Identification of the Luciferin–Luciferase System and Quantification of Coelenterazine by Mass Spectrometry in the Deep-Sea Luminous Ostracod *Conchoecia pseudodiscophora*

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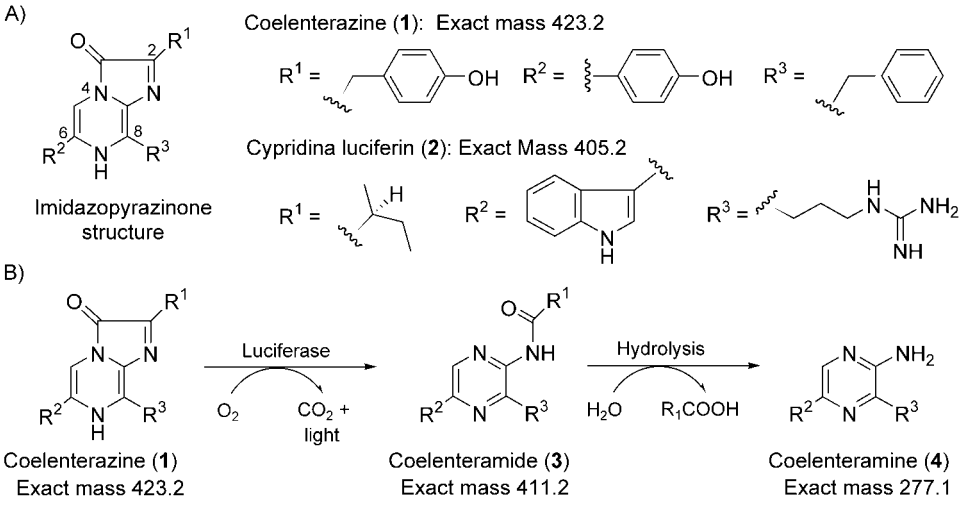
The bioluminescence system of the ostracod Conchoecia pseudodiscophora*, which is abundant in the Sea of Japan, has been characterized. The luminescence (λ_{max} = 463 nm) is produced by a luciferin–luciferase reaction, and the luciferin has been identified as coelenterazine. Coelenterazine, coelenteramide, and coelenteramine from* C. pseudodiscophora *were quantified by LC-ESI-MS/MS analysis. The coelenterazine content was estimated to be approximately 230 pg per animal by using a calibration curve of synthetic coelenterazine. The reaction between homogenates of* C. pseudodiscophora *and synthetic coelenterazine showed luminescence activity; this suggests that a coelenterazine-type luciferase is present.*

Introduction

The phenomenon of bioluminescence in marine organisms has been investigated,[1,2] and light emission has been found to be produced principally through a luciferin–luciferase reaction. Several luciferins have been identified in marine organisms,[3] and one well-characterized type is represented by imidazopyrazinone (3,7-dihydroimidazopyrazin-3-one) compounds such as coelenterazine (**1**)[4] and Cypridina luciferin (=Vargula luciferin; **2**),[5,6] as shown in Scheme 1 A. Coelenterazine (**1**) is widely distributed in luminous and nonluminous marine organisms,[7–11] and has been identified as a luciferin in various luminous organisms including *Renilla*,[12] *Oplophorus*,[13] *Periphylla*,[14] and *Gaussia*.[15] It is also known as a component of calcium-sensitive photoproteins such as aequorin[16] and obelin.[17] Cypridina luciferin (**2**) was isolated from the luminescent ostracod *Cypridina hilgendorfii* (now known as *Vargula hilgendorfii*)[5,6,9,11] and is also used for luminescence reactions of luminous fish[18] in the Osteicthyes *Porichthys notatus*,[19–21] *Apogon*,[22–23] and *Parapriacanthus*.[24]



Scheme 1. *Imidazopyrazinone structure (A) and the luminescent reaction of coelenterazine by luciferase (B).*

In the class Ostracoda, luminous species have been found in the families Cypridinidae and Halocypridae,[25,26] and are present in the genera of *Cypridina* (Cypridinidae), *Vargula* (Cypridinidae), and *Conchoecia* (Halocypridae).[9,27] In *Conchoecia*, coelenterazine has been detected in twelve species.[9] These findings indicate that *Vargula* and *Conchoecia*, both classed as Ostracoda, utilize different luciferins based on the same imidazopyrazinone component structure. This is an interesting observation in relation to events in evolution.

