Tetrahedron. Vol. 29, pp. 2035 to 2039. Pergamon Press 1973. Printed in Great Britain

CYPRIDINA BIOLUMINESCENCE—VIII

THE BIOLUMINESCENCE OF *CYPRIDINA* LUCIFERIN ANALOGS[a]

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(*Received in Japan* 17 *January* 1973; *Received in the UK for publication* 20 *February* 1973)

Abstract—The relative rates of bioluminescence, as well as chemiluminescence, among *Cypridina* luciferin analogs, and the relative light yield between bioluminescence and chemiluminescence of each of the analogs have been measured with reference to *Cypridina* luciferin.

Studies on the bioluminescence of *Cypridina hilgendorfii* (Japanese name: umihotaru) have been carried out from various aspects since 1917, when Harvey[1] observed the luciferin–luciferase reaction with the crustacean. Among the bioluminescent systems studied *Cypridina* has been regarded as one of the simplest, which requires only *Cypridina* luciferin (1) (substrate), *Cypridina* luciferase (enzyme) and oxygen for the light emission in aqueous solution. The luciferin was isolated in a crystalline state[2] and its structure elucidated as 1,[3] which is obviously derived biogenetically from tryptamine, isoleucine and arginine.

When dissolved in aprotic solvents, luciferin (1) shows chemiluminescence,[4] the mechanism of which was suggested as follows:[5] one electron transfer from the luciferin anion to the oxygen molecule produces the radical pair (2), which rapidly combines to give the hydroperoxide anion (3). The hydroperoxide decomposes through the dioxetane intermediate (4) to afford the acylaminopyrazine anion (oxyluciferin anion) (5) in a singlet excited state, which then gives light. The reaction is first-order with respect to the luciferin under a constant oxygen pressure; the first step being the rate-determining step. The imidazolone ring is destroyed during the luminescence of luciferin. The structure essential for the luminescent reaction is considered to be the 3,7-dihydroimidazo[1,2-a]-pyrazin-3-one nucleus, and this assumption has been verified by synthesis of the parent compound, 3,7-dihydroimidazo[1,2-a]pyrazin-3-one (6), which showed chemiluminescence in aprotic solvents.[4]

However, since the parent compound does not bioluminesce in the presence of luciferase, the three substituents attached to the nucleus must play some important roles supporting the bioluminescent system of *Cypridina*. Hydrolysis of the guanidino group in the luciferin to the corresponding amino group [luciferamine (7)] made the bioluminescent activity considerably lower. Thus, the guanidino group must be very important in the bioluminescent system. Hence the specificity of the enzyme was tested first by making a change in the chain length between the guanidine and the imidazopyrazine ring; a dramatic change in the rate of bioluminescence being observed, whilst the length of the chain has no effect on the chemiluminescence rate as shown in Table 1. It is interesting that the bioluminescence rate of the 5-methylene compound is faster than that of the 4-methylene compound. A zig-zag shape of the methylene side chain might be responsible for this anomally. Displacement of the indolyl moiety of luciferin by a phenyl group makes the bioluminescence rate markedly slower, whilst the chemiluminescence rate becomes rather faster.

The ratio of light yield in the Table corresponds to luminescence efficiency. It is noted that in the case of luciferin analogs having an indole and a guanidino group, bioluminescence efficiency is about 10 times better than chemiluminescence efficiency. When the indolyl group is changed to a phenyl group, the ratio of light yield becomes

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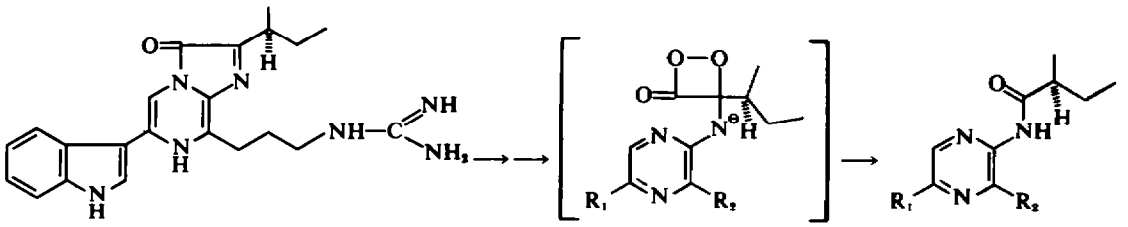
[a]Preliminarily reported in *Pure and Appl. Chem.*, 17, 421 (1968); preceding paper: *Tetrahedron Letters*, 4299 (1969).



