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Development of simple firefly luciferin analogs emitting blue, green, red, and near-infrared biological window light

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ABSTRACT

Simple firefly luciferin analogs emitting blue, green, and red light were developed. The longest emission maximum was observed at 675 nm, which belongs to the NIR biological window (650–900 nm), useful for deep site bioimaging of living animals. The analogs showed a slow rise of emission intensity compared with the rapid emission of natural luciferin. The light emission of the adenylated analogs was strongly enhanced compared with those of analogs themselves.

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1. Introduction

Firefly luciferase is well known for its highly efficient light emission by catalyzing the oxidation of substrate D-luciferin (D-LH_2). The accepted catalytic mechanism of firefly bioluminescence is shown in Fig. 1.[1–3] This enzymatic light emission process does not require an external light source to fluorescence, thereby an extremely high signal-to-noise ratio is provided by this process. Because of its substrate specificity and high sensitivity, firefly luciferase and luciferin combination is widely used in the detection of ATP, and biological studies, with luciferase as a reporter gene in cell culture systems,[4] and recently in noninvasive whole-body bioimaging.[5] For the bioimaging technique, emissions of light with various colors are useful. In particular, red, or more desirably, the light around the 650–900 nm region termed the near-infrared (NIR) biological window,[6] is suitable for noninvasive whole-body imaging. Since the light of the NIR window is not strongly absorbed by oxygenated hemoglobin and melanin in animal tissues, the light is expected to be useful for deeper site imaging.

In addition to Lampyridae (firefly) luciferases, several different isozymes are known to emit light in different colors using the same

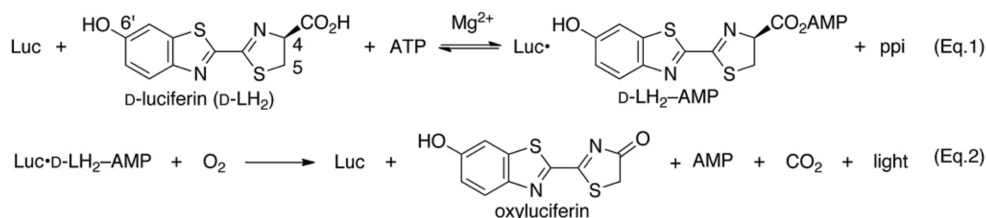

Fig. 1. Proposed reaction catalyzed by firefly luciferase. In the first step of the reaction, luciferase (Luc) catalyzes adenylation of D-LH_2 with ATP in the presence of Mg^{2+} to generate the intermediate luciferyl–AMP (D-LH_2–AMP) accompanied by pyrophosphate (ppi) (Eq. 1). Then the oxidative decarboxylation of the intermediate gives excited-state oxyluciferin, which then releases visible light in the course of relaxation to the ground state (Eq. 2).

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substrate D-LH_2.[7] Based on a bioengineering study, several mutant luciferases emitting various colors were developed.[8,9] Among various luciferases including their mutants, the shortest emission maximum wavelength was 534 nm and the longest was 623 nm.[7] Theoretical study of the light emitter oxyluciferin indicated an expected emission range from natural D-LH_2 to be 421–626 nm.[10] This study suggested that a new substrate scaffold, other than the natural luciferin chromophore, is required in order to obtain a wider emission range and longer maximum emission wavelength with Lampyridae luciferases including their mutants.

After the structure of luciferin was elucidated and synthesized in the 1960s, some modified luciferin analogs, such as 6′-aminoluciferin,[11] were synthesized and a few of them functioned as light-emitting substrates.[3] More recently, *N*-alkylated 6′-aminoluciferins were found to act as substrates[12] and cyclic alkylaminoluciferin was reported to show red light emission (607 nm) by using the mutant of *Photuris pennsylvanica* firefly luciferase (Ultra-Glo).[13] Some modified luciferins were newly developed for color change and specific purposes.[14–22] However, most synthetic bioluminescent luciferin analogs consist of benzothiazole and 4-carboxythiazolin-2-yl rings unchanged from natural luciferin, except for quinolyl-, coumaryl-, naphthylluciferins,[17,23a] and very recently published[23b] heterocyclic luciferin analogs.

In this study, we have substituted a benzothiazole moiety with a simplified aromatic structure to investigate the effect of π conjugation to the emission wavelength (Fig. 2).

