Biosci. Biotechnol. Biochem., **70** (6), 1528–1532, 2006

Communication

Stereoselective Incorporation of Isoleucine into Cypridina Luciferin in *Cypridina hilgendorfii* (*Vargula hilgendorfii*)

Shin-ichi KATO,[1] Yuichi OBA,[1] Makoto OJIKA,[1,†] and Satoshi INOUYE[2]

[1]*Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan*
[2]*Yokohama Research Center, Chisso Co., 5-1 Okawa, Kanazawa-ku, Yokohama 236-8605, Japan*

Received February 6, 2006; Accepted March 27, 2006; Online Publication, June 23, 2006
[doi:10.1271/bbb.60066]

The emission of light in the marine ostracod *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*) is produced by the Cypridina luciferin-luciferase reaction in the presence of molecular oxygen. Cypridina luciferin has an asymmetric carbon derived from isoleucine, and the absolute configuration is identical to the C-3 position in L-isoleucine or D-alloisoleucine. To determine the stereoselective incorporation of the isoleucine isomers (L-isoleucine, D-isoleucine, L-alloisoleucine, and D-alloisoleucine), we synthesized four ^{2}H-labeled isoleucine isomers and examined their incorporation into Cypridina luciferin by feeding experiments. Judging by these results, L-isoleucine is predominantly incorporated into Cypridina luciferin. This suggests that the isoleucine unit of Cypridina luciferin is derived from L-isoleucine, but not from D-alloisoleucine.

Key words: bioluminescence; biosynthesis; luciferin; isotopic label; imidazopyradinone

The luminous marine ostracod *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*) lives near the coast around Japan. The luminescence system has been investigated extensively, since Harvey reported the luciferin-luciferase reaction in 1917.[1)] When the specimen is stimulated physically or electrically, it expels Cypridina luciferin (**1**) and Cypridina luciferase into the seawater to produce a brilliant bluish luminescence. The chemical structure of Cypridina luciferin (**1**), which possesses an imidazopyrazynone skeleton with side residues of isoleucine, arginine, and tryptophan, was determined by Kishi *et al.* (Fig. 1).[2–4)] An asymmetric carbon of the isoleucine unit in **1** was determined to be (*S*)-configuration by chemical and enzymatic procedures.[3)] Recently, it has been demonstrated that **1** can be biosynthesized from three L-amino acids (L-arginine, L-isoleucine, and L-tryptophan), but not from tryptamine or D-tryptophan.[5–8)] But it was not determined whether the origin of the single asymmetric carbon in **1** was from L-isoleucine or D-alloisoleucine. In this study, we

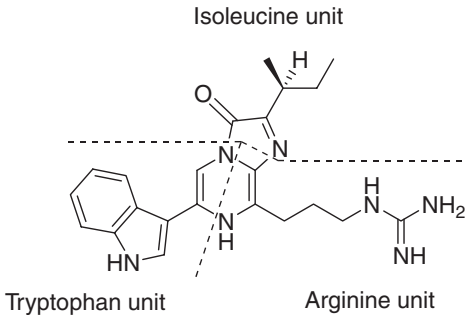


Fig. 1. Chemical Structure of Cypridina Luciferin and Its Biosynthetic Units.

