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Identification of the biosynthetic units of Cypridina luciferin in *Cypridina* (*Vargula*) *hilgendorfii* by LC/ESI-TOF-MS

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Abstract—In a luminous ostracod *Cypridina* (*Vargula*) *hilgendorfii*, Cypridina luciferin with an imidazopyrazinone structure (3,7-dihydroimidazopyrazin-3-one) is utilized for the luminescence reaction. To identify the biosynthetic units of Cypridina luciferin, the stable isotope labeled compounds were examined by feeding experiments with living *Cypridina* specimens. The incorporation of the labeled compounds into Cypridina luciferin was identified by the method of LC/ESI-TOF-MS analyses and these results suggested that L-tryptophan, L-arginine and L-isoleucine are structural units of Cypridina luciferin.

1. Introduction

In bioluminescent marine organisms, Cypridina luciferin (**1**) and coelenterazine (**2**) having an imidazopyrazinone structure (3,7-dihydroimidazopyrazin-3-one, Fig. 1) are used for the luminescence reaction as follows;

$$\text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + CO_2 + \text{Light}$$

Cypridina luciferin (**1**) is isolated from a luminous ostracod crustacean, *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*), living near the Japanese coast.[1] It is also utilized for the luciferase reaction in certain kinds of bioluminescent fishes including *Apogon*, *Parapriacanthus and Porichthys*.[2] On the other hand, coelenterazine (**2**) is widely distributed in luminous and non-luminous coelenterates, fishes, shrimps and squids,[3] and is known as *Watasenia* preluciferin, *Renilla* luciferin and *Oplophorus* luciferin.[4] Further, **2** serves as the chromogenic compound of photoproteins including aequorin and obelin.[5]

The luminescence system of *Cypridina* has been investigated extensively, since Harvey reported the luciferin-luciferase reaction with extracts of the specimens in 1917.[6] When *Cypridina* specimens are stimulated physically or electronically, it expels Cypridina luciferin (**1**) and luciferase directly into the seawater to produce a brilliant bluish luminescence ($\lambda_{max}=460$ nm). After the luminescent reaction, **1** is converted to oxyluciferin (**3**), which is then hydrolyzed to etioluciferin (**4**) (Fig. 2).[7] The chemical mechanism of the luminescence reaction catalyzed by

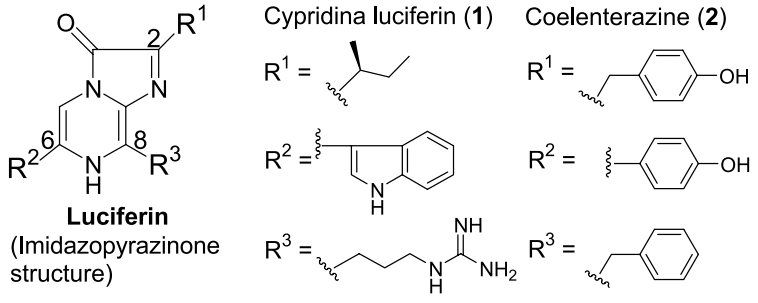

Figure 1. Structures of imidazopyrazinone, Cypridina luciferin (**1**) and coelenterazine (**2**).

Keywords: Bioluminescence; Stable isotope; Imidazopyrazinone; Luciferase; Biosynthesis.
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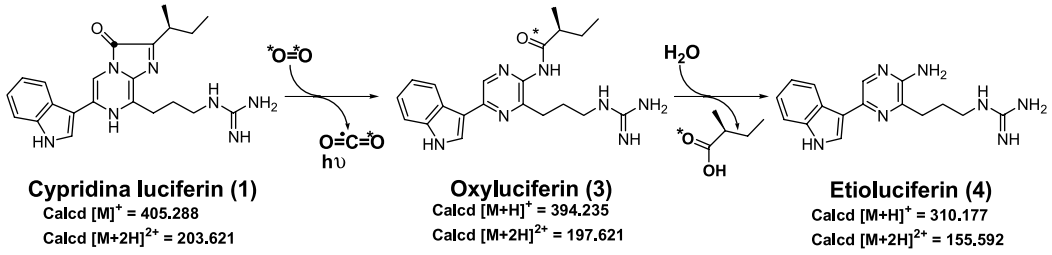

Figure 2. Luminescence reaction of Cypridina luciferin by Cypridina luciferase and the hydrolysis of oxyluciferin. Oxygen atoms derived from O_2 and a carbon atom at the C3 position of Cypridina luciferin are represented by asterisks and dot, respectively.

