Proc. Nat. Acad. Sci. USA
Vol. 70, No. 1, pp. 120-123, January 1973

Structure and Chemical Synthesis of a Biologically Active Form of *Renilla* (Sea Pansy) Luciferin*

(bioluminescence/pyrazine derivative/coelenterates)

KAZUO HORI AND MILTON J. CORMIER

Department of Biochemistry, University of Georgia, Athens, Ga. 30601

Communicated by William D. McElroy, October 30, 1972

ABSTRACT **The structure of a biologically active form of *Renilla* (sea pansy) luciferin has been elucidated; this structure, confirmed by total chemical synthesis, is 3,7-dihydro-2-methyl-6-(*p*-hydroxyphenyl)-8-benzylimidazo [1,2-*a*] pyrazin-3-one. In the natural compound the methyl group at the 2 position is replaced by an unknown, more complex group. For this reason the synthetic compound is 10% as active as the natural compound in producing light with *Renilla* luciferase. However, the spectral properties of the two compounds are identical. In addition the rates of the luminescent reaction with both compounds are similar, and the color of the light produced is identical in each case.**

A compound isolated from the calcium-triggered photoprotein aequorin has been identified by Shimomura and Johnson [(1972) *Biochemistry* 11, 1602] to be 2-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine. This compound forms an integral part of the structure of *Renilla* luciferin. This, and other evidence, suggests that the structure elucidated for *Renilla* luciferin is a more general one associated with the luciferins of most, if not all, bioluminescent coelenterates.

From about 40,000 sea pansies (*Renilla reniformis*), obtained by dredging the ocean bottom at depths of 10–20 meters, one can obtain about 0.5 mg of pure *Renilla* luciferin. It is for this reason that elucidation of the structure of *Renilla* luciferin has been a difficult problem. We have accumulated a considerable amount of absorption data (U.V., visible, I.R.), as well as chemical and high-resolution mass spectroscopy data, over the last 10 years (1–3). These data, and the observation that several of the physical and chemical properties of *Renilla* luciferin were similar to those of *Cypridina* luciferin (1), led us to propose a partial tentative structure for *Renilla* luciferin that was similar to the structure of *Cypridina* luciferin (1). Due to the small amount of *Renilla* luciferin available and the ease with which luciferin is autooxidized good NMR data have never been available to us. Thus, several alternative structures were possible based on the mass spectroscopy data.

A choice between the various possible structures might have been a more difficult problem if it were not for several observations made by us and by other investigators in the field of bioluminescence who were working on problems that seemed at first unrelated. The key observations and ideas that led to the proper choice of structure are outlined below.

When the luminous tissues of bioluminescent coelenterates are extracted with EDTA-containing buffers, a protein can be isolated that exhibits a bluish luminescence upon the addition of calcium ions (4–7). Such proteins were termed "photoproteins." Recently, the existence of calcium-triggered photoproteins have been demonstrated in extracts of a wide variety of coelenterates such as *Obelia*, *Aequorea*, *Pelagia*, *Renilla*, *Mnemiopsis*, *Campanularia*, *Clytia*, *Phialidium*, *Lovenella*, *Ptilosarcus*, and *Diphyes* (7). Furthermore, the characteristics of these photoproteins were for the most part similar to those described for *Aequorea*.

These and other observations led us to look for possible biochemical similarities in the bioluminescence of coelenterates. Such similarities were indeed found. For example, components required for luminescence in *Renilla* were also found in several bioluminescent coelenterates examined such as *Aequorea*, *Obelia*, *Cavernularia*, *Ptilosarcus*, *Stylatula*, *Acanthoptilum*, *Parazoanthus*, and *Mnemiopsis* (8). Depending on the organism, these included one or more of the following: luciferyl sulfate, luciferase, photoprotein, and luciferin sulfokinase. The luciferyl sulfates isolated, including the one from *Aequorea*, produced light when added to the *Renilla* system, suggesting that the luciferins and luciferases from these coelenterates were either identical or very similar.

When *Aequorea* photoprotein is treated with urea and mercaptoethanol, a compound is released that has been designated AF-350 (9). We have noted that the absorption and fluorescence characteristics reported for AF-350 are very similar to those observed by us for *Renilla*-autooxidized luciferin (1).

