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View Article Online
DOI: 10.1039/C7CC09720D

Journal Name

COMMUNICATION

An Allylated Firefly Luciferin Analogue with Luciferase Specific Response in Living Cells

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Yuma Ikeda,[a] Tsuyoshi Saitoh,[b] Kazuki Niwa,[c] Takahiro Nakajima, [d] Nobuo Kitada, [e] Shojiro A. Maki,[e] Moritoshi Sato,[d] Daniel Citterio,*[a] Shigeru Nishiyama[f] and Koji Suzuki*[a]

An allylated firefly luciferin was succesfully synthesized and its bioluminescence properties were evaluated. When applied to cellular imaging in combination with Eluc, which is one of the commercially available luciferases, this analogue displayed a luciferase-specific bioluminescence signal with prolonged emission (>100 min).

Bioluminescence-based assays with luciferin-luciferase pairs are widely used as *in vitro* and *in vivo* reporters of biologically relevant substances, gene expression, and tumour progression, among others.[1,2] Since bioluminescence-based assays do not require excitation light, they exhibit much lower background signals than fluorescence-based assays and are applicable to highly sensitive bioimaging.[3] Among the available systems, the firefly luciferin-luciferase reaction is one of the most well-studied emission systems, due to its high quantum yield (φ_{BL} = 0.23~0.61) and relatively long emission wavelength (λ_{em} 530~620 nm).[4] In the firefly bioluminescence reaction, the luciferase catalyses the oxidation of D-luciferin in the presence of adenosine triphosphate (ATP) and Mg^{2+} acting as co-factors to release a light photon. (Fig. 1a).

A variety of luciferases has been discovered[5–7] and cloned, and many of them have been adapted to "spy" on cells in whole animals utilizing their different bioluminescence colours and superior properties, such as prolonged light emission and high luminescence efficiency, among others.[8,9] However, it is widely known that D-luciferin is the only substrate that results in light emission in combination with these luciferases. Although different luciferases can be selectively expressed to a specific target, they all result in bioluminescence emission upon introduction of D-luciferin. For this reason, it is basically not possible to distinguish between multiple targets within a single cell. Although the development of luciferases has been a very active field of research, there are unfortunately only few reports of successful development of synthetic luciferins, presumably due to the difficulty in designing and synthesizing bioluminescence-active luciferins.

Many details regarding bioluminescence of the firefly system still remain unknown, and random modifications of the luciferin structure may lead to the loss of the luminescence activity. Therefore, we referred to previously performed studies on the structure-activity relationship of firefly luciferin to more efficiently design analogues. The molecular structure of D-luciferin is mainly composed of two subunits, the thiazoline and

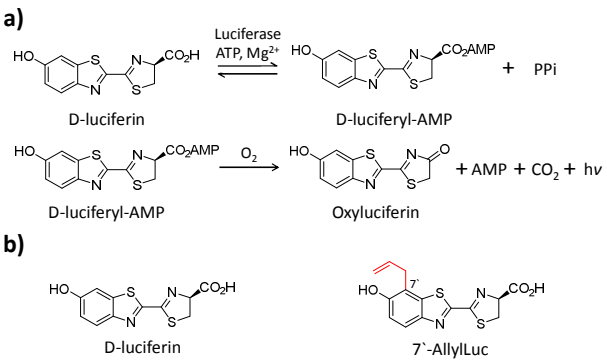

Fig. 1 a) Firefly bioluminescence reaction: Luciferase catalyses the adenylation and oxidation of its substrate D-luciferin to release a photon. b) Chemical structures of D-luciferin and 7`-AllylLuc.

