Briggs, M., Kamp, P.-K., Robinson, N. C., and Capaldi, R. A. (1975), *Biochemistry 14,* 5123.
Capaldi, R. A. (1974), *Arch. Biochem. Biophys. 163,* 99.
Capaldi, R. A., and Vanderkooi, G. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 930.
Carroll, R. G., and Eytan, G. A. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 2036.
Crane, F. L., Glenn, J. L., and Green, D. E. (1956), *Biochim. Biophys. Acta 64,* 475.
Das Gupta, V. D., and Rieske, J. S. (1973), *Biochem. Biophys. Res. Commun. 54,* 1247.
Davies, K. A., Hatefi, Y., Poff, K. L., and Butler, W. L. (1972), *Biochem. Biophys. Res. Commun. 46,* 1984.
Fish, W. W., Reynolds, J. A., and Tanford, C. (1970), *J. Biol. Chem. 245,* 5166.
Gellerfors, P., and Nelson, B. S. (1975), *Eur. J. Biochem. 52,* 433.
Goldberger, R., Smith, A. L., Tisdale, H., and Bonstein, R. (1961), *J. Biol. Chem. 236,* 2788.
Guerrieri, F., and Nelson, B. D. (1975), *FEBS Lett. 54,* 339.
Hare, J. F., and Crane, F. L. (1974), *Sub. Cell. Biochem. 3,* 1.
Hatefi, Y., Haavik, A. G., and Griffiths, D. E. (1962), *J. Biol. Chem. 237,* 1681.
Lorenz, B., Kleinow, W., and Weiss, H. (1974), *Hoppe-Seyler's Z. Physiol. Chem. 355,* 300.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 93,* 265.
Mason, T. L., Poynton, R. O., Wharton, D. C., and Schatz, G. (1973), *J. Biol. Chem. 248,* 1346.
Ohnishi, K. (1966), *J. Biochem. (Tokyo) 59,* 1.
Rieske, J. S. (1967), *Methods Enzymol. 10,* 239.
Rieske, J. S., MacLennan, D. H., and Coleman, R. (1964), *Biochem. Biophys. Res. Commun. 15,* 338.
Ross, E., Ebner, E., Poynton, R. O., Mason, T. L., Ono, B., and Schatz, G. (1974), in The Biogenesis of Mitochondria, Kroon, A. M., and Saccone, C., Ed., London, Academic Press, p 477.
Schatz, G., and Mason, T. L. (1974), *Annu. Rev. Biochem. 43,* 53.
Silman, H. I., Rieske, J. S., Lipton, S. H., and Baum, H. (1967), *J. Biol. Chem. 242,* 4867.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1196.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39,* 462.
Trumpower, B. L., and Katki, A. (1975), *Biochemistry 14,* 3635.
Tzagoloff, A., Akai, A., and Sierra, M. (1972), *J. Biol. Chem. 247,* 6511.
Weiss, H., and Ziganke, B. (1974), *Eur. J. Biochem. 41,* 63.
Wikström, M. K. F. (1973), *Biochim. Biophys. Acta 301,* 155.
Williams, J. R. (1964), *Arch. Biochem. Biophys. 107,* 537.
Yamashita, S., and Racker, E. (1969), *J. Biol. Chem. 244,* 1220.
Yu, C. A., Yu, L., and King, T. E. (1972), *J. Biol. Chem. 247,* 1012.
Yu, C. A., Yu, L., and King, T. E. (1974), *J. Biol. Chem. 249,* 4905.

Structural Identification and Synthesis of Luciferin from the Bioluminescent Earthworm, *Diplocardia longa*†

Hiroko Ohtsuka, Noel G. Rudie, and John E. Wampler*

ABSTRACT: For the first time, luciferin from a bioluminescent earthworm has been purified, identified, and synthesized. This luciferin from the North American species, *Diplocardia longa,* is a simple aldehyde compound, *N*-isovaleryl-3-aminopropanal, with an amide functional group. It is a clear, odorless oil at room temperature. It is nonvolatile and has no near-uv–visible absorption or fluorescence. Derivatives of this compound were made to facilitate its identification: the luciferin 2,4-dinitrophenylhydrazone (mp 174 °C), a yellow crystalline solid; and the luciferin alcohol, a clear oil. Synthesis of *Diplocardia* luciferin yielded an oil of identical spectroscopic (proton nuclear magnetic resonance (NMR), ^{13}C NMR, mass, and ir), chemical (dinitrophenylhydrazone and alcohol derivatives, bioluminescence activity), and physical (thin-layer chromatography, volatility) properties to those of the purified native *Diplocardia* luciferin.