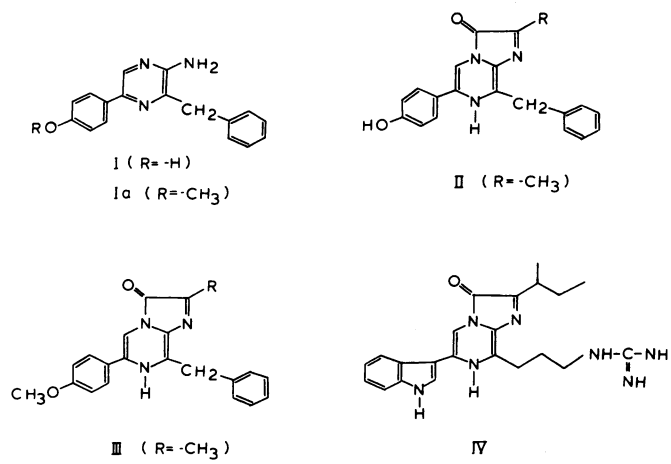

FIG. 1. Luciferin structures and some of their derivatives.

* This is paper no. XII of a series entitled, "Studies on the Bioluminescence of *Renilla reniformis*."

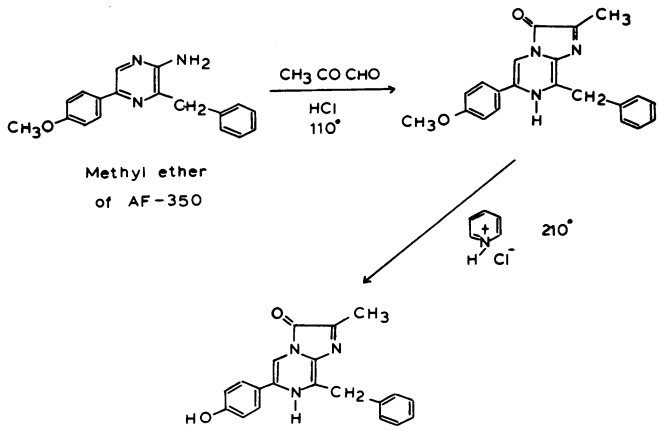

FIG. 2. Synthetic scheme for the synthesis of a biologically active form of *Renilla* luciferin.

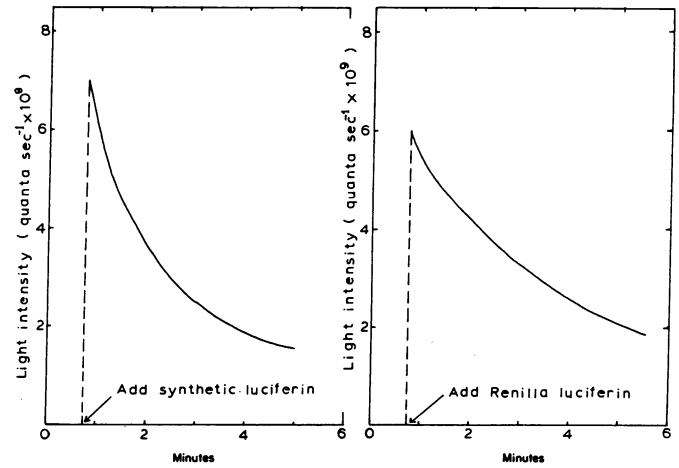

FIG. 3. A comparison of the rates of the luminescent reaction upon initiation with synthetic luciferin and *Renilla* luciferin. In each case, 1 nmol of luciferin was injected into a solution of *Renilla* luciferase.

The reported molecular weight of AF-350 is 277, which is also the strongest peak observed when *Renilla* luciferin is analyzed in a mass spectrometer. Further, the mass spectroscopy patterns of AF-350 and *Renilla* luciferin are essentially the same up to a mass of 277.

The structure of AF-350 (I in Fig. 1) was reported recently (10), and this structure has been confirmed by synthesis (11).

We noted that with minor modifications of our proposed structure of *Renilla* luciferin (1, 2) AF-350 becomes an integral part of the structure of *Renilla* luciferin.

Based on the above observations, as well as a consideration of the energetics of the luminescent reaction, we felt that a likely ring structure for *Renilla* luciferin would involve fusing an imidazole ring to structure I, thus converting it to structure II. With this in mind, structure II was synthesized and was found to be biologically active. This paper describes the synthesis and some of the biological properties of this newly synthesized luciferin.

