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Syntheses and evaluation of the bioluminescent activity of (*S*)-Cypridina luciferin and its analogs

Chun Wu,[a] Kosei Kawasaki,[b] Satoru Ohgiya[b] and Yoshihiro Ohmiya[a,*]

[a]*Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology, Osaka 563-8577, Japan*
[b]*Research Institute for Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology, Sapporo 062-8517, Japan*

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Abstract—Cypridina luciferin is the substrate in the bioluminescence of a luminous ostracod *Cypridina* (*Vargula*) *hilgendorfii*. Cypridina luciferin contains a chiral center in the *sec*-butyl moiety. Here, we report a convenient method for the preparation of (*S*)-Cypridina luciferin by the condensation of (*S*)-1,1-diethoxy-3-methylpentan-2-one with ethioluciferin. The light yield of the synthesized (*S*)-luciferin in the presence of Cypridina luciferase was about 1.7 times as active as that of racemic form. Furthermore, several luciferin analogs prepared by the same condensation with different α-ketoacetal derivatives showed moderate light yield with Cypridina luciferase. These readily available Cypridina luciferin and analogs are applicable to the bioluminescent detection of Cypridina luciferase.

Cypridina luciferin, which was first isolated from a luminous ostracod *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*), is a substrate in an enzymatic oxidation that generates visible light. Cypridina luciferin (**1**) was shown to be an imidazopyrazinone compound having three functional groups at the C2, C6, and C8 positions (Fig. 1).[1] It is obvious that luciferin **1** derives biogenetically from three amino acids or its equivalent: L-arginine, L-isoleucine, and L-tryptophan (or tryptamine). Recently, a feeding experiment has indicated that Cypridina luciferin may be biosynthesized from these three amino acids,[2] but the enzymes involved in the biosynthesis of **1** are still unknown. Cypridina luciferin (**1**) contains a chiral center in the *sec*-butyl moiety at the C2 position. The natural luciferin is the (*S*)-configuration.[1] The first total synthesis of (*S*)-Cypridina luciferin (**1**) was achieved by the condensation of reduced ethioluciferin (**2**) with (+)-α-keto acid (**3**) in three steps,[1] but the yield (~1%) is too low to use this route to prepare (*S*)-**1** in a large quantity (Fig. 1). A new route using the condensation of **2** with racemic ketoaldehyde (**4**) in place of **3** in the presence of acidic catalyst gave the racemic form **1** in a moderate yield (70%).[3] Another route by using a racemic keto acetal **5** provided the racemic form **1** in 24% yield and the synthesized **1** showed a light yield of about 60% of that of (*S*)-**1** in the bioluminescence of Cypridina luciferase.[4] A practical synthetic method for (*S*)-**1** or optical resolution of the racemic form **1** has not to our knowledge been reported yet.

The secreted Cypridina luciferase has a big advantage as a non-destructive bioluminescent reporter. However, there are only a few examples in the literature of this application because of the problem of the supply of the luciferin.[5] Recently, Cypridina luciferase from *Cypridina noctiluca* has been cloned[6a] and functionally expressed in mammalian cells[6a] and yeast.[6b] This live yeast cell-based bioluminescent assay system is very useful for high-throughput screening for drugs or hormone-disrupting chemicals. Therefore, we are interested in developing an efficient synthetic method for (*S*)-**1** for the Cypridina luciferase bioluminescent assay. Here, we reported our recent results in developing a simple method of preparing (*S*)-**1** and syntheses of several Cypridina luciferin analogs having different alkyl groups at the C2-position. Bioluminescent activities of the synthesized (*S*)-**1** and its analog were evaluated with recombinant Cypridina luciferase.[6]

The approach taken for the preparation of (*S*)-**1** luciferin is shown in Figure 1. We chose the chiral (*S*)-ketoacetal

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* Corresponding author. E-mail: y-ohmiya@aist.go.jp