Bioluminescent earthworm species (Oligochaete) are found worldwide but few detailed studies of their luminescence system have been carried out. Harvey (1952) has reviewed the published work on earthworm bioluminescence and since that time only a few additional studies have been performed (Johnson et al., 1966; Cormier et al., 1966; Bellisario et al., 1972). In general these reports have indicated that earthworm luminescence originates from exuded coelomic fluid and that it involves a classical luciferin–luciferase reaction. More recently the work on the North American species, *Diplocardia longa,* has indicated that, at least in the case of this worm, the luminescence system requires

† From the Bioluminescence Laboratory, Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received September 19, 1975.* This work was supported by National Science Foundation Grant GB-43804. The University of Texas HRM spectrometer was purchased with funds provided by the National Science Foundation (GP-8509). The Joel PFT-100 spectrometer was purchased by the University of Georgia with matching funds from the National Science Foundation.

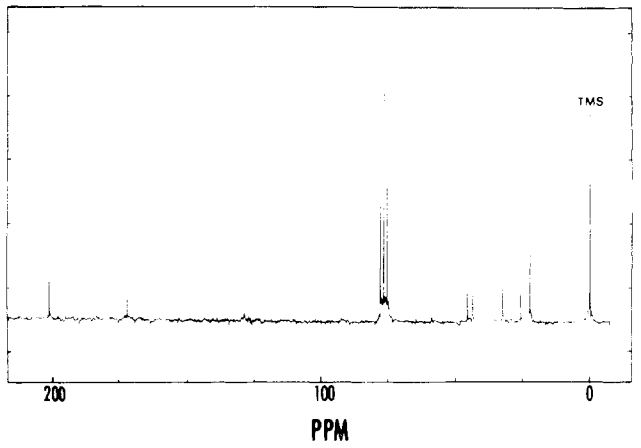


FIGURE 1: Carbon-13 nuclear magnetic resonance spectrum of *Diplocardia* luciferin. Approximately 15 mg of native luciferin was taken up in $CDCl_3$ and added to a microtube along with the internal standard (tetramethylsilane, 0 ppm). The spectrum is a scan average from several hours of scanning.

hydrogen peroxide as the primary oxidant, not molecular oxygen. The oxygen requirement of the in vivo reaction of other worms (Johnson et al., 1966; Gilchrist, 1919; Harvey, 1926) may be explained, as in the case of *Diplocardia* (Bellisario and Cormier, 1971), by the presence of a peroxide generating system in vivo.

With *Diplocardia* Bellisario et al. (1972) have shown that the in vitro light reaction requires only purified luciferase, luciferin, and hydrogen peroxide. *Diplocardia* luciferase is a large protein (300 000 mol wt.) containing copper and made up of several subunits (Bellisario and Cormier, 1971; Bellisario et al., 1972). *Diplocardia* luciferin has recently been isolated and purified in our laboratory (Rudie et al., 1976). The first step toward a more detailed understanding of the light reaction of this and other earthworms is the identification of the luciferin molecule. Identification of *Diplocardia* luciferin as *N*-isovaleryl-3-amino-1-propanal and its synthesis is reported here.

Materials and Methods

Materials. Spectrograde solvents were used throughout the synthesis and purification procedures. The organic and inorganic chemicals used were reagent grade or better. Chromatographic media were obtained from the following suppliers: DEAE-cellulose (Whatman), silica gel 90–200 mesh for chromatography (Gallard Schlessinger), silica gel 10–40 μ grain size for thin-layer chromatography (Merck), LH-20 (Pharmacia), and alumina (Merck).

Diplocardia longa were collected between May and September 1974 at the University of Georgia Range Grazing Project. Coelomic cells were collected using electrical stimulation of the worms in small batches as previously described (Rudie et al., 1976). The cells were washed and frozen until needed.

