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Identification of 3-enol sulfate of Cypridina luciferin, Cypridina luciferyl sulfate, in the sea-firefly *Cypridina (Vargula) hilgendorfii*

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ABSTRACT

Cypridina luciferin from the luminous ostracod *Cypridina (Vargula) hilgendorfii* has an imidazopyrazinone core structure (3,7-dihydroimidazopyrazin-3-one), which is identical to that of coelenterazine. Cypridina luciferyl sulfate (3-enol sulfate of Cypridina luciferin) was isolated for the first time and the chemical structure was identified by LC/ESI–TOF–MS analysis. Furthermore, Cypridina luciferyl sulfate was chemically synthesized, and its absorption and MS/MS spectra were in agreement with that of Cypridina luciferyl sulfate isolated. Using the crude extracts of *Cypridina* specimens, Cypridina luciferyl sulfate could be converted to Cypridina luciferin in the presence of adenosine 3′,5′-diphosphate (PAP), and Cypridina luciferin was converted to Cypridina luciferyl sulfate in the presence of 3′-phosphoadenosine 5′-phosphosulfate (PAPS). These results suggested that a sulfotransferase catalyzes the reversible sulfation of Cypridina luciferin in *Cypridina hilgendorfii*. In aqueous solution, Cypridina luciferyl sulfate was more stable than Cypridina luciferin and might be a storage form of Cypridina luciferin.

1. Introduction

The luminescence of the ostracod *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*) is produced by a luciferin–luciferase reaction in the presence of molecular oxygen.[1] Cypridina luciferin (**1**) is an imidazopyrazinone compound[2–4] and is catalyzed by Cypridina luciferase to produce oxyluciferin (**2**), CO_2, and blue light (λ_{max}=460 nm) (Fig. 1A). The resultant oxyluciferin is hydrolyzed to etioluciferin and 2-methylbutylic acid.[5] Cypridina luciferase displays narrow substrate specificity and is highly specific for (*S*)-Cypridina luciferin.[6–8] On the other hand, coelenterazine (**3**) is another imidazopyrazinone-type luciferin and is widely distributed in marine animals[9–13] (Fig. 1B). Until the present time, two sulfate derivatives for coelenterazine have been reported: (i) Coelenterazine disulfate (**4**) has two sulfates at the hydroxy groups of the C2 and C6-phenyl moieties and is known as a luciferin for the firefly squid *Watasenia scintillans*.[14] (ii) Coelenterazine 3-enol sulfate (**5**) was found in the sea pansy *Renilla reniformis*[15,16] and was proposed to be a storage form of coelenterazine[17] (Fig. 1C). Coelenterazine 3-enol sulfate was enzymatically converted to coelenterazine by transferring the sulfate group of coelenterazine 3-enol sulfate to adenosine 3′,5′-diphosphate (PAP) to give coelenterazine and 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Coelenterazine 3-enol sulfate can be converted non-enzymatically to coelenterazine by heat treatment with 0.1 N HCl.[18]

It has been reported that Cypridina luciferin and coelenterazine are biosynthesized from free L-amino acids in living animals.[19–21] However, the biosynthetic mechanism of these luciferins has not been elucidated, and a biosynthetic intermediate has not been reported. During our studies on the biosynthesis of Cypridina luciferin, we found an acid-labile derivative of Cypridina luciferin in the crude extracts of *Cypridina* specimens. The derivative was converted to Cypridina luciferin by acid treatment and was used in the luminescence reaction of Cypridina luciferase. In this manuscript, we report that a sulfate derivative of Cypridina luciferin isolated from *C. hilgendorfii* was identified as 3-enol sulfate of Cypridina luciferin, and was named 'Cypridina luciferyl sulfate' (**6**, Fig. 1C).

