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Synthesis of *Latia* luciferin benzoate analogues and their bioluminescent activity

Mitsuhiro Nakamura,[a,b] Mizuki Masaki,[a] Shojiro Maki,[a] Ryo Matsui,[a] Minako Hieda,[a] Masashi Mamino,[a] Takashi Hirano,[a] Yoshihiro Ohmiya[b] and Haruki Niwa[a,*]

[a]*Department of Applied Physics and Chemistry, The University of Electro-Communications, Chofu, Tokyo 182-8585, Japan*
[b]*The Special Division for Human Life Technology, National Institute of Advanced Industrial Science and Technology (AIST), Ikeda, Osaka 563-8577, Japan*

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Abstract—The bioluminescent system of the univalve shell *Latia neritoides* exhibits a luciferin–luciferase reaction. We study the enol formate structure of *Latia* luciferin, which is expected to be important for luminescent activity. The *Latia* luciferin analogues with an enol substituted benzoate moiety were synthesized and their bioluminescent activity was measured. The *Latia* luciferin benzoate analogues delay emission for natural luciferin in bioluminescence, indicating that the *Latia* bioluminescent activity can be controlled by the design of the enol ester.

The univalve shell *Latia neritoides*, which inhabits clear and shallow streams on the North Island in New Zealand, secretes a green luminescence mucus (536 nm) by physical or chemical stimulation.[1] The *Latia* bioluminescent system exhibits a luciferin–luciferase reaction in the presence of oxygen.[2] The low water solubility of *Latia* luciferin decreases the luminous efficiency ($\phi = 0.003$–0.007).[2b] While the luciferin of the firefly and sea firefly having bioluminescence are derived from amino acids, the *Latia* luciferin (**1**) is a terpenoid including the 2,6,6-trimethylcyclohexene and enol formate.[2,3] For the bioluminescent system of the firefly and sea firefly, the fluorophore is oxy-luciferin. In the bioluminescent system of *Latia*, the oxy-luciferin (**2**) does not have a fluorophore site, therefore, *Latia* luciferin is thought to be the energy source in this bioluminescent reaction (Scheme 1). It indicates that the *Latia* bioluminescent system is different from known mechanisms. For the *Latia* bioluminescent mechanism, it is proposed that when the luciferin is oxidized to the oxy-luciferin (**2**), CO_2 and formic acid in the luciferase, forming the singlet-excited oxy-luciferin transfers energy to an internal emitter for emitting green fluorescence (Scheme 1).[2c] However, the detailed profiles of this reaction still remain to be elucidated.

Previously, we reported that the *Latia* luciferin analogues had been synthesized and evaluated.[4] The *Latia* luciferin analogues, which replaced the 2,6,6-trimethylcyclohexene moiety with benzene or dihydrogenated 2,6,6-trimethylcyclohexane, did not have the luminescent activity. It is expected that the 2,6,6-trimethylcyclohexene moiety is important for the structure recognition of luciferase. Whereas, although the *E*- and *Z*-enol acetate analogue **3** had bioluminescent activity, no emission was found in the case of the methyl enol ether analogue. The bioluminescent activity of *E*-**3** was stronger than that of *Z*-**3** as a natural luciferin.[3b] From these results, it is implied that the enol ester moiety is important for the bioluminescent activity. In this study, several benzoate analogues **5**–**8** (Scheme 2) were synthesized for investigating the bioluminescent activity.

The synthetic method for *Latia* luciferin (**1**) was previously described, and luciferin analogues were synthesized by a procedure similar to that of **3**.[4] Briefly, selective hydrogenation of β-ionone **9** with Bu_3SnH followed by the Wittig olefination of the resulting ketone **2** (*Latia* oxy-luciferin) afforded the methyl enol ether **10** as a mixture of isomers (E:$Z = 1$:1) as shown in Scheme 3. The enol ether **10** was hydrolyzed in the 80% aq. acetone including *p*-TsOH, giving aldehyde **11** in

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* Corresponding author. Tel.: +81-424-435484; fax: +81-424-86-1966; e-mail: niwa@pc.uec.ac.jp

