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Toward Bioluminescence in the Near-Infrared Region: Tuning the Emission Wavelength of Firefly Luciferin Analogues by Allyl Substitution

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ABSTRACT

The synthesis and bioluminescence of allyl-substituted luciferin derivatives as substrates for firefly luciferase are reported. The allylation of luciferins induced bathochromic shift (15–40 nm) of the bioluminescence emission. Upon combination with other chemical modifications for bioluminescence wavelength tuning, novel red emitting luciferin analogs were obtained with emission maxima at 685 and 690 nm.

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Luminescent (e.g. fluorescent and bioluminescent) systems that emit light in the red or near-infrared (NIR) region have recently been reported as powerful chemical probes for the observation of biological phenomena and other applications.[1–4] In particular, the demand for NIR-luminescent systems for non-invasive and operationally simple diagnosis techniques, such as *in vivo* imaging, has grown drastically.[5] The optical window (650–950 nm) is expected to allow sensitive detection during *in vivo* imaging by avoiding unexpected absorption of visible and IR light by living specimen. While various NIR-fluorescent systems have been developed, including cyanines[6,7] or boron-dipyrromethenes (BODIPYs),[8] only few NIR-bioluminescent systems have been reported.[9–16] In the luciferin bioluminescence reaction, photons are emitted from the excited state of oxyluciferin (**2**), which is a metabolic product of the firefly luciferin (**1a**) after adenylation by firefly luciferase, followed by a reaction with molecular oxygen (Figure 1).[17] The excited state of **2** emits only at *ca*. 560 nm in the presence of natural luciferase. During the course of our studies on the development of luciferin analogues,[10,11,16] we discovered that the replacement of benzothiazole with other aromatic rings causes a dramatic shift of the emission wavelength. We have described a method to tune the emission wavelength of luciferins (Figure 1), in which the extension of the π-conjugation between the thiazoline moiety and the aromatic ring affords a large bathochromic shift (*ca*. 100 nm), while substitution of the hydroxide moiety with dimethylamine provides a smaller bathochromic shift (*ca*. 30

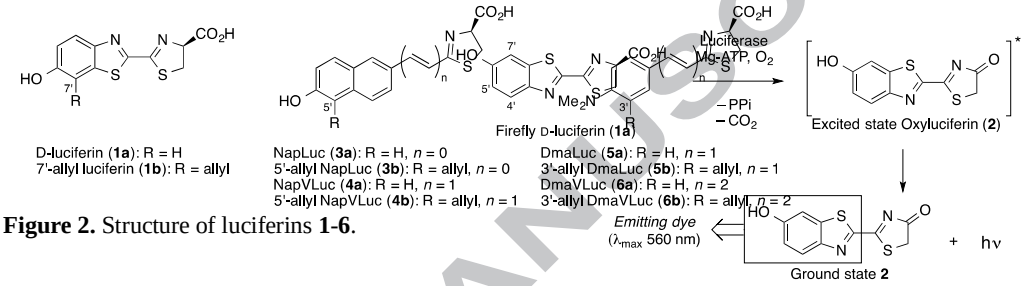

Figure 2. Structure of luciferins **1**-**6**.

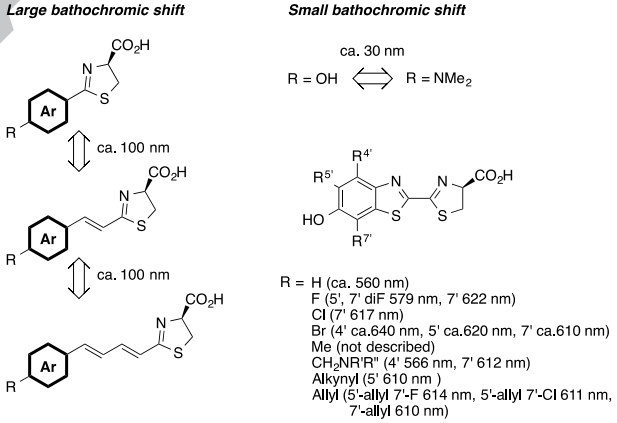

Figure 1. Bioluminescence reaction mechanism and structure–wavelength relationship for luciferins.