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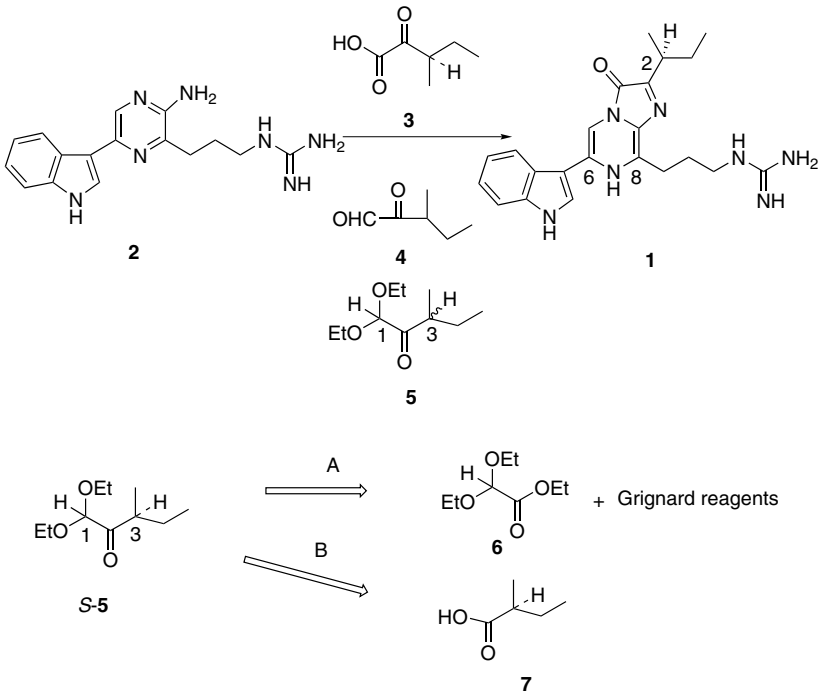
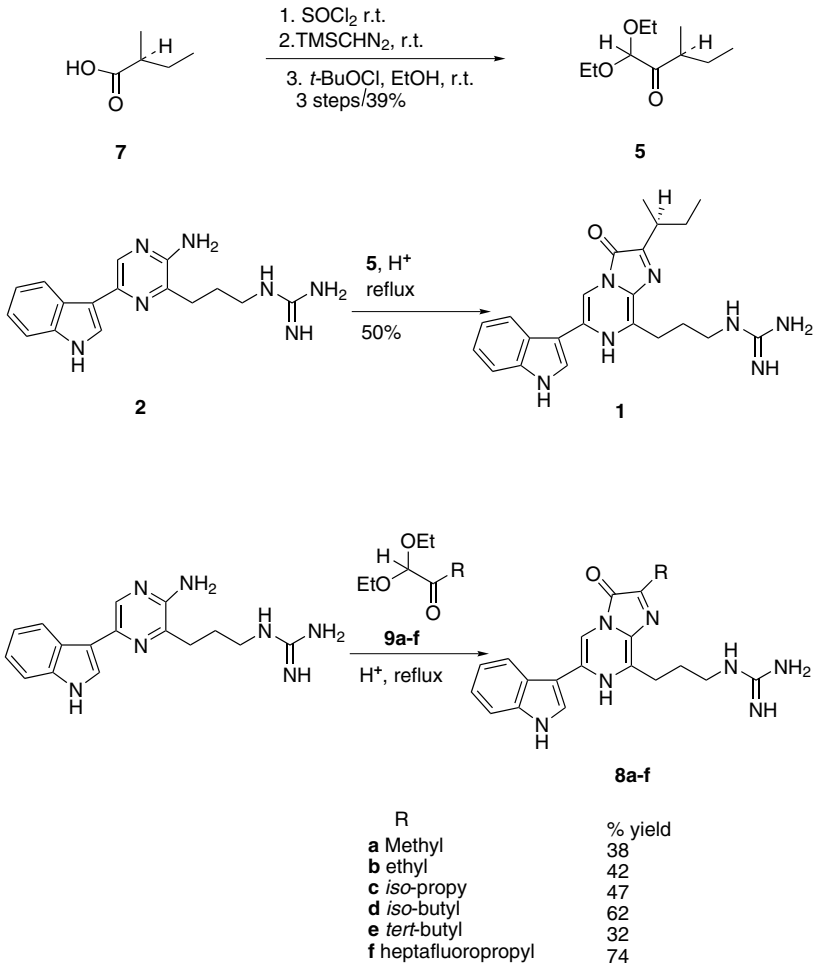


Figure 1. Three routes for the synthesis of Cypridina luciferin (**1**).



R	% yield
a Methyl	38
b ethyl	42
c *iso*-propy	47
d *iso*-butyl	62
e *tert*-butyl	32
f heptafluoropropyl	74

Scheme 1. Synthesis of **1** and its analogs.

compound **5** because the acetal group was expected to avoid the racemization due to the difference of pK_a values of protons between the C1 and C3 positions. The general approach for the preparation of α-ketoacetal derivatives is via a Grignard reaction from esters **6** or its amides (route A).[7] However, this route is not suitable for the preparation of (*S*)-**5**, which was prepared from the commercially available reagent **7** (route B).[8]