Luciferase solutions for the in vitro reaction were prepared according to the method of Bellisario et al. (1972). For this reaction, 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5), 0.1 ml of luciferase solution, and 0.02 ml of luciferin solution were mixed at room temperature; the light reaction was initiated by injection of 0.2 ml of 0.022 M H_2O_2; and the flash of luminescence was recorded using a photometer circuit of our own design. The flash peak was used as a measure of activity with intensity calibrated in quanta per second using the luminol standard (Lee et al., 1966).

Diplocardia luciferin was purified by the procedure of Rudie et al. (1976). In this procedure an organic extract of luciferin is purified by silica gel and Sephadex LH_{20} chromatography until it is judged pure by the absence of fluorescence, the lack of near-uv and visible absorption, and migration as a single spot on thin-layer chromatography in a variety of solvents. This highly purified luciferin is a clear oil.

Characterization of the Compounds. Thin-layer chromatography was done on glass slides coated with silica gel. Spots were located with uv lamps and I_2 staining. Absolute bioluminescence emission spectra were determined using an on-line spectrofluorimeter system (Wampler and DeSa, 1971). Infrared spectra were obtained on a Perkin-Elmer IR-10 spectrophotometer; low resolution mass spectral data on a Bell and Howell 21-490 at various temperatures; and high resolution mass spectra were obtained by Dr. C. Cone of the University of Texas at Austin. Proton nuclear magnetic resonance (NMR) spectra were taken with a Varian Associates HA-100 and ^{13}C NMR on a Joel PFT-100 spectrometer with the EC-100 data system. For ^1H NMR and ^{13}C NMR samples were dissolved in $CDCl_3$ containing tetramethylsilane as an internal standard. Melting points, determined using a Fisher-Johns melting point apparatus, are given uncorrected.

Reduction of Native Luciferin. Diplocardia luciferin (5 mg) in 95% ethanol (4 ml) was treated with sodium borohydride (10 mg). After stirring for 1 h, the reaction mixture was acidified with acetic acid and evaporated to dryness. The residue was suspended in methyl acetate (2 ml) and filtered through a short alumina column which was washed with methyl acetate (15 ml). The effluents were combined and evaporated to give a colorless oil (3.2 mg) which had no activity in the in vitro *Diplocardia* reaction. Ir: $\nu_{max}(CHCl_3)$ 3480, 3035, 2990, 2905, 1650, 1527, 1470, and 1375 cm^{-1}.

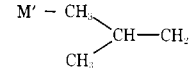
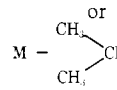
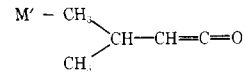
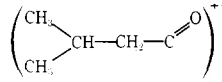
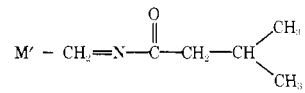
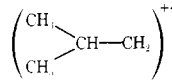
2,4-Dinitrophenylhydrazone of Native Luciferin. The dinitrophenylhydrazone of luciferin was prepared as described previously (Rudie et al. 1976). Yellow crystalline solid (mp 174 °C): ir ν_{max}(KBr) 3308, 3100, 2960, 1642, 1620, 1596, 1555, 1520, 1426, 1330, 1220, 1136, and 1077 cm^{-1}; mass *m/e* 337, 260, 236, 145, 139, 129, 111, 98, 97, 85, 83, 69, 57, and 55.

Synthesis of Luciferin. A solution of isovaleryl chloride (1.2 g) in 5 ml of benzene was added dropwise to a mixture of 3-amino-1-propanol (2.7 g) and potassium carbonate (0.7 g) in 45 ml of acetonitrile at −20 °C. The reaction mixture was stirred for 30 min at −20 °C and 30 min at room temperature, filtered, and evaporated to dryness. The remaining oily residue was dissolved in chloroform and washed with 1 N HCl, 10% potassium carbonate, and water. The chloroform solution was then dried for 3 h over sodium sulfate, filtered, and evaporated to dryness yielding *N*-isovaleryl-3-amino-1-propanol as an oil (1.51 g, 95%).

Ir spectra of this alcohol were identical with those of the reduction product of native luciferin. Mass: *m/e* 159, 142, 117, 102, 100, 89, 87, 85, 84, 57, NMR δ 0.95 (d, 6 H, J = 7 Hz), 1.69 (m, 2 H), 2.07 (m, 2 H), 3.37 (q, 2 H, J = 6 Hz), 3.63 (t, 2 H, J = 6 Hz), 4.14 (s, 1 H), 7.06 (s, 1 H).

