Tetrahedron 72 (2016) 7505–7508

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Development of a luminescence-controllable firefly luciferin analogue using selective enzymatic cyclization

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ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 26 September 2016
Accepted 29 September 2016
Available online 29 September 2016

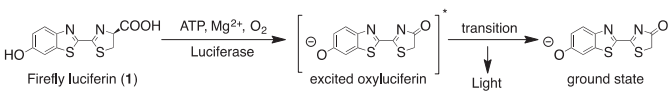
Keywords:
Firefly luciferin derivative
Bioluminescence
Organic synthesis
Enzyme detection
Firefly luciferase

ABSTRACT

In this study, a new firefly luciferin analog that can switch firefly bioluminescence (BL) activity from 'off' to 'on' state was designed and synthesized. BL inactive *N*-Ac-γ-glutamate luciferin **3** contains an acyclic precursor of the thiazoline moiety. Enzymatic treatment of **3** with aminoacylase resulted in a smooth removal of the acyl protecting group and concomitant cyclization to provide BL active carboluciferin **2**.

1. Introduction

Bioluminescence (BL) is a widely used for biological studies.[1,2] The firefly BL is commonly used because it emits a yellow-green light (λ_{max}=553–559 nm)[3] with a high quantum efficiency (Φ_{BL}=41%).[4] Light is emitted because of the reaction of firefly luciferin **1**[5,6] with firefly luciferase in the presence of Mg^{2+}, ATP, and O_2 (Scheme 1).[7–9] The high-efficiency and simple reaction conditions required for firefly BL have led to its wide application in the field of bioimaging,[10–12] food hygiene control[13,14] and reporter gene assays.[15,16]



Scheme 1. Firefly BL pathway.

A number of firefly luciferin analogues have been developed in order to detect life processes spatiotemporally (Fig. 1).[17–21] Z-DEVD-aminoluciferin,[18,19,21] in which the NH_2 group of aminoluciferin is substituted by the DEVD peptide sequence, does not show BL in the presence of luciferase. However, the DEVD peptide is selectively cleaved in the presence of Caspase 3/7 substrates, resulting in aminoluciferin that can emit light. Similarly, 6-*O*-β-D-galactopyranosyl-luciferin,[17,20] in which the OH group of firefly luciferin is bound to the C1 position of β-D-galactose, generates BL

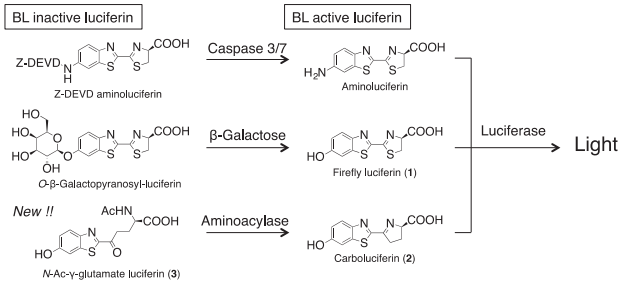


Fig. 1. Novel sensing luciferins. *N*-Ac-γ-glutamate luciferin **3** does not contain the thiazoline moiety, whereas the other luciferins shown here do.

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http://dx.doi.org/10.1016/j.tet.2016.09.069
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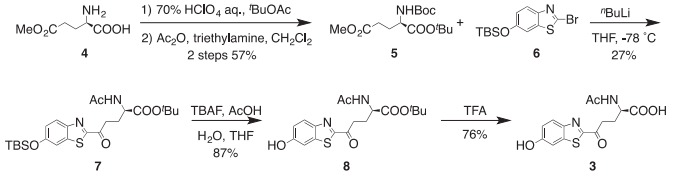
activity in the presence of β-galactosidase. Despite such luciferin-based sensing molecules having been considered as effective tools for specific monitoring of enzyme activity, these synthetic compounds have the disadvantage of possessing a D-absolute configuration in the thiazoline moiety,[22,23] which easily epimerizes under physiological conditions, and the resulting L-luciferin analogues show strong inhibitory activities against firefly luciferase.[7] Furthermore, facile enzymatic or aerobic oxidation of the thiazoline moiety[24] was suggested to promote the reaction of BL-inactive derivatives with luciferase without concomitant light emission. In this study, we report a novel linear precursor of a firefly luciferin analogue that can be activated using a specific enzyme. Our previous study reported that luciferin analogues wherein the thiazoline moiety was replaced with an acyclic structure, did not produce BL and were not recognized by firefly luciferase.[25] The ability to convert a BL-inactive acyclic luciferin to the corresponding BL-active cyclic species by a specific enzyme would provide a new 'off' to 'on' control system. To confirm the applicability of this hypothesis, we used carboluciferin **2**[25] as the BL-active cyclic luciferin, *N*-Ac-γ-glutamate luciferin **3** as the BL-inactive acyclic luciferin, and aminoacylase[26,27] as the specific enzyme. **3** was synthesized and examined to determine if it cyclizes and generates BL when a free NH_2 group is generated by reacting **3** with aminoacylase.

2. Results and discussion

N-Ac-γ-glutamate luciferin precursor **7** was synthesized by the condensation reaction of **5** and **6** by using ^{n}BuLi. In order to obtain **5**, the α-carboxylic acid and NH_2 group of precursor **4**[28] were protected by ^{t}Bu and Ac groups, respectively. Compound **6** was prepared using a known procedure.[25] Successive deprotection of the TBS ether and ^{t}Bu ester of **7**, afforded *N*-Ac-γ-glutamate luciferin **3** (Scheme 2).



Scheme 2. Synthesis of *N*-Ac-γ-glutamate luciferin **3**.

