Oplophorus Oxyluciferin and a Model Luciferin Compound Biologically Active with *Oplophorus* Luciferase

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The luciferin of the bioluminescent decapod shrimp, *Oplophorus gracilorostris*, was purified and studied with respect to u.v. spectrum, fluorescence spectrum, mass spectrum and luminescent cross-reaction with the enzyme luciferase of the bioluminescent ostracod, *Cypridina hilgendorffii*. On the basis of these results, an empirical formula $C_{10}H_{13}N_3O_3$ and an imidazo[1,2-*a*]pyrazin-3-one structure are proposed for luciferin. Of three model luciferin compounds, 3-hydroxy-2-methylimidazo[1,2-*a*]pyridine is biologically active with both *Oplophorus* and *Cypridina* luciferase, indicating that a pyrazine structure is not essential for biological activity with *Cypridina* luciferase.

In spite of great progress in research on bioluminescence, the majority of bioluminescence systems are chemically unidentified. The structures of only a few luciferins, namely those from *Photinus* (White *et al.*, 1963), *Cypridina* (Kishi *et al.*, 1966a), *Latia* (Shimomura & Johnson, 1968) and *Renilla* (Hori & Cormier, 1973), have been clarified. The structures of the light-emitting moieties of a photoprotein from *Aequorea* (Shimomura & Johnson, 1973) and of *Watasenia* (Goto *et al.*, 1974) were also determined. As far as the structures of luciferins from sea organisms are concerned, it can be tentatively suggested that the 2-aminopyrazine skeleton is essential for luminescence. Generally, it has been recognized that difficulties with chemical studies of bioluminescence are ascribed to scarcity of bioluminescent materials and instability of luciferin and luciferase. In particular, it was known that *Oplophorus* luciferin (Johnson *et al.*, 1966) was very unstable and its isolation was difficult. Preliminary experiments on the extraction of luciferin from the defatted powder of freeze-dried specimens showed that *Oplophorus* luciferin was about ten times as unstable as *Cypridina* luciferin. *Oplophorus* luciferin is a luciferin of the luminescent system of a deep-sea shrimp, *Oplophorus gracilorostris*. As part of the chemical investigation of the bioluminescence, the isolation and structure of *Oplophorus* luciferin was studied.

In the present paper, emphasis has been placed on finding an improved method to identify the chemical structure of an unstable material by isolation, under an inert-gas atmosphere, followed by mass-spectral analysis, by examining the cross-reaction of *Oplophorus* luciferin with *Cypridina* luciferase, and by studying the substrate specificity of *Oplophorus* luciferase.

Experimental

Materials

Specimens of *Oplophorus gracilorostris* were collected in Suruga Bay, Japan, over a period of 1 year, with the aid of fishermen who seasonally trawl for the edible shrimp, 'Sakura ebi'. As the shrimps migrated to the surface at night, they were caught in the same net haul and the one or two specimens of *Oplophorus* usually present were picked out from among the rest of the catch and placed on ice. On returning to port, the specimens were immediately stored at -35°C . The specimens were freeze-dried in batches of several hundred each. Subsequently they were ground to a fine powder and defatted for 10h in a stream of cold benzene recycled through a modified Soxhlet apparatus (see the acknowledgements). The powder was then extracted for an additional 5h with cold diethyl ether in the same apparatus. The extraction apparatus was continuously flushed with argon to exclude O_2 , which might oxidize luciferin. The powder was air-dried overnight and stored in air-tight containers containing anhydrous CaCl_2 .

Cypridina hilgendorffii was collected at Yokosuka and Tateyama at the gate of Tokyo Bay. In the evening, strings with fish scraps tied to their ends were thrown into the sea. *Cypridina* would rise from the sandy sea bottom to feed on those scraps and could be collected by pulling up the scraps and scraping the *Cypridina* off by hand into a bucket. They were frozen with solid CO_2 for transfer to the laboratory. Specimens were then kept in the refrigerator in a tightly sealed bottle with a CaCl_2 bag.

Euphausia japonica was caught at the same time and place and in the same ways as *Oplophorus* shrimps with the help of shrimp fishermen. After rapid

freeze-drying, the specimens were stored in air-tight bottles containing anhydrous CaCl_2 .

All chemicals used were of reagent-grade quality or of the highest quality available commercially. Distilled water was used throughout the study.