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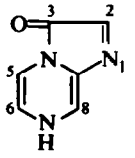

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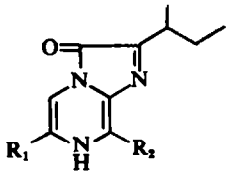
Fig 1.

Table 1.



	R_1	R_2	Relative rate of luminescence Biol.	Chem.	Ratio of Light yield Bio./Chem.
#	β-Indolyl	—H	0·1	30	0·5
23a	β-Indolyl	—CH_2CH_2—G*	1	100	10
1	β-Indolyl	—$CH_2CH_2CH_2$—G	*100*†	*100*†	10
23b	β-Indolyl	—$CH_2CH_2CH_2CH_2$—G	1	100	10
23c	β-Indolyl	—$CH_2CH_2CH_2CH_2CH_2$—G	4	100	10
23d	β-Indolyl	—$CH_2CH_2CH_2CH_2CH_2CH_2CH_2$—G	0·6	100	10
7	β-Indolyl	—$CH_2CH_2CH_2NH_2$	0·2	100	0·5
24	Phenyl	—$CH_2CH_2CH_2$—G	0·4	200	1
#	Phenyl	—H	0	25	0

*—G: —NH—C(=NH)NH_2.

†Standard.

#These were prepared from 2-amino-5-phenylpyrazine and 2-amino-5-(3-indolyl)pyrazine [S. Sugiura, S. Inoue, Y. Kishi, and T. Goto, *Yakugaku Zasshi*, **89**, 1646 (1969)].

almost unity. Thus, in the latter case no enhancement of luminescence efficiency by the enzyme is observed. This indicates that the indolyl group is necessary for the high quantum yield of bioluminescence.

For synthesis of the luciferin analogs, we employed the original scheme[6] for the total synthesis of luciferin. The synthetic route is shown below. The last step, condensation of etioluciferin analogs with the ketoacid, proceeds less than 1%, but a highly sensitive instrument for the measurements of the luminescence makes the kinetic measurement possible without isolation of the luciferin analogs. Large amounts of the starting materials present in the testing solutions do not interfere with the light production.

EXPERIMENTAL

Aminoalkanal acetals (**8**). The following acetals were prepared according to the literatures indicated: 3-aminopropanal diethyl acetal (**8a**),[7] 58–59·5°/7 mmHg; 5-aminopentanal ethylene acetal (**8b**),[8] 65°/0·65 mmHg; 6-aminohexanal ethylene acetal (**8c**),[8] 65–68°/0·16 mmHg; and 8-aminooctanal ethylene acetal (**8d**),[8] 101–102°/1 mmHg.

8-Benzoylaminooctanal ethylene acetal (**9d**). To a soln of **8d** (10 g) in 5% KOH aq (100 ml) was added benzoyl