Furthermore, the cyclization of **3** was examined by the reaction of *N*-Ac group with aminoacylase. The retention times of **2** and **3** were confirmed using high-performance liquid chromatography (HPLC), and the following results were obtained: 17.8–17.9 min for **2** and 25.0–25.4 min for **3** (Fig. 2a–c). The reaction of **3** and aminoacylase was monitored for 1 h at 37 °C using HPLC under the same solvent conditions as those used for **2** and **3** (Fig. 2d). The results showed that **3** was converted almost completely to **2**, indicating that the *N*-Ac group of **3** was hydrolyzed by aminoacylase, and **2** was generated by the subsequent cyclization.

The rate of the aminoacylase-promoted cyclization reaction was obtained by evaluating the fluorescence of **2**, **3**, and the reaction mixture of **2** and **3**. Cyclic **2** emitted fluorescence at 565 nm (green light), whereas the acyclic precursor **3** emitted fluorescence at 525 nm (yellow light) upon irradiation with an excitation light of 350 nm (Fig. 3a). The fluorescence intensities of **2** and **3** were detected at wavelengths of 565 nm and 525 nm, respectively, and at both wavelengths for the reaction mixture of **3** and aminoacylase (5 min at 37 °C), as shown in Fig. 3b. The 565 nm fluorescence of the acyclic structure decreased with increase in the enzyme concentration. In contrast, the 525 nm fluorescence of the cyclic structure increased with increase in the enzyme concentration. Removal of the *N*-Ac group of **3**, generating an NH_2 group, resulted in **3** being

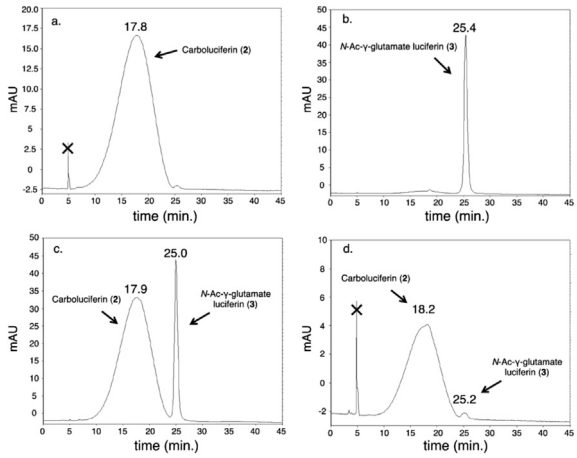


Fig. 2. HPLC results. a) Carboluciferin **2**, b) *N*-Ac-γ-glutamate luciferin **3**, c) Mixture of **2** and **3**, d) Mixture of **3** and aminoacylase after reaction.

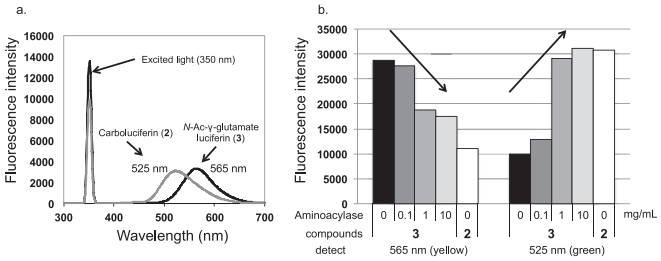


Fig. 3. Fluorescence results. a) The fluorescence wavelength spectra of **2** (525 nm) and **3** (565 nm). b) The fluorescence intensities at 565 nm and 525 nm were measured after the reaction of **3** with aminoacylase solution for 5 min at 37 °C.

immediately cyclized to afford **2** as judged from the fluorescence value attained by the reaction mixture, which was same as that attained by an equivalent molar solution of **2** within 5 min.

The potential of **3** or the reaction mixture of **3** and aminoacylase to display BL was finally examined. The total photon counts are shown in Fig. 4a. There was no significant difference between the

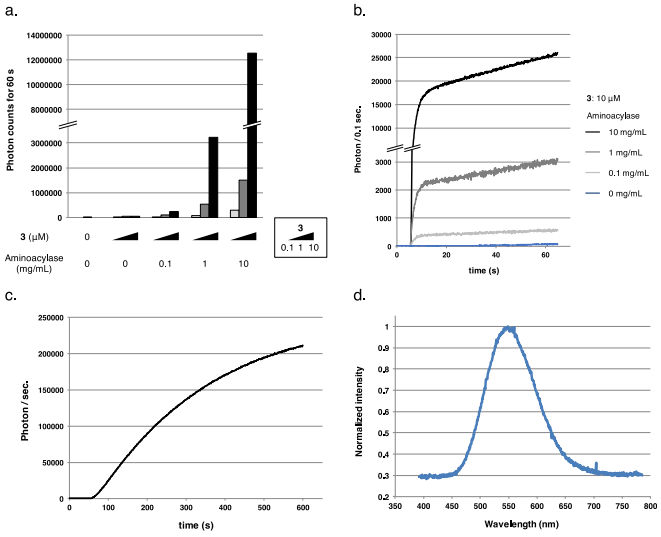


Fig. 4. BL results. a) The total photons measured during 60 s emitted by the BL of **3** or a mixture of **3** and aminoacylase reacted for 5 min at 37 °C. b) BL change of **3** by reaction with aminoacylase solution for 5 min at 37 °C. c) Aminoacylase solution was added to a mixture of **3**, luciferase and ATP-Mg^{2+} for 50 s, and the photon count was the measured for 510 s. d) The wavelength spectrum of the reaction mixture of **3** and aminoacylase. The maximum wavelength was 545 nm.