Cypridina luciferase was proposed.[8] Its luciferase gene was isolated[9] and has been utilized as a reporter protein.[10] The isolation, structural determination and total synthesis of Cypridina luciferin (**1**) was achieved[1] and Kishi et al. proposed that **1** should be biosynthesized from three amino acids or their equivalents: L-arginine, L-isoleucine and L-tryptophan (or tryptamine).[1]

Recently, we reported that L-tryptophan is one of the structural units of Cypridina luciferin (**1**) in its biosynthesis.[11] After feeding of the deuterium labeled L-tryptophan as bait to *Cypridina* specimens for 6 days, the ethanol extract from living specimens was analyzed by LC/ESI-TOF-MS and the incorporation of the deuterium labeled L-tryptophan into Cypridina luciferin was identified. In this study, other possible amino acids for the biosynthetic units of Cypridina luciferin (**1**) were examined and we concluded that natural amino acids of L-arginine, L-isoleucine and L-tryptophan are structural units, but not D-tryptophan and tryptamine.

Table 1. Monovalent and divalent ions of natural and synthetic Cypridina luciferin (**1**) by ESI-TOF-MS

Ions	Ion state	*m/z* (relative intensity, %)		Calculated mass (%)
		Natural[a]	Synthetic[b]	
Monovalent	$[M]^+$	405.227 (100)	405.226 (100)	405.228 (100)
	$[M+1]^+$	406.233 (39.6)	406.227 (51.6)	406.230 (27.4)
Divalent	$[M+2H]^{2+}$	203.619 (100)	203.614 (100)	203.621 (100)
	$[M+1+2H]^{2+}$	204.120 (27.5)	204.120 (29.8)	204.123 (27.4)

[a] Extracted from *C. hilgendorfii*.
[b] Chemical synthesized.[12]

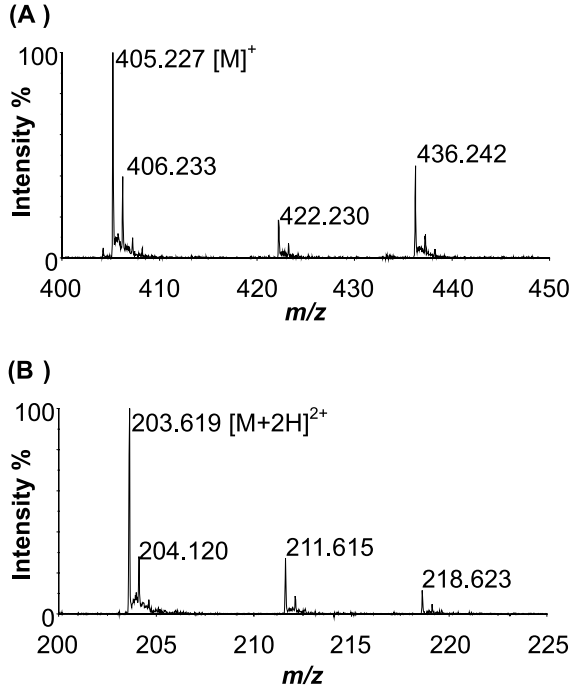

Figure 3. Mass spectra of Cypridina luciferin (**1**) extracted from *C. hilgendorfii* by ESI-TOF-MS (+). (A) monovalent ions, (B) divalent ions.

2. Results and discussion

2.1. ESI-TOF-MS analyses of natural and synthetic Cypridina luciferin (1)

Before identifying the incorporation of stable isotopic compounds into Cypridina luciferin (**1**) by LC/ESI-TOF-MS, **1** was analyzed by the infusion method with ESI-TOF-MS. As the authentic compounds, chemically synthesized *dl*-Cypridina luciferin (**1**) and natural **1** were used and the peaks of the monovalent and divalent ions were detected in the positive mode (Table 1 and Fig. 3). The mass value of monovalent ion corresponding to Cypridina luciferin (**1**) was mainly observed at *m/z* 405.2 as $[M]^+$, but not at *m/z* 406.2 as $[M+H]^+$. Similar peak patterns were observed by FD-MS[12] and MALDI-TOF-MS (data not shown). The intensities of *m/z* 406.2 $[M+1]^+$ as an isotopic peak for natural and synthetic Cypridina luciferin (**1**) were 39.6 and 51.6%, respectively. These intensities were inconsistent with the calculated value of 27.4%. On the other hand, the intensity of the isotopic peak (*m/z* 204.1) of the divalent ion was good agreement with the calculated value. Thus, the divalent ion was chosen for determining the number of isotope atoms in Cypridina luciferin (**1**). In ESI-TOF-MS analysis of Cypridina luciferin (**1**), the monovalent peaks at *m/z* 422.2 and *m/z* 436.2 were also detected and these mass