Abbreviations: PAP, adenosine 3′,5′-diphosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RP-HPLC, reversed-phase HPLC; RLU, relative light units; TFA, trifluoroacetic acid; LC/ESI–TOF–MS, liquid chromatography electrospray ionization time-of-flight mass spectrometry.

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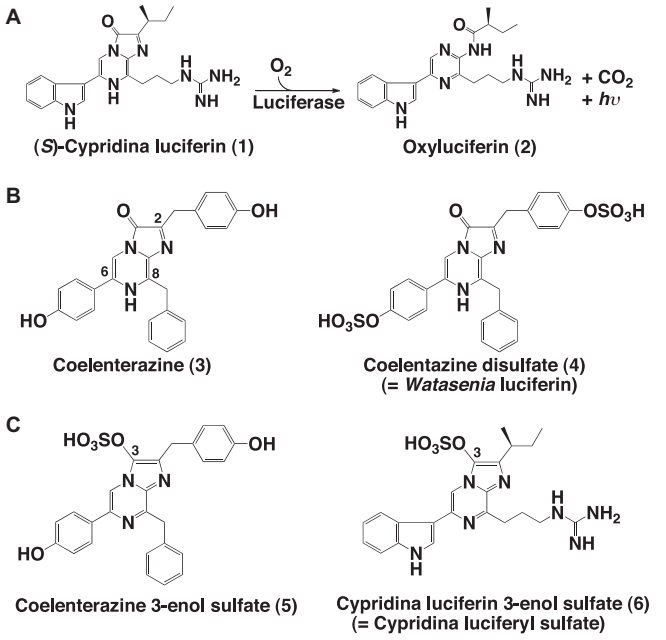

Fig. 1. Luminescence reaction of Cypridina luciferase with Cypridina luciferin and the chemical structures of imidazopyrazinone-type luciferins and their sulfate derivatives. A, The luminescence reaction of Cypridina luciferase catalyzes the oxidation of (*S*)-Cypridina luciferin (**1**) to produce oxyluciferin (**2**), CO_2, and light. B, The chemical structures of coelenterazine (**3**) and coelenterazine disulfate (*Watasenia* luciferin) (**4**). C, The chemical structures of coelenterazine 3-enol sulfate (**5**) and 3-enol sulfate of Cypridina luciferin (Cypridina luciferyl sulfate) (**6**).

2. Results and discussion

2.1. Identification of an acid-labile derivative of Cypridina luciferin in the extracts of *C. hilgendorfii*

The extracts of *C. hilgendorfii* were prepared by homogenizing with 0.1 M glycine–NaOH buffer (pH 8.5) and acidified by addition of 1% TFA. After incubating at 25 °C for various times, the acidic extracts were neutralized and used for the luminescence reaction with the extracts of *C. hilgendorfii* as a source of Cypridina luciferase. The luminescence activity was significantly stimulated by addition of the acid-treated fraction (Fig. 2). These results suggested that Cypridina luciferin was produced by acid hydrolysis of a Cypridina luciferin derivative and/or released from a denatured protein, such as a luciferin-binding protein. In *Renilla*, the coelenterazine-binding protein has been reported[22] and can stabilize coelenterazine in aqueous solution.[23]

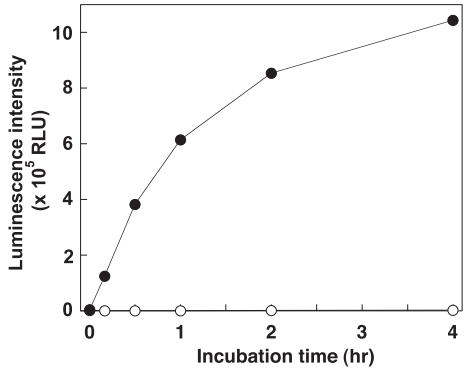

Fig. 2. Stimulation of the luminescence activity of Cypridina luciferase by the acid-treated fractions of the extracts of *C. hilgendorfii*. The extracts of *C. hilgendorfii* were incubated in 1% TFA for various times, neutralized, and then used in the luminescence reaction of Cypridina luciferase. The assay conditions are described in Materials and methods.

