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Chapter

Near-Infrared Luciferin Analogs for In Vivo Optical Imaging

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Abstract

The firefly bioluminescence reaction has been exploited for in vivo optical imaging in life sciences. To develop highly sensitive bioluminescence imaging technology, many researchers have synthesized luciferin analogs and luciferase mutants. This chapter first discusses synthetic luciferin analogs and their structure–activity relationships at the luminescence wavelength of the firefly bioluminescence reaction. We then discuss the development of luciferin analogs that produce near-infrared (NIR) light. Since NIR light is highly permeable for biological tissues, NIR luciferin analogs might sensitively detect signals from deep biological tissues such as the brain and lungs. Finally, we introduce two NIR luciferin analogs (TokeOni and seMpai) and a newly developed bioluminescence imaging system (AkaBLI). TokeOni can detect single-cell signals in mouse tissue and luminescence signals from marmoset brain, whereas seMpai can detect breast cancer micro-metastasis. Both reagents are valid for in vivo bioluminescence imaging with high sensitivity.

Keywords: Firefly bioluminescence, Bioluminescence imaging, Structure–activity relationships, Multicolor, Near-infrared light

1. Introduction

In Japan, watching the light of fireflies has been a summer tradition for over one thousand years. Modern fireflies are known to glow yellow-green, but in ancient times they emitted a dark green luminescence, as confirmed by recent molecular biology techniques [1]. The detailed mechanism of firefly bioluminescence is described in previous chapters. This chapter focuses on synthetic substrates of firefly luciferase, which are employed in firefly bioluminescence imaging (BLI).

In recent biological research, BLI technology has observed biological events in vivo [2–8]. For example, in cancer research, BLI has been applied to real-time monitoring of gene expression, cell numbers, and other biological events in transgenic mouse models [9–16]. Our group has developed firefly substrate analogs for use in these research fields.

The firefly bioluminescence reaction proceeds via the oxidation of D-luciferin (1, LH2, Figure 1) catalyzed by firefly luciferase (Fluc) in the presence of adenosine triphosphate (ATP), Mg$^{2+}$ and O$_2$ by a two-step reaction. In the first step, LH2 is adenylated with ATP, and is then oxidized by O$_2$, forming excited-state oxyluciferin that relaxes to the ground state with yellow-green light emission ($\lambda_{max} = 560$ nm) [17–19]. However, yellow-green light is not able to easily penetrate biological tissues [20], and is useful only for imaging shallow tissues such as subcutaneous tissues. To detect signals from deep tissues such as brain and lung [21], near-infrared (NIR)
light should be used, as it is highly permeable to biological tissues [20] and is suitable for in vivo deep tissue imaging [21]. Recently, many synthetic luciferin analogs have been reported. Our group has synthesized various luciferin analogs and compared them with 1. By studying the structure–activity relationships of these analogs and Photinus pyralis (Ppy) luciferase, we have developed luciferin analogs that produce wide-spectrum light (from blue to red), along with NIR luciferin analogs (AkaLumine, TokeOni, seMpai) for BLI. Our different analogs are described in this chapter.

2. Luciferin analogs of firefly luciferase

Many researchers have synthesized luciferin analogs, and different substrates reacted with luciferases exhibit different luminescence activities [22–24]. Most luciferin analogs are formed by modifying the benzothiazole moiety of 1. Analogs of 1 were first synthesized by White et al. in 1966. They showed that aminoluciferin (2, Figure 1), in which the hydroxyl group of benzothiazole is replaced with an amino group, can function as a substrate of Fluc and emit red bioluminescence [25].