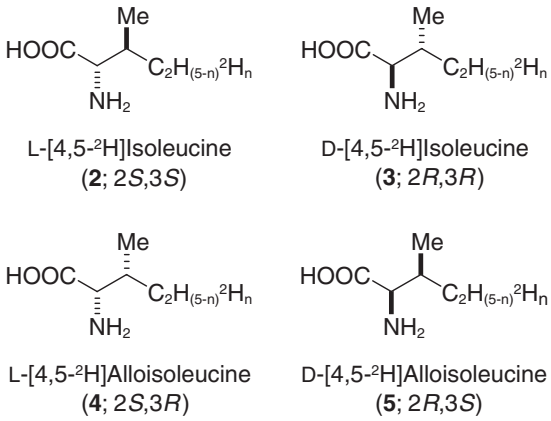
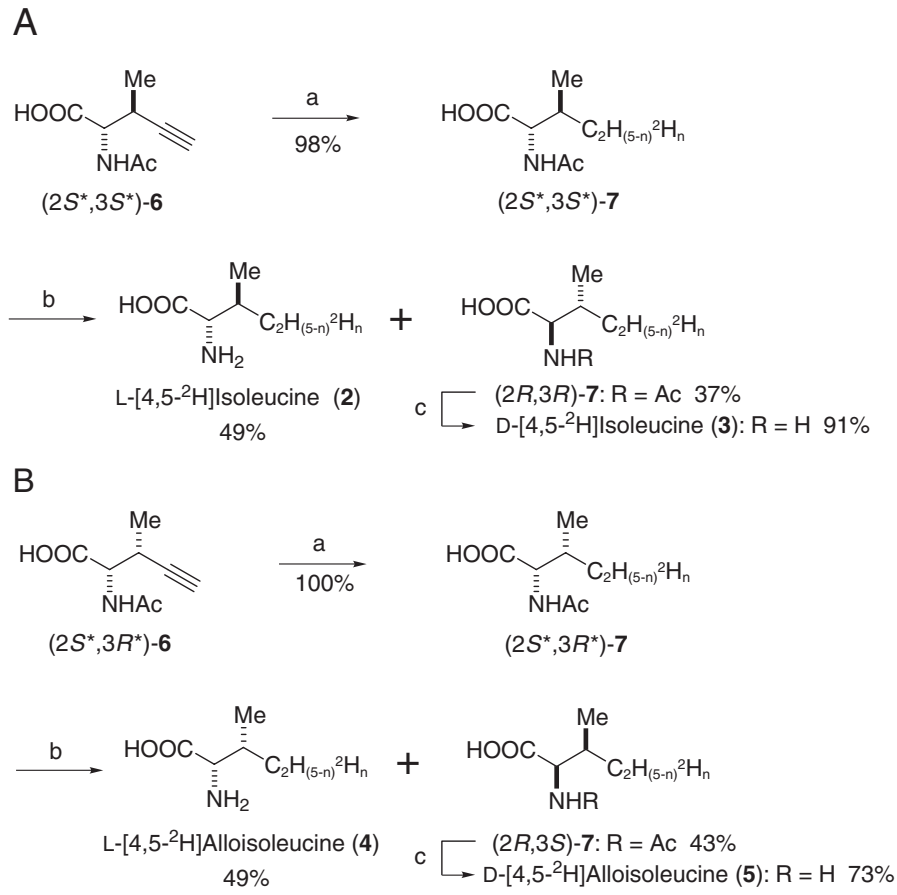


Fig. 2. [4,5-^{2}H]-Labeled Isoleucine Isomers Used for the Incorporation Studies.

examined the stereoselective incorporation of four ^{2}H-labeled isoleucine isomers **2**–**5** (Fig. 2) into Cypridina luciferin by feeding experiments using live animals.

Four stereoisomers of ^{2}H-labeled isoleucine (Fig. 2) were synthesized chemically and enzymatically from

† To whom correspondence should be addressed. Tel/Fax: +81-52-789-4284; E-mail: ojika@agr.nagoya-u.ac.jp
Abbreviations: HPLC, high performance liquid chromatography; ESI, electrospray ionization; IT, ion trap; MS, mass spectrometry; U, units



Scheme 1. Synthesis of ^{2}H-Labeled Isoleucines.