The amino alcohol was oxidized without further purification by adding it (936 mg in 10 ml of methylene chloride) to a solution of chromium trioxide (3 g)–pyridine (5.1 ml) in methylene chloride (45 ml). The solution was stirred for 90

Table I: Mass Spectral Data for *Diplocardia* Luciferin.

At 50 °C			At 100 °C		
Peak *m/e*	Assignment	Empirical Formula	Peak *m/e*	Assignment	Empirical Formula[a]
157	M	$C_8H_{15}NO_2$	296	M′	$C_{16}H_{28}N_2O_3$
131	?		278	M′ − H_2O	$C_{16}H_{26}N_2O_2$
128	M − CHO	$C_7H_{14}NO$	254	M′ − CH_2=CH−CH_3	$C_{13}H_{22}N_2O_3$
115	M − CH_2=CH−CH_3	$C_5H_9NO_2$	239	M′ − 	$C_{12}H_{19}N_2O_3$
100	M − CH_2−CH_2CHO	$C_5H_{10}NO$			
	or		194		
	M − 	$C_4H_6NO_2$		M′ − 	$C_{11}H_{18}N_2O$
87	M − CO + CH_2=CH−CH_3	C_8H_9NO			
85		C_5H_9O	183[b]	M′ − 	$C_{10}H_{17}NO_2$
72	$(NH-CH_2CH_2CHO)^{+\cdot}$	C_3H_6NO	157	M	$C_8H_5NO_2$
57		C_4H_9			
	or				
	$(CH_2CH_2CHO)^{+\cdot}$	C_3H_5O			

[a] Empirical formulas determined from high resolution mass spectra. [b] Product of allylic cleavage with hydrogen rearrangement.

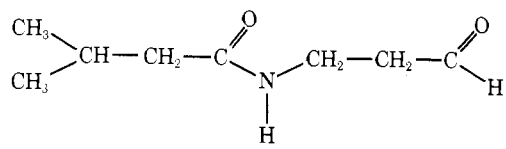
min at room temperature, then filtered through a silica gel column (6 × 14 cm). The residue was washed twice with 30 ml of methylene chloride and each wash filtered through the silica gel column. The column was washed with 30% ethanol in hexane. All effluents were combined and evaporated to dryness yielding a brown viscous oil. This oil was purified by two passes through a silica gel column (2 × 50 cm) eluted with 2.5% methanol in chloroform to give *N*-isovaleryl-3-aminopropanol as an oil (562 mg, 60.7%): ir ν_{max}($CHCl_3$) 3448, 3000, 2960, 2870, 2725, 1727, 1653, and 1520 cm^{-1}; ^1H NMR δ 0.95 (d, 6 H, J = 7 Hz), 2.0 (m, 3 H), 2.72 (dt, 2 H, J = 1 and 6 Hz), 3.54 (q, 2 H, J = 6 Hz), 6.68 (s, 1 H), 9.75 (t, 1 H, J = 1 Hz); mass *m/e* 157, 128, 115, 100, 87, 85, 72, 57.

Synthetic luciferin (53 mg) when treated with 2,4-dinitrophenylhydrazine (80 mg) in ethanol following the same procedure as that used for native luciferin gave a hydrazone (62 mg), mp 172∼174 °C, with an identical ir spectrum. The mixture melting point test (172∼174 °C) showed no depression.

Results

Diplocardia luciferin is fairly stable at room temperature in many solvents and is nonvolatile up to 40 °C under vacuum. However, it is subject to slow air oxidation and concentrated solutions or the neat oil will turn slightly yellow if precautions are not taken to prevent oxidation. The pure compound is a colorless oil with no fluorescence or uv-visible absorption. It is soluble in polar solvents (methanol, ethanol, acetone, and methyl acetate) but insoluble in nonpolar solvents like hexane and carbon tetrachloride.

