioluminscence imaging) for **5** is 55%, which is higher than the values for D-luciferin and aminoluciferin (23% and 41%, respectively). Analogue **5** has been used for bioluminescence imaging in human breast adenocarcinoma cells (MDA-MB-468) and in nude mice with subcutaneous tumor xenograft of a MDA-MB-468 cell line, both of which were constitutively expressing FLuc.

Very recently, Prescher and co-workers developed two types of luciferin analogues, that is, luciferins **6** and **7** with a benzimidazole scaffold and **8** and **9** with an imidazoline scaffold, through replacement of the benzothiazole and thiazoline units in D-luciferin with benzimidazole and imidazoline rings, respectively (Scheme 3).[7] Incubated with the enzyme, ATP, and coenzyme A (to reduce product inhibition) at pH 7.4, **6** and **7** showed strong emission. By contrast, no light emission was observed with **8** and only minimal light



Scheme 3. Structures of luciferin analogues **6**–**9**.

emission was observed with **9**, an observation that can be attributed to poor binding to Fluc, lower efficiencies of light production, or a combination of those and other factors. It is noteworthy, that **7** emits the largest amount of blue light among the known Fluc substrates, thus suggesting that **7** may be useful for multicomponent imaging applications, as its emission can be readily resolved from those of other luciferins through the use of appropriate filter sets. Bioluminescence imaging experiments in Fluc-expressing HEK 293 cells indicated that the benzimidazole scaffolds are sufficiently biocompatible for use in cellular imaging studies.

Although the above-mentioned hydroxy- or amino-substituted luciferin analogues show strong emission, no light is emitted when the 6′-hydroxy or 6′-amino groups are replaced with methoxy, ethoxy, or *N*-acetylamino groups.[12] These results provide the opportunity to develop various Turn-ON bioluminescence probes in a simplified method.

Figure 2 shows the general design of a Turn-ON bioluminescence probe. The modification of (amino)luciferin with a trigger at the 6′-hydroxy or 6′-amino group prevents light

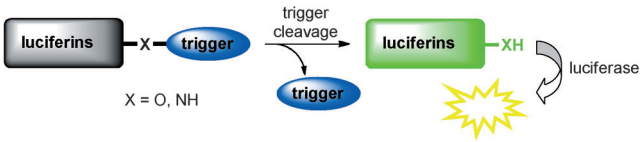


Figure 2. General mechanism for modular Turn-ON bioluminescence probes.

emission. However, removal of the trigger releases (amino)-luciferin for subsequent reaction with the firefly luciferase to produce a photon. Through this approach, appealing bioluminescence probes should be available by utilizing the experiences accumulated in the development of fluorescent probes.[13] For example, three luciferin-based bioluminescence probes for imaging of caspase-1 activity, β-lactamase activity, and hydrogen peroxide production, have been independently reported by the groups of Kindermann,[14a] Rao,[14b] and Chang,[14c] respectively.

Apart from the aforementioned simple alteration of atoms of the original D-luciferin, replacement of the entire benzothiazole core of D-luciferin with a quinoline, naphthalene, or coumarin unit provides an alternative approach to luciferin analogues.[15a] In a publication of 2010, Nagano and co-workers reported a class of luciferin analogues that bear an amino group on their benzothiazole, quinoline, naphthalene, or coumarin scaffold.[15b] These analogues emit biolumines-

cence in various colors: red, orange, yellow, and green. The authors utilized one of these analogues, coumarylaminoluciferin, as an energy-donating substrate and yellow-fluorescent protein (YFP) as the energy-accepting fluorophore to construct a bioluminescence resonance energy transfer (BRET) system with click beetle red luciferase (CBRluc), and obtained a clearly bimodal bioluminescence spectrum.

In this Highlight, we summarized recent progress on luciferin analogues with strong emission. These analogues are expected to be used in the development of bioluminescence probes for numerous biological applications, such as probing biologically important species species and imaging biologically important processes.

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