(A) ^{2}H-labeled L- and D-isoleucines, (B) ^{2}H-labeled L- and D-alloisoleucines. Reagents: (a) Platinum black, $^2H_2$, r.t., 40 min, (b) aminoacylase I, 0.1 M sodium phosphate buffer (pH 7.0), r.t., 24 h, (c) D-aminoacylase, 0.1 M sodium phosphate buffer (pH 7.5), 37 °C, 12 h.

racemic mixtures of (2*S**,3*S**)-**6** or (2*S**,3*R**)-**6**, which were prepared according to the previous report[9] (Scheme 1). In brief, compound (2*S**,3*S**)-**6** was labeled using platinum black under a deuterium atmosphere to give (2*S**,3*S**)-**7**. Enantioselective hydrolysis of (2*S**,3*S**)-**7** was accomplished using aminoacylase I to give L-[4,5-^{2}H]isoleucine (**2**) and (2*R*,3*R*)-**7**. D-[4,5-^{2}H]Isoleucine (**3**) was given by hydrolysis of (2*R*,3*R*)-**7** using D-aminoacylase. L-[4,5-^{2}H]Alloisoleucine (**4**) and D-[4,5-^{2}H]alloisoleucine (**5**) were prepared from (2*S**,3*R**)-**6** by the same procedures as above. The structures and purity of the stereoisomers were determined by NMR. The enantio purity of the stereoisomers was analyzed by HPLC with a chiral column: Chiralpak MA(+) (4.6 × 50 mm, Daicel Chemical Industries, Osaka, Japan); mobile phase, 2 mM $CuSO_4$ aqueous solution; flow rate, 0.5 ml/min; UV detection, 254 nm. Each product contained trace of the diasteromer (0.4% in **2**, 0.9% in **3**, and 1.6% in **4** and **5**), but not the enantiomer (Fig. 3). The efficiency of ^{2}H-labeling was determined by flow injection analysis on an ESI-ion trap-mass spectroscopy (ESI-IT-MS): Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with an Esquire 3000 (ESI-IT-MS; Bruker Daltonics, Billerica, MA) in the positive mode, and the mobile phase was 50% methanol containing 0.1% formic acid at a flow rate of 0.2 ml/min (Table 1).

For feeding experiments, the specimens were collected at Mukaishima, Hiroshima, Japan on 25 July 2005, and were kept in an aquarium at 25 °C. Each isomer of ^{2}H-labeled isoleucine (25 mg) was dissolved in aqueous extracts of porcine liver (1 ml) and gelled in 3% agarose (Type VII, Sigma, St. Louis, MO). After feeding of a piece of the gel for 5 d, three specimens were frozen in liquid nitrogen and extracted in 3 times weight volume of ethanol. A portion (1 µl) of the extract was analyzed on LC/ESI-IT-MS (Table 2): Agilent 1100 HPLC system with Esquire 3000; column, a Cadenza CD-C18 (2.0 × 75 mm; Imtakt, Kyoto, Japan) with an Unison US-C18 precolumn (2.0 × 5 mm, Imtakt); mobile phase, methanol-water containing 0.1% formic acid, linear gradient from 25% to 65% methanol for 20 min; flow rate, 0.2 ml/min; UV detection, 280 nm; MS analysis, ESI-IT-MS in the positive ion mode. The results of the mass spectral analyses showed that the incorporation efficiency of L-[4,5-^{2}H]isoleucine (**2**), L-[4,5-^{2}H]alloisoleucine (**4**), and D-[4,5-^{2}H]alloisoleucine (**5**) into Cypridina luciferin were 20.1, 1.1, and 0.3%