2.1 Development of luciferin analogs based on LH2

As mentioned above, many luciferin analogs are prepared by modifying the benzothiazole moiety of 1 [22–24]. For instance, N-cycloaminolucifersins (2a–f, Figure 2A) are prepared by cyclizing the NH$_2$ of 2. These analogs were reported by two independent groups, who synthesized them by different routes [26, 27] (Figure 2B–C). When reacted with Fluc, 2a–f show longer wavelengths than 1, probably reflecting the electron donation effect of cycloamine substitutes. Comparing the bioluminescence activities and emission wavelengths of analogs 2e and 2f on Fluc and Fluc mutant luciferase R218K, it was found that 2e/Fluc and 2f/R218K produced light at 604 and 614 nm, respectively, whereas 2e/R218K and 2f/Fluc produced no light [26]. The interaction between the active site of luciferase and the substrate is very critical, indicating that the structures of both reactants play essential controlling roles in luminescence activity.

Miller et al. synthesized CycLuc1 (7a, Figure 3) by fusing N-cycloalikylation of 2 with benzothiazole [28]. Analog 7a exhibited a longer luminescence wavelength on Fluc (599 nm) than 1 on Fluc, and was emitted more intensely than 1 in a Photuris pennsylvanica firefly luciferase mutant (Ultra-Glo). The BLI of 7a detects the signals from deep organs such as brains and lungs [21, 29]. Li et al. synthesized CybLuc (7b, Figure 3) by substituting the hydroxy group of 2 with a cycloamino group. Analog 7b produced light at 603 nm and its BLI detected the signals from mouse brain [30].

Iwano et al. developed luciferin analogs 8a–g (Figure 4A) by substituting the benzothiazole moiety of 1 with a simple benzene ring and extended π-conjugations [31]. Olefins were extended by the Wittig reaction from 10c–d and 12e–f as starting
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In this synthesis, hydrolysis was stepwise followed by condensation with D-Cys(STrt)-OMe, thiazoline cyclization, and methyl ester deprotection (Figure 4B). The obtained analogs 8a–f produced luminescent colors over a wide range (blue to red) [31]. Among these, AkaLumine (8e), which produces light at 675 nm, is a leading compound for NIR luciferin analogs, as described in Section 2.2. Later, analog 8e was used as a reagent for BLI. In the same paper, 3-hydroxyl analog 8g (Figure 4A) was also synthesized, but this analog produced no light [31]. Therefore, the position of the OH substituent is critical in the firefly bioluminescence reaction.

In contrast, the thiazoline site is rarely modified. Conley et al. synthesized a seleno-aminoluciferin analog 13a (Figure 5A) in which the S of the thiazoline ring of 2 was replaced with Se [32], and Ioka et al. synthesized O- or C-substituted analogs 13b–c (Figure 5A) [33]. Analog 13a, which produced light at 600 nm, was synthesized by the cyclization reaction of selenocysteine (Figure 5B) [32]. Analog 13b was obtained by synthesizing an amide 16b synthesizing an amide from D-serine, cyclizing it with diethylaminosulfur trifluoride (DAST), and hydrolyzing it with Amano lipase (Figure 5C). Analog 13c was prepared by coupling with bromothiazole 19 and pyrrolidione carboxylate 21 to form glutamate-linked benzothiazole 16c, and cyclizing 16c with trifluoroacetic acid (TFA) (Figure 5D). Interestingly, 13c produces light at 547 nm, whereas 13b is non-bioluminescence [33] but shows chemiluminescent ability. This result indicates that the thiazoline of 1 is an essential moiety for recognizing the activity site in luciferase.

Figure 2.
Structures and synthetic routes of luciferin analogs 2a–f. (A) N-cycloaminoluciferin analogs 2a–f, (B) the synthetic route reported by Miller et al. [28], and (C) the synthetic route reported by Hirano et al. [29].

Figure 3.
Structures of CycLuc1 (7a) and CybLuc (7b).
2.2 Structure–activity relationships for developing NIR luciferin analogs

Based on these structure–activity relationships, additional luciferin analogs have been designed and synthesized for NIR light production. For example, Anderson et al. synthesized iLH2 (22, Figure 6) by inserting an olefin into the structure of 1. Analog 22 produced NIR light at 706 nm [34]. However, the luciferase used at that time was a mutant (S284T), and the luminescence wavelength on Fluc was 670 nm. The same authors developed an in vivo dual-imaging technique that combines 1 and 22 with two different luciferases. This system can potentially observe new biological events by tracking two processes simultaneously [35]. Hall et al. synthesized NH2–NpLH2 (23, Figure 6) by extended conjugation of 2. Analog 23 produced no light with Fluc, but its luminescence wavelength was extended to 743 nm by reaction with CBR2, a mutant luciferase of click beetles (Pyrophorus plagiophthalamus) [36]. All of these studies achieved long-wavelength emissions from mutant luciferases, but their luminescence activity is much lower than that of combinations of 1 and wild type Ppy luciferase.