To understand the luciferin–luciferase system in *Conchoecia*, we focused on the specimen readily collectable in Japan and so chose *Conchoecia pseudodiscophora* for analyses. The Halocypridae *C. pseudodiscophora* (body length less than 1.5 mm) is abundant in the mesopelagic zone (300–800 m depth) of the Sea of Japan.[28] Studies on the population and life cycle of

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C. pseudodiscophora having been reported in detail by Ikeda and Imamura.[29] However, the bioluminescent properties of *C. pseudodiscophora* have not been described in these reports.

In this manuscript we describe the isolation and identification of coelenterazine as a luciferin and its oxidized products (coelenteramide (**3**) and coelenteramine (**4**); Scheme 1 B). Amounts of coelenterazine were determined by mass spectrometry. Because of the low concentrations of coelenterazine in *C. pseudodiscophora*, we employed reversed-phase HPLC in tandem with electrospray ionization triple quadrupole (ESI/3Q) mass spectrometer, in product ion scan and multiple reaction monitoring (MRM) modes.[30,31] The presence of a luciferase in *C. pseudodiscophora* was also confirmed by a method based on the luminescence cross-reaction of the homogenate of *C. pseudodiscophora* with synthetic coelenterazine.

Results and Discussion

Identification of bioluminescence in *C. pseudodiscophora*

Live specimens of *C. pseudodiscophora* (Figure 1 A) from the Sea of Japan were collected from seawater at 333 m depth and were kept at 4 °C. When the specimens were stimulated physically, blue luminescence was observed with the naked eye. Light emission did not last more than a few seconds. As previously reported by Angel in 1968,[25] eleven *Conchoecia* species (*C. alata*, *C. bispinosa*, *C. curta*, *C. daphnoides*, *C. elegans*, *C. imbricata*, *C. magna*, *C. rhynchena*, *C. secernenda*, *C. spinifera*, and *C. subarcuata*) are luminous. The luminescence types of species were classified as secretion or/and as retention within the carapace.[25] In *C. pseudodiscophora*, luminescence was only observed inside of the carapace and was not secreted outside of the bodies. Interestingly, the blue luminescence occurred in the same particles as blue fluorescence, scattered through the carapace (Figure 1 B). For measurement of the luminescence spectrum, the frozen animals were thawed at room temperature, and the blue luminescence was recorded. As shown in Figure 2, the emission peak of the luminescence was at 463 nm, which is similar to that seen for other coelenterazine-type luminescence, such as seen with *Renilla* luciferase and photoproteins.[32]

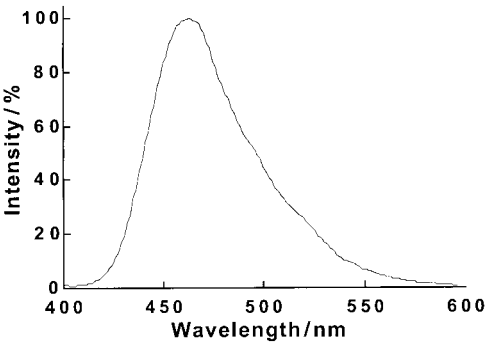

Figure 2. *Bioluminescence spectrum obtained from frozen and subsequently thawed* C. pseudodiscophora. *Scan speed; 500 nm min*$^{-1}$.