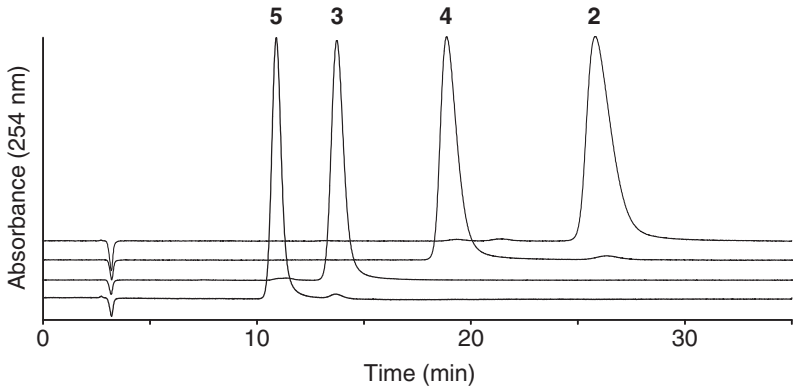


Fig. 3. HPLC Analysis of the Synthetic [4,5-^{2}H]Isoleucines on the Chiral Column.
L-[4,5-^{2}H]Isoleucine (**2**), D-[4,5-^{2}H]isoleucine (**3**), L-[4,5-^{2}H]alloisoleucine (**4**), and D-[4,5-^{2}H]alloisoleucine (**5**).

Table 1. Relative Intensity of Mass Ion Peaks in [4,5-^{2}H]-Labeled Isoleucines

No. of stable isotope atom	Relative peak intensity (%)				
	Non-labeling L-Ile	L-[4,5-^{2}H]Ile (**2**)	D-[4,5-^{2}H]Ile (**3**)	L-[4,5-^{2}H]alloIle (**4**)	D-[4,5-^{2}H]alloIle (**5**)
+0	100.00 ± 0.38	16.13 ± 0.33	16.22 ± 0.56	8.69 ± 0.66	8.19 ± 0.55
+1	7.17 ± 0.35	56.33 ± 0.61	56.28 ± 0.92	40.53 ± 0.92	38.14 ± 1.77
+2	0.90 ± 0.06	95.75 ± 0.78	98.21 ± 0.82	89.55 ± 1.00	83.80 ± 0.86
+3	—	100.00 ± 0.43	100.00 ± 0.25	100.00 ± 0.69	100.00 ± 2.20
+4	—	58.46 ± 0.82	57.07 ± 0.96	60.36 ± 0.87	56.71 ± 0.73
+5	—	15.56 ± 0.32	15.49 ± 0.36	14.40 ± 0.17	14.33 ± 0.39

Data are expressed as the mean ± standard error of three independent measurements. Ile, isoleucine; alloIle, alloisoleucine.

Table 2. Incorporation of [4,5-^{2}H]-Labeled Isoleucines into Cypridina Luciferin (Divalent ion)

No. of stable isotope atom	Relative peak intensity (%)				
	Non-feeding	L-[4,5-^{2}H]Ile (**2**)	D-[4,5-^{2}H]Ile (**3**)	L-[4,5-^{2}H]alloIle (**4**)	D-[4,5-^{2}H]alloIle (**5**)
+0	100.00 ± 0.46	100.00 ± 0.93	100.00 ± 0.74	100.00 ± 0.54	100.00 ± 0.88
+1	24.82 ± 0.35	30.76 ± 0.70**	22.99 ± 0.68	24.13 ± 0.40	24.43 ± 0.61
+2	3.29 ± 0.29	12.43 ± 0.54**	3.38 ± 0.18	3.99 ± 0.18*	3.45 ± 0.24*
+3	0.37 ± 0.09	9.99 ± 0.68**	0.32 ± 0.06	1.11 ± 0.04*	0.62 ± 0.06
+4	—	5.75 ± 0.45	—	0.48 ± 0.12	0.23 ± 0.06
+5	—	1.86 ± 0.12	—	0.24 ± 0.02	0.09 ± 0.04
Total	128.48	160.79	126.69	129.95	128.82
Incorporation[a]		20.1%	—	1.1%	0.3%

Data are expressed as the mean ± standard error of three independent measurements. Asterisks indicate statistical significance compared to the non-feeding specimens group at: $^*P < 0.05$, $^{**}P < 0.01$. Ile, isoleucine; alloIle, alloisoleucine. [a]Incorporation efficiency (%) = $(\text{Total}_{\text{feeding}} - \text{Total}_{\text{non-feeding}})/\text{Total}_{\text{feeding}} \times 100$

respectively (Table 2), whereas no detectable incorporation of D-[4,5-^{2}H]isoleucine (**3**) was observed. The small incorporation of **4** might be attributed to L-[4,5-^{2}H]isoleucine (**2**) in synthesized **4** (Fig. 3). In conclusion, L-isoleucine is stereoselectively incorporated into Cypridina luciferin (**1**). This suggests that the isoleucine unit of Cypridina luciferin (**1**) is derived from L-isoleucine, but not from D-alloisoleucine.

