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BIOSYNTHESIS OF CYPRIDINA LUCIFERIN IN *CYPRIDINA NOCTILUCA*

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Abstract – Luminescence in the marine ostracods *Cypridina noctiluca* and *Vargula hilgendorfii* is produced by Cypridina luciferin-luciferase reaction. In this study, a feeding experiment with stable isotope-labeled amino acids was performed in *C. noctiluca*. The results of LC/ESI-MS analyses indicated that *C. noctiluca* is able to synthesize Cypridina luciferin *de novo* from three amino acids (L-tryptophan, L-isoleucine, and L-arginine).

Luminescence in living animals is produced principally through luciferin-luciferase reaction.[1] In marine organisms, the chemical structures of several luciferins have been determined, including coelenterazine,[2] *Watasenia* luciferin,[3] Cypridina luciferin,[4] krill luciferin,[5] and dinoflagellate luciferin.[6] The well-characterized luciferins are imidazopyrazinone (3,7-dihydroimidazopyrazin-3-one) compounds such as coelenterazine and Cypridina luciferin, and they are distributed in various marine animals.[1,7,8,9] Luminous ostracods have been found in the genera of *Vargula*, *Cypridina*, *Pyrocypris* and *Conchoecia*.[1,10,11] *Vargula*, *Cypridina* and *Pyrocypris* are classified in the family Cypridinidae and the luciferin for luciferase reaction is Cypridina luciferin.[10] *Conchoecia* is classified in the family Halocyprididae and the luciferin for luciferase reaction is coelenterazine.[8,9,12] As previously proposed,[4,13] imidazopyrazinone-type luciferins would be biosynthesized from three amino acids (i.e., arginine, isoleucine and tryptophan for Cypridina luciferin; phenylalanine and two tyrosines for coelenterazine). Recently, we found that Cypridina luciferin in *Vargula hilgendorfii* (formerly *Cypridina hilgendorfii*) was biosynthesized from three L-amino acids (L-arginine, L-isoleucine and L-tryptophan; Figure 1).[14-16] However, the biosynthesis of Cypridina luciferin in other species such as *Cypridina* and *Pyrocypris* has

not been reported. In this study, we quantified Cypridina luciferin in *Cypridina noctiluca* and determined its biosynthetic units by the methods of LC/MS.

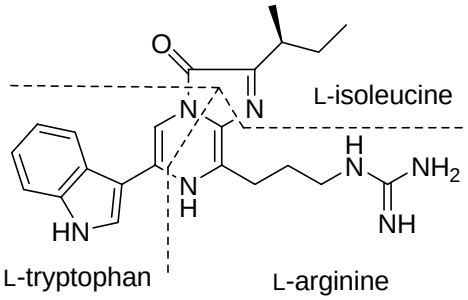

Figure 1. Chemical structure and biosynthetic units of Cypridina luciferin.

The specimens of *C. noctiluca* (*ca*. 0.7 mm of body length) were collected using a plankton-net (mesh size, 0.18~0.23 mm) at Shimoda, Shizuoka, Japan on 9 October 2005.

For quantification of Cypridina luciferin in *C. noctiluca*, three frozen specimens were extracted with 30 μL of ethanol, and an aliquot (2 μL) was analyzed by a reversed phase (RP)-HPLC connected to an electrospray-ionization (ESI) triple quadrupole tandem mass spectrometer (RP-HPLC/ESI-QqQ-MS/MS) on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with API 2000 (Applied Biosystems, Foster City, CA). The RP-HPLC conditions were as follows: column, Cadenza CD-C18 (2 × 75 mm; Imtakt, Kyoto, Japan) connected to a Unison US-C18 pre-column (2 × 5 mm, Imtakt); mobile phase, methanol-water containing 0.1% formic acid, a linear gradient from 25% to 88% methanol for 7 min and then isocratic elution with 88% methanol for 3 min; flow rate, 0.2 mL/min; UV detection, 280 nm. The ESI-MS/MS mode was positive and the source temperature was 350°C. The authentic *dl*-Cypridina luciferin for the calibration was chemically synthesized,[17] and was eluted at 8.3 min under the HPLC conditions described above. The amount of Cypridina luciferin was determined by monitoring the mass values of *m/z* 406 (parent ion) and 389 (fragment ion) using the multiple reaction monitoring (MRM) mode.[12] The results showed that the total amount of Cypridina luciferin in *C. noctiluca* was 17 to 46 pmol per specimen (n=3). This amount is comparable to the reported value for the congeneric species *Cypridina dentata* (13 to 28 pmol), which was estimated by luciferin-luciferase reaction.[9] In *V. hilgendorfii*, the amount of Cypridina luciferin was estimated to be 75 pmol (*ca*. 1.0 mm of body length) to 7.8 nmol (*ca*. 3.0 mm) per specimen.

