is therefore possible the biological effects of not only vanadium(IV) but also vanadium(V) will depend on the compartmentalization of the vanadium and the particular proteins vanadium affects under these conditions.

Conclusions

We have developed an acid or alkaline phosphatase assay to determine vanadium concentrations in the 10^{-5}-10^{-7} M range. The method is cheap, simple, and fast and has been applied to the interactions of vanadium(IV) and (V) with organic molecules. In general, we find that vanadium interactions are very important not only for in vitro studies of vanadyl cation but also for in vitro studies with vanadate. Many of the molecules present in biological studies such as TEA interact strongly with both vanadyl cation and vanadate, whereas others such as Tris interact only weakly at these low vanadium concentrations. The vanadyl cation was usually found to interact more strongly with a ligand than vanadate. However, one ligand, Tricine, was found to form vanadate complexes 8 times *more* stable than the vanadyl complex.

Carbohydrates or polyalcohols are enzyme substrates or cofactors not likely to interact with vanadate at low vanadate concentrations unless, for example, the carbohydrate and vanadate are enclosed in the active site of an enzyme or a hydrophobic pocket generated by a membrane and/or a protein. Other enzyme substrates such as α-hydroxy carboxylic acids, including citrate, malate, and glycerate, complex vanadate even at very low vanadate concentrations. Most amino acids with the possible exception of proline are observed to interact weakly with vanadate and vanadyl cation even at 10^{-5} M vanadate concentrations. The high affinity of the amine group in lysine for interaction with vanadate has not previously been recognized. Random interaction with proteins such as bovine serum albumin was observed at 10^{-5} M vanadate concentrations. Enzymes including ribonuclease, myosin, acetylcholinesterase, acid phosphatase, aldolase, pepsin, and chymotrypsin were all found to interact with vanadate with greater affinity than bovine serum albumin.

The results presented in this paper suggest that both vanadyl and vanadate at 10^{-5} M concentrations can interact with many organic molecules including substrates, cofactors, amino acids, peptides, and proteins in in vitro biological studies. Such spontaneous interaction may be important for the mechanisms by which vanadate acts in vivo and must be considered in studies with vanadium.

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Structure of Dinoflagellate Luciferin and Its Enzymatic and Nonenzymatic Air-Oxidation Products

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Contribution from the Departments of Chemistry and of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543. *Received February 14, 1989*

Abstract: Dinoflagellate luciferin (**1**) was isolated from cultured *Pyrocystis lunula*, and its structure was elucidated primarily by comparing the spectroscopic data of **1** with those of krill fluorescent substance F (**5**). From the crude extract of luciferin, air-oxidation product **3** with a characteristic blue color was isolated. Air oxidation of dinoflagellate luciferin in methanol in the absence of luciferase proceeded without emission of light to yield **2**, whereas air oxidation in water in the presence of luciferase proceeded with the emission of light at 474 nm to yield **4**.

Dinoflagellates have recently drawn much attention because they produce a variety of bioactive substances including neurotoxins and antineoplastic compounds.[1] They often reach abnormally high concentrations in the ocean, forming the so-called red tides. Some of them are toxic, often causing serious health and economic problems such as shellfish and fish poisoning.[2] Many species of dinoflagellates are bioluminescent; they are ubiquitous in the oceans of the world and are responsible for much of the sparkling luminescence at night elicited by disturbing surface waters, recognized as the "phosphorescence" of the sea.[3] Many factors are involved in the regulation of dinoflagellate bioluminescence, including the endogenous circadian clock.[4]

The chemical process of light emission involves air oxidation of dinoflagellate luciferin catalyzed by dinoflagellate luciferase.[5] Luciferin and luciferase from several different dinoflagellate species were proven to cross-react. Interestingly, the dinoflagellate bioluminescent system was shown also to cross-react with the krill bioluminescent system,[6] composed of a photoprotein and krill fluorescent substance F.[7] Krill fluorescent substance F appears to function both as a catalyst for air oxidation of the photoprotein and as the light emitter but not as a substrate for enzymatic air