nm).[10] This approach has been applied to other luciferin derivatives, such as naphthyl luciferin[18] (NapLuc, **3a**; $n = 0$) and dimethylanilyl luciferin (DmaLuc, $n = 0$) to obtain the red-emitting vinyl analogues NapVLuc (**4a**), DmaVLuc (**5a**), and DmaDVLuc (**6a**) (Figure 2). Especially **6a** exhibits promising bioluminescence properties in the NIR region (λ_{em} = 680 nm) that could potentially be exploited for the *in vivo* imaging of deep tissue.[19,20] However, the further extension of the π-conjugation of luciferin analogues renders the compound instable, resulting in a dramatic suppression of the luminescence activity. Another type of NIR luciferins has been developed based on the modification of D-aminoluciferin. Cyclic alkylaminoluciferins (CycLucs)[14] and *N*-cycloalkylaminoluciferins such as cybLuc[15] exhibit red (600–650 nm) bioluminescence and these luciferins can be used for *in vivo* bioimaging of deep tissue such as brain tissue.[15,21] Modifications of D-luciferin (**1a**) by halogenation,[22–24] alkylation,[25] and alkynylation[26] at the C-4', -5', and -7' positions have also been reported to produce intriguing bioluminescence features. Recently, we have reported the synthesis of C-7' ally luciferin (**1b**) and its interesting bio-orthogonal luminescence properties.[27] The C-7' allylation of **1a** induced a bathochromic shift (40 nm) of the emission wavelength, implying a weaker enzymatic recognition of the benzothiazole ring and the formation of an excited state with lower energy. However, the detailed effects of such an allyl substitution on the bioluminescence emission of other known luciferase substrates remain to be elucidated. Based on these observations, we herein report the application of said allylation method for the further tuning of the bioluminescence wavelength of known luciferins.

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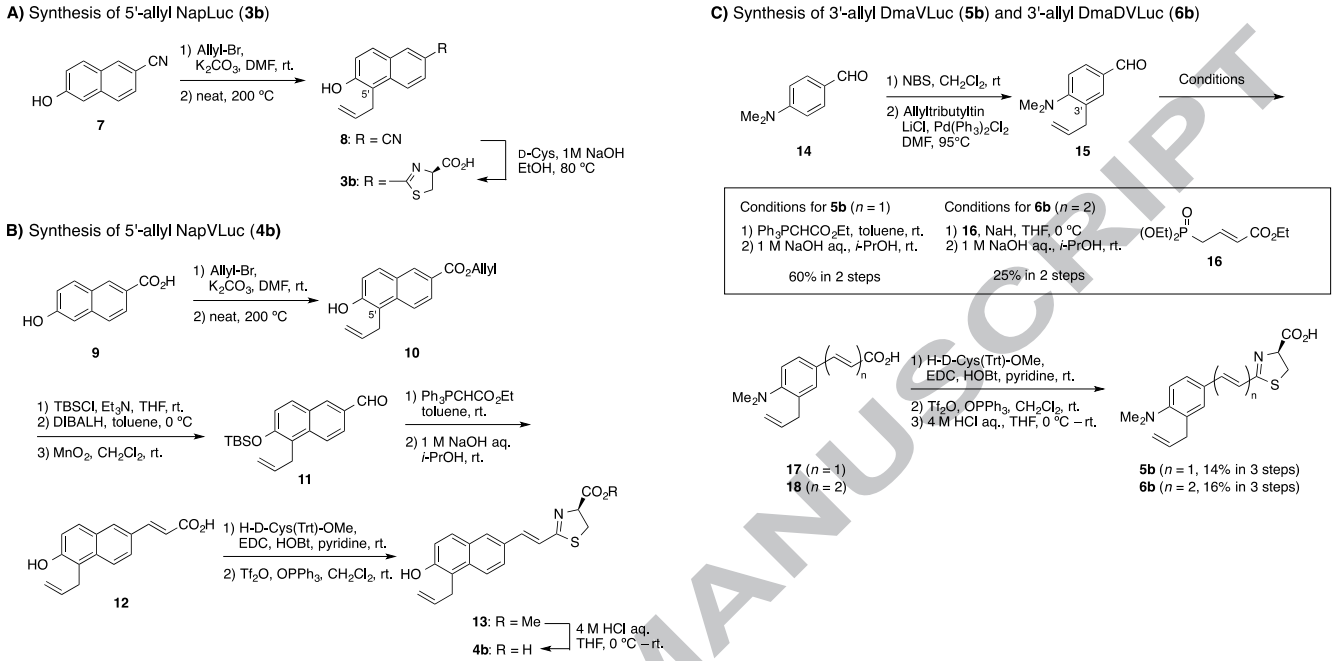
The effect of allyl substitution on the firefly bioluminescence of luciferin derivatives (**3b**, **4b**, **5b**, and **6b**) was investigated (Figure 2). The synthesis of **3b** started with the *O*-allylation of 6-cyano-2-naphthol (**7**) to afford an allyl ether, followed by a Claisen rearrangement, which selectively proceeded at the C-7' position to afford **8** in high yield. A cyclocondensation with D-cysteine