To demonstrate the incorporation of free amino acids into Cypridina luciferin in *C. noctiluca*, three stable isotope-labeled amino acids were used for the feeding experiments as follows: L-[indole-$^{2}H_{5}$]tryptophan,[14] L-[$^{13}C_{6}$]isoleucine (Cambridge Isotope Laboratories, Inc., Andover, MA), and L-[guanidino-$^{15}N_{2}$]arginine (Mass Trace, Inc., Woburn, MA). The composition of stable isotopes in the

labeled amino acids was determined by LC/ESI-ion trap (IT)-MS on an Agilent 1100 HPLC system equipped with an Esquire 3000 (ESI-IT-MS; Bruker Daltonics, Billerica, MA); mobile phase, 50% methanol-water containing 0.1% formic acid; flow rate, 0.2 mL/min (Table 1).

Table 1. Relative intensity of mass ion peaks (monovalent) in stable isotope-labeled amino acids

No. of stable isotope atom	Relative intensity (%)		
	L-[indole-$^{2}H_5$]Trp	L-[$^{13}C_6$]Ile	L-[guanidino-$^{15}N_2$]Arg
+0	1.07	1.19	1.47
+1	0.47	0.89	0.97
+2	0.85	0.26	100.00
+3	7.24	0.75	6.63
+4	38.53	1.91	0.53
+5	100.00	11.01	-
+6	11.19	100.00	-
+7	1.51	1.70	-

Feeding experiments were performed using a 24-well microplate. A stable isotope-labeled amino acid (25 mg) was dissolved in aqueous extracts of porcine liver (0.5 mL), and gelled with 3% agarose (Type VII, Sigma). The specimens (3~7 bodies) in a single-well fed with a piece of the gel for 10 days were frozen in liquid nitrogen, and then extracted with a 4-5 times weight volume of ethanol on dry ice. An aliquot (2 μL) was analyzed by LC/ESI-IT-MS under the conditions described above. The incorporation efficiencies of L-[indole-$^{2}H_5$]tryptophan, L-[$^{13}C_6$]isoleucine and L-[guanidino-$^{15}N_2$]arginine into Cypridina luciferin were 15.4%, 2.8% and 2.4%, respectively (Table 2). These results suggested that *C. noctiluca* synthesizes Cypridina luciferin from these three amino acids.

Table 2. Incorporation of stable isotope-labeled amino acids into Cypridina luciferin (divalent ion) in *Cypridina noctiluca*

No. of stable isotope atom	Relative intensity (%)			
	Non-feeding	L-[indole-$^{2}H_5$]Trp	L-[$^{13}C_6$]Ile	L-[guanidino-$^{15}N_2$]Arg
+0	100.00	100.00	100.00	100.00
+1	25.29	29.30	26.00	24.69
+2	3.72	4.33	3.13	6.44
+3	0.68	2.31	0.64	0.86
+4	-	3.52	0.19	0.37
+5	-	11.55	-	-
+6	-	2.12	2.62	-
+7	0.29	0.58	1.18	0.77
Total	129.98	153.71	133.76	133.13
Incorporation*	-	15.44%	2.83%	2.37%

* Incorporation = $(Total_{feeding}-Total_{non\text{-}feeding})/Total_{feeding} \times 100$

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