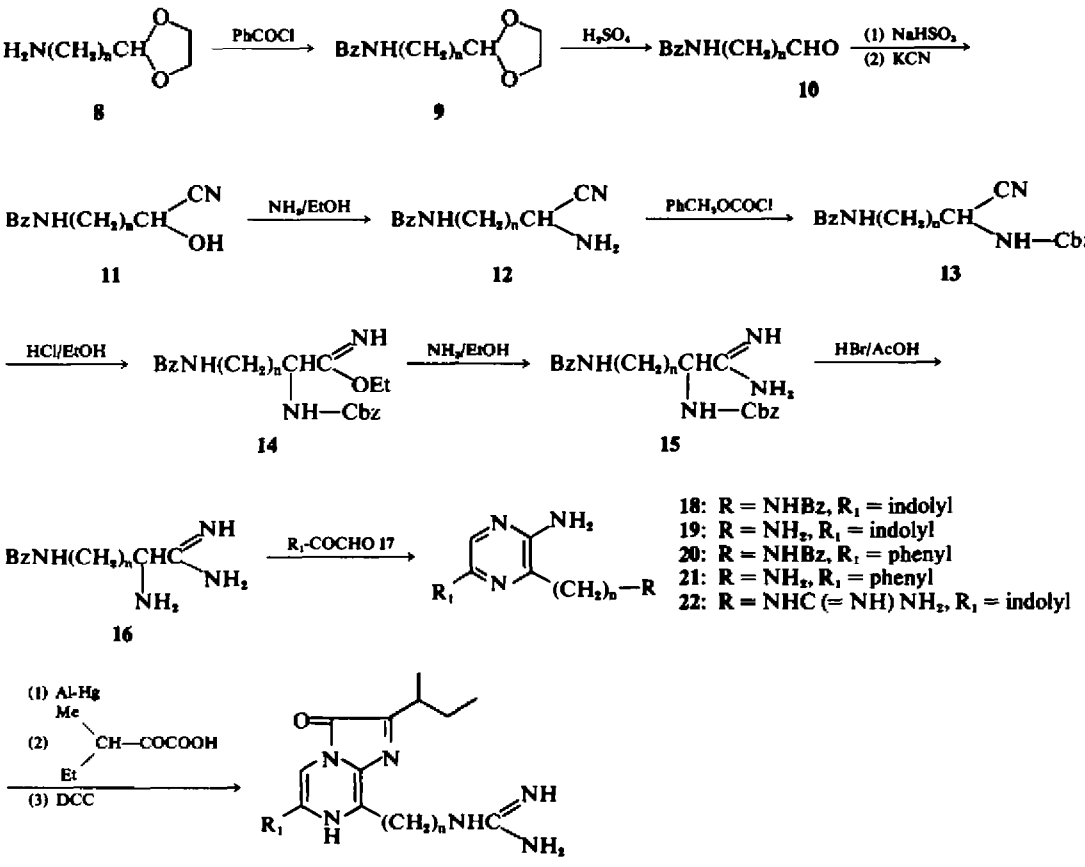


Fig 2

chloride (10 g) at −5° to 0° with stirring in an ice bath. After the addition was complete, the mixture was kept at 0–5° for 1 hr and then gradually warmed to room temp. The ppt was collected by filtration, rinsed with water and dried *in vacuo* over $CaCl_2$. Crystallization from a mixture of benzene and benzine gave white needles, m.p. 64–64·5°, yield 82%. (Found: C, 70·07; H, 8·66; N, 4·79. $C_{17}H_{25}NO_3$ requires: C, 70·07; H, 8·65; N, 4·81%).

2-*Amino*-9-*benzoylaminononanenitrile* (**12d**). A soln of **9d** (25·0 g) in a mixture of 1N H_2SO_4 (150 ml) and dioxane (100 ml) was shaken at 36° for 24 hr and then extracted with ether. The ether extracts were washed with sat NaCl, dried over Na_2SO_4, and evaporated *in vacuo* to give the corresponding free aldehyde **10d** (23 g) as a waxy solid. It was dissolved in a mixture of water and dioxane (1 : 1) and the soln was added with stirring to a sat $NaHSO_3$ aq (8 g in water) under cooling in an ice bath, when the bisulfite adduct of the aldehyde was precipitated. The adduct was collected by filtration and dissolved in water and to this soln was added dropwise a cold KCN aq (7 g in 7 ml of water) with stirring in a period of 1 hr. After being allowed at room temp for 1 hr, the mixture was saturated with NaCl and extracted with ether. The extracts were evaporated to give the corresponding cyanohydrin **11d** as a colorless oil (31 g). The crude cyanohydrin (28 g) and EtOH (120 ml) saturated with ammonia at 0° were placed in a sealed tube and heated at 60° for 5 hr. After being cooled, the solvent and excess ammonia were evaporated completely *in vacuo* and the residual oil was dissolved in cold EtOAc (200 ml). The soln was treated with 5% HCl (50 ml), when the aminonitrile hydrochloride **12d** crystallized out. The crude crystals were collected and recrystallized from EtOH to give white rods, m.p. 125–129·5° (17 g, 54%). (Found: C, 61·88; H, 7·81; N, 13·53; Cl, 11·58. $C_{16}H_{24}ON_3Cl$ requires: C, 62·02; H, 7·81; N, 13·56; Cl, 11·45%).

9-*Benzoylamino*-2-*carbobenzoxyaminononanenitrile* (**13d**). To a mixture of **12d** (15 g), acetone (30 ml), $NaHCO_3$ (5 g), and water (5 ml) was added with stirring at −5° to 0° a toluene soln of carbobenzoxy chloride (1·1 eq) during a period of 30 min, and the stirring was continued overnight at room temp. The solvents were evaporated gently and the residual oil was rinsed with water. When being triturated with a mixture of MeOH and water the residue gave crystals, which were collected and recrystallized from benzene to give colorless needles, m.p. 86·5–87°, yield 96%. (Found: C, 70·88; H, 6·99; N, 10·17. $C_{24}H_{29}N_3O_3$ requires: C, 70·73; H, 7·17; N, 10·31%).