Identification of coelenterazine and its oxidized products by mass spectrometry

To identify whether the luciferin was coelenterazine or Cypridina luciferin, the *C. pseudodiscophora* specimens were extracted with methanol, and the extracts were analyzed by LC-ESI-MS/MS. As shown in Figure 3, MRM analysis exhibited the presence of coelenterazine, coelenteramide, and coelenteramine, with the same HPLC retention times and mass values as the authentic compounds. Cypridina luciferin and its oxidative products were not detected in the methanol extracts. Further, product ion scan analysis of $m/z = 424.0$, corresponding to the calculat-

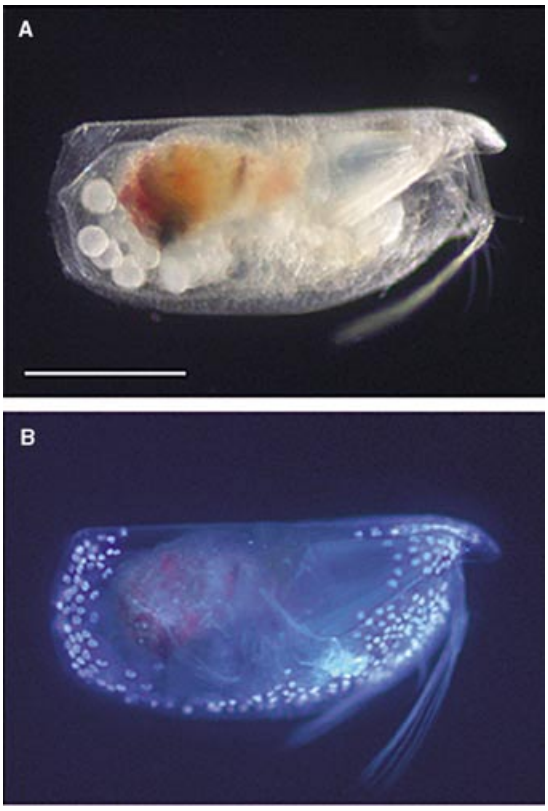

Figure 1. *Photographs of* Conchoecia pseudodiscophora *(A) and of its fluorescence (B) under a microscope. Scale bar; 0.5 mm.*

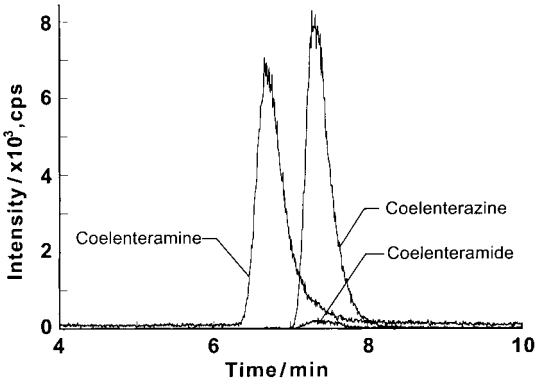

Figure 3. *Mass chromatogram for methanol extracts of* C. pseudodiscophora *by MRM analysis. Coelenterazine, coelenteramide, and coelenteramine were monitored at* m/z *= 302.0, 278.0, and 200.1, respectively.*

ed $[M+H]^+$ mass value of coelenterazine, showed that the fragment ion peaks of the sample were predominantly consistent with those of the synthetic coelenterazine (Figure 4). These results indicated that the compound with a mass value of $m/z = 424.0$ in *C. pseudodiscophora* was coelenterazine.