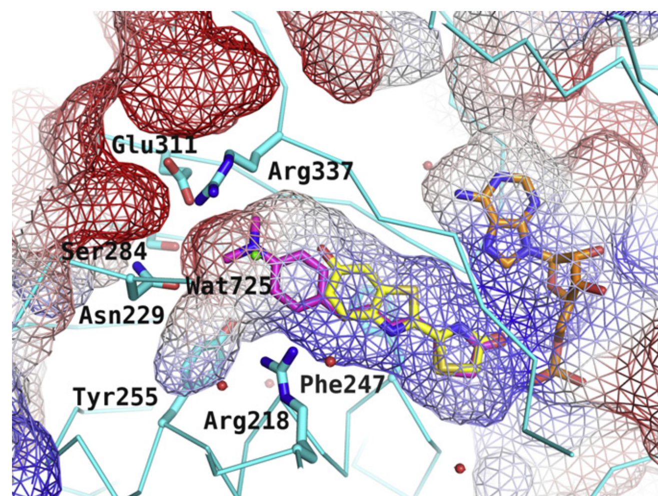

Fig. 3. Structure around plausible ligand-binding site of *P. pyralis* luciferase and overlay model of oxy-**3b**, oxyluciferin and AMP. The backbone structure of luciferase (4G36) is shown in cyan, and the electrostatic surface is presented as a wire mesh with negative and positive charges in red and blue, respectively. The original ligand DLSA is omitted from the figure for clarity. Side chains of Arg218, Ans229, Phe247, Tyr255, Ser284, Glu311, and Arg337 are shown in stick form. The view was sliced to show the binding pocket and some residues were omitted from the figure for clarity. Water molecules are shown as red spheres, except Wat725 is colored green. The ligand atoms are displayed in stick form with the carbon atom of oxy-**3b** in magenta, oxyluciferin in lemon yellow and AMP in orange. Nitrogen is colored blue, oxygen is colored red, sulfur is colored yellow, and phosphorus is colored deep orange.

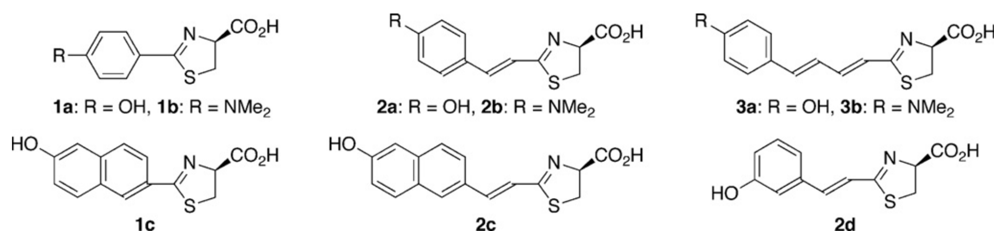

Fig. 2. Chemical structure of synthetic substrates (**1**–**3**).

2. Results and discussion

2.1. Molecular design and synthesis of luciferin analogs

Because the scaffolds of these analogs are different from those of existing substrates, we have used molecular modeling to predict the interaction between intermediate species of one of the longest analogs, **3b**, and luciferase. Fig. 3 shows a plausible location of the model compound oxy-**3b** and AMP at the active site of the *Photinus pyralis* luciferase. The crystal structures used (PDB accession number 4G36[23c] and 2D1R[32]) were downloaded from the RSCB protein data bank. Structural alignment, generation of electrostatic surface potential and construction of the figure were carried out using PyMOL (DeLano Scientific; http://www.pymol.org). The coordinates of the DLSA-bound *P. pyralis* luciferase structure (chain B of 3G36) superimposed closely on the oxyluciferin and AMP-bound *Luciola cruciata* luciferase structure (2D1R), giving a root-mean-square deviation of 1.1 Å based on 3227 atoms. The local minimum conformation model of oxy-**3b** was obtained using Spartan '04 (Wavefunction; http://www.wavefun.com) by Hartree–Fock 3-21G calculation. The 4,5-dihydro-4-oxothiazol-2-yl moiety in oxy-**3b** was manually aligned onto both a 4,5-dihydro-4-oxothiazol-2-yl ring of the oxyluciferin and a thiazole ring of DLSA. As shown in Fig. 3, the extended *E*,*E*-form of oxy-**3b** fitted into the narrow and deep substrate-binding site. Several polar amino acid side chains (Arg218, Ans229, Tyr255, Ser284, Glu311, and Arg337) faced the bottom of the binding pocket and provided the rather negative electrostatic surface expected in a cation-stabilizing environment. Notably, the nitrogen atom of oxy-**3b** closely overlapped the water molecule (Wat725) of the 4G36 crystal structure. This preliminary evaluation indicated that it is structurally possible to locate the designed analogs on the native luciferin binding site.

As a simple bioluminescence chromophore for luciferase substrate, we chose a 4-hydroxyphenyl group as an aromatic part. This part was connected to a 4-carboxythiazolin-2-yl ring directly (analog **1a**), or was connected through one (analog **2a**) or two (analog **3a**) double bonds to study the effect of π conjugation on the emission wavelength (Fig. 2). A 4-(dimethylamino)phenyl group was also selected as an aromatic part (**1b**–**3b**) with an expectation of a red shift of light emission, through the electron donating effect of the alkylamino group. In addition, 6-hydroxynaphthalen-2-yl analogs **1c**[23a] and **2c** were also anticipated as shifting emission maxima toward red, because of longer π-conjugated systems than that of the corresponding 4-hydroxyphenyl analogs. The 3-hydroxystyryl-type luciferin analog **2d** was also prepared to evaluate the importance of the hydroxy group position for bioluminescence activity.

The synthesis of luciferin analogs was conducted as shown in Scheme 1. We utilized D-cysteine or (*S*)-trityl-D-cysteine methyl ester for constructing the chiral thiazoline ring. Thus, the analogs **1a**, **1b**, **1c**, and **2a** were synthesized directly by the coupling of D-cysteine with the corresponding nitriles, **4a**, **4b**, **4c**, and **6**,

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