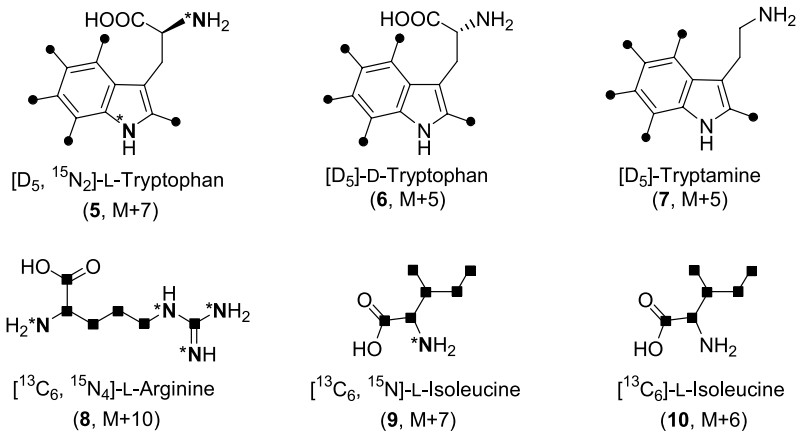

Figure 4. Stable isotope labeled compounds for the feeding experiments in *C. hilgendorfii*. Positions of stable isotopes are indicated as ^{13}C (■), ^{15}N (*N) and D (●).

values were corresponded to that of luciferinol and luciferyl methyl ether,[13] respectively. They might be generated during measuring with ESI-TOF-MS in our conditions.

2.2. Preparation of [D_5,$^{15}N_2$]-L-tryptophan (5), [D_5]-D-tryptophan (6) and [D_5]-tryptamine (7)

The deuterium labeled tryptophan (**5** and **6**) and tryptamine (**7**) (Fig. 4) were prepared by the deuterium-exchange method as previously reported.[11] Briefly, [D_5,$^{15}N_2$]-L-tryptophan (**5**) was prepared from [$^{15}N_2$]-L-tryptophan by heating with DCl in D_2O. [D_5]-D-Tryptophan (**6**) and [D_5]-tryptamine (**7**) were prepared from D-tryptophan and tryptamine by the same method, respectively. The purity of the deuterium labeled compounds was determined to be >99% by HPLC analysis.[11] The optical purity of these compounds was >95% ee, and slight decrease of optical purity was observed during the labeling procedures. To confirm the deuteration efficiency of the labeled compounds, [D_5,$^{15}N_2$]-L-tryptophan (**5**), [D_5]-D-tryptophan (**6**) and [D_5]-tryptamine (**7**) were analyzed by ESI-TOF-MS (Table 2). The peak ratios of [D_5]:[D_4] for L-tryptophan (**5**), D-tryptophan (**6**) and tryptamine (**7**) were 100: 23.3, 100: 53.0 and 100: 21.6, respectively (Table 2). The labeling efficiency of these compounds was suitable for the feeding experiments (Table 2, Fig. 4).

2.3. Identification of biosynthetic units in Cypridina luciferin (1) by LC/ESI-TOF-MS analysis

To identify the biosynthetic units in Cypridina luciferin (**1**), the feeding experiments were performed as described in Section 4.5. After feeding with isotope labeled compounds (Fig. 4) for 15 days, 2–5 specimens were extracted with ethanol and the incorporation of the stable isotopes into Cypridina luciferin (**1**) was identified by LC/ESI-TOF-MS. Cypridina luciferin (**1**) on HPLC was confirmed using *dl*-synthetic **1** (Fig. 5). Oxyluciferin (**3**) and etioluciferin (**4**)[1,7] were also detected in ethanol extracts. The mass spectral data (Fig. 6) indicated that [D_5,$^{15}N_2$]-L-tryptophan (**5**), [$^{13}C_6$,$^{15}N_4$]-L-arginine (**8**), [$^{13}C_6$,^{15}N]-L-isoleucine (**9**) and [$^{13}C_6$]-L-isoleucine (**10**) were incorporated into Cypridina luciferin as biosynthetic units (Table 3). Based on the peak intensity of mass spectra, the incorporation efficiency of [D_5,$^{15}N_2$]-L-tryptophan (**5**), [$^{13}C_6$,$^{15}N_4$]-L-arginine (**8**), [$^{13}C_6$,^{15}N]-L-isoleucine (**9**) and [$^{13}C_6$]-L-isoleucine (**10**) into **1** in living animals were estimated to 36.4%, 19.2%, 29.4% and 10.1%, respectively. The difference of the incorporation efficiency between [$^{13}C_6$,^{15}N]-L-isoleucine (**9**) and [$^{13}C_6$]-L-isoleucine (**10**) might be due to the animal conditions. On the other hand, Cypridina luciferin (**1**) obtained by feeding with [D_5]-D-tryptophan (**6**) and [D_5]-tryptamine (**7**) did not show the signification incorporation of stable isotopes. Thus, Cypridina luciferin (**1**) is