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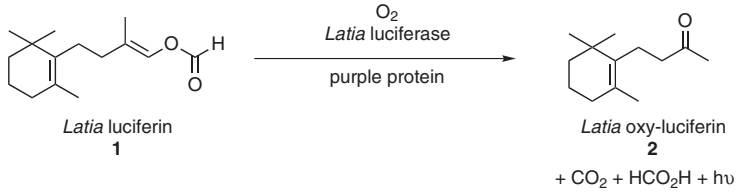
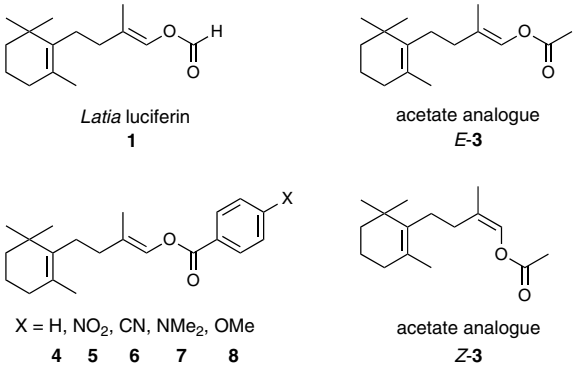


Scheme 1. Proposed bioluminescent reaction of *Latia neritoides*.

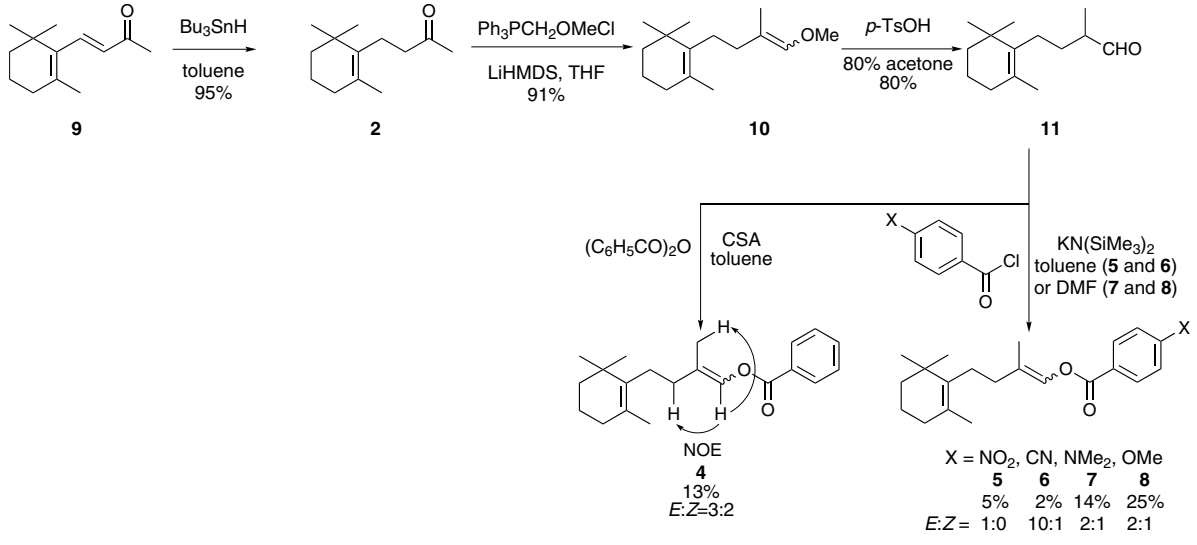


Scheme 2. The structure of *Latia* luciferin and its analogues.

80% yield. The aldehyde **11** was achieved by treatment with benzoic anhydrous and CSA to give the desired enol ester **4**. The aldehyde **11** was also converted into the enol ester analogues (**5**–**8**) by enol esterification using KHMDS and the corresponding substituted benzoyl chlorides. The *E*- and *Z*-isomers of these benzoate analogues were separated using a preparative TLC plate. The *E*- and *Z*-conformations of the *Latia* luciferin analogues (**4**–**8**) were characterized by the NOE experiments.

The luciferase used for this study was prepared as follows; A frozen *Latia* sample was homogenized in 50 mM Tris–HCl buffer (pH 7.6) at 0 °C and the homogenized mixture was centrifuged at 7000 rpm for 20 min at 4 °C. The supernatant was diluted 10 times, and used for the bioluminescence measurement as a crude luciferase. Because the *Latia* luciferin (**1**) and the synthesized analogues were hydrophobic, the bioluminescent activity was evaluated in a buffer containing various amount of ethanol. The bioluminescent activity was measured by mixing 100 μL of the crude luciferase solution with 100 μL of *Latia* luciferin or the analogue solution (58 μM) in various ethanol concentrations (final concentration; 0.7%, 10%, 25% and 50%, see Fig. 1). The generated photons were counted by a luminometer[5] for 2 h. As a consequence, for the 10% ethanol-buffer condition, the bioluminescent activity of the natural luciferin (**1**) exhibited the best result (Fig. 1). Because the luciferase activity was inhibited by an out of the limit concentration of ethanol, the natural luciferin exhibited a decreased bioluminescence, though the solubility was raised by the addition of ethanol. The bioluminescent activity of other luciferin analogues exhibited almost the same results (data not shown). Therefore, the bioluminescent activity of *Latia* luciferin and its analogues were measured in 10% ethanol as the optimized condition.