Chemical synthesis

3-Hydroxy-2-methylimidazo[1,2-a]pyridine (3). 2-Aminopyridine (1g) and 40% (w/v) methylglyoxal in water (1.9g) were dissolved in 4ml of conc. HCl-ethanol (1:1, v/v) in a sealed tube. The mixture was heated for 1.5h at 110°C , then left at room temperature (20°C) for 15h, and finally dissolved in 5ml of ethanol and concentrated in a rotary evaporator. The residue was dissolved in 5–10ml of ethanol-acetone (1:1, v/v) and kept in a refrigerator, and the crystalline compound *N*-(2-pyridyl)- α -alanine hydrochloride was obtained (yield 53.4%). It was esterified by refluxing for 6h in a solution of benzene-ethanol (7:3, v/v). After distillation, *N*-(2-pyridyl)- α -alanine ethyl ester was obtained (yield 59.9%). This ester (1.3g) was heated at 140°C for 6h under reduced pressure (4mmHg; 0.5kPa), giving an orange-yellow-coloured crystalline product, which was washed with dry ether (yield 40.0%).

3,7-Dihydro-2-methylimidazo[1,2-a]pyrazin-3-one (4) and 3,7-dihydro-2-ethylimidazo[1,2-a]pyrazin-3-one (5). They were synthesized by the procedures described by Hori & Cormier (1973), with less than 2% yield. Compound (5) had the following properties. U.v. spectrum in water (ϵ values in parentheses): λ_{max} 212 (9000), 260 (13000), 315 (3600), 419 (9600)nm. Fluorescence emission spectrum in water excited at 365nm: λ_{max} 523nm. I.r. spectrum in KBr disc: ν_{max} 3400, 3050, 1680, 1620, 1530, 1415, 1350, 1290, 1220, 1195, 1120, 1050, 1000, 920, 860, 775cm^{-1} . N.m.r.: $\delta^2\text{H}_2\text{O}$ 1.22 (3H), 2.80 (2H), 7.3–8.5 (3H) p.p.m., 2,2-dimethyl-2-silapentane-5-sulphonate was used for the internal n.m.r. standard. M.p. $195\text{--}196^\circ\text{C}$ (decomp.) (Found: C, 58.88; H, 5.53. $\text{C}_8\text{H}_9\text{N}_3\text{O}$ requires C, 58.88; H, 5.56%).

Methods

Preparation of crude *Oplophorus luciferin*. Defatted *Oplophorus* powder (13g) was rapidly added to 100ml of boiling acetic acid (pH4), which was continuously flushed with argon, and refluxed for 30min. After thorough chilling in an ice bath, the mixture was vacuum filtered and the filtrate containing the luciferin stored under argon in a glass vessel with a small side-port fitted with a rubber stopper (see the

acknowledgements) so allowing luciferin solution to be withdrawn with a needle and syringe as needed.

Partial purification of *Oplophorus luciferin*. De-fatted *Oplophorus* powder (50 g) was placed in a 1-litre three-necked round-bottom flask with a glass inlet tube, a stopper and a sintered-glass filter in the necks. The flask was continuously flushed with argon and 500ml of argon-saturated methanol and 3 g of KOH were added. The flask was sealed and left at room temperature with occasional shaking for 24 h. The extract was thoroughly chilled in a solid CO₂-methanol bath and filtered under positive argon pressure into a 1-litre flask (step I). A portion was removed from the flask and freeze-dried for assay. The filtrate was rapidly evaporated to about 60 ml with a flash evaporator while the temperature was maintained at less than 31°C and a fine stream of argon was passed through. The mixture was chilled in a solid CO₂-methanol bath, divided into three equal volumes and each was centrifuged at 7300g for 10 min at 0°C. The resulting combined supernatant was mixed with 200 ml of 0.3 M-NaHCO₃ and was flushed with argon and the solution extracted seven or eight times each with 40 ml portions of cold freshly distilled diethyl ether, or until a colourless extract was obtained. The alkaline solution was finally extracted with 2 × 40 ml and 1 × 20 ml portions of cold butan-1-ol, which were combined (step II). A sample was immediately removed and freeze-dried for assay. The remaining butan-1-ol solution of luciferin was divided into three equal volumes in 100 ml ampoules, evacuated to dryness with a vacuum pump, and the ampoules were sealed under argon.