Table 2. Relative intensity of mass ion peaks in stable isotope labeled compounds

Numbers of stable isotope atom	Relative intensity (%)					
	[D_5,$^{15}N_2$]-L-Trp (**5**)	[D_5]-D-Trp (**6**)	[D_5]-Tryptamine (**7**)	[$^{13}C_6$,$^{15}N_4$]-L-Arg (**8**)	[$^{13}C_6$,^{15}N]-L-Ile (**9**)	[$^{13}C_6$]-L-Ile (**10**)
+0	3.3					
+1						
+2		3.2				
+3	2.0	13.9	3.3			
+4	2.4	53.0	21.6			
+5	5.2	100.0	100.0		2.0	6.6
+6	23.3	11.9	9.4		13.8	100.0
+7	100.0				100.0	1.1
+8	16.3					
+9	2.8			5.1		
+10				100.0		
+11						

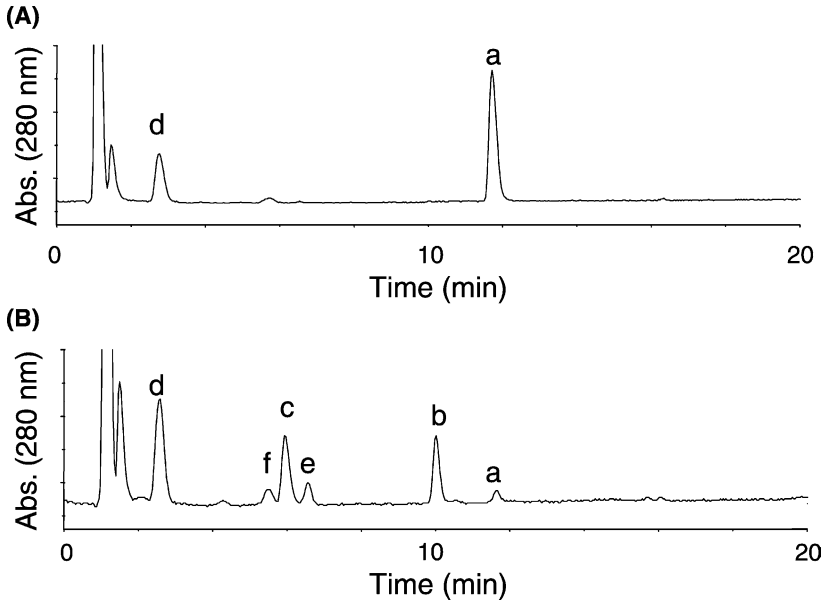

Figure 5. HPLC chromatogram of ethanol extracts from *C. hilgendorfii* (A) and its air oxidation products (B). a, Cypridina luciferin (**1**); b, oxyluciferin (**3**); c, etioluciferin (**4**); d, tryptophan; e, unknown (*m/z*=311); f, unknown (*m/z*, not detected).

biosynthesized from L-tryptophan, L-arginine and L-isoleucine in living animals, and tryptamine and D-tryptophan do not take part in the structural units. As previously reported,[1–4] Cypridina luciferin (**1**) and coelenterazine (**2**) are used for luciferase reaction in many marine organisms. In a luminous fish *Porichthys notaus*, the recycling system of Cypridina luciferin (**1**) was suggested.[15] However, the biosynthetic pathway and the animal species of Cypridina luciferin synthesis have not been demonstrated. Thus, the present study is the first demonstration of the biosynthesis of Cypridina luciferin (**1**) from three amino acids and suggests that coelenterazine (**2**) may be synthesized from two moles of L-tyrosine and one mole of phenylalanine in some marine organisms.