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(1) For recent reviews, see; Faulkner, D. J. *Nat. Prod. Rep.* **1984**, *1*, 251, 551; **1986**, *3*, 1; **1987**, *4*, 539.
(2) For example, see: *Toxic Dinoflagellates*; Anderson, D. M.; White, A. W.; Baden, D. G., Eds.; Elsevier: New York, 1985.
(3) Harvey, E. N. *Bioluminescence*; Academic: New York, 1952.
(4) (a) Johnson, C. H.; Hastings, J. W. *Am. Sci.* **1986**, *74*, 29. (b) Roenneberg, T.; Nakamura, H.; Hastings, J. W. *Nature* **1988**, *334*, 432.
(5) (a) Hastings, J. W.; Sweeney, B. M. *J. Cell. Comp. Physiol.* **1957**, *49*, 209. (b) Hastings, J. W.; Bode, V. C. In *Light and Life*; McElroy, W. D.; Glass, B., Eds.; Johns Hopkins: Baltimore, 1961; p 294. (c) Fogel, M.; Hastings, J. W. *Arch. Biochem. Biophys.* **1971**, *142*, 310.
(6) Dunlap, J. C.; Hastings, J. W.; Shimomura, O. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1394.
(7) (a) Shimomura, O.; Johnson, F. H. *Biochemistry* **1967**, *6*, 2293. (b) Shimomura, O.; Johnson, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *59*, 475. (c) Shimomura, O. *FEBS Lett.* **1980**, *116*, 203.

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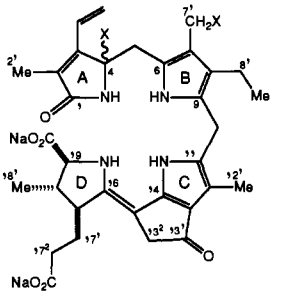
oxidation. Nonetheless, dinoflagellate luciferin and krill fluorescent substance F share common chemical and spectroscopic properties.[6,7] They are both unstable in the presence of oxygen, which made studies on these bioluminescent systems difficult. On the basis of spectroscopic and degradation experiments, we have recently succeeded in elucidating the structure of krill fluorescent substance F (**5**), isolated from *Euphausia pacifica*, and its air-oxidation product oxy-F (**6**).[8] In this paper, we would like to report the structure of dinoflagellate luciferin and its enzymatic and nonenzymatic air-oxidation products.

Luciferin was isolated from *Pyrocystis lunula*, which is known to contain luciferin in larger amounts than other dinoflagellate species.[9] Unialgal but not axenic cultures of *P. lunula* were grown at 20 °C for 40 days under a 12:12 light/dark cycle. Cells were harvested during the light phase, and luciferin was extracted in boiling phosphate buffer (pH 8.5) containing 2-mercaptoethanol. Purification was performed on a DEAE-cellulose column, followed by an alumina column at 5 °C under argon. As in the case of krill fluorescent substance F, HPLC on TSK DEAE-5PW was very effective for purification of dinoflagellate luciferin. However, the process of desalting and concentration of HPLC eluents caused substantial decomposition. On the basis of ^{1}H NMR spectroscopy, the purity of dinoflagellate luciferin thus isolated was estimated to be around 60–70%. Nonetheless, it exhibited chemical and spectroscopic properties, including UV spectrum (λ_{max} 388 nm), chromatographic behavior, and bioluminescence capacity, consistent with those reported by Hastings and co-workers.[5,9]