Reaction of the starting material **7** with thionyl chloride followed by treatment of the resulting acid chloride with $TMSCHN_2$ provided a homologation product (Scheme 1). Treatment of the homologation product with *tert*-butylhyperchlorite in the presence of ethanol gave compound **5**. The specific optical rotations of (*S*)-**5** was +22 (in dichloromethane), which was almost the same as that of the starting material +19 (in dichloromethane). Compound **2** was prepared according to the method described by Nakamura et al.[4] Reaction of **2** with **5** proceeded smoothly in the presence of acidic catalyst affording (*S*)-**1** in moderate yield (50%).[9] Luciferin **1** was purified on a reversed-phase HPLC column (Fig. 2). The specific optical rotation of the synthetic (*S*)-**1** was +22, which was almost the same as that of the natural luciferin **1**+19.[10] Therefore, the optical purity of the synthetic **1** could be 100%. This is the first report on the optical rotation of Cypridina luciferin. The (*dl*)-Cypridina luciferin was synthesized by using a racemic ketoacetal **5** under the same conditions.[4] Condensation of **2** with ketoacetal derivatives **9a**–**f** that were prepared from commercially available acids by using the same route afforded these desired Cypridina luciferin analogs **8a**–**f** in 32–74% yield (Scheme 1).[11]

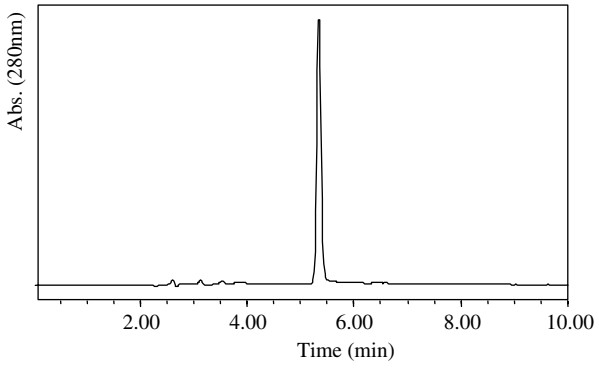


Figure 2. A HPLC profile of the synthetic **1**. (Cosmosil C18-column 4.6 × 250 mm, 69:30:1 water/acetonitrile/10% TFA, 1.0 mL/min, detected at 280 nm).

Table 1. Luminescent properties of Cypridina luciferin (**1**) and its analogs (**8a**–**f**)

	(*S*)-**1**	(*dl*)-**1**	**8a**	**8b**	**8c**	**8d**	**8e**	**8f**
Relative light yield	100	58	3	12	67	10	2	2
λ_{max}/nm	460	460	460	460	460	460	N.D.	N.D.

Bioluminescence was performed at 1×10^{-7} M of luciferins in 50 mM Tris–HCl pH 8 with recombinant Cypridina luciferase (10 ng/mL). N.D. not determined.

The bioluminescent activities of **1** and its analogs were measured in the presence of recombinant Cypridina luciferase (Table 1). The light yield of (*S*)-**1** was 1.7 times greater than that of (*dl*)-**1**. The light yield generated from the analog having an isopropyl group was a little higher than that of the racemic **1**. Analogs with ethyl and isobutyl groups showed about 10% of the light yield of (*S*)-**1**. Analogs with methyl, butyl, and heptafluoropropyl group showed 1–3% of the light yield of (*S*)-**1**. The wavelength of the light emission maxima of **1** and its analogs were similar to each other. In previous studies, the change of indolyl group and the chain length of alkylguaydinyl group of **1** caused a dramatic decrease in the light yield.[4,12] Our results indicated that the light yield also depends on the chirality and the size of the substituent at the C2 position. This high substrate specificity should contribute to the highly efficient bioluminescence of Cypridina luciferase, which is about 0.28 absolute light yield.

The commercially available firefly luciferase and *Renilla* luciferase from the sea pansy are widely used to monitor gene expression in eukaryotic cells. The secreted Cypridina luciferase has a potentially large advantage over these luciferases as a non-destructive genetic reporter. Our method of preparing (*S*)-Cypridina luciferin is simple and efficient. These readily available Cypridina luciferin and analogs are applicable to the bioluminescent detection of Cypridina luciferase.