MATERIALS AND METHODS

Luciferase was purified as described (12), with elimination of the acetone powder step. Native *Renilla* luciferyl sulfate was isolated as described (2), and was then converted to luciferin (15).

Absorption spectra were determined on a Cary model 14 Spectrophotometer. Absolute bioluminescence and fluorescence emission spectra were determined by use of an on-line spectrofluorimeter system (14) derived from a component fluorimeter (13).

Low-resolution mass spectral data were obtained on a Dupont Instrument model 21-490 single-focusing mass spectrometer equipped with a solid sample inlet probe, variable ionizing voltage control, and electrical ion detection. High-resolution mass spectral data were obtained with a CEC 21-110B mass spectrometer with photoplate recording of the spectra.

The methyl ether of AF-350 (Structure I*a*) was synthesized as described (11). A biologically active *Renilla* luciferin derivative (Structure II) was synthesized from I*a* by modification of the final steps involved in the synthesis of *Cypridina* luciferin (16). The method used was as follows: Compound I*a* was reacted with methyl glyoxal in ethanol–12 N HCl 1:1 under an argon atmosphere in a sealed tube at 110° for 1.5 hr. The hydrochloride of Compound III was obtained in good yield (85%). It was converted to Compound II by treatment with anhydrous pyridine hydrochloride at 210° for 2 hr under an argon atmosphere in a sealed tube. The hydrochloride of Compound II was isolated from the reaction mixture in good yield (90%) by chromatography of the reaction mixture through LH-20 (2). The elution solvent was 80% methanol made 0.1 N with HCl. The hydrochloride of II was converted to free luciferin (II) by neutralizing it with phosphate buffer. Yellow crystals of II were obtained by heating an aqueous suspension of II at pH 2 under a hydrogen atmosphere, and allowing the solution to slowly cool. It should be noted that by use of derivatives of glyoxal other than methyl any desired substitution can be made at the 2-position of luciferin (II).

RESULTS

Structure II was synthesized from AF-350 (Structure I) according to procedures outlined under *Methods*. The synthetic scheme is shown in Fig. 2.

Absorption and fluorescence properties of II are identical to those observed for *Renilla* luciferin (1). In methanol solutions at pH 7.0, they both absorb at 262 and 433 nm and they both exhibit the same bathochromic shifts in acidic and basic environments.

The molar extinction coefficients of these two transitions were essentially the same as those reported for *Renilla* luciferin (1). In methanol solution at pH 7.0, both *Renilla* luciferin and II exhibit identical yellow–green fluorescence emissions (λ_{max} = 538 nm).

The molecular weight of II is 331. The mass spectral pattern of this compound was identical to that of *Renilla* luciferin up to a mass of 331. High-resolution mass analysis of *Renilla* luciferin, however, shows it to have a molecular weight of 513. Thus, the R group of II is rather complex in natural luciferin. However, it does not influence the spectral characteristics of luciferin, as judged by the absorption, fluorescence, and bioluminescence emission of luciferin as compared with II.

The synthetic compound (II) was biologically active, while I was totally inactive. When II was added to *Renilla* luciferase, light production occurred. As shown in Fig. 3, the rates of the light reaction were similar whether we used II or *Renilla* luciferin. Furthermore, the color of bioluminescence was identical

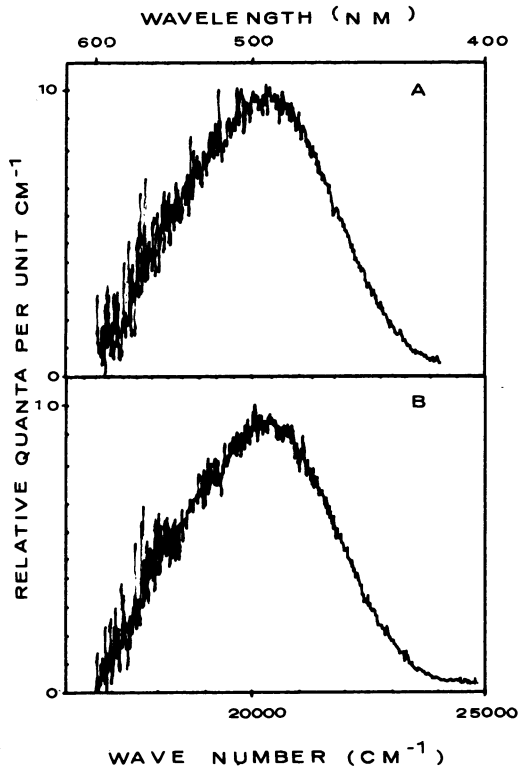

FIG. 4. A comparison of the color of bioluminescence upon initiation of the reaction with synthetic luciferin (*A*) and *Renilla* luciferin (*B*). In each case, the reaction was initiated by injection of about 0.1 μmol of luciferin into a solution of luciferase. These spectra were corrected for instrument spectral response with quinine bisulfate as spectral standard.