Mild chromic acid oxidation of dinoflagellate luciferin (CrO_3/$KHSO_4$/H_2O/RT) yielded 3-methyl-4-vinylmaleimide (**7**), 3-methyl-4-ethylmaleimide (**8**), and aldehyde **9**, whereas vigorous chromic acid oxidation (CrO_3/2 N H_2SO_4/90 °C) gave hematinic acid (**10**).[10] These results suggested structural similarity between dinoflagellate luciferin and krill fluorescent substance F except for a methyl group on ring B in dinoflagellate luciferin instead of a hydroxymethyl group found in krill fluorescent substance F.[8]

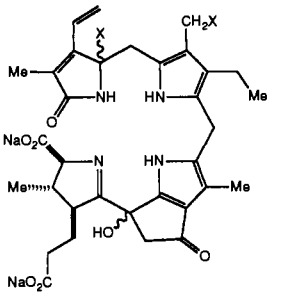
Like krill fluorescent substance F, dinoflagellate luciferin was slowly oxidized in methanol-d_6 at −20 °C in the presence of a small amount of air, to yield air-oxidation product **2**. On the other hand, during the purification process of luciferin, the formation of a product with a characteristic deep blue color was observed.[11] This substance was separated from dinoflagellate luciferin at the stage of HPLC on TSK DEAE-5PW and further purified by HPLC on Hamilton PRP-1 to yield air-oxidation product **3**. Compared with dinoflagellate luciferin itself, both air-oxidation products **2** and **3** were relatively stable and could be isolated in pure form. Hence, careful spectroscopic studies were conducted first on **2** and **3**.

Air-oxidation product **2** exhibited a UV absorption at 237 and 300 (shoulder) nm, almost identical with that of oxy-F, suggesting that the air oxidation of dinoflagellate luciferin (λ_{max} 388 nm) and krill fluorescent substance F (λ_{max} 388 nm) involved the same chemical change on their UV chromophore.[8] The high-resolution FAB mass spectrum established the molecular formula of **2** to be $C_{33}H_{38}O_7N_4Na_2$ [m/z 649.2620, $(M + H)^+$, Δ 0.6 mmu, and m/z 671.2484, $(M + Na)^+$, Δ 5.1 mmu]. The NMR spectra of **2** characterized the nature of all hydrogen and carbon atoms (Table I). On comparison with those of oxy-F, the NMR data confirmed the presence of a methyl group [δ_H 1.76 (s), δ_C 9.9 (q)]