Experimental

Platinum black, aminoacylase I (from porcine kidney, EC 3.5.1.14, 4300 U/mg) and D-aminoacylase amano (from *Escherichia coli*, EC 3.5.1.81, 10.1 MU/g) were purchased from Wako Pure Chemical (Tokyo), and $^2H_2$ gas was purchased from Shoko (Tokyo). ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded on a Bruker AMX2-600 spectrometer. Chemical shifts of ^{1}H-NMR were given in ppm relative to the solvent peak of CD_2HOD (δ 3.5) or the internal standard of 1,4-dioxane (δ 3.75). Chemical shifts of ^{13}C-NMR were given in ppm relative to the solvent peak of CD_3OD (δ 49.0) or the internal standard of 1,4-dioxane (δ 69.3). Elementary analyses were performed on a

Series II CHNS/O Analyzer 2400 (Perkin Elmer, Wellesley, MA). Optical rotations $[\alpha]_D$ were determined on a DIP-370 polarimeter (Jasco, Tokyo).

[4,5-^{2}H]-(2S,3S*)-2-Acetamido-3-methylpentanoic acid ((2S*,3S*)-7): Procedure I.* A mixture of (2S*,3S*)-2-acetamido-3-methylpent-4-ynic acid ((2S*,3S*)-**6**; 365 mg, 2.16 mmol) and platinum black (19.1 mg) in ethyl acetate (11 ml) was stirred under $^2H_2$ at room temperature for 40 min. The mixture was filtered, and the filtrate was evaporated under reduced pressure. The residue was dissolved in MeOH and evaporated again to give 374 mg (98% yield) of [4,5-^{2}H]-(2S*,3S*)-2-acetamido-3-methylpentanoic acid ((2S*,3S*)-**7**). ^{1}H-NMR (600 MHz, CD_3OD) δ: 1.11 (1.7H, m), 1.14 (3H, d, J = 6.6 Hz), 1.43 (0.5H, m), 1.70 (0.5H, m), 2.06 (1H, m), 2.19 (3H, s, CH_3CO), 4.55 (1H, d, J = 6.0 Hz). ^{13}C-NMR (150 MHz, CD_3OD) δ: 11.4 (m), 16.0, 22.3 (m), 38.2, 58.3, 173.4, 174.9.