[a] Department of Applied Chemistry Faculty of Science and Technology, Keio University, Hiyoshi 3-14-1, Kohoku-ku, Yokohama, Kanagawa, 223-8522, Japan. E-mail: citterio@applc.keio.ac.jp, suzuki@applc.keio.ac.jp
[b] International Institute for Integrative Sleep Medicine, University of Tsukuba, Tennodai 1-1-1, Tsukuba-shi, Ibaraki, 305-8577, Japan
[c] National Institute of Advanced Industrial Science and Technology, Tsukuba-shi, Ibaraki, Japan
[d] Graduate School of Arts and Science, The University of Tokyo,3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
[e] Department of Engineering Science, Graduate School of Informatics and Engineering, University of Electro-Communications, Chofu, Tokyo, 182-8585, Japan
[f] Department of Chemistry, Faculty of Science and Technology, Keio University, Hiyoshi 3-14-1, Kohoku-ku, Yokohama, Kanagawa, 223-8522, Japan

† Electronic Supplementary Information (ESI) available: Detailed Synthetic procedure and spectral characterization of compounds. Additional information on absorbance and fluorescence spectra, bioluminescence properties as noted in text and summarized luminescence properties. See DOI: 10.1039/x0xx00000x

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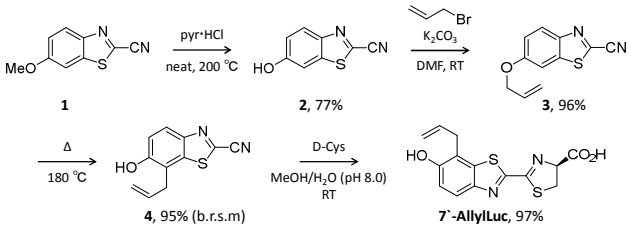

Scheme1 Synthesis of 7`-AllylLuc

the benzothiazole substructures. The position and the stereochemistry of the carboxyl group on the thiazoline ring is a crucial factor for the luminescence activity.[10–12] In contrast to the luminescence activity of the D-form, it is widely known that the optical isomer L- luciferin works as a potential luminescence inhibitor.[13] Additionally, many of the heteroatom-variants and substituent modifications of this thiazoline ring have resulted in a dramatic reduction or loss of luminescence activity,[14,15] and thus, the thiazoline ring subunit is regarded as an essential site for substrate recognition by the luciferase enzyme and the luminescence activity. On the other hand, it has been reported that most analogues with modification at the benzothiazole ring subunit retain their luminescent activities. Actually, analogues with heteroatom-substitution in the benzothiazole ring[15,16] and modification of its chemical structure[17-19] maintained the luminescence activity with shifts in the emission wavelength. Therefore, it can be said that the benzothiazole ring is the modifiable site of D-luciferin. A recent study revealed that modifications of the benzothiazole ring at the C-7` position with sterically demanding residues potentially lead to bolstered bioluminescence properties.[20] Furthermore, it was also shown that mutant luciferases can effectively result in substrate-specific luminescent properties with structurally modified luciferin analogues.[21,22]

In order to expand the applications of bioluminescent systems, we have developed a firefly luciferin analogue modified with an allyl group at the C-7` position of its benzothiazole core (Fig. 1b), exhibiting bioluminescence emission with various luciferases in analogy to D-luciferin, and confirmed its utility for bioluminescence imaging.

To access the desired allyl-modified luciferin structure, focus was set on the Claisen rearrangement for introduction of an allyl-group (Scheme 1). The synthesis of allyl luciferin begins with pyridine hydrochloride promoted thermal deprotection of commercially available 2-cyano-6-methoxybenzothiazole (**1**), which is transformed to its phenol form, 2-cyano-6-hydroxybenzothiazole (**2**). It is then converted to the allyloxy-benzothiazole (**3**) by treatment with allyl bromide/K_2CO_3, which on regioselective Claisen rearrangement of the allyl group results in compound (**4**). The coupling reaction of 2-cyano-benzothiazole derivative (**4**) with D-cysteine generated **7`-AllylLuc** via the standard procedure. Thus, **7`-AllylLuc** can be prepared in 4 steps from commercially available starting materials in 68% overall yield.