Figure 4.
Structures of 8a–g (A) and their synthetic routes 8a–f (B).
Meanwhile, Maki’s group has developed a number of analogs based on the structure of 8e, which are expected to produce NIR light. Miura et al. formed a mother skeleton by a coupling reaction, and thus synthesized biphenyl analogs 24a–c (Figure 7 and 8A) [37]. Analog 24a produced light at 675 nm, but the luminescence intensity was weak. Although its conjugation was more extended than in 8e, the luminescence wavelength of 24a did not change as that of 8e (675 nm). This result suggests that the biphenyl moiety rotates and reduces the fluorescent intensity.

Kiyama et al. synthesized cyclic amino analogs of 8e (25a–d, Figure 7) [38] from 4-fluorobenzaldehyde 32 as the starting material. They replaced the F group with various secondary amines, and conducted the Horner–Wadsworth–Emmons
reaction, condensation and cyclization to obtain the final compounds 25a–d (Figure 8B). Despite containing an electron-donating amino group, 25a–d produced luminescence at almost the same wavelengths (656–667 nm) as 8e (668 nm). However, the luminescence intensity of 25a was approximately four times stronger than that of 8e. The fluorescence quantum yields of 8e and these cyclic amino analogs 25a–d were almost identical, suggesting that the luminescence intensity largely depends on the reactivity with luciferase.

The luminescent wavelength can be lengthened not only by extending the $\pi$-conjugations and introducing an electron donate substituent, but also by introducing an allyl group. Kitada et al. synthesized allyl analogs 26a–b (Figure 7) by introducing allyl groups into 8c, 8e and naphthol analogs 27a–d (Figure 7). The analogs were introduced by two routes: Pd-catalyzed Stille coupling (Figure 8C) and Claisen rearrangement (Figure 8D) [39]. Although these analogs delivered very low luminescence intensities, their wavelength shift was long (approximately 15–35 nm). As the allyl group itself does not affect the $\pi$-conjugations of the substrate structure, it was considered that induce fitting was occurred at the luciferase active site and stabilized the substrate metabolite to lower energy state conformation. To develop a long-wavelength, Kitada et al. synthesized NIR analog (28 in Figure 7) by introduced both an electron-donating NMe$_2$ and an allyl group. When reacted with Fluc, 28 produced NIR light at a sufficiently long-wavelength (705 nm), but the luminescence intensity was only 1.3% of that of 8e. Although the allyl group extends the luminescent wavelength, it greatly reduces the luminescence intensity, which is a major disadvantage.

The aromatic ring site has also been targeted in the development of potential NIR emitters. Saito et al. synthesized three analogs 29a–c (Figure 7) in which the
aromatic ring of 8e was replaced with an N-heteroaromatic ring [40]. Interestingly, the luminescence wavelengths of three analogs depended on the positions and numbers of their N atoms; 29a produced red light at 645 nm, seMpai (29b) produced NIR light at 675 nm, and 29c produced orange light at 625 nm. This result highlights the importance of interactions between the luciferase active site and the N atoms of the heterocycle. Although the luminescence wavelength of all three analogs were shorter than 700 nm, the wavelength was changed with a single atom, suggesting that interaction with the luciferase active site is an important part of molecular design.