Acknowledgments

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9. A mixture of ethioluciferin **2** (0.03 mmol) and (*S*)-**5** (0.02 mL) in 1:50:49 47% aq HBr/ethanol/water (0.2 mL) were heated to reflux for 1 h. The mixture was cooled to room temperature and was purified by RP-HPLC. (Cosmosil C18-column 4.6 × 250 mm, 69:30:1 water/acetonitrile/10% TFA, 1.0 mL/min.) The synthetic (*S*)-**1**: $[\alpha]_D^{24}$ +22 (0.046, acetonitril/water/10% TFA 39:60:1); The natural Cypridina luciferin **1**[10] $[\alpha]_D^{24}$ +19 (0.026, acetonitril/water/10% TFA 39:60:1); LR-FABMS *m*/*z* 404 (M^-); ^{1}H NMR (500 MHz, CD_3OD) δ 0.95 (3H, t, $J = 8$ Hz), 1.45 (3H, d, $J = 8$ Hz), 1.80–1.99 (2H, m), 2.33 (2H, quintet, $J = 8$ Hz), 3.17 (1H, sextet, $J = 8$ Hz), 3.41 (2H, q, $J = 8$ Hz), 3.44 (2H, q, $J = 8$ Hz), 7.18–7.21 (2H, m), 7.50 (1H, d, $J = 8$ Hz), 8.01 (1H, s), 8.07 (1H, d, $J = 8$ Hz), 8.44 (1H, s); IR (KBr) 3376, 3175, 1652, 1545, 1446 cm^{-1}.
10. Extraction and purification of natural Cypridina luciferin was according to the below reference: Haneda, Y.; Johnson, F. H.; Masuda, Y.; Saiga, Y.; Shimomura, O.; Sie, H.-C.; Sugiyama, N.; Takatsuki, I. *J. Cell. Comp. Physiol.* **1961**, *57*, 55. The purity of the natural luciferin **1** was judged by the HPLC analysis.[9]
11. Compound **8a**: LR-FABMS *m*/*z* 362 (M^-); ^{1}H NMR (500 MHz, CD_3OD) δ 2.30 (2H, quintet, $J = 8$ Hz), 2.54 (3H, s), 3.30 (2H, t, $J = 8$ Hz), 3.43 (2H, t, $J = 8$ Hz), 7.17–7.22 (2H, m), 7.47 (1H, d, $J = 8$ Hz), 7.98 (1H, s), 8.01 (1H, d, $J = 8$ Hz), 8.31 (1H, s); Compound **8b**: LR-FABMS *m*/*z* 376 (M^-); ^{1}H NMR (500 MHz, CD_3OD) δ 1.41 (3H, t, $J = 8$ Hz), 2.31 (2H, quintet, $J = 8$ Hz), 2.95 (2H, q, $J = 8$ Hz), 3.35 (2H, t, $J = 8$ Hz), 3.43 (2H, t, $J = 8$ Hz), 7.16–7.22 (2H, m), 7.47 (1H, d, $J = 8$ Hz), 7.99 (1H, s), 8.02 (1H, d, $J = 8$ Hz), 8.35 (1H, s); Compound **8c**: LR-FABMS *m*/*z* 390 (M^-); ^{1}H NMR (500 MHz, CD_3OD) δ 1.50 (6H, t, $J = 8$ Hz), 2.29 (2H, quintet, $J = 8$ Hz), 3.32 (1H, sextet, $J = 8$ Hz), 3.37 (2H, q, $J = 8$ Hz), 3.43 (2H, q, $J = 8$ Hz), 7.11–7.14 (2H, m), 7.40 (1H, d, $J = 8$ Hz), 7.96 (1H, s), 7.94 (1H, d, $J = 8$ Hz), 8.22 (1H, s); Compound **8d**: LR-FABMS *m*/*z* 404 (M^-); ^{1}H NMR (500 MHz, CD_3OD) δ 1.03 (6H, t, $J = 8$ Hz), 2.18 (1H, m), 2.33 (2H, quintet, $J = 8$ Hz), 2.78 (2H, d, $J = 8$ Hz), 3.35 (2H, t, $J = 8$ Hz), 3.43 (2H, t, $J = 8$ Hz), 7.16–7.22 (2H, m), 7.47 (1H, d, $J = 8$ Hz), 7.99 (1H, s), 8.04 (1H, d, $J = 8$ Hz), 8.36 (1H, s); Compound **8e**: LR-FABMS *m*/*z* 404 (M^-); ^{1}H NMR (500 MHz, CD_3OD) δ 1.55 (9H, s), 2.33 (2H, quintet, $J = 8$ Hz), 3.41 (2H, t, $J = 8$ Hz), 3.43 (2H, t, $J = 8$ Hz), 7.16–7.22 (2H, m), 7.47 (1H, d, $J = 8$ Hz), 8.00 (1H, s), 8.02 (1H, d, $J = 8$ Hz), 8.39 (1H, s); Compound **8f**: ^{1}H NMR (500 MHz, CD_3OD) δ 2.29 (2H, quintet, $J = 8$ Hz), 3.01 (2H, d, $J = 8$ Hz), 3.41 (2H, t, $J = 8$ Hz), 7.14–7.20 (2H, m), 7.43 (1H, d, $J = 8$ Hz), 7.87 (1H, s), 8.02 (1H, s), 8.08 (1H, d, $J = 8$ Hz).
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