N-*Benzoyltetrakishomoethioluciferamine* [2-*amino*-3-(7'-*benzoylaminoheptyl*)-5-(3"-*indolyl*)*pyrazine*] (**18d**). Dry HCl gas was introduced for 3 hr with stirring into a cold soln of **13d** (2·0 g) in abs EtOH (6 ml). The mixture

was diluted with anhyd ether and the resulting white ppt was collected and washed well with anhyd ether. The crude iminoether **14d** thus obtained was then dissolved in EtOH (20 ml) saturated with ammonia and the soln kept overnight at 5°. Addition of ether to the mixture gave the amidine **15d** as a yellowish ppt, which was collected and dried over P_2O_5 *in vacuo*, yield 1·4 g. To a soln of the crude **15d** (1·4 g) in gracial AcOH (8 ml) AcOH (10 ml) saturated with anhyd HBr gas at 0° was added, and the mixture was heated at 35–50° for 5 hr. The mixture was diluted with anhyd ether to give a white ppt, which was separated from the mother liquor by repeated addition of ether and decantation of the supernatant. The crude aminoamidine ·2HCl **16d** thus obtained was used to the next step without further purification. The amidine **16d** and $NaHSO_3$ adduct of indolylglyoxal (0·90 g) were dissolved in MeOH (180 ml) and water (90 ml) and the soln was made basic by dropwise addition of 30% KOH aq at −10° with stirring. After being kept at −10° for 3 hr, it was allowed to stand overnight at room temp, and then concentrated *in vacuo* to *ca* 7 ml. After standing at 5° overnight, the concentrate precipitated a solid, which was collected and reprecipitated from MeOH and water (0·88 g). For elemental analysis this compound was converted to its picrate and recrystallized from EtOH, m.p. 206–219° (dec). (Found: C, 58·26; H, 4·85; N, 16·81. $C_{32}H_{32}O_8N_8$ requires: C, 58·53; H, 4·91; N, 17·07%).

Tetrakishomoethioluciferamine (**19d**). A mixture of the benzamide **18d** (100 mg) in MeOH (10 ml) and KOH (6 g) in water (4 ml) was refluxed until the hydrolysis was completed (monitored by TLC; *ca* 4 hr). The mixture was concentrated to dryness, dissolved in water (3 ml) and extracted with ether continuously until no fluorescent spot could be detected on TLC of the extract. The ether extracts were evaporated to dryness to give yellow powder (68 mg, 92%). Attempted crystallization failed, but the following physical data identified this compound. High-resolution mass spectrum shows a parent peak at *m/e* 323·211 (calcd. for $C_{19}H_{25}N_5$: 323·211) and main fragment peaks at 237(**),† 224(*****), 197(*), 155(*), 142(***), 130(*), and 129(*), indicating the correct structure;[9] IR (KBr disc) 3350s, 3150w, 2900s, 2800m, 1620s, 1545s, 1455vs, 1390m, 1210m, 740s cm^{-1}; UV (MeOH) 218, 272, 358 nm; (MeOH—HCl) 220, 275, 305, 405 nm.

6-Benzoylaminohexanal ethylene acetal (**9c**). This compound was prepared from **8c** as white needles, m.p. 64·5–65°, yield 90%. (Found: C, 68·26; H, 7·92; N, 5·38. $C_{15}H_{21}O_3N$ requires: C, 68·41; H, 8·04; N, 5·32%).

7-Benzoylamino-2-carbobenzoxyaminoheptanenitrile (**13c**). The acetal **9c** (39 g) was hydrolyzed in 1N H_2SO_4 (400 ml) and dioxane (50 ml) at 36° for 24 hr to the aldehyde **10c** (oil, 32 g), which was converted through its bisulfite adduct into the cyanohydrin **11c** (32 g as a crude colorless oil). **11c** (32 g) and aminated to give aminonitrile **12c** as an oily residue, which was extracted below 5° as quickly as possible with 5% HCl (50 ml) and then water (50 ml × 3). The combined extracts were made alkaline with a cold NaOH aq and extracted with EtOAc. The organic extracts were worked up as usual to give crude **12c** (18 g, 61%). **12c** (10 g) was carbobenzoxylated and crystallized from benzene as white needles, m.p. 91–92° (15 g, 92%). (Found: C, 69·55; H, 6·54; N, 11·32. $C_{22}H_{25}N_3O_3$ requires: C, 69·63; H, 6·64; N, 11·08%).