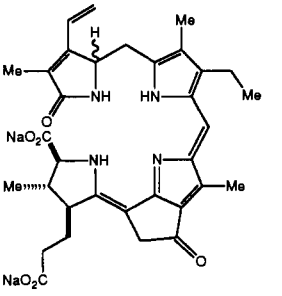
1 : X=H

5 : X=OH

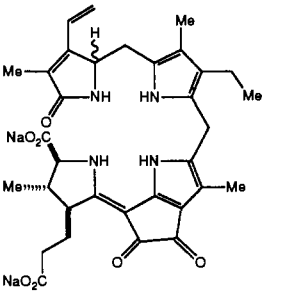


2 : X=H

6 : X=OH



3



4

on ring B as suggested from the degradation experiment and also revealed the presence of a methine group [δ_H 4.54 (m), δ_C 58.9 (d)] on ring A instead of a tertiary carbinol carbon found in krill oxy-F. The proton resonance of this methine group showed spin couplings with the C.5 methylene protons [δ_H 2.71 (dd, J = 15, 6 Hz) and 3.02 (dd, J = 15, 5 Hz), δ_C 31.5 (t)] and the C.2^1 methyl protons [δ_H 1.76 (br s), δ_C 8.6 (q)]. The presence of a hydrogen atom at the C.4 position was further supported by the following experimental observations: unlike the case of krill fluorescent substance F, 3-methyl-4-vinylmaleimide (**7**) or the alkaline degradation product **11b**, corresponding to the alkaline degradation product **11a** in the krill series, was not formed from luciferin under basic or acidic conditions, and one ^{18}O atom was incorporated into **7** in chromic acid oxidation using $H_2{}^{18}O$ and $^{18}O_2$.[10,12] On comparison with two compounds (**12** and **13**) known in the literature, the ^{13}C chemical shift [δ_C 8.6 (q)] of the C.2^1 methyl group established the substitution pattern as shown.[13] The connectivity between ring A and ring B was based on the detection of a long-range spin coupling between the C.5 methylene protons [δ_H 3.02 (dd)] and the C.7^1 methyl protons [δ_H 1.76 (s)].[14] The connectivity between ring B and ring C was also supported by the observation that the C.12^1 methyl protons [δ_H 2.13 (br s)] was spin coupled with the C.10 methylene protons [δ_H 3.78 (d, J = 16 Hz) and 3.81 (d, J = 16 Hz)].[14] The carbon and proton resonances observed for the remaining C.10 to C.20 portion of **2** were almost superimposable on those of krill oxy-F ($\Delta\delta_H$ <0.07 ppm and $\Delta\delta_C$ <0.3 ppm). On the basis of the evidence presented, the gross structure of the air-oxidation product was formulated as **2**.[15]

Blue air-oxidation product **3** showed UV–visible absorption maxima at 633 and 590 (shoulder) nm, suggesting the presence of a chromophore more conjugated than the chromophore found in luciferin (**1**) and air-oxidation product **2**. The high-resolution

(8) Nakamura, H.; Musicki, B.; Kishi, Y.; Shimomura, O. *J. Am. Chem. Soc.* **1988**, *110*, 2683.

(9) Dunlap, J. C.; Hastings, J. W. *Biochemistry* **1981**, *20*, 983.

(10) Early structural studies of dinoflagellate luciferin were primarily based on degradation reactions and functional group tests. See: Dunlap, J. C.; Hastings, J. W.; Shimomura, O. *FEBS Lett.* **1981**, *135*, 273.

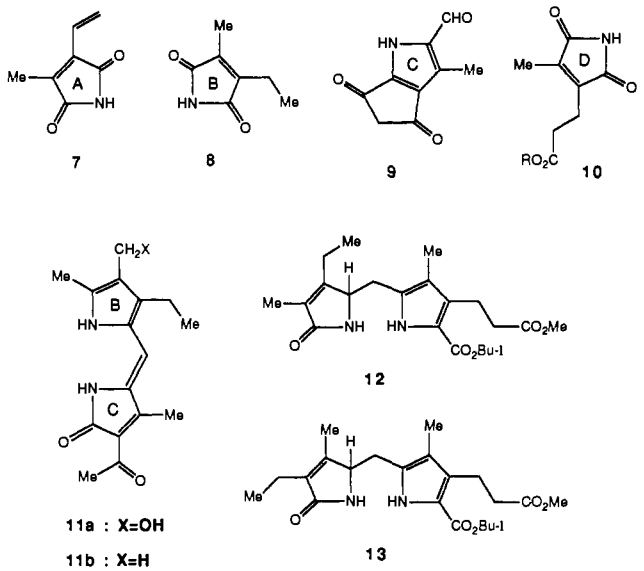
(11) Although formation of the blue compound was always observed in the purification of luciferin (**1**), a method for selectively converting **1** into this blue compound is not yet established. When luciferin was stored in the presence of 2-mercaptoethanol, no formation of the blue compound was observed. I_2 oxidation[10] of luciferin in aqueous acetone yielded a mixture of inseparable blue compounds, which contained two structurally closely related major products (^{1}H NMR). Upon treatment with 2-mercaptoethanol, the blue compound (**3**) gave a colorless product(s), which was not identical with luciferin (**1**) since it did not show bioluminescent activity, or to air-oxidation product **2**.