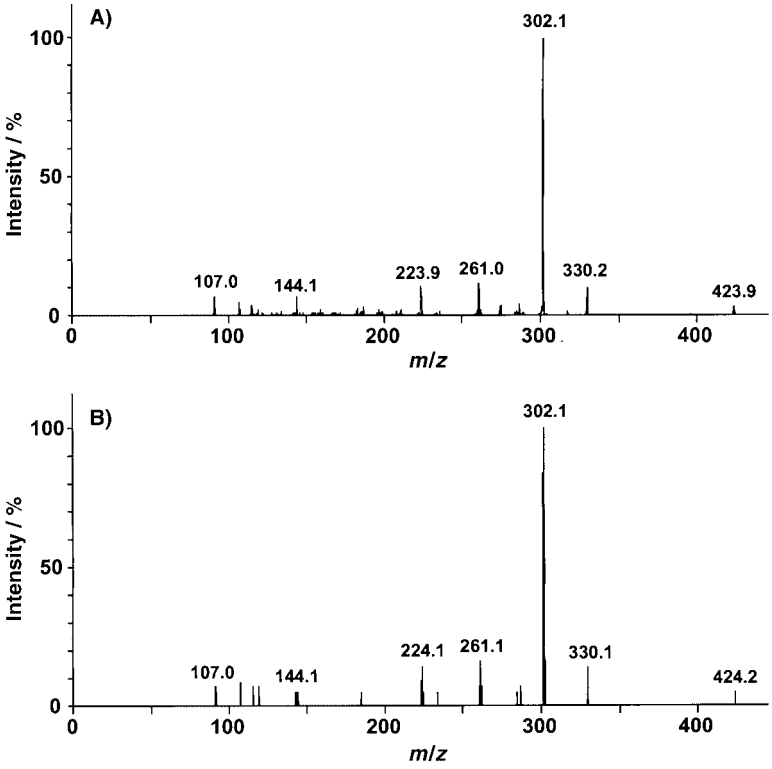

Figure 4. *Identification of coelenterazine in methanol extracts of* C. pseudodiscophora *by the product ion scan analysis of* m/z *424. A) Synthetic coelenterazine. B) Methanol extracts of* C. pseudodiscophora.

Calculation curve for coelenterazine, coelenteramide, and coelenteramine

To estimate the concentrations of coelenterazine, coelenteramide, and ceolenteramine in *C. pseudodiscophora*, standard curves were prepared by the MRM analysis method by using the authentic compounds. As shown in Figure 5, these compounds showed linear relationships; linearity of quantification for coelenteramide and coelenteramine was observed from 0.1 to 1000 ng mL^{-1}. Meanwhile, since the lower concentration of coelenterazine was decomposed by air oxidation to give coelenteramide, the linearity of coelenterazine was observed only from 30 to 1000 ng mL^{-1}. Under our MRM analysis conditions, the detection limits of coelenterazine, coelenteramide, coelenteramine, and Cypridina luciferin were approximately 10, <0.1, <0.1, and 10 ng mL^{-1}, respectively.

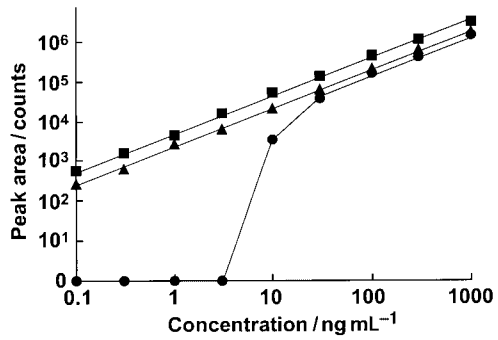

Figure 5. *Calibration curve for coelenterazine (●), coelenteramide (▲), and coelenteramine (■) by MRM analysis.*

Quantification of coelenterazine in *C. pseudodiscophora*

The MRM quantification analyses revealed that the levels of coelenterazine, coelenteramide, and coelenteramine in *C. pseudodiscophora* were approximately 230, <0.2, and 80 pg per individual specimen, respectively (Table 1). As coelenteramide and coelenteramine are oxidized products of coelenterazine (Scheme 1), the contents of these compounds might fluctuate in each specimen. Since the specimens had been affected physically during collection and had produced luminescence, the actual amount of coelenterazine might have been affected. On the other hand, when methanol extracts from *C. pseudodiscophora* were left at room temperature for 1 h, coelenterazine in the extracts partially decomposed into coelenteramine via coelenteramide by air oxidation (data not shown).

Table 1. *Quantitation of coelenterazine and related compounds in* C. pseudodiscophora *by MRM analysis.*

	Quantity (pg per specimen)[a]
Coelenterazine	230 ± 80
Coelenteramide	<0.2
Coelenteramine	80 ± 19

[a] Three independent samples were measured.

Identification of the luciferase-luciferin system in *C. pseudodiscophora*

Coelenterazine was identified in *C. pseudodiscophora* by mass spectral analyses. Further, the presence of coelenterazine was confirmed by the luminescence reaction with recombinant *Renilla* luciferase, a coelenterazine-type luciferase specific for coelenterazine as the luciferin.[32] Methanol extracts from *C. pseudodiscophora* were fractionated by reversed-phase HPLC, and individual fractions were assayed by incubation with *Renilla* luciferase. Only the peak fractions corresponding to coelenterazine on HPLC showed luminescence activity (open bars in Figure 6).