*L-[4,5-^{2}H]Isoleucine (**2**) and D-[4,5-^{2}H]isoleucine (**3**): Procedure II.* Compound (2S*,3S*)-**7** (282 mg, 1.59 mmol) was dissolved in 0.1 M sodium phosphate buffer (16 ml, pH 7.0). Aminoacylase I (3 mg, 12,900 U) was added, and the mixture was incubated at room temperature for 24 h. After incubation, the solution was adjusted to pH 5 with 1 M HCl, and the enzyme was denatured at 60 °C for 10 min and then filtered. The filtrate was acidified to pH 1 with 1 M HCl and washed with ethyl acetate. The aqueous layer was applied to a cation exchange column (20 × 20 mm, Biorad AG 50W-X8 resin, H^+-form), which was rinsed with water to make it neutral, and then eluted with 1 M NH_4OH. The eluate was evaporated under reduced pressure to give L-[4,5-^{2}H]isoleucine (**2**; 105 mg, 49%) as a white solid. $[\alpha]_D^{27}$ +38.7° (*c* 0.1, 6 M HCl). ^{1}H-NMR (600 MHz, D_2O) δ: 0.91 (1.8H, m), 1.00 (3H, d, J = 6.6 Hz), 1.23 (0.4H, m), 1.44 (0.5H, m), 1.97 (1H, m), 3.65 (1H, d, J = 3.6 Hz). ^{13}C-NMR (150 MHz, D_2O) δ: 13.4 (m), 17.4, 27.1 (m), 38.5, 62.3, 176.9. Found: C, 54.96; H, 9.78; N, 10.52. Calcd. for $C_6H_{13}NO_2$: C, 54.94; H, 9.99; N, 10.68. The ethyl acetate layer was washed with 1 M HCl and dried over Na_2SO_4. The solvent was evaporated under reduce pressure to give [4,5-^{2}H]-(2R,3R)-2-acetamido-3-methylpentanoic acid ((2R,3R)-7; 103 mg, 37%) as a white solid. Compound (2R,3R)-7 (103 mg, 0.58 mmol) was dissolved in 0.1 M phosphate buffer (pH 8.0). D-Aminoacylase amano (1 mg, 10.1 kU) was added, and the mixture was incubated at 37 °C for 12 h. After incubation, the solution was adjusted to pH 5 with 1 M HCl, and the enzyme was denatured at 60 °C for 10 min and then filtered. The filtrate was acidified to pH 1 with 1 M HCl and extracted with ethyl acetate. The aqueous layer was applied to the cation exchange column as described above. The eluate was evaporated under reduced pressure to give D-[4,5-^{2}H]isoleucine (**3**; 72 mg, 91%) as a white solid. $[\alpha]_D^{27}$ −32.7° (*c* 0.1, 6 M HCl). ^{1}H-NMR (600 MHz, D_2O) δ: 0.91 (1.8H, m), 0.99 (3H, d, J = 6.6 Hz), 1.23 (0.5H, m), 1.44 (0.5H, m), 1.94 (1H, m), 3.62 (1H, d, J = 4.2 Hz). ^{13}C-NMR (150 MHz, D_2O) δ: 13.6 (m), 17.4, 27.1 (m), 38.6, 62.4, 177.4. Found: C, 54.93, H, 9.98, N, 10.65. Calcd. for $C_6H_{13}NO_2$: C, 54.94; H, 9.99; N, 10.68.

[4,5-^{2}H]-(2S,3R*)-2-Acetamido-3-methylpentanoic acid ((2S*,3R*)-7).* Compound (2S*,3R*)-**7** was synthesized from (2S*,3R*)-**6** (211 mg, 1.25 mmol) according to Procedure I and was obtained as a white solid in quantitative yield (222 mg, 1.25 mmol). ^{1}H-NMR (600 MHz, CD_3OD) δ: 1.10 (1.6H, m), 1.13 (3H, d, J = 6.6 Hz), 1.42 (0.4H, m), 1.59 (0.4H, m), 2.15 (1H, m), 2.20 (3H, s), 4.71 (1H, s). ^{13}C-NMR (150 MHz, CD_3OD) δ: 11.6 (m), 15.1, 22.3, 27.0 (m), 38.1, 56.9, 173.6, 175.3.

*L-[4,5-^{2}H]Alloisoleucine (**4**) and D-[4,5-^{2}H]alloisoleucine (**5**).* Compound **4** was synthesized from (2S*,3R*)-**7** according to Procedure II and was obtained as a white solid in 49% yield (75 mg). $[\alpha]_D^{27}$ +37.0° (*c* 0.1, 6 M HCl). ^{1}H-NMR (600 MHz, D_2O) δ: 0.92 (3H, d, J = 7.2 Hz), 0.93 (1.8H, m), 1.30 (0.4H, m), 1.40 (0.4H, m), 2.05 (1H, m), 3.71 (1H, d, J = 3.6 Hz). ^{13}C-NMR (150 MHz, D_2O) δ: 13.4 (m), 16.0, 28.1 (m), 38.2, 61.3, 177.5. Found: C, 54.95; H, 9.95; N, 10.56. Calcd. for $C_6H_{13}NO_2$: C, 54.94; H, 9.99; N, 10.68. D-[4,5-^{2}H]Alloisoleucine (**5**) was obtained as white solid in 31% yield. (2 steps, 48 mg) $[\alpha]_D^{27}$ −32.0° (*c* 0.1, 6 M HCl). ^{1}H-NMR (600 MHz, D_2O) δ: 0.92 (3H, d, J = 7.2 Hz), 0.93 (2.2H, m), 1.29 (0.4H, m), 1.39 (0.4H, m), 2.00 (1H, m), 3.64 (1H, d, J = 4.2 Hz). ^{13}C-NMR (150 MHz, D_2O) δ: 13.5 (m), 16.0, 28.2 (m), 38.6, 61.4, 178.7. Found: C, 54.92; H, 9.85; N, 10.59. Calcd. for $C_6H_{13}NO_2$: C, 54.94; H, 9.99; N, 10.68.