(12) In the case of fluorescent substance F and oxy-F, no ^{18}O was incorporated into **7** under the same conditions.[7]

(13) The ^{13}C resonance of the C.2^1 methyl group in **12** was observed at 8.42 ppm while that of the C.3^1 methyl group in **13** was at 12.14 ppm: Wray, V.; Gossauer, A.; Gruning, B.; Reifenstahl, G.; Zilch, H. *J. Chem. Soc., Perkin Trans. 2* **1979**, 1558. In our literature search, there was no exception for this trend.

(14) These long-range couplings were confirmed by 2D-COSY experiments.

(15) In order to assign the stereochemistry of bridging olefinic bond(s), we made substantial efforts on NOE measurements without conclusive results.



FAB mass spectrum of **3** established the molecular formula to be $C_{33}H_{36}O_6N_4Na_2$ for **3** [m/z 609.2672, $(M - Na + 2H)^+$, Δ 1.7 mmu, and m/z 631.2524, $(M + H)^+$, Δ 1.5 mmu]. Compared with that of **2**, the ^1H NMR spectrum of **3** demonstrated the presence of one additional olefinic proton [δ_H 6.88 (s)] but one less isolated methylene protons. Since the C.5 methylene protons [δ_H 3.33 (dd, J = 14, 4 Hz) and 3.56 (dd, J = 14, 6 Hz)] as well as the C.13[2] methylene protons [δ_H 3.37 (d, J = 20 Hz) and 3.56 (d, J = 20 Hz)] remained intact, the additional olefinic bond must be incorporated at the C.10 position, giving gross structure **3**,[15] a dehydrated form of **2**, for the blue oxidation product.

Dinoflagellate luciferin showed a UV absorption identical with that of krill fluorescent substance F (λ_{max} 388 nm). At the early stage of study, the analysis of ^1H and ^{13}C NMR spectra of dinoflagellate luciferin was hampered by the unavailability of a pure sample. However, we overcame this difficulty by adding a small amount of 2-mercaptoethanol to the HPLC eluents before the steps of desalting and concentration. With this procedure, 131 g wet weight of cells from 950 L of culture medium gave 3–4 mg of dinoflagellate luciferin with at least 90% purity, which allowed us to make unambiguous assignment for all the proton and carbon resonances of **1** (Table I). Thus, it became possible to compare the ^1H and ^{13}C NMR spectra of dinoflagellate luciferin (**1**) to those of krill fluorescent substance F (**5**).[8,16] In connection with the ^1H NMR spectrum, it is interesting to note that deuterium exchange of two NH protons, resonating at δ_H 10.7 (s) and 11.9 (s), respectively, was very sluggish.[16,17] The FAB mass spectrum of dinoflagellate luciferin in the presence of 2-mercaptoethanol showed clear pseudomolecular ions at m/z 633 [$(M + H)^+$] and 655 [$(M + Na)^+$], accompanied with the corresponding peaks of air-oxidation product **2**.[18] On the basis of these spectroscopic data of dinoflagellate luciferin and krill fluorescent substance F, coupled with the chemical reactivities observed for them, the structure of dinoflagellate luciferin was formulated as **1**. The stereochemistry of the C.15–C.16 olefinic bond,[15] the C.4 stereochemistry relative to the stereocenters of ring D, and the absolute configuration of chiral centers on ring D have not been experimentally established. However, considering its probable biogenesis, the same absolute configuration found in chlorophylls was tentatively assigned for the ring D portion. Dinoflagellate luciferin (**1**), as well as krill fluorescent substance F (**5**), represents a new type of plant bile pigment, apparently derived from chlorophylls and/or biogenetically related substances.[19]