In the feeding experiments with [D_5,$^{15}N_2$]-L-tryptophan (**5**, M+7) and [$^{13}C_6$,^{15}N]-L-isoleucine (**9**, M+7), the mass spectral data of Cypridina luciferin (**1**) (Table 3, Fig. 6) indicated that the relative peak intensity of [M+7]-luciferin was decreased, by comparing the ratio of the relative intensity for [M+7]: [M+6] in [D_5,$^{15}N_2$]-L-tryptophan (**5**) and [$^{13}C_6$,^{15}N]-L-isoleucine (**9**) (Table 3, Fig. 6). To explain mass decrease in these labeled Cypridina luciferins, the labeled amino acids used for the feeding experiments were recovered from the animals and were analyzed by LC/ESI-TOF-MS (Table 4). The mass data indicated that the ratios of [M+6]:[M+7] for the labeled tryptophan and isoleucine from the animals were 100: 57.1 and 100: 33.6, respectively, and both intensities of [M+7] were decreased (Table 4). Thus, one stable isotope atom of D, ^{15}N or ^{13}C in the labeled amino acids was exchanged by a non-isotope atom after feeding. As previously reported,[11] the replacement of deuterium atoms on the indol ring of labeled tryptophan with hydrogen atoms from H_2O could not occur in living animals. When [$^{13}C_6$]-L-isoleucine (**10**, M+6) was used as bait, the loss of a ^{13}C atom was not detected (Table 3). These results suggested that the ^{15}N-atom of amino group in [D_5,$^{15}N_2$]-L-tryptophan (**5**) and [$^{13}C_6$,^{15}N]-L-isoleucine (**9**) were replaced with a ^{14}N-atom in living animals. Regarding [$^{13}C_6$,$^{15}N_4$]-L-arginine (**8**, M+10), the signal peak intensity of recovered arginine was lower and the significant exchange in four ^{15}N-atoms was not detected (Table 4). The replacement of amino group in L-tryptophan and L-isoleucine could occurred enzymatically, catalyzing by an enzyme like aminotransferase (EC 2.6.1.-) or dehydrogenase (EC 1.4.1.9).[16] From above results, we summarized the biosynthetic structural units of Cypridina luciferin (**1**) in Fig. 7, even if the biosynthetic pathway of Cypridina luciferin (**1**) from three amino acids in *C. hilgendorfii* is not clear.

2.4. Air oxidation of Cypridina luciferin (1) extracted from *C. hilgendorfii*

It is known that Cypridina luciferin (**1**) decomposes non-enzymatically into oxyluciferin (**3**) and CO_2 by air oxidation, and oxyluciferin (**3**) is further hydrolyzed to etioluciferin (**4**) with release of 2-methyl butyric acid, as similar to the Cypridina luciferase reaction[1,7] (Fig. 2). In our experiments with air oxidation procedures of labeled Cypridina luciferin, the formation of oxyluciferin (**3**) and etioluciferin (**4**)[1,7] was identified by LC/ESI-TOF-MS. As previously reported by Shimomura and Johnson,[17] the formation of CO_2 in the Cypridina luciferin-luciferase reaction was determined by incorporation of one oxygen atom from $^{18}O_2$ into CO_2. Thus, the carbon atom of CO_2 is considered to be the carbonyl carbon in the isoleucine moiety. The elimination of one carbon atom from Cypridina luciferin (**1**) was confirmed using [$^{13}C_6$]-Cypridina luciferin labeled with [$^{13}C_6$]-L-isoleucine (**10**). After air oxidation, [$^{13}C_6$]-Cypridina luciferin gave [$^{13}C_5$]-Cypridina oxyluciferin which was then converted to non-labeled etioluciferin (Table 3). On the other hand, the air oxidation products of Cypridina luciferin obtained from [D_5,$^{15}N_2$]-L-tryptophan