The luminous intensity of the benzoate analogues was approximately 1/25 to 1/125 times lower than the natural luciferin (Fig. 2, Table 1). However, the total luminous energy of the benzoate luciferin analogues for 2 h was from nearly equal to approximately 1/6 times lower than the natural luciferin. From the tendency of the time at the peak height, it can be understood that the reaction



Scheme 3. Synthesis of *Latia* luciferin benzoate analogues.

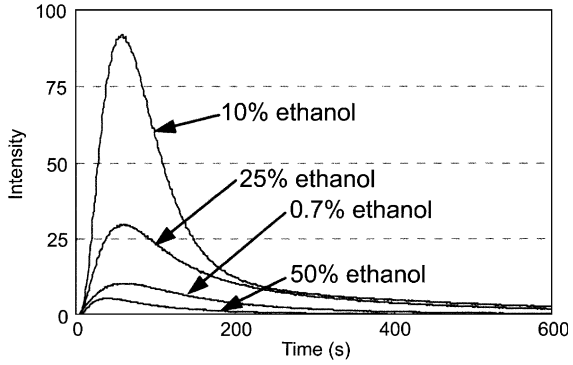


Figure 1. The bioluminescent activity of *Latia* luciferin in various ethanol concentrations. The ethanol concentration values indicate the final concentration.

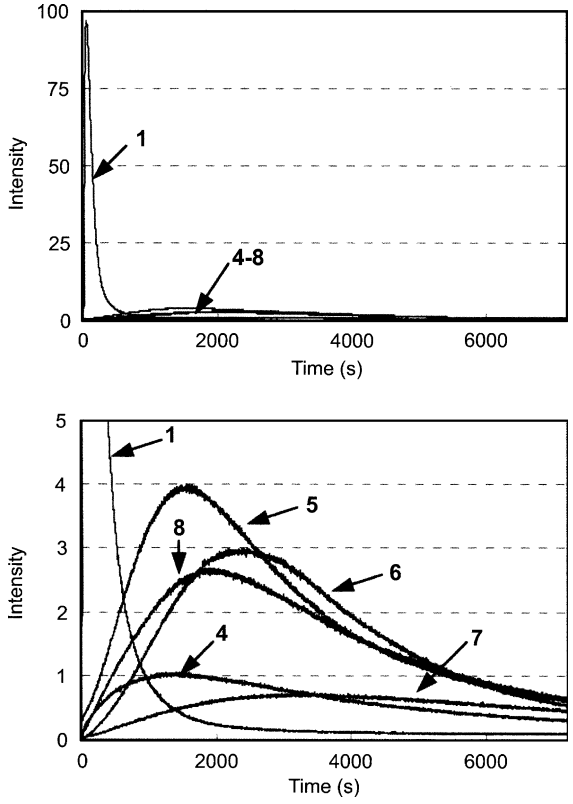


Figure 2. The bioluminescent activity of *Latia* luciferin and its analogues. Below is a magnified figure of above.

Table 1. Relative bioluminescent activity of *Latia* luciferin analogues

Substrate	Luminous intensity[a]	Total luminous energy[b]	Emission max. (nm)
E-**1**	100	100	536
E-**4**	1.1	28	536
E-**5**	4.2	85	536
E-**6**	3.2	71	536
E-**7**	0.79	16	536
E-**8**	2.9	69	536

[a] The peak height was recorded as the luminous intensity.
[b] Total luminous energy was obtained by integrating the bioluminescent measured for 2 h.

speed of the bioluminescent activity for the *Latia* luciferin benzoate analogues is slower. Delay of the bioluminescent activity of benzoate analogues was thought to be caused by various factors like their bulky structure, electronic state and cleavage rate of enol ester moiety and charge of modified moiety. The bioluminescent activities of the benzoate analogues **4** and **7** were approximately 1/5 times lower than the other benzoate analogues. This indicates that the difference in the substituent affects the bioluminescent activity. The recognition activity for luciferase was different from the enol ester substituents, so that the generation rate of the excited state of luciferin, probably oxygenated luciferin, might be influenced. The wavelength at the maximum luminescence of the benzoate analogues was identical to that of the natural luciferin (Table 1). This indicates that the enol ester site of luciferin does not affect the wavelength of the luminescence. As a result, it can be considered that the difference in the enol ester site produced a different bioluminescent activity, but did not affect the wavelength at the maximum luminescence.

In conclusion, the *Latia* luciferin benzoate analogues exhibited delayed bioluminescence compared to the natural luciferin. It is indicated that the *Latia* bioluminescent activity can be controlled by the design of the enol ester.

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