Scheme 1. Syntehsis allyl-substituted luciferins (**3b**, **4b**, **5b**, **6b**)

ultimately converted **8** into 5'-allyl NapLuc (**3b**) (Scheme 1A). To access 5'-allyl NapVLuc (**4b**), a twofold *O*-allylation of commercially available 6-hydroxy-2-naphthoic acid (**9**) was carried out, followed by a Claisen rearrangement to afford 5'-allyl naphthalene **10**. After protection of the hydroxyl group with TBS, the allyl ester was reduced with DIBALH and subsequently oxidized into aldehyde **11** (Scheme 1B). A Wittig reaction then furnished a vinyl ester, which yielded carboxylic acid **12** upon hydrolysis with NaOH aq. Subsequently, **12** was condensed with H-D-Cys(Trt)-OMe, followed by the formation of a thiazoline ring with Tf_2O and $OPPh_3$[28] to furnish **13**. The desired luciferin **4b** was ultimately isolated following the hydrolysis of the methyl ester moiety. Similarly, the synthesis of 3'-allyl DmaLuc (**5b**) and DmaVLuc (**6b**) started with the bromination of commercially available 4-dimethylaminobenzaldehyde (**14**), followed by a Pd-catalyzed Stille coupling with allyltributyltin to afford allyl arene **15** (Scheme 1C). A Wittig or Horner–Wadsworth–Emmons reaction of the aldehyde with phosphonic acid diester **16** afforded the corresponding vinyl and dienyl esters, which yielded the carboxylic acids **17** and **18** upon hydrolysis with NaOH aq. The desired luciferins **5b** and **6b** were isolated in the same manner as **4b**.

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The bioluminescence (BL) spectra of the synthetic luciferins with recombinant firefly luciferase (Ppy; *Photinus pyralis*) are

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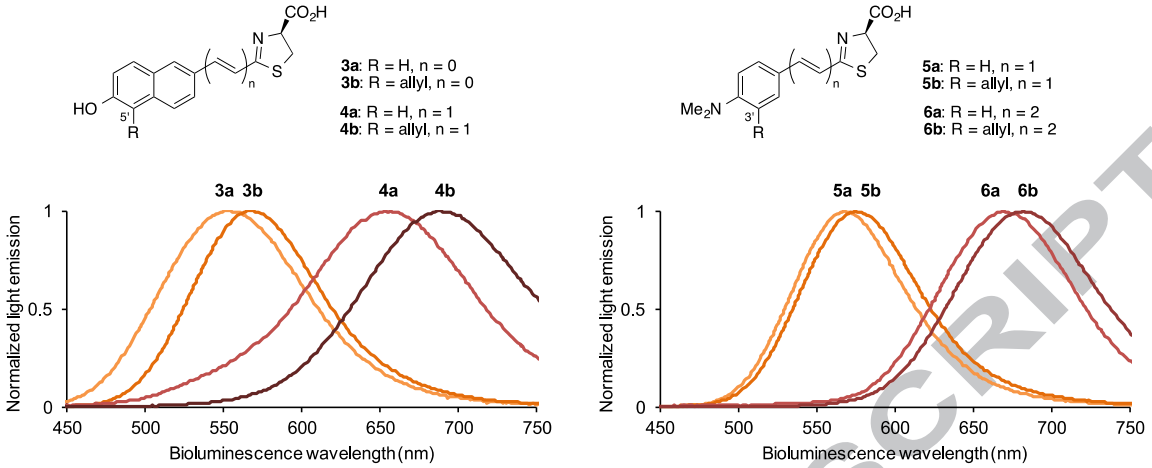

Figure 3. Normalized bioluminescence emission spectra for **3a** (λ_{max} = 555 nm), **3b** ((λ_{max} = 570 nm), **4a** (λ_{max} = 655 nm), **4b** (λ_{max} = 690 nm), **5a** (λ_{max} = 565 nm), **5b** (λ_{max} = 575 nm), **6a** (λ_{max} = 670 nm), and **6b** (λ_{max} = 685 nm).