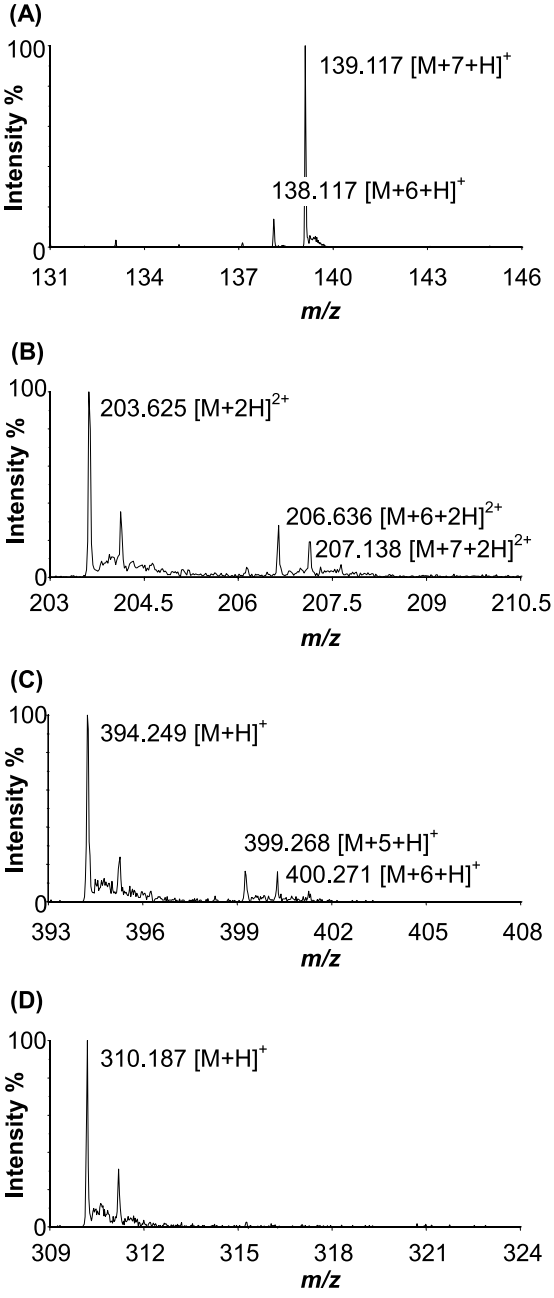

Figure 6. Mass spectra of Cypridina luciferin (**1**) labeled with [$^{13}C_6$,^{15}N]-L-isoleucine (**9**), its oxyluciferin and etioluciferin by LC/ESI-TOF-MS. (A) [$^{13}C_6$,^{15}N]-L-isoleucine (**9**), (B) Cypridina luciferin (**1**), (C) oxyluciferin (**3**), (D) etioluciferin (**4**).

(**5**) or [$^{13}C_6$,$^{15}N_4$]-L-arginine (**8**) retained the labeled isotopes in their molecules (Table 3).

3. Conclusion

After feeding experiments of the stable isotope labeled compounds to *C. hilgendorfii*, Cypridina luciferin (**1**) extracted from the living specimens was analyzed by LC/ESI-TOF-MS. From the mass spectral analysis, we concluded that Cypridina luciferin (**1**) is biosynthesized from three amino acids, L-tryptophan, L-arginine and L-isoleucine in *C. hilgendorfii*.

Table 3. Incorporation of the isotope labeled amino acids into Cypridina luciferin (**1**), oxyluciferin (**3**) and etioluciferin (**4**)

Numbers of stable isotope atom	Relative intensity (%)											
	[D_5,$^{15}N_2$]-L-Trp (**5**)			[$^{13}C_6$,$^{15}N_4$]-L-Arg (**8**)			[$^{13}C_6$,^{15}N]-L-Ile (**9**)			[$^{13}C_6$]-L-Ile (**10**)		
	1	**3**	**4**	**1**	**3**	**4**	**1**	**3**	**4**	**1**	**3**	**4**
+0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
+1	25.0	27.8	20.4	24.5	28.2	19.8	35.3	23.9	31.1	25.5	21.4	17.4
+2	8.2	8.4		8.6	8.4		7.6	5.6		7.0	7.0	
+3								3.0				
+4							5.4	16.4			7.8	
+5	9.5	7.2					28.3	16.0		11.5	3.7	
+6	24.3	37.7	43.0				19.2	5.6		3.4		
+7	30.5	36.4	40.1				6.7					
+8	11.9	17.7	21.6									
+9				5.1								
+10				19.8	16.9	23.0						
+11				6.8								

Table 4. Replacement of amino group in labeled amino acid after feeding experiments

Numbers of stable isotope atom	Relative intensity (%)		
	[D_5,$^{15}N_2$]-L-Trp (**5**)	[$^{13}C_6$,$^{15}N_4$]-L-Arg (**8**)	[$^{13}C_6$,^{15}N]-L-Ile (**9**)
+0	79.3	100.0	58.9
+1	22.1	24.3	14.3
+2			
+3			
+4	3.6		5.3
+5	26.6		10.3
+6	100.0		100.0
+7	57.1		33.6
+8	8.3		
+9	3.7	2.4	
+10		14.1	
+11			