N-Benzoylbishomoethioluciferamine (**18c**). The nitrile **13c** (6 g) was converted to **15c** (crude, 5·85 g), which in turn on hydrolysis followed by condensation with indolylglyoxal afforded **18c** (0·23 g) identified by its picrate, m.p. 211·5–215° (dec). (Found: C, 56·82; H, 4·43; N, 17·34. $C_{30}H_{28}O_8N_8$ requires: C, 57·32; H, 4·49; N, 17·83%).

Bishomoetioluciferamine (**19c**). Hydrolysis of **18c** was carried out according to the method described for **19d**. The high-resolution mass spectrum shows a parent peak at *m/e* 295·1818 (calcd. for $C_{17}H_{21}N_5$: 295·1798) and main fragment peaks at 278(*), 265(*), 251(*), 237(**), 224(***), 197(*), 155(*), 142(*), 140(*), and 130(*), indicating the correct structure;[9] IR (KBr disc) 3350, 3150, 2900, 2800, 1615, 1545, 1450, 1390, 1220, 1145, 750 cm^{-1}.

6-Benzoylamino-2-carbobenzoxyaminohexanenitrile (**13b**). The acetal **8b** was converted into **13b** by a method similar to the preparation of **13c**. Crystallization from MeOH and water gave crude crystals, which were recrystallized from benzene, m.p. 94–95°. (Found: C, 68·92; H, 6·36; N, 11·37. $C_{21}H_{23}O_3N$ requires: C, 69·02; H, 6·34; N, 11·50%).

Homoetioluciferamine (**19b**). The nitrile **13b** (0·5 g) was converted to N-benzoylhomoluciferamine (**18b**) by essentially the method described in the preparation of **18d**. Hydrolysis of the benzamide was carried out with KOH as described for **19d**. The structure was deduced from mass, UV and IR spectra.

4-Benzoylamino-2-carbobenzoxyaminobutanenitrile (**13a**). The acetal **8a** (20 g), on benzoylation, gave **9a** (32 g, 92%), which (25 g) was hydrolyzed with 5% H_2SO_4 (66 ml) to give crude **10a** (17 g, 98%). A mixture of the aldehyde **10a** (16 g) and $NaHSO_3$ (13 g) was treated with KCN aq (6 g) and the oil separated was extracted with EtOAc to give crude cyanohydrin **11a** (19 g). The cyanohydrin and EtOH saturated with ammonia (19 g) were placed in an autoclave, heated for 30 min at 90° and the solvent was removed *in vacuo*. The residue was treated to give crude aminonitrile **12a**, which on carbobenzoxylation gave **13a** as a waxy solid. It was crystallized from MeOH-toluene, m.p. 120°, yield 16 g. (Found: C, 68·37; H, 5·57; N, 12·75. $C_{19}H_{19}N_3O_3$ requires: C, 67·64; H, 5·68; N, 12·46%).

4-Benzoylamino-2-carbobenzoxyaminobutanoamidine hydrochloride (**15a**). To a mixture of **13a** (10 g), abs EtOH (35 ml) and anhyd ether (35 ml), HCl gas was introduced under cooling until the solid dissolved. Ether was added to the mixture, the supernatant liquid was decanted, and the residue was treated with EtOH (50 ml) saturated with ammonia at −5°. After standing overnight at room temp, the mixture was diluted with ether. The supernatant soln was decanted and the residue was crystallized from water, m.p. 97–100°, yield 6 g. (Found: C, 58·01; H, 5·91; N, 14·60. $C_{19}H_{23}N_4OCl$ requires: C, 58·46; H, 5·9; N, 14·35%).

N-benzoylnoretioluciferamine (**18a**). The benzoylaminoamidine **15a** was hydrolyzed with HBr in AcOH and then condensed with indolylglyoxal to give **18a**, which was converted to its picrate, m.p. 235°, for analysis. (Found: C, 55·41; H, 3·64; N, 18·90. $C_{27}H_{22}O_8N_8$ requires: C, 55·29; H, 3·75; N, 19·10%).

Noretioluciferamine (**19a**). The benzamide **18a** (150 mg) was hydrolyzed with KOH to give crude **19a** (100 mg) which was used for the preparation of noretioluciferin without further purification.