Chromatography of *Oplophorus luciferin*. Washed Sephadex G-15, suspended in 10% (v/v) ethanol, saturated with argon, was used to form a column (1.5 cm × 20 cm) in a chromatographic apparatus provided with inlet and outlet tubes above and below the Sephadex bed for circulating argon. This arrangement made it possible to exclude O₂ from the chromatographic column, eluent and effluent. The luciferin (0.6–0.8 g) was introduced into the column dissolved in a few ml of argon-saturated 10% (v/v) ethanol and eluted with the same solvent; 5 ml fractions were collected. Each fraction was immediately placed in a solid CO₂ bath and freeze-dried. The residue was dissolved in 5 ml of argon-saturated acetic acid, pH 4, and a sample removed for assay. The active fractions were combined and a sample removed and freeze-dried for assay (step III). The luciferin solution was also checked for purity by paper chromatography, by using Toyo filter paper no. 51 and a developing solvent consisting of 3% aq. NH₃, methanol and ethanol (1:1:2, by vol.). The chromatography was carried out in an argon atmosphere in a sealed tube (3 cm × 30 cm) and the position of luciferin was located with a u.v. lamp. The presence of impurities on the paper was

further checked with the ninhydrin reagent (0.5% in butan-1-ol).

Assay of *Oplophorus luciferin*. Each residue was dissolved in 1.0–5.0 ml of argon-saturated acetic acid, pH 4, and a 0.5 ml portion was removed and diluted with 0.5 ml of 0.125 M-Tris-HCl buffer, pH 8.2. The mixture was then injected into a reaction cell containing 0.5 ml of partially purified luciferase diluted with 0.5 ml of 0.125 M-Tris-HCl buffer, pH 8.2. The total integrated light was measured with a photomultiplier photometer.

Light measurement. The photometer used for light measurement was a modified Hitachi model 139 photomultiplier photometer, equipped with a Hitachi QPD-53 recorder. The measurements were expressed in arbitrary light units.

Preparation of crude *Oplophorus luciferase*. De-fatted *Oplophorus* powder (20 g) was stirred for a few minutes in 250 ml of cold water with 50 g of ice and homogenized for 5 min in a Waring-type blender. After centrifuging at 23 500g for 1 h at 0°C in a Hitachi 20PR preparative centrifuge, the supernatant (about 200 ml) was dialysed for 48 h at 4°C against four changes of water of about 2 litres each. The dialysed solution was centrifuged again as described above and the enzyme preparation was either used directly or stored at –35°C, under which conditions it retained most of its activity for many months.

Partial purification of *Oplophorus luciferase*. De-fatted *Oplophorus* powder (12 g) was stirred for a few minutes in 250 ml of cold 0.01 M-sodium phosphate buffer, pH 7.0, and homogenized for 5 min in a Waring-type blender. Small portions of shaved ice were added as needed to keep the temperature low (4°C). The mixture was centrifuged at 18 600g for 1 h at 0°C in a Hitachi 20PR preparative centrifuge. The supernatant (about 280 ml) was placed on a column (2.5 cm × 40.0 cm) of DEAE-cellulose previously equilibrated against 0.01 M-sodium phosphate buffer, pH 7.0. The column was washed with 200 ml of 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.1 M-NaCl. The luciferase was then eluted from the column with 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.3 M-NaCl. Fractions (10 ml) were collected with a fraction collector and luciferase activity was determined by diluting 1.0 ml of each fraction with 0.5 ml of 0.85 M-Tris-HCl buffer, pH 8.2, and injecting it into a mixture of 1.0 ml of crude or partially purified luciferin and 0.5 ml of 0.85 M-Tris-HCl buffer, pH 8.2. The partially purified luciferin was prepared by dissolving 0.45 g of the luciferin in 100 ml of 0.1 M-acetic acid. The initial light intensity was used as a measure of luciferase activity. The active fractions were combined and dialysed for 48 h at 4°C against many changes of water. The most active fraction had a protein absorption spectrum with a peak at 277 nm and was 87-fold more active than the initial crude extract.