4. Experimental

4.1. Materials

[$^{13}C_6$,^{15}N]-L-Isoleucine (**9**)(L-[U–$^{13}C_6$,U–^{15}N; >98%]isoleucine; chemical purity >95%) was purchased from Spectra Stable Isotopes (Columbia, MD, USA). [$^{15}N_2$]-L-Tryptophan (L-[U–$^{15}N_2$; 96–99%]tryptophan, chemical purity >98%), [$^{13}C_6$,$^{15}N_4$]-L-arginine (**8**)(L-[U–$^{13}C_6$; >99%, U–$^{15}N_4$; >99%]arginine), [$^{13}C_6$]-L-Isoleucine (**10**)(L-[U–$^{13}C_6$; >98%]isoleucine, chemical purity >98%) and D_2O (99.9 atom % D) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). DCl (20 wt% solution, 99.5 atom% D) and mercaptoacetic acid were from Aldrich. (1-Fluoro-2,4-dinitrophenyl)-5-L-alaninamide was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). *dl*-Cypridina luciferin as an authentic compound was chemically synthesized.[12]

4.2. Preparation of deuterium labeled indol compounds, [D_5,$^{15}N_2$]-L-tryptophan (5), [D_5]-D-tryptophan (6) and [D_5]-tryptamine (7)

The procedures for deuterium exchange at the indol ring in [$^{15}N_2$]-L-tryptophan, D-tryptophan and tryptamine were carried out as previously described.[11] A compound (20–40 mg/ml) was dissolved in 2 ml of D_2O containing 4% DCl and 5% of mercaptoacetic acid, and the mixture was degassed three times in a methanol-dry ice bath. The reaction mixture was heated at 110 °C for 3–4 h. After removing the reagents and solvent by evaporation, this labeling procedure was repeated one times. The resultant solution was concentrated, resolved in H_2O and dried up again. The deuterium labeled compound was obtained quantitatively as hydrochloride salt. The purity was confirmed by HPLC with a multi-wavelength UV detector and any other products were not detected. The labeling efficiency was determined by ESI-TOF-MS analysis and the peak ratios of [D_5]:[D_4] for [$^{15}N_2$]-L-tryptophan, D-tryptophan and tryptamine were 100:23.3, 100:53.0 and 100:21.6, respectively. The absolute configuration was determined by the Marfey's method to be >95% ee for both [D_5,$^{15}N_2$]-L-tryptophan (**5**) and [D_5]-D-tryptophan (**6**).

4.3. Determination of enantio excess of amino acids by the Marfey's method

Absolute configuration of the deuterium labeled tryptophan was determined by the Marfey's method.[14] Fifty microliters of 50 mM [D_5,$^{15}N_2$]-L- or [D_5]-D-tryptophan was added to 20 µl of 1 M $NaHCO_3$, and then the mixture was incubated with 100 µl of 1% (w/w) (1-fluoro-2,4-dinitrophenyl)-5-L-alaninamide in acetone for 1 h at 37 °C. The reaction was terminated by adding 20 µl of 1 N HCl and the mixture was subjected to reversed-phase HPLC analysis with a Develosil ODS-SR-5 (4.6×250 mm) column by a linear gradient of 30–60% acetonitrile in 0.1 M ammonium acetate (pH 3) in 45 min at a flow rate of 0.8 ml/min. The elution was monitored at 340 nm using a multiwave-length detector (MD-2010 plus, JASCO). Retention times of L- and D-derivatives were 25.8 and 28.3 min, respectively.

4.4. LC/ESI-TOF-MS analyses

The ethanol extracts from frozen specimens of *C. hilgendorfii* were analyzed by LC/ESI-TOF-MS with an Agilent 1100 HPLC system (Hewlett–Packard) connected to a Mariner Biospectrometry Workstation (Applied Biosystems). The column was Cadenza CD-C18 (2.0×75 mm, Imtakt) and the mobile phase was water–methanol containing 0.1% of formic acid, from 25 to 65% for 20 min at a flow rate of 0.2 ml/min. The monitoring was performed at 280 nm. The ESI-TOF-MS was carried out in the positive mode at the sprit ratio of 1:40 (5 µl/min). The calibration of

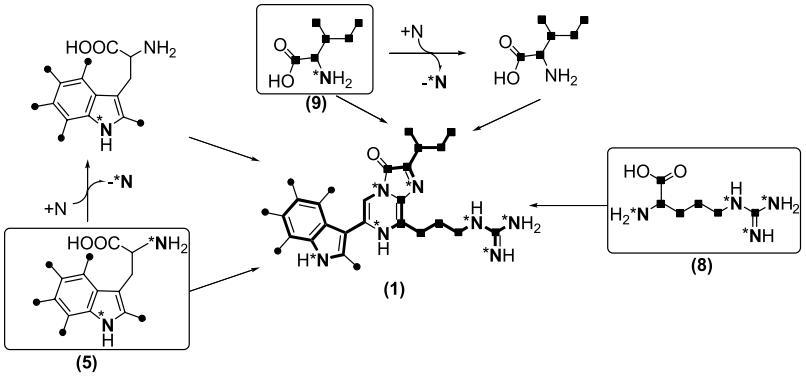

Figure 7. Biosynthesis of Cypridina luciferin (**1**) from L-tryptophan, L-arginine and L-isoleucine, and plausible nitrogen exchange of amino group in *C. hilgendorfii*.