in both cases, as shown in Fig. 4. In each case the typical emission spectrum of the *Renilla in vitro* reaction was observed.

Because of the differences in the R group of II and that of *Renilla* luciferin as outlined above, II is only 10% as active as *Renilla* luciferin in producing light with *Renilla* luciferase. We compared the two compounds by comparing the peak intensities of the bioluminescent flash when equimolar amounts were injected into solutions of luciferase.

It was interesting to note that the methylated form of II (structure III in Fig. 1) was totally inactive in producing light with luciferase. Upon demethylation, biological activity was restored.

DISCUSSION

From the data reported here it appears that, with the exception of the R group in II, the structure of II and *Renilla* luciferin are identical. It is interesting to compare the structure of II with that of *Cypridina* luciferin (IV), whose structure and synthesis has also been accomplished (16–18). They both contain a pyrazine ring with a fused imidazole nucleus. The side chains, however, are considerably different. Side chains derived from tryptophan and arginine in *Cypridina* luciferin are replaced by those derived from tyrosine and phenylalanine in II. Since the heterocyclic ring structures of both compounds are the same, it is not surprising that we noted striking similarities in the absorption and fluorescence properties when *Renilla* and *Cypridina* luciferins were compared (1). A published tentative structure of *Renilla* luciferin (1, 2), which included an indole nucleus, was based on mass spectral data and a color test when *Renilla* luciferin is reacted with *p*-dimethylaminobenzaldehyde (3). However, the mass spectral data on *Renilla* luciferin can now be easily explained based on structure II.

As mentioned above, AF-350 (I) can be isolated when the calcium-triggered photoprotein of *Aequorea* is treated with urea and mercaptoethanol (9). It is most interesting to find that this structure is an integral part of the structure of *Renilla* luciferin. This observation reinforces our recent proposal that the luciferins and luciferases of bioluminescent coelenterates are similar or identical (8). In this connection, it should be mentioned that both *Renilla* luciferin and II will produce light

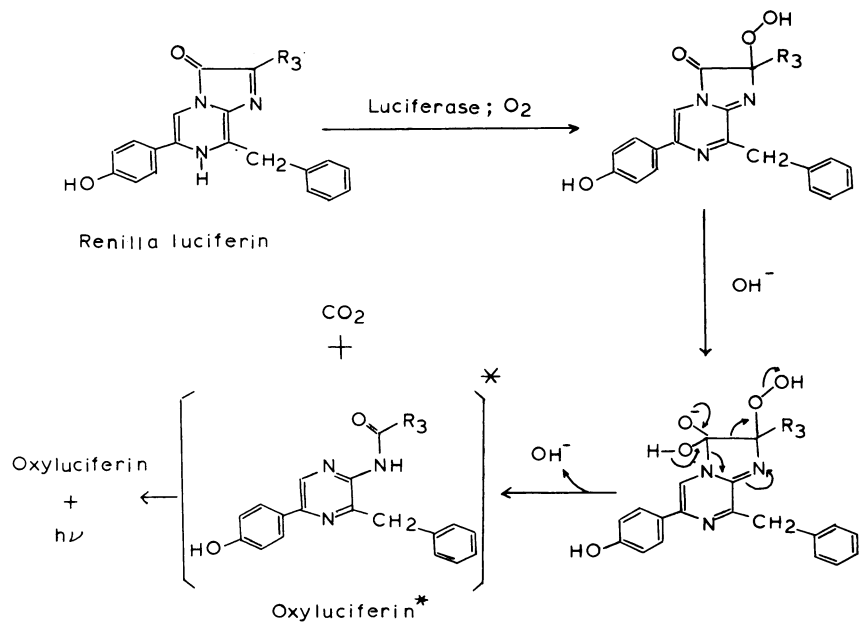

FIG. 5. Mechanism of the *Renilla* bioluminescence reaction.

when mixed with luciferases isolated from several coelenterates such as *Renilla*, *Ptilosarcus*, and *Stylatula*.