Luminescent cross-reactions between *Oplophorus*, *Euphausia japonica* and *Cypridina* systems. *Cypridina* luciferin was prepared from 50 g of defatted *Cypridina* powder by the same method used to prepare partially purified *Oplophorus* luciferin (step II). Purified *Cypridina* luciferase was prepared by the method of Tsuji & Sowinski (1961). *E. japonica* luciferin was prepared from 3.0 g of the ground powder of freeze-dried organisms with 5 ml of argon-saturated methanol by using a mini-extraction apparatus (see the acknowledgements). *E. japonica* luciferase was prepared from 4 g of freeze-dried organisms, which were homogenized in cold water with a syringe-type homogenizer, and the mixture was centrifuged at 3000 rev./min for 30 min. The supernatant was used directly for the experiment. For the cross-reaction test, 1.0 ml of crude *Oplophorus* luciferin was mixed with 0.5 ml of 0.85 M-Tris-HCl buffer, pH 8.2, and injected into a reaction cell containing 1.0 ml of *Cypridina* luciferase (33 ng/ml) and 0.5 ml of 0.85 M-Tris-HCl buffer, pH 8.2. In the reciprocal test, 1.0 ml of *Cypridina* luciferin (0.84 mg/ml in 0.1 M-acetic acid) was mixed with 0.5 ml of 0.85 M-Tris-HCl buffer, pH 8.2, and injected into a cell containing 1.0 ml of 0.85 M-Tris-HCl buffer, pH 8.2. Cross-reaction tests between *Cypridina* and *E. japonica* and between *Oplophorus* and *E. japonica* were performed in the same manner.

Test of luciferin model compounds with *Oplophorus* and *Cypridina* luciferases. Each compound was dissolved in water to give a concentration of 5 mM; 0.5 ml of the solution was placed in a reaction cell and into this a mixture of 1.0 ml of crude luciferase and 1.0 ml of 0.125 M-Tris-HCl buffer, pH 8.6, was injected. As a second test, 10 μ l of the solution was placed in a reaction cell and into it was injected a mixture of 1.0 ml of dimethylsulphoxide and 10 μ l of 0.5 M-potassium tertiary butoxide (Sugiura *et al.*, 1970). In each case, the total light was integrated with a photomultiplier photometer.

Measurement of u.v., fluorescence and mass spectra. U.v. spectra were measured with a Hitachi EPS-3T spectrophotometer. Fluorescence spectra were measured with the same instrument equipped with the L-3 fluorescence attachment, by using the 365 nm line of a Hitachi high-pressure mercury lamp for excitation. Mass spectra were measured with a C.E.C. 110B high-resolution mass spectrometer.

Results

Purification of luciferin

The last pale-yellow band migrating in the Sephadex column was luciferin. It was eluted as a single, sharp peak in about 20 ml (four fractions) of eluent, with some tailing in ten additional fractions. Recoveries of luciferin obtained during the purifi-

Table 1. Purification steps for *Oplophorus luciferin*

Details of purification steps and assay for luciferin are given under 'Methods'. Total light yield is equal to total integrated light produced by a unit weight of residue multiplied by the total weight of residue estimated to be present at the end of each purification step.

Steps	Total light units	Specific activity (light units/mg of residue)
Defatted powder	8.0×10^8	1.6×10^4
(I)	7.1×10^6	6.7×10^2
(II)	6.3×10^6	5.7×10^3
(III)	3.4×10^6	2.7×10^6

Fig. 1. U.v. spectrum of *Oplophorus luciferin*

For details see the text. —, In neutral methanol; ----, in 0.1 M-HCl-methanol; — · —, in 0.1 M-KOH-methanol.

cation procedure are shown in Table 1. The results show considerable protection from oxidation during the purification steps and a high degree of purification during the final chromatographic step. In contrast, chromatography with powdered cellulose (eluting solvent: upper layer of ethyl acetate-ethanol-water, 5:2:3, by vol.) or DEAE-cellulose (eluting solvent: 50% ethanol in 0.01 M-sodium phosphate buffer, pH 7.0, containing 0.02 M-NaCl) gave low activities and poor recoveries. Paper chromatography with a mixture of 3% aq. NH_3 , methanol and ethanol as developing solvent gave a R_F of 0.55. Examination of the paper chromatogram with u.v. light revealed the presence of oxyluciferin. Ninhydrin reagent sprayed on the paper showed no evidence of protein contamination.