mass value was performed using 1 μM each of angiotensin I (m/z=324.9272, 432.9003), bradykinin (m/z=354.1949) and neurotensin (m/z=558.3111) in 50% acetonitrile containing 1% acetic acid as external standards. The labeling efficiency was calculated from peak intensity of labeled ions in comparison with that of non-labeled luciferin ions.

4.5. Feeding experiments and extraction of Cypridina luciferin (1) from specimens

(i) Cypridina specimens. The specimens of *C. hilgendorfii* were collected at night using a bottle trap with a porcine liver as bait, at Mukaishima, Hiroshima in Japan, on 20 Dec. 2001, 13 Sept. 2002, 26 Dec. 2002, 7 Aug. 2003 and 7 Apr. 2004. The living specimens were kept in an aquarium (12 L) with aeration under the control of temperature at 20 °C.

(ii) Preparation of feeding gel for incorporation experiments. The preparation of the feeding gel containing a stable isotopic labeled amino acid was as follows; two grams of well-washed porcine liver were soaked in 15 ml distilled water for 15 min. The soaked extract was mixed with a labeled amino acid at the concentration of 50 mg/ml, except for 25 mg/ml of [$^{13}C_6$]-L-isoleucine (**10**) and [$^{13}C_6$,^{15}N]-L-isoleucine (**9**). The mixture was melt in 3% agarose (type VII, Sigma) at 75 °C and then was gelled in a plastic dish.

(iii) Feeding experiments. A feeding gel containing a labeled amino acid was cut into 7 mm cubic and fed to 2–5 specimens (2.0–3.0 mm in body size) in a small Petri dish (ϕ35 mm) for 1 h once a day. The feeding was monitored by blue staining of the stomach with trypan blue dye in feeding gel.

(iv) Ethanol extraction of Cypridina luciferin (**1**) from the specimens. After feeding for 15 days, the specimens were collected and immediately frozen in liquid nitrogen. The frozen specimens (24.8 and 27.8 mg wet weight/4 specimens for [D_5,$^{15}N_2$]-L-tryptophan (**5**) and [$^{13}C_6$,^{15}N]-L-isoleucine (**9**), respectively; 39.5 mg/5 specimens and 7.5 mg/2 specimens for [$^{13}C_6$,$^{15}N_4$]-L-arginine (**8**) and [$^{13}C_6$]-L-isoleucine (**10**), respectively) were homogenized with 3 times weight volume of ethanol by a plastic pestle on dry ice. The homogenate was sonicated (UT-105, Sharp) for 30 s, centrifuged at 12,000*g* for 10 min, and filtrated using an Ultrafree-MC filter (pore size 0.45 μm, Amicon). Two microliters of the filtrate were served to LC/ESI-TOF-MS analysis.

4.6. LC/ESI-TOF-MS analysis of stable isotope labeled amino acid extracted from *C. hilgendorfii*

Tryptophan in the ethanol extracts from *C. hilgendorfii* was analyzed by LC/ESI-TOF-MS as described in Section 4.4. Arginine and isoleucine in the extracts were analyzed by the same procedures except for the mobile phase and the detection wavelength at 210 nm on HPLC conditions. For arginine, the mobile phase was 40% methanol–water containing 0.05% heptafluorobutyric acid (HFBA) and arginine was detected at 3.6 min in the mass chromatogram. For isoleucine, the elution was performed stepwise with 35% methanol–water containing 0.05% HFBA (0–3 min) and then 100% methanol containing HFBA (3–10 min), and isoleucine was detected at 6.1 min in the mass chromatogram.

4.7. Air oxidation and hydrolysis of Cypridina luciferin (1) extracted from *C. hilgendorfii*

For air oxidation of Cypridina luciferin (**1**), five microliters of ethanol extracts from *C. hilgendorfii* in a plastic tube were left at room temperature (20–25 °C) for 3 days, and then analyzed by LC/ESI-TOF-MS as described in Section 4.4.

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