2-Amino-3-(γ-benzoylaminopropyl)-5-phenylpyrazine (**20**). A mixture of phenylglyoxal monohydrate (0·5 g), α-amino-δ-benzoylaminovaleroamidine dihydrobromide (1·5 g), MeOH (100 ml) and water (50 ml) was stirred under cooling at −10°. To this mixture was added dropwise a

†Number of asterisks indicates the intensity of the peak.

soln of KOH (1 g) in water (5 ml) and the stirring was continued for 1 hr at this temp and 2 hr at room temp. When the mixture was concentrated to *ca* 30 ml, a crystalline mass was obtained which was filtered and recrystallized from benzene to afford yellow needles (0·8 g) melting at 150–150·5°. (Found: C, 72·06; H, 5·98; N, 17·13. $C_{20}H_{20}N_4O$ requires: C, 72·27; H, 6·07; N, 16·86%); UV (MeOH) 339 nm (ε 40,000).

2-Amino-3-(γ-aminopropyl)-5-phenylpyrazine (**21**). A mixture of **20** (0·58 g) in MeOH (50 ml) and KOH (22 g) in water (15 ml) was refluxed for 14 hr until TLC analysis gave no spot of the benzamide. The mixture was then evaporated *in vacuo* to 5 ml, from which was obtained a crystalline mass. It was collected and purified by repeated precipitations from a mixture of MeOH and water to afford pale yellow crystals, m.p. 111–116°. The high-resolution mass spectrum gave a molecular peak at *m/e* 228·137 (calcd. for $C_{13}H_{16}N_4$: 228·138); NMR (DMSO) 1·9 (2H,m), 2·6 (4H,m), 6·3 (2H, NH), 7·35 (3H,m), 7·8 (2H,m), 8·35 (1 H,s).

Bishomoetioluciferin [*2-amino-3-(5'-aminopentyl)-5-(3''-indolyl)pyrazine*] (**22c**). S-Methylisothiourea sulfate (30 mg) in MeOH (2 ml) was mixed with KOH (18 mg), and the mixture was added to a soln of **19c** (40 mg) in MeOH (4 ml). Stirring was continued overnight at room temp. TLC analysis indicated that the reaction was proceeding. The mixture was evaporated *in vacuo* to dryness and extracted with EtOH. The solvent was replaced with BuOH to which a small amount of HBr was added through a capillary tube. A yellow ppt appeared which was collected on a filter and rinsed with ether, yield 12 mg. The structure was assumed by comparison of IR and UV with those of etioluciferin and from a positive Sakaguchi reaction for a monosubstituted guanidine. Other etioluciferin analogs were prepared similarly.

Condensation of bishomoetioluciferin (**22c**) *with α-keto-β-methylvaleric acid.* A soln of **22c** (1·85 mg) in 90% EtOH (2 ml) was placed in a reaction apparatus, whose inside was filled with argon. On the other hand, aluminum foil (*ca* 3 cm^2) was washed with ether (2 times) and EtOH (3 times), treated for 30 sec with 1% $HgCl_2$ (1 ml) and rinsed with a little water (2 times) and EtOH (3 times). This activated aluminum was then added into the soln in the apparatus. After 15 min at 65°, the mixture was filtered under argon and the filtrate was treated with 2% ethanolic solution (200 μl) of *d-α*-keto-β-methylvaleric acid for 10 min and then with DCC (*ca* 20 mg). After the mixture had been allowed to stand overnight at room temp, the measurements of chemi- and bioluminescence activities were carried out without isolation of bishomoluciferin (**23c**). The yield of **23c** may be less than 1%. Other luciferin analogs were prepared similarly.

Measurements of luminescence. Bioluminescence was measured as follows: a sample soln (10 μl) was placed in a test tube (1·8φ × 9 cm), which was faced to the photomultiplier. To this tube was added at once with a pipette a soln of luciferase[10] in phosphate buffer (0·05M, pH 7·0) containing 0·1M NaCl and the integrated light yield was recorded on a recorder. Chemiluminescence was measured by using diethylene glycol dimethyl ether (2 ml, purified by refluxing with Na) containing 0·6M acetate buffer (pH 5·6; 10 μl) in place of the luciferase soln in the case of bioluminescence. Photomultiplier Toshiba MS9SY was used and no spectral correction was made.

Acknowledgements—Some experiments were carried out by Mr. Y. Abe, to whom thanks are due. We are indebted to Mr. S. Miyamoto and Mr. H. Miyamoto for collecting *Cypridina hilgendorfii*, and to JEOL Co. for high-resolution mass spectra. We thank Mr. N. Naruse, Torey Basic Research Laboratories, for valuable suggestions.

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