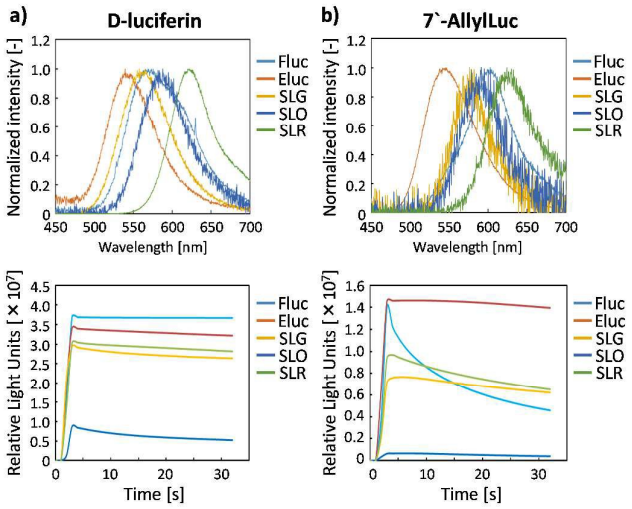

Fig. 2 Normalized bioluminescence emission spectra and light emission time courses obtained with various luciferases for a) D-luciferin and b) 7`-AllylLuc at pH 8.0. Additional experimental details are described in the ESI.

To explore the potential emission ability of **7`-AllylLuc**, a conventional chemiluminescence assay was first conducted. This non-enzymatic emission process is a useful method for verifying the luminescence ability of the substrate itself. When **7`-AllylLuc** was subjected to chemiluminescence assay conditions, luminescence was observed (Fig. S1). This suggested that **7`-AllylLuc** is a potential emitter for luciferases. Next, it was investigated, whether firefly or beetle luciferases are able to recognize **7`-AllylLuc** as their substrate to release a photon of light. Bioluminescence measurements with commonly used luciferases were carried out using commercially available enzymes, including firefly luciferase (Fluc) from *Photinus pyralis*, Emerald Luc (Eluc) from *Pyrearinus termitilluminans*, Stable Luciferase Green (SLG) from *Rhagophthalmus ohbai*, as well as Stable Luciferase Orange (SLO) and Stable Luciferase Red (SLR) from *Phrixothrix hirtus*. **7`-AllylLuc** exhibited bioluminescence catalysed by all of these luciferases similar to that of the native luminescent substrate D-luciferin (Fig. 2). Upon using Fluc as the luciferase, the bioluminescence spectra of **7`-AllylLuc** showed a significant red-shift (maximum emission wavelength at 605 nm) compared with that of D-luciferin (Fig. 2 and S2), despite having almost identical fluorescence spectra (Fig. S3), whereas such a shift was not observed when using the other luciferases (Eluc, SLG, SLO and SLR). Moreover, the bioluminescence spectra obtained under several pH conditions with Fluc, known to be a pH sensitive luciferase, were completely identical like previously reported C-7` modified analogues (Fig. S4).[17]

In the crystalline structure of *Luciola cruciate* luciferase, which is a type of luciferase with an amino acid sequence resembling that of Fluc, complexed with an inhibitor (5`-*O*-[*N*-(dehydroluciferyl)-sulfamoyl]adenosine; DLSA) structurally similar to D-luciferyl-AMP, it has been clarified that luciferin is tightly sandwiched in the extremely hydrophobic microenvironment of the enzyme.[23] However, collapse of the planar structure of luciferin by structural modification causes a disruption of the hydrogen-bonding network around the luciferin, [17] which may result in a more polar

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microenvironment (Fig. S5). Finally, the energy level of the excited state oxyluciferin is stabilized, and red-shifted bioluminescence emission is observed. Actually, previous studies have described

Table 1 Kinetic properties of allyl luciferin in combination with various luciferases

Luciferase	*Apparent* K_m [μM]	*Apparent* V_{max} [a] [×10^{8}photons/sec]
Fluc	23.8±6.9	7.69
Eluc	18.8±6.4	6.93
SLG	40.6±8.4	0.0725
SLO	10.2±4.8	0.0292
SLR	12.5±7.2	0.0751