The assignment of luciferin as *N*-isovaleryl-3-aminopropanal (**1**) is based on a variety of consistent spectroscopic



1

analysis. To the authors' knowledge this compound has not been previously reported. Its ir spectrum reveals the aldehyde (2725 and 1727 cm^{-1}) and amide groups (3448, 1653, and 1520 cm^{-1}) and the presence of both of these functional groups is supported by ^{13}C NMR. The ^{13}C NMR spectrum (Figure 1) shows seven different carbon signals. Those at 301.4 (doublet) and 172.5 ppm (singlet) are easily assigned to the aldehyde and amide carbons, respectively, by virtue of their chemical shifts. The other five signals are assigned to saturated carbon atoms: one or two methyl groups (22.4 ppm, q), three methylene (32.8, t; 44.0, t; 46.0, t, ppm), and one methyne carbon (26.1 ppm, d). This assignment is supported by proton NMR and off resonance decoupling techniques. The proton NMR spectrum also reveals the aldehyde and amide functional groups (δ 9.75 and 6.68, respectively). Deuterium oxide causes an ^1H NMR quartet at 3.54 ppm (methylene) to change to a triplet while the amide peak (6.68 ppm) disappears, and irradiation of the aldehyde peak (9.75 ppm) and the 3.54-ppm peak each cause changes in the 2.72 ppm double-triplet (another methylene; change to clear triplet, J = 6 Hz, and doublet, J = 1 Hz, respectively). All of these data support an aminopropanal amide as part of the luciferin structure.

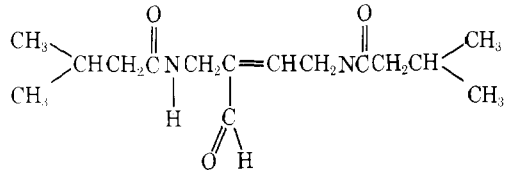
A multiplet at 2.0 ppm representing three protons on integration is assigned to overlapping bands of a methyne and a methylene carbon. Irradiation of this peak causes the 0.95-ppm peak (six protons on integration, two methyl groups) to change from a doublet to a singlet. Since the relative intensity of the 22.4 ppm peak from ^{13}C NMR is consistent with two identical methyl groups and since only seven different carbons are seen, the isovaleryl moiety is also supported by the spectroscopic data.

Structure **1** is also supported by the mass spectral data. Low resolution spectra at 50 °C show fragmentation leading to major loss from structure **1** of the aldehyde group (M − CHO, *m/e* 128), the isovaleryl chain (M − $(CH_3)_2CH$-CH_2, *m/e* 100), and the aminopropanal moiety (M − $NHCH_2CH_2CHO$, *m/e* 85). Table I shows the assignments of the mass spectrum as fragments of structure **1**.

Table II: Chemical and Physical Properties of Synthetic and Native *Diplocardia* Luciferin.

Synthetic	Native
Description: Nonvolatile colorless oil	Nonvolatile colorless oil
^{13}C NMR: 301.4, 172.5, 46.0, 44.0, 32.8, 22.4, 26.1	301.4, 172.5, 46.0, 44.0, 32.8, 22.4, 26.1
Mass spectrum, apparent molecular ion (*m/e*)	
50 °C: 157	157
100 °C: 296	296
Alcohol derivative	
Mass (*m/e*): 159	159
Dinitrophenylhydrazone	
Description: Yellow crystalline solid	Yellow, crystalline solid
Mass (*m/e*): 337	337
Bioluminescence activity (light units per mg): 10.7 ± 1.2	10.4 ± 0.4
Emission spectrum, *in vitro* reaction	
Peak, nm 490	490
Half-width, nm: 76	74

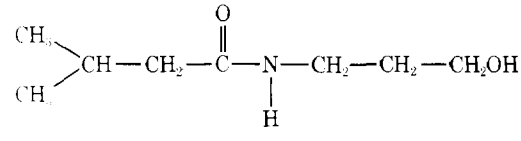
As the temperature is increased, a new molecular ion peak appears at *m/e* 296 with major fragments at 278, 254, 239, 194, 183, 157, 115, and a base peak at *m/e* 57. High resolution mass spectra give the empirical formulas for the major fragments listed in Table I. These spectra are consistent with the formation at higher temperatures of a luciferin dimer **2**, by aldol condensation of **1** followed by dehydration.



2

Fragmentation of this dimer **2** can result in the peaks above *m/e* 157 shown in Table I.