When the problem of the nature of photoproteins is considered, it is also interesting that we have isolated from *Aequorea*, and several other bioluminescent coelenterates, a *Renilla*-like luciferyl sulfate (8). Since *Renilla* extracts also contain luciferyl sulfate, as well as calcium-triggered soluble photoprotein, it seems reasonable to assume that luciferin, derived from luciferyl sulfate, somehow becomes an integral part of the photoprotein complex in coelenterates. This is an important consideration when one considers the overall energetics of the *Renilla* luminescent reaction, since about 70 kcal/mol quanta are required for the creation of the electronically excited state of the emitter. Based on structure II and on previous work on the mechanism of the luminescent reaction in *Renilla* with ^{18}O (19), a mechanism that satisfies the energy requirements and predicts the structure of the emitter (oxyluciferin) is shown in Fig. 5. Hydrolysis of the peptide bond in oxyluciferin would lead to the formation of AF-350 (I).

We thank Dr. R. E. Lovins of this department for providing the low- and high-resolution mass spectral data and Dr. J. E. Wampler for providing the absolute fluorescence and bioluminescence emission data. This work was supported by grants from the National Science Foundation and the U.S. Atomic Energy Commission. M. J. C. is a Career Development Awardee no. 1-K3-6M-333-07 of the U.S. Public Health Service. This is contribution no. 235 from the University of Georgia Marine Institute, Sapelo Island, Ga.

1. Hori, K. & Cormier, M. J. (1973) in *Chemiluminescence and Bioluminescence*, eds. Cormier, M. J., Hercules, P. M. & Lee, J. (Plenum Press, New York), in press.
2. Hori, K., Nakano, Y. & Cormier, M. J. (1972) *Biochim. Biophys. Acta* **256**, 638–644.
3. Hori, K. & Cormier, M. J. (1966) *Biochim. Biophys. Acta* **130**, 420–425.
4. Shimomura, O., Johnson. F. H. & Saiga, Y. (1962) *J. Cell. Comp. Physiol.* **59**, 223–240.
5. Shimomura, O., Johnson, F. H. & Saiga, Y. (1963) *J. Cell. Comp. Physiol.* **62**, 1–8.
6. Shimomura, O., Johnson, F. H. & Saiga, Y. (1963) *J. Cell. Comp. Physiol.* **62**, 9–16.
7. Morin, J. G. & Hastings, J. W. (1971) *J. Cell Physiol.* **77**, 305–311.
8. Cormier, M. J., Hori, K., Karkhanis, Y. D., Anderson J. M., Wampler, J. E., Morin, J. G. & Hastings, J. W. (1973) *J. Cell. Physiol.*, in press.
9. Shimomura, O. & Johnson, F. H. (1969) *Biochemistry* **8**, 3991–3997.
10. Shimomura, O. & Johnson, F. H. (1972) *Biochemistry* **11**, 1602–1608.
11. Kishi, Y., Tanino, H. & Goto, T. (1972) *Tetrahedron Lett.* **27**, 2747–2749.
12. Karkhanis, Y. D. & Cormier, M. J. (1971) *Biochemistry* **10**, 317–326.
13. Cormier, M. J. & Prichard, P. M. (1968) *J. Biol. Chem.*, **243**, 4706–4714.
14. Wampler, J. E. & DeSa, R. J. (1971) *Appl. Spectros.* **25**, 623–627.
15. Cormier, M. J., Hori, K. & Karkhanis, Y. D. (1970) *Biochemistry* **9**, 1184–1189.
16. Inoue, S., Sugiura, S., Kakoi, H. & Goto, T. (1969) *Tetrahedron Lett.* **20**, 1609–1610.
17. Kishi, Y., Goto, T., Hirata, Y., Shimomura, O. & Johnson, F. H. (1966) *Tetrahedron Lett.* **29**, 3427–3436.
18. Kishi, Y., Goto, T., Inoue, S., Sugiura, S. & Kishimoto, H. (1966) *Tetrahedron Lett.* **29**, 3445–3450.
19. DeLuca, M., Dempsey, M. E., Hori, K., Wampler, J. E. & Cormier, M. J. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1658–1660.