[a] V_{max} values are within 5% error

similar red-shifted behaviour for sterically hindered luciferins (e.g. alkyn- or halogen-substituted) with Fluc.[24,25]The results obtained in this study suggested that allyl modification at the C-7` position influences the hydrophobic environment of the active site of Fluc, only. While continuous emission was observed for D-luciferin in all luciferases, the emission of **7`-AllylLuc** with Fluc gradually decreased with time, with the emission intensity reaching half of its original value 12 seconds after the start of the reaction (Fig. 2b). This decrease in bioluminescence intensity with Fluc may be due to inhibition by the reaction product. Surprisingly, the emission intensity with ELuc was the highest among all the luciferases, and the maximum emission intensity was sustained (Fig. 2b). Since bioluminescence imaging generally requires long-term exposure, prolonged luminescence is a very important characteristic for both *in vitro* and *in vivo* imaging.

To further understand the enzymatic reaction, the apparent Michaelis constants K_m (defined as the concentration at half of the maximum reaction rate) and the maximum velocities V_{max} for **7`-AllylLuc** with each luciferase were evaluated. The initial luminescence intensity was measured as a function of luciferin concentration (Fig. S6). All **7`-AllylLuc** - luciferase pairs showed typical Michaelis-Menten plots. The K_m and V_{max} values were calculated from the Lineweaver-Burk plots. Little variation among K_m values was observed for allyl luciferin (Table 1). On the other hand, there were notable differences in the V_{max} values, which displayed a strong correlation with the emission intensity shown in Fig. 2. Moreover, the V_{max} values were 3~100-fold lower compared with that of the D-luciferin - Fluc pair, the most commonly used luciferin-luciferase pair (data not shown). In order to further discuss the difference in luminescence efficiency between D-luciferin and **7`-AllylLuc**, bioluminescence quantum yield measurements with Fluc and Eluc were performed (Fig. S7). They revealed that **7`-AllylLuc** has about 10% of the luminous efficiency with both luciferases (Fluc and Eluc) compared to the natural substrate D-luciferin (Table S1). These results suggest that while allyl modification at the C-7` position enables enzyme recognition, it may interfere with the catalysis necessary to emit light. Steric hindrance by modification with an allyl group may prevent effective luminescence processing of allyl luciferin, like it has been previously reported in the case of brominated luciferins.[25]

Encouraged by these results *in vitro*, the bioluminescent properties of **7`-AllylLuc** were further evaluated in live luciferase-expressing COS-7 monkey kidney tissue cells. The cells were cultured in 96-well microplates and monitored with a luminometer over 100 minutes with various substrate