On the other hand, to identify the presence of a coelenterazine-type luciferase in *C. pseudodiscophora*, the homogenate of *C. pseudodiscophora* was assayed as a source of luciferase with

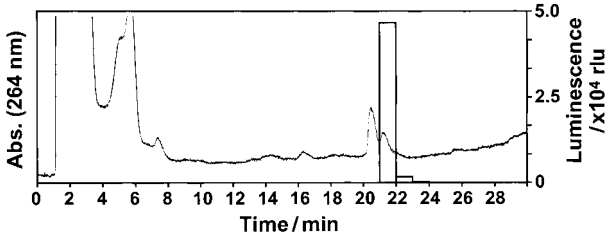

Figure 6. *HPLC chromatogram of methanol extracts of* C. pseudodiscophora *and its luminescence activity. UV absorbance was monitored at 264 nm. Luminescence activities are shown by open bars.*

synthetic coelenterazine. As shown in Table 2, luminescence activity was detected in the homogenate from *C. pseudodiscophora* with coelenterazine. Thus *C. pseudodiscophora* has a luciferase that will catalyze the oxidation of coelenterazine, similarly to other previously reported luminous *Conchoecia sp.* (*C. hiura*, *C. ametra*, *C. kampta*, and *C. rhynchena*).[9] Recently, phylogenetic analyses have suggested that the genera *Conchoecia* and *Vargula* both belong to the same group of Ostracoda (Crustacea).[33] It is of interest that their luminous species utilize different luciferins—coelenterazine and Cypridina luciferin—despite their evolutionary relationship.

Table 2. *Luciferase activity of the homogenate from* C. pseudodiscophora.

Coelenterazine	Homogenate[a]	Luminescence activity (I_{max} [rlu]) 1st	2nd
−	−	2	2
+	−	10	7
−	+	24	19
+	+	783	479

[a] Homogenate corresponds to five specimens.

Conclusion

In conclusion, *C. pseudodiscophora* living in the Sea of Japan is a luminous species, and its luminescence is produced by a typical luciferase–luciferin reaction. The luciferin is coelenterazine, and a coelenterazine-type luciferase is present. The amount of coelenterazine was estimated to be approximately 230 pg per specimen, and the oxidized products—coelenteramine and coelenteramide—were also quantified by LC-ESI-MS/MS analyses. In these studies, the quantification and qualification of coelenterazine were performed with a single specimen of *C. pseudodiscophora*. It is planned to apply this method to the detection of coelenterazine in other luminous and nonluminous marine organisms.

Experimental Section

Specimens: Specimens of *C. pseudodiscophora* were collected from the drain filter of deep-sea water pumped up from 333 m depth at Toyama Bay in Japan on November 12, 2002, January 15, 2003, and April 16, 2003. Live animals were kept on ice and isolated by use of a syringe (5 mL). After removal of excess seawater, the collected animals (size: 0.7 to 1.5 mm in body length) in a tube (1.5 mL) were weighed, frozen in liquid nitrogen, and stored at −80 °C.

Chemicals: Coelenterazine and Cypridina luciferin were chemically synthesized.[4,34] Coelenteramide and coelenteramine were obtained as a gift from Dr. K. Teranishi (Mie University, Japan). Recombinant *Renilla* luciferase was prepared as previously described.[32] All other chemicals were of the highest grade commercially available.

Fluorescence microscopy: The fluorescence photograph of a living specimen was taken with a digital camera (Coolpix 995, Nikon, Tokyo, Japan) under a fluorescence microscope (Eclipse TE200, Nikon) with a mercury lamp as light source and a UV-2A filter (Nikon).

Determination of bioluminescence spectrum: The bioluminescence spectrum of *C. pseudodiscophora* was measured on a Jasco FP-777W fluorescence spectrophotometer (Jasco, Tokyo, Japan) at 25 °C with the excitation light source turned off. Frozen animals (50 specimens) in a cuvette were thawed at room temperature, and the luminescence spectrum was recorded.