U.v. and fluorescence spectra

Column-chromatographed luciferin dissolved in neutral methanol had absorption peaks at 211, 230, 257, 300 and 330 nm (Fig. 1). The solution, although highly active with luciferase, appeared to contain a mixture of luciferin (2) and oxyluciferin (1), as

shown by an analysis of the observed absorption maxima. Since the structures of *Oplophorus* luciferin and *Cypridina* luciferin resemble each other, it is quite likely that the 230 and 330 nm peaks are due to the 2-aminopyrazine skeleton of oxyluciferin (1) and the 211, 257 and 300 nm peaks are due to the imidazo [1,2-*a*]pyrazin-3-one group in luciferin. So this spectrum was recognized as that of the mixture of luciferin (2) and oxyluciferin (1), and the latter compound would be more abundant because a peak between 410 and 440 nm could not be found. In acidified methanol, luciferin had peaks at 228 and 326 nm, corresponding closely to the peaks reported for 2-aminopyrazine in acidified methanol (Cheeseman, 1960). In methanol made alkaline with 0.1 M-KOH, luciferin showed no peaks. The peaks in the neutral and acidic methanol solutions gradually decreased on standing, probably owing to air oxidation.

The fluorescence emission spectrum of *Oplophorus* luciferin dissolved in methanol, excited at 365 nm, had a peak at 410 nm, which decreased rapidly during the measurement (Fig. 2). In contrast, 2-aminopyrazine, when excited at 365 nm, gave a fluorescence emission peak at 395 nm in water, 396 nm in methanol, and 418 nm in 0.1 M-HCl. The decrease in the emission peak at 410 nm is presumably due to the conversion of oxyluciferin (1), whose fluorescence emission peak is also at 410 nm, into oxyluciferin carboxylic acid (6) with a shoulder at 380 nm. These results suggest

Fig. 2. Changes in fluorescence intensity of column-chromatographed *Oplophorus* luciferin in methanol at room temperature

Curve (a), zero time; (b), 3 min; (c) 6 min; (d) 9 min; (e), 12 min; and (f), 15 min.

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Fig. 3. Fluorescence spectra of (a) aqueous mixture of column-chromatographed *Oplophorus* luciferin and partially purified *Oplophorus* luciferase, and (b) aqueous solution of luciferase

The mixture in (a) was left for a few minutes at room temperature for the luminescence reaction to go to completion.

that oxyluciferin probably has a 2-aminopyrazine skeleton. It is noteworthy, however, that the fluorescence emission peak of *Oplophorus* luciferin was neither at 535 nm nor 510 nm, which are the fluorescence emission peaks for *Cypridina* luciferin and oxyluciferin respectively, dissolved in 0.05 M-sodium phosphate-0.1 M-NaCl, pH 7.4 (Shimomura *et al.*, 1969). Moreover, with *Cypridina* oxyluciferin, the fluorescence emission peak is at 477 nm in butanol.

Fig. 3 shows the fluorescence emission spectrum, excited at 365 nm, after the addition of partially purified *Oplophorus* luciferase to a water solution of column-chromatographed *Oplophorus* luciferin and leaving the mixture to stand. The maximum of the fluorescence emission spectrum is at 455 nm. Such a shift in the fluorescence emission peak has been observed previously with *Cypridina* luciferin, in which the fluorescence peak is shifted from 535 nm (excited at 430 nm) to 465 nm (excited at 360 nm) on the addition of luciferase to the solution (Shimomura *et al.*, 1969). The new peak is due to oxyluciferin bound to luciferase and corresponds exactly to the peak of the bioluminescence emission spectrum. The fluorescence emission peak of oxyluciferin (7) for the *Oplophorus* luciferin-luciferase mixture was 455 nm, and corresponded roughly to the peak for a *Cypridina* luciferin-luciferase mixture (465 nm).

Mass-spectral analysis

Two samples of *Oplophorus* luciferin were analysed by the direct introduction method. Both

Fig. 4. Hypothetical fragmentation pattern of *Oplophorus luciferin*

For details see the text.

samples showed a M^+ peak at 223. By using three values with the smallest error from each analysis, the best element combination was given by $\text{C}_{10}\text{H}_{13}\text{N}_3\text{O}_3$. The selection of this element combination was also favoured because of the probability that the partial chemical structure of the *Oplophorus luciferin* molecule would be similar to, if not identical with, the *Cypridina luciferin* molecule (Kishi *et al.*, 1966b). The possible pathways for the fragmentation of *Oplophorus luciferin* are partially given in Fig. 4.