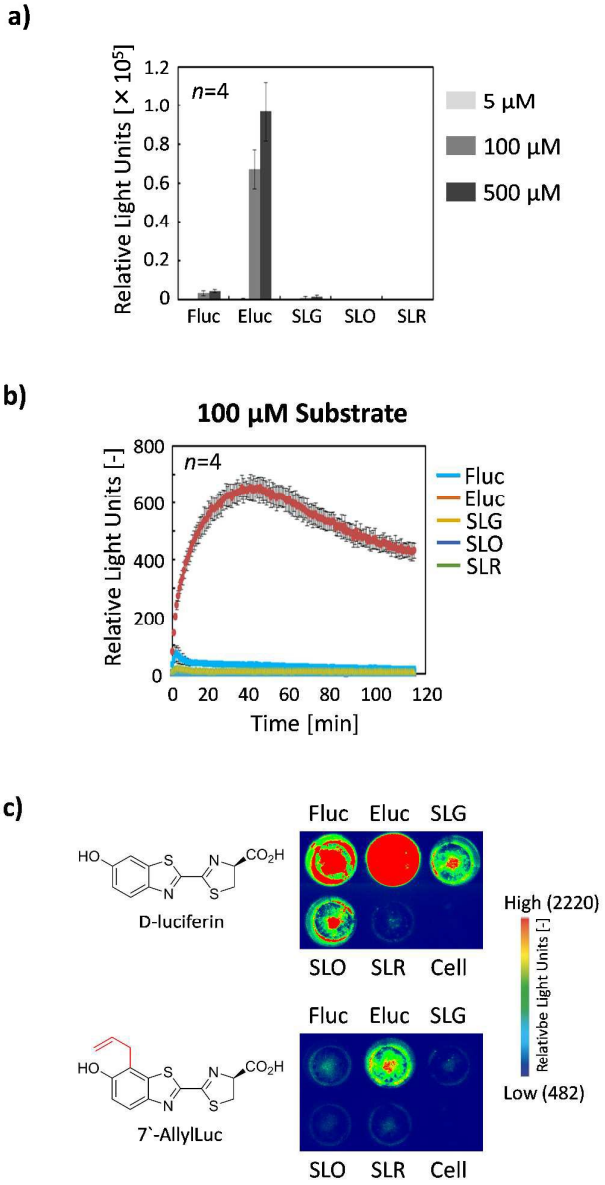

Fig. 3 Light emission from luciferase-expressing COS-7 cells treated with 7`-AllylLuc: a) total intensities of dose-response emission; b) time courses at 100 μM substrate; Error bars represent the standard deviation of 4 experiments; c) imaging of luciferase-expressing COS-7 cells treated with D-luciferin or 7`-AllylLuc.

concentrations. While the light output from **7`-AllylLuc** was weaker compared to D-luciferin (Fig. 3, Fig. S8 and S9), a dose-dependent response was observed (Fig. 3a). No detectable signal could be obtained at low concentrations (5 μM) of luciferin, presumably due to the low cell membrane permeability of the luciferins. Surprisingly, specific light

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emission was observed with Eluc-expressing cells, only (Fig. 3). Furthermore, the **7`-AllylLuc**-based light emission from Eluc-expressing cells was sustained like in the case of the native D-luciferin emitting system and lasted over 100 minutes, which is a desirable property for bioluminescence applications, as already noted before. Cell viability evaluation by a standard MTT assay revealed that there was no significant cytotoxicity of **7`-AllylLuc** even after treatment with a high dosage of **7`-AllylLuc** (500 µM) for 24 h (Fig. S10). Despite **7`-AllylLuc** having weaker emission intensity, a significant benefit of this analogue is its high luciferase specificity for Eluc, making it a potentially promising tool for multi-target imaging.

In conclusion, a novel luciferin analogue modified with an allyl group at the C-7` position was successfully synthesized. In cellular imaging, this **7`-AllylLuc** displayed a luciferase-selective bioluminescence signal in combination with Eluc, which is one of the commercially available beetle luciferases from *Pyrearinus termitilluminans*. Studies are now in progress to develop artificial beetle luciferases that display stronger bioluminescence emission with **7`-AllylLuc** compared to that of D-luciferin, and to apply its selective response to bioluminescence-based reporter assays.

In addition, besides of being a promising luciferase substrate by itself, the terminal olefin function of the allyl group is available as a linker for further covalent modifications. It is convertible to aldehyde groups by Lemieux-Johnson oxidation, which can then easily react with various functional groups.[17] Therefore, allyl group introduction into firefly luciferin enables further useful possibilities, such as the development of various analogues and labelling applications.

It is believed that this new synthetic luciferin will contribute to the expansion of bioluminescence imaging applications both *in vitro* and *in vivo*.

This study was supported by a Grant-in Aid for Scientific Research (S) (Grant No. 24225001) to K.S. from the Japan Society for the Promotion of Science (JSPS).

Conflicts of interest

There are no conflicts of interest to declare.