2.2. Isolation of an acid-labile derivative of Cypridina luciferin from the extracts of *C. hilgendorfii*

To isolate an acid-labile derivative of Cypridina luciferin, the extracts of *C. hilgendorfii* were applied on a reversed-phase silica gel column. An acid-labile compound was identified in the methanol fraction by measuring the luminescence activity after acid hydrolysis. In addition, an acid-labile derivative of Cypridina luciferin was separated under neutral pH conditions by RP-HPLC using an ODS column and then was analyzed by LC–TOF–MS in ESI mode. The mass spectrum of the isolated compound showed *m*/*z* 484.1799 ($[M-H]^-$) in negative ion mode (Fig. 3A), and *m*/*z* 508.1768 ($[M+Na]^+$) in positive ion mode (Fig. 3B). These results indicated that the molecular weight of the acid-labile derivative of Cypridina luciferin was 485. In the MS/MS spectrum of *m*/*z* 486 in positive ion mode, the ion peak at *m*/*z* 406 was generated without collision energy (data not shown). The fragmentation pattern in the MS/MS spectra of *m*/*z* 406 was identical to that of the synthetic (*S*)-Cypridina luciferin. Furthermore, the retention time of Cypridina luciferin on HPLC was different from that of the acid-labile compound at 6.9 min. These results suggested that the ion peak at *m*/*z* 406 was the fragment ion of *m*/*z* 486, and that the acid-labile compound was expected to be Cypridina luciferin modified with an adduct of 80 Da.

2.3. Identification of an acid-labile derivative of Cypridina luciferin as 3-enol sulfate of Cypridina luciferin

The difference of 80 Da between the acid-labile compound and Cypridina luciferin suggested that the functional group in the acid-labile derivative of Cypridina luciferin could be either sulfate (SO_3, calculated mass=79.96) or phosphate (PO_3H, calculated mass=79.97). The observed mass of the acid-labile derivative of Cypridina luciferin was closer to the calculated mass of luciferyl sulfate than that of luciferyl phosphate (Table 1). It is known that the natural sulfur shows a ^{34}S peak with 4.5% to a ^{32}S peak (100%), and a sulfur-containing compound can be identified by the ratio of the third to the first isotopic peak. The relative intensities of the third isotopic peak for the acid-labile compound were close to the calculated mass of luciferyl sulfate (Table 1). The MS/MS spectrum of the acid-labile compound showed the mass peaks at *m*/*z* 79.9 (100%) and *m*/*z* 81.9 (3.5%) (Fig. 4B). These values were close to those of $MgSO_4$ (Fig. 4C), but not NaH_2PO_4 (Fig. 4D). From these results, we concluded that the acid-labile compound was the sulfate form of Cypridina luciferin.

As described above, the MS/MS spectrum of the acid-labile compound showed a characteristic ion with a neutral loss of 80 Da. Yi et al. suggested that the sulfated aromatic alcohols or enols are dissociated by the heterolytic cleavage to produce a characteristic ion peak with a neutral loss of 80 Da, which forms an ion peak at *m*/*z* $[M-H-80]^-$.[24] The hemolytic cleavage of the sulfate group in other sulfated compounds dose not give a characteristic ion peak at *m*/*z* $[M-H-80]^-$. Therefore, the acid-labile compound has the structure of an enol sulfate in Cypridina luciferin. From these results, we concluded that the acid-labile derivative of Cypridina luciferin is the 3-enol sulfate of Cypridina luciferin, and we named it Cypridina luciferyl sulfate.

2.4. Chemical synthesis of Cypridina luciferyl sulfate from (*S*)-Cypridina luciferin

Cypridina luciferyl sulfate as an authentic compound for mass spectrometry was chemically synthesized according to the similar