Preparation of methanol extracts from *C. pseudodiscophora* for MS analyses: The general method for preparation of extracts from *C. pseudodiscophora* is as follows. Frozen animals (50 specimens) were homogenized on dry ice with methanol (50 μL) containing dithiothreitol (DTT; 6.5 μM) by use of a plastic pestle. The homogenate was sonicated for 30 s (US-4, Iuchi, Osaka, Japan) and was centrifuged at 17400×g for 10 min at 4 °C. The resultant supernatant was filtered with Ultrafree-MC (0.45 μm, Amicon, Bedford, MA) and the filtrate was stored at −80 °C before analysis.

Preparation of *C. pseudodiscophora* homogenate for luciferase analyses: To determine the luciferin–luciferase reaction, a homogenate of *C. pseudodiscophora* as a source of luciferase was prepared as follows. Frozen animals (50 specimens) were homogenized (pestle) on ice in Tris-HCl (1 mL, pH 7.6, 30 mM)/EDTA (10 mM) for 1 min. A potion of the homogenate (100 μL = 5 animals) was used immediately for determination of the luminescence activity.

Mass spectrometry: A methanol extract from frozen animals (50 specimens, wet weight 16.7 mg) was prepared and analyzed by LC-ESI-MS/MS on an Agilent 1100 HPLC system (Agilent, Wilmington, DE) connected to an API 2000 (Applied Biosystems, Foster City, CA). The column was a Cadenza CD-C18 (2.0×75 mm, Imtakt, Kyoto, Japan), and the mobile phase was water/methanol containing formic acid (0.1 %) with a linear gradient of methanol from 25 to 95 % (2 % per min). The flow rate was 0.2 mL min^{-1}, and monitoring of peaks was performed by mass chromatogram and UV absorption at 264 nm. The ESI-MS/MS mode was positive and the source temperature was 500 °C.

For product ion scan analysis, aliquots (40 μL) of the methanol extracts were used. Tandem mass spectrometric analysis was performed with nitrogen as the collision gas (collision energy, 45 V).

For MRM analysis, aliquots (10 μL) of the methanol extracts were used. Tandem mass spectrometric analysis was performed with nitrogen as the collision gas (collision energy, 30 V). The equipment and the analytical conditions were the same as for the LC-ESI-MS/MS analysis, except the methanol gradient was from 50 to 100 % (5 % per min). The mass values for parent/fragment ions were monitored as follow: coelenterazine (424.0/302.0), coelenteramide (412.0/278.0), coelenteramine (278.1/200.1), and Cypridina luciferin (406.2/347.2).

Isolation of coelenterazine by HPLC: Methanol extracts of *C. pseudodiscophora* (100 specimens, wet weight 27.8 mg) were prepared, dried under nitrogen gas, and then dissolved in methanol (10 μL). This solution was subjected to HPLC for separation. The equipment and the analytical conditions were the same as those used in the case of LC-ESI-MS/MS analysis, except for the lack of ESI-MS/MS. Monitoring of elution was performed at 264 nm and the eluate was fractionated. Methanol solution (2 μL) containing dithiothreitol (65 μM) was added to the fractions. The fractions were concentrated in vacuo by centrifugation (EC-57CST, Sakuma, Tokyo, Japan) and the solution was lyophilized and stored at −80 °C.

Luminescence assay: Luminescence activities were determined by use of an Atto (Tokyo, Japan) model AB-2200 luminometer. i) For identification of coelenterazine, the lyophilized fractions from the HPLC separation were dissolved in methanol (20 μL), and 1 μL was used. The luminescence reaction was started by the addition of buffer (30 mM Tris-HCl, 10 mM EDTA, pH 7.5, 99 μL) containing recombinant *Renilla* luciferase (0.5 μg) at 25 °C. The luminescence activity was measured and integrated for an initial 10 s. ii) For detection of luciferase activity, synthetic coelenterazine (1 μg) dissolved in methanol was added to the homogenate of *C. pseudodiscophora* (100 μL), and the luminescence was measured for 10 s. The initial intensity (I_{max}) of 1 ng of the purified recombinant aequorin showed 6.4×10^4 relative light units (rlu).

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Keywords: coelenteramide · coelenteramine · Cypridina luciferin · luminescence · mass spectrometry

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