Notes and references

1 J. A. Prescher and C. H. Contag, *Curr. Opin. Chem. Biol.*, 2010, **14**, 80–89.
2 S. T. Adams and S. C. Miller, *Curr. Opin. Chem. Biol.*, 2014, **21**, 112–120.
3 M. A. Paley and J. A. Prescher, *Med Chem Commun*, 2014, **5**, 255–267.
4 P. Colepicolo-Neto, C. Costa and E. J. H. Bechara, *Insect Biochem.*, 1986, **16**, 803–810.
5 K. Niwa, Y. Ichino, S. Kumata, Y. Nakajima, Y. Hiraishi, D. Kato, V. Viviani and Y. Ohmiya, *Photochem. Photobiol.*, 2010, **86**, 1046–1049.
6 V. R. Viviani, A. C. R. Silva, G. L. O. Perez, R. V. Santelli, E. J. H. Bechara and F. C. Reinach, *Photochem. Photobiol.*, 1999, **70**, 254–260.
7 V. R. Viviani, E. J. H. Bechara and Y. Ohmiya, *Biochemistry (Mosc.)*, 1999, **38**, 8271–8279.
8 B. R. Branchini, T. L. Southworth, N. F. Khattak, E. Michelini and A. Roda, *Anal. Biochem.*, 2005, **345**, 140–148.
9 Y. Nakajima, T. Yamazaki, S. Nishii, T. Noguchi, H. Hoshino, K. Niwa, V. R. Viviani and Y. Ohmiya, *PLoS ONE*, 2010, **5**, e10011.
10 E. H. White, F. McCapra and G. F. Field, *J. Am. Chem. Soc.*, 1963, **85**, 337–343.
11 E. H. White, H. Wörther, G. F. Field and W. D. McElroy, *J. Org. Chem.*, 1965, **30**, 2344–2348.
12 E. H. White, E. Rapaport, T. A. Hopkins and H. H. Seliger, *J. Am. Chem. Soc.*, 1969, **91**, 2178–2180.
13 N. Lembert, *Biochem. J.*, 1996, **317**, 273–277.
14 S. Ioka, T. Saitoh, S. Iwano, K. Suzuki, S. A. Maki, A. Miyawaki, M. Imoto and S. Nishiyama, *Chem. - Eur. J.*, 2016, **22**, 9330–9337.
15 D. C. McCutcheon, M. A. Paley, R. C. Steinhardt and J. A. Prescher, *J. Am. Chem. Soc.*, 2012, **134**, 7604–7607.
16 C. C. Woodroofe, P. L. Meisenheimer, D. H. Klaubert, Y. Kovic, J. C. Rosenberg, C. E. Behney, T. L. Southworth and B. R. Branchini, *Biochemistry (Mosc.)*, 2012, **51**, 9807–9813.
17 H. Takakura, R. Kojima, T. Ozawa, T. Nagano and Y. Urano, *ChemBioChem*, 2012, **13**, 1424–1427.
18 S. Iwano, R. Obata, C. Miura, M. Kiyama, K. Hama, M. Nakamura, Y. Amano, S. Kojima, T. Hirano, S. Maki and H. Niwa, *Tetrahedron*, 2013, **69**, 3847–3856.
19 C. Miura, M. Kiyama, S. Iwano, K. Ito, R. Obata, T. Hirano, S. Maki and H. Niwa, *Tetrahedron*, 2013, **69**, 9726–9734.
20 K. A. Jones, W. B. Porterfield, C. M. Rathbun, D. C. McCutcheon, M. A. Paley and J. A. Prescher, *J. Am. Chem. Soc.*, 2017, **139**, 2351–2358.
21 K. R. Harwood, D. M. Mofford, G. R. Reddy and S. C. Miller, *Chem. Biol.*, 2011, **18**, 1649–1657.
22 S. T. Adams, D. M. Mofford, G. S. K. K. Reddy and S. C. Miller, *Angew. Chem. Int. Ed.*, 2016, **55**, 4943–4946.
23 T. Nakatsu, S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata and H. Kato, *Nature*, 2006, **440**, 372–376.
24 R. C. Steinhardt, J. M. O'Neill, C. M. Rathbun, D. C. McCutcheon, M. A. Paley and J. A. Prescher, *Chem. - Eur. J.*, 2016, **22**, 3671–3675.
25 R. C. Steinhardt, C. M. Rathbun, B. T. Krull, J. M. Yu, Y. Yang, B. D. Nguyen, J. Kwon, D. C. McCutcheon, K. A. Jones, F. Furche and J. A. Prescher, *ChemBioChem*, 2017, **18**, 96–100.