Table 1. Luminescence wavelength and kinetic properties of the synthetic luciferins **1**-**6**

Comp.	BL λ_{max} [nm][a]	CL λ_{max} [nm][b]	rel. V_{max} [e]	K_m [f] [μM]
1a	570	585[c]	100	160 ± 58[g]
1b	610	580[c]	4.1	370 ± 50
3a	555	515[c]	0.096	58 ± 13
3b	570	520[c]	0.12	28 ± 5.6
4a	655	605[c]	0.10	3.2 ± 0.88
4b	690	610[c]	0.13	3.3 ± 0.54
5a	565	580[d]	6.3	4.4 ± 2.0
5b	575	580[d]	0.10	71 ± 27
6a	670	665[d]	1.9	2.0 ± 0.3
6b	685	660[d]	0.12	17 ± 4.1

[a] Bioluminescence emission maxima; [b] Chemiluminescence emission maxima; [c] Chemiluminescence was measured using 1.25 mM luciferin methyl ester and 125 mM *t*-BuOK in DMSO; [d] Chemiluminescence was measured using 1 mM luciferin, T_3P, and Et_3N in DMF;[29 e,f] The kinetic constants are apparent values, determined by measurement of the initial rates of light emission from 0.02 μM to 200 μM luciferin, 2 μg Ppy and 80 μM ATP-Mg.[30,31] The rel. V_{max} values were shown by comparison with the value for **1a**. [g] The K_m value strongly depends on the measurement conditions. The value of **1a** obtained in this study is essentially consistent with previously reported data that were measured under identical conditions.[18,31]

shown in Figure 3. The allylated NapLuc **3b** displays a bathochromic shift (15 nm) of the emission maximum compared to that of **3a** (Table 1). On the other hand, allylated NapVLuc **4b** exhibits a larger bathochromic shift (35 nm) of its peak maximum relative to that of **4a**, with an emission wavelength similar to that of **1b**. Regarding the effect of allyl substitution on the DmaLuc derivatives, **5b** (10 nm) and **6b** (15 nm) display bathochromically shifted peak maxima compared to the non-allylated compounds. We then measured the chemiluminescence (CL) spectra, which reflect the energy level of the excited states without any effect of the enzyme, in order to evaluate the effect of allyl substitution. However, the observed chemiluminescence maxima of allylated luciferins (**1**–**6b**) are not significantly changed relative to those of the original luciferins (**1**–**6a**), indicating that allyl substitution on the aromatic rings of luciferin does not significantly affect the energy levels of the oxyluciferins in excited singlet state. Because relative V_{max} (rel. V_{max}) values were determined by using the data of emitted photons with a constant concentration of the luciferase, a rel. V_{max} value is proportional to the product of the rate constant of the overall reaction (k_{cat}) and the bioluminescence quantum yield (Φ_{bl}). The rel. V_{max} values of **3a** and **3b** are similar to each other, and **4a** and **4b** also have similar rel. V_{max} values, indicating that the allyl modification to the hydroxyl-substituted analogues has an insignificant effect on the k_{cat} and Φ_{bl} properties. Interestingly, the rel.

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V_{max} values of **5b** and **6b** are one order of magnitude less than those of **5a** and **6a**, respectively. This result indicates that the allyl modification to the dimethylamino-substituted analogues affects an interaction between the substrate and the luciferase, resulting in a decrease of the reaction rate and/or the efficiency of photon production. The bathochromic shifts observed in **5b** and **6b** are probably related to the K_m values, which are higher than those of **5a** and **6a**, implying a weaker enzymatic recognition similar to **1b**.[27] However, even though NapLuc derivatives also showed bathochromic shifts, the kinetic properties indicated equally strong enzymatic recognition. This result probably implies a different binding mode for NapLuc derivatives with luciferase, which stabilized the conformation of lower energy state of their metabolites. The *in vivo* bioimaging with NIR substrates **4b** and **6b** was investigated in CAG-ffLuc-cp156 transgenic mice[33] (Fig. 4A). Low but detectable bioluminescence was observed for each substrate. Interestingly, the overall photon output of **4b** and **6b** was 2.3 and 3.4%, respectively, compared to that of **1a** in spite of the lower V_{max} values (Fig. 4B). **6a** also exhibited brighter *in vivo* than *in vitro* bioluminescence, due to the higher membrane permeability, lower saturation concentration, and NIR bioluminescence.[19] The photon output of **4b** and **6b** should accordingly also be enhanced due to these bioluminescence and pharmacokinetic properties.