Acknowledgments

The authors thank Dr. K. Yasui and Dr. N. Yamaguchi of Mukaishima Marine Biological Laboratory of Hiroshima University in Japan for assistance in the collection of animals, and Mr. S. Kitamura of the analytical laboratory of Nagoya University for elementary analyses. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a JSPS fellowship (to S.K.).

References

1) Harvey, E. N., Studies on bioluminescence. IV. The chemistry of light production in a Japanese ostracod crustacean, *Cypridina hilgendorfii*, Müller. *Am. J. Physiol.*, **42**, 318–341 (1917).
2) Shimomura, O., Goto, T., and Hirata, Y., Crystalline *Cypridina* luciferin. *Bull. Chem. Soc. Jpn.*, **30**, 929–933 (1957).
3) Kishi, Y., Goto, T., Hirata, Y., Shimomura, O., and Johnson, F. H., Cypridina bioluminescence. I. Structure of *Cypridina* luciferin. *Tetrahedron Lett.*, **7**, 3427–3436 (1966).
4) Kishi, Y., Goto, T., Inoue, S., Sugiura, S., and Kishimoto, H., Cypridina bioluminescence. III. Total synthesis of

Cypridina luciferin. *Tetrahedron Lett.*, **7**, 3445–3450 (1966).

5) Oba, Y., Kato, S., Ojika, M., and Inouye, S., Biosynthesis of luciferin in the sea firefly, *Cypridina hilgendorfii*: L-tryptophan is a component in Cypridina luciferin. *Tetrahedron Lett.*, **43**, 2389–2392 (2002).
6) Kato, S., Oba, Y., Ojika, M., and Inouye, S., Identification of the biosynthetic units of Cypridina luciferin in *Cypridina* (*Vargula*) *hilgendorfii* by LC/ESI-TOF-MS. *Tetrahedron*, **60**, 11427–11434 (2004).
7) Kato, S., Oba, Y., Ojika, M., and Inouye, S., Biosynthesis of Cypridina luciferin from free amino acids in *Cypridina* (*Vargula*) *hilgendorfii*. In "Bioluminescence and Chemiluminescence: Progress and Perspectives," eds. Tsuji, A., Matsumoto, M., Maeda, M., Kricka, L. J., and Stanley, P. E., World Scientific, Danvers, pp. 121–125 (2005).
8) Toya, Y., Biosynthesis of *Vargula hilgendorfii* luciferin, arisen from L-arginine, L-tryptophan, and L-isoleucine. In "Bioluminescence and Chemiluminescence: Progress and Perspectives," eds. Tsuji, A., Matsumoto, M., Maeda, M., Kricka, L. J., and Stanley, P. E., World Scientific, Danvers, pp. 129–132 (2005).
9) Hasegawa, H., Arai, S., Shinohara, H., and Baba, S., Synthesis of (2*S*,3*S*)-2-amino-3-methylpent-4-ynoic acid, a precursor amino acid for the preparation of tritium- or deuterium-labeled peptide in the isoleucine residue. *J. Chem. Soc., Perkin Trans. 1*, 489–494 (1993).