3. Solubility enhancement of TokeOni and seMpai (29b) for sensitive in vivo imaging

As mentioned in the previous section, our research group has developed multicolor luciferin analogs for in vivo imaging. The luminescence activities of AkaLumine (8e) and seMpai (29b) are potentially suitable for BLI. Therefore, the usefulness of these analogs as reagents for in vivo BLI was evaluated in further animal experiments.
3.1 Development of AkaBLI (TokeOni and AkaLuc)

Prior to administering the reagent into the animal models, we increased the aqueous solubility of 8e (which is inherently low) and developed an HCl salt of 8e, AkaLumine-HCl (Tokeoni) [21]. In ultrapure water, the solubility of TokeOni was 40 mM, approximately 20 times higher than that of 8e (2 mM). Therefore, TokeOni enable to administered to experimental animals with a smaller solution volume and higher concentration than 8e. TokeOni/Fluc BLI was performed with significantly higher sensitivity than LH2/Fluc and CycLuc1/Fluc in the lungs [21] and brain [41] of mice. However, as the in vitro luminescence intensity of TokeOni/Fluc was lower than that of LH2/Fluc, it must be improved before imaging large animals such as marmosets.

Accordingly, Iwano et al. developed a mutant luciferase Akaluc specialized for TokeOni. They developed the artificial bioluminescence system AkaBLI, which combines TokeOni with Akaluc [42] (a mutation of 28 amino acid residues on Ppy luciferase). The AkaBLI luminescence intensity was approximately 10-fold higher in cells (in vitro), 52-fold higher in mouse lungs (in vivo), and 1400-fold higher in mouse brain tissue (in vivo) than LH2/Fluc luminescence intensity. In addition, AkaBLI detected single-cell signals from mouse lung and to quantified 1–10 cells. For large animal imaging, the authors inserted the Akaluc gene into an adenovirus (AAV) vector, and introduced the recombinant AAV into the striatal neurons of marmosets. AkaBLI achieved video-rate real-time imaging of marmoset brains.

3.2 BLI with seMpai

seMpai (29b) was developed to improve two weak points in TokeOni: neutral pH and disturbance by hepatic background signals. As TokeOni is acidic [43], it may cause acidosis when injected; moreover, TokeOni detects the hepatic background signals, which are not detected by LH2 [43, 44]. Due to the effect of N atom, seMpai was sufficiently soluble for administration to experimental animals and 69 mM was dissolved in phosphate buffered saline (pH 7.4) [40]. In Fluc-expressing lung cancer model mice, the sensitivities of seMpai and TokeOni were not significantly different [40], but seMpai detected no hepatic background signals and seMpai BLI detected breast cancer micro-metastasis [43]. When repeated with TokeOni and LH2, this experiment was unsuccessful. Although seMpai/Fluc was less sensitive than AkaBLI for single-cell imaging, its imaging sensitivity could be improved mutant luciferases such as Akaluc.

Fukuchi et al. monitored the expression of brain-derived neurotrophic factor (BDNF) in Bdnf-luc transgenic mice with LH2, TokeOni, and seMpai [45]. TokeOni achieved the most sensitive BLI, and seMpai and LH2 were comparable. The result probably reflects the different abilities of the compounds to penetrate the blood–brain-barrier. This result also indicates the necessary of evaluating the imaging reagent in terms of both its luminescence activity and pharmacokinetics. Additionally, biocompatibility such as cytotoxicity of TokeOni and seMpai has not been reported yet. No acute toxicity or adverse side effects were observed in mice when these compounds were administrated at a concentration of 33 mM [40], however, preliminary experiments are recommended when using new analogs, not limited to TokeOni and seMpai.

4. Conclusion

By investigating the structure–activity relationship of luciferin analogs, researchers have developed various methods for tuning the luminescence
wavelengths of these analogs. However, the intensity of the luminescence is poorly controlled. If the luminescence intensity and structure–activity relationships could be associated by a predictable law, we could synthesize new luciferin analogs with high luminescence intensity, and further develop an imaging technology with greater usefulness than conventional technologies.

For a practical imaging technology, both the luminescent substrate/enzyme activity and the pharmacokinetics are very important. Improving the various properties of the substrates and enzymes will enhance the sensitivity of bioluminescence imaging.

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Conflict of interest

The authors declare no conflict of interest.

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