In conclusion, we have synthesized a series of allylated luciferins as prospective bioluminescence probes. Allylic substitution on the aromatic moieties induces small but detectable bathochromic shifts (15–40 nm) of the emission wavelength. This approach can be combined with other known methods for the tuning of the bioluminescence wavelength. The luciferin analogues **4b** and **6b** exhibit NIR emission at 690 and 685 nm, respectively. To our knowledge, **4b** is thus the substrate with the longest emission wavelength for native firefly luciferase. Recently, a significant improvement of luminous efficacy of synthetic luciferins with mutant luciferases has been reported.[14,25,34] We have also reported the enzyme specificity of 7'-allyl luciferin (**1b**), i.e., the combination with **1b** and Eluc, a click beetle luciferase, improved the luminous efficacy.[27] Although the NIR luciferins reported here exhibit much lower emission intensity, it should be possible to improve the luminous efficacy with mutant luciferase(s). Most importantly, the modification strategy for known luciferins should provide access to the next generation of NIR bioluminescence tools.

Acknowledgments

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Supplementary data

Supplementary data (characterization, bio- and chemiluminescence assays and bioluminescence imaging) associated with this article can be found in the online version at XXX.

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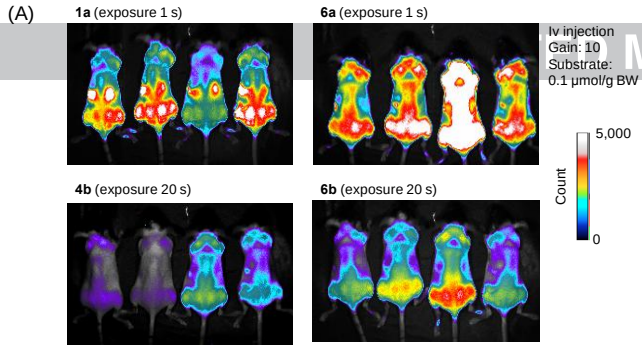

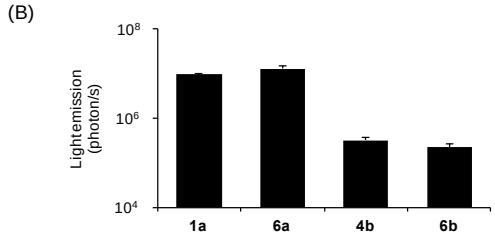

Figure 4. In vivo bioluminescence with luciferin analogues. A) Luciferins (0.1 μM/g BW) were administered into CAG-ffLuc-cp156 transgenic mice. Bioluminescent images at respective exposure time are shown. B) Photon counts of the images during 1s exposure. Error bars represent the standard error of the mean (n = 4).

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Highlights

- Allyl-substituted luciferin derivatives were designed and synthesized.
- Bioluminescence properties of allyl luciferin derivatives were evaluated.

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- The allylation of luciferins induced bathochromic shift of their bioluminescence.
- The longest near-infrared bioluminescence emission wavelength was achieved.

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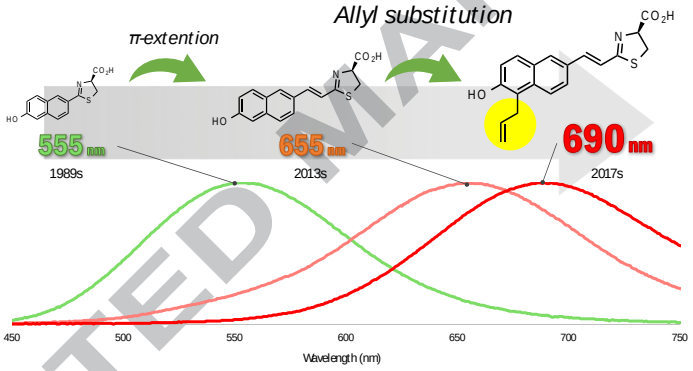

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