The luciferin alcohol (**3**) gave a molecular ion in mass spectra at *m/e* 159. In contrast to the luciferin data, this spectrum was not temperature variant. Reaction of luciferin with 2,4-dinitrophenylhydrazine gave the molecular ion *m/e* 337 expected for the luciferin phenylhydrazone.



3

Confirmation of the structure of luciferin as *N*-isovaleryl-3-aminopropanal (**1**) was obtained by synthesis of the active compound following the procedure outlined under Materials and Methods. When isovaleryl chloride was treated with 3-amino-1-propanol, the luciferin alcohol (*N*-isovaleryl-3-amino-1-propanol, **3**) was produced quantitatively. Its identity was varified by ir and mass spectra. The subsequent oxidation of luciferin alcohol with chromium trioxide–pyridine complex gave luciferin (**1**) in 60% yield. The spectroscopic data (ir, NMR, and mass spectra) of this product were identical with those of native luciferin. The mass spectrum showed the same temperature dependence, the in vitro bioluminescence emission spectra were identical (Table II), and the specific activity of the synthesized compound (10.7 light units/mg) in a standard assay system was the same as that of native luciferin (10.4 light units/mg).

Discussion

From the data presented, *Diplocardia* luciferin is *N*-isovaleryl-3-aminopropanal (**1**), a clear, nonvolatile, odorless oil. Synthetic luciferin is identical with the purified luciferin by all of the analytical criteria and in its bioluminescence activity (Table II). Since synthetic luciferin gives the same mass spectrum temperature dependence as the native compound, and since both native and synthetic luciferin give the expected phenylhydrazone and corresponding alcohol derivatives, the 296 peak in the mass spectra at high temperatures is considered to be an artifact created on heating the sample in the spectrometer.

Earthworm bioluminescence has been a difficult process to study for several reasons. It is "catalyzed" by a large protein which, in vitro at least, does not turn over, but is instead inactivated in a rapid side reaction with the oxidant, hydrogen peroxide (Bellisario et al., 1972). The emission spectrum is on the red side of known bioluminescence spectra, suggesting an emitter which absorbs in the visible region. As yet we have no evidence for visible absorption bands or fluorescence chromophores associated with luciferase; certainly neither luciferin (**1**) nor any of its common analogues should absorb or emit that far out in the green. However, with the structure of luciferin now known, we are now investigating both the chemical mechanism and the nature of the emitter. Synthesis of a variety of analogues of luciferin is currently in progress in our laboratory to aid in these studies.

Acknowledgment

The authors thank Dr. M. J. Cormier, Dr. R. E. Lovins, and Dr. Kazuo Hori of this department for valuable aid and discussions. Our thanks also to Mr. Roy Holland of the University Range Grazing Project for help in establishing the earthworm collection program. Our thanks also to Dr. C. Cone of the University of Texas at Austin for the high resolution mass spectrum of luciferin and Dr. Richard Cox, Department of Chemistry, University of Georgia, for ^{13}C NMR and proton NMR spectra.

References

Bellisario, R., and Cormier, M. J. (1971), *Biochem. Biophys. Res. Commun. 43*, 800–805.

Bellisario, R., Spencer, T. E., and Cormier, M. J. (1972), *Biochemistry 11*, 2256–2266.

Cormier, M. J., Kreiss, P., and Prichard, P. M. (1966), in Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N.J., Princeton University Press, pp 363–384.

Gilchrist, J. D. (1919), *Trans. R. Soc. S. Afr. 7*, 203–212.

Harvey, E. N. (1926), *Biol. Bull. (Woods Hole, Mass.) 51*, 89–97.

Harvey, E. N. (1952), Bioluminescence, New York, N.Y., Academic Press, pp 233–241.

Johnson, F. H., Shimomura, O., and Haneda, Y. (1966), in Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N.J., Princeton University Press, pp 385–389.

Lee, J., Wesley, A. S., Ferguson, J. F., and Seliger, H. H. (1966), in Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N.J., Princeton University Press, pp 35–43.

Rudie, N. G., Ohtsuka, H., and Wampler, J. E. (1976), *Photochem. Photobiol.* (in press).

Wampler, J. E., and DeSa, R. J. (1971), *Appl. Spectrosc. 25*, 623–627.