Luminescent cross-reactions between *Oplophorus*, *Euphausia japonica* and *Cypridina* systems

The luciferin and luciferase of *Oplophorus* reacted reciprocally with the luciferin and luciferase of *Cypridina*. The reaction appeared to be far greater, in terms of the amount of emitted light by a factor of about ten, between *Oplophorus luciferase* and *Cypridina luciferin* than between *Cypridina luciferase* and *Oplophorus luciferin*. *Cypridina* and *Euphausia* systems also cross-reacted, but *Oplophorus* and *Euphausia* systems could not cross-react.

Synthetic model luciferin compounds

Of the three compounds synthesized, only compound (3) (3-hydroxy-2-methylimidazo[1,2-*a*]-pyridine) showed activity with *Oplophorus luciferase* (Table 2). However, all three compounds (3, 4 and 5) were active with *Cypridina luciferase* and were chemiluminescent in a mixture of dimethylsulphoxide and potassium tertiary butoxide. The activity of compound (3) was not only strong in terms of light emission, but produced approximately the same amount of light with both *Oplophorus luciferase* and *Cypridina luciferase*.

Discussion

The results presented above suggest that the structure of *Oplophorus luciferin* is probably similar to that of compound (2), which is a closely related structure of *Cypridina luciferin* (Kishi *et al.*, 1966a). Observations that support this conclusion are

Table 2. Specific activities (light units/mg of compound) in the bioluminescence and chemiluminescence of luciferin model compounds

Compound	<i>Oplophorus</i> luciferase	<i>Cypridina</i> luciferase	Dimethylsulfoxide + potassium <i>t</i> -butoxide
(3)	2.0×10^2	2.6×10^2	4.4×10^6
(4)	—	4.3×10	8.7×10^6
(5)	—	6.6×10	7.4×10^6

as follows. Similarities in the u.v. spectra of *Oplophorus luciferin* and *Cypridina luciferin*, similarities in the u.v. spectra of *Oplophorus luciferin* and 2-aminopyrazine at acid pH, similarity in the fluorescence emission maximum of a *Oplophorus luciferin*–luciferase mixture with that of a *Cypridina luciferin*–luciferase mixture, a molecular weight of 223 based on mass-spectral analysis, and a positive reciprocal cross-reaction between the luciferins and luciferases of *Oplophorus* and *Cypridina*.

The tertiary alcohol compound (compound 1) of *Oplophorus luciferin* detected by the mass spectrometer is more difficult to explain, but it may have originated from the reaction of a hydroperoxide molecule with a luciferin molecule.

The light emission produced by compound (3) with both *Oplophorus luciferase* and *Cypridina luciferase* suggest that the 3,7-dihydroimidazo[1,2-*a*]-pyrazin-3-one (of *Cypridina luciferin*) and 3-hydroimidazo[1,2-*a*]pyridine (of compound 3) are effective moieties at the active sites of the enzymes.

The results of the present study show that the *Oplophorus* bioluminescence reaction is of the luciferin–luciferase type (Johnson *et al.*, 1966). The enzyme, when partially purified and dissolved in water, was stable for several months at -35°C . The luciferin, however, was highly unstable in the presence of molecular O_2 and had to be kept in an argon atmosphere to preserve activity. The luciferin therefore appears to be less stable than *Cypridina luciferin*, but more stable than either of the two components in the *Euphausia* bioluminescence

cence system. The great sensitivity of luciferin to molecular O₂ made it extremely difficult to obtain good i.r. and nuclear-magnetic-resonance spectra, and eventually these attempts had to be abandoned.

The question as to whether the bioluminescence reaction systems of *Oplophorus* and *Cypridina* are related to the system in *Euphasia* is a matter of interesting speculation. Taxonomically, the euphausiids differ from decapods only in that the thoracic appendages (feet) are split into two parts in the former and not in the latter (Harvey, 1852). Further, the fact that methanol and cold-water extracts of *Euphausia* give weak but distinct light-emitting cross-reactions with methanol and cold-water extracts of *Cypridina* suggests that the systems may have some kind of biological relationship.

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used in extracting and chromatographing the *Oplophorus* luciferin, which were designed by me. Finally, this work is taken from the Ph.D. Thesis of I. Yamaguchi, Tokyo Kyoiku University at Tokyo, 1974.

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