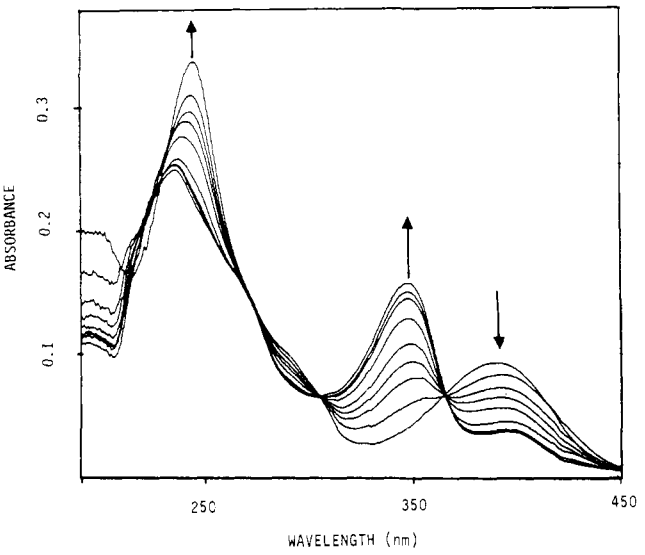


Figure 1. Transient absorption spectra of the in vitro bioluminescence of dinoflagellate luciferin in the presence of luciferase in 0.2 M phosphate buffer, pH 6.3, containing 0.1 mM EDTA and bovin serum albumin (0.1 mg/mL). Luciferin was oxidized by three successive additions of luciferase (approximately 10-min intervals), and the absorption spectrum was recorded before the first addition of luciferase and several times after each addition (see Experimental Section).

Dinoflagellate luciferin was the substrate in the bioluminescent reaction catalyzed by luciferase isolated from the dinoflagellate *Gonyaulax polyedra*.[20–22] At a relatively high concentration of luciferin,[23] the bioluminescent reaction could be monitored by UV spectroscopy. Light emission, triggered by three successive additions of luciferase, was accompanied by decreases in absorption at 388 nm and concomitant increases at 350 nm (Figure 1). After completion of the reaction, the resulting mixture was subjected to purification by HPLC on a TSK DEAE-5PW column, to yield a single product (4) in approximately 50% yield.

This product exhibited a UV absorption maximum at 350 nm and was not fluorescent at room temperature. The high-resolution FAB mass spectrum established the molecular formula of **4** to be $C_{33}H_{36}O_7N_4Na_2$ [m/z 647.2463, $(M + H)^+$, Δ 0.6 mmu, and m/z 669.2252, $(M + Na)^+$, Δ 2.5 mmu], suggesting an incorporation of one oxygen atom and a loss of two hydrogen atoms during the enzymatic air oxidation of luciferin (**1**). The ^1H NMR spectrum of **4** revealed that the oxidation took place on one of the isolated methylene groups; the resonance with a large spin-coupling constant (>18 Hz), detected in the ^1H NMR spectrum of **1** and assignable to C.13[2] protons, was not observed in the ^1H NMR spectrum of **4**. The ^{13}C NMR spectrum showed 31 different carbon signals (2 carbon signals were not identified probably because of signal overlapping), 16 of which were found to bear

(16) By use of this improved method of purification, krill fluorescent substance F (**5**) was isolated in better than 95% purity. ^1H NMR spectrum of **5** showed NH resonances at δ_H 11.1 (s) and 11.9 (s), and the ^{13}C NMR spectrum showed 33 carbon signals at 174.2 (s, C.1), 130.7 (s, C.2), 8.4 (q, C.2[1]), 152.1 (s, C.3 or C.14), 128.1 (d, C.3[1]), 122.7 (t, C.3[2]), 90.0 (s, C.4), 35.6 (t, C.5), 125.7 (s, C.6, C.7, C.8, or C.9), 119.3 (s, C.7, C.6, C.8, or C.9), 55.6 (t, C.7[1]), 120.4 (s, C.8, C.6, C.7, or C.9), 18.5 (t, C.8[1]), 17.4 (q, C.8[2]), 123.5 (s, C.9, C.6, C.7, or C.8), 22.2 (t, C.10), 137.3 (s, C.11), 123.4 (s, C.12), 9.7 (q, C.12[1]), 108.5 (s, C.13), 193.4 (s, C.13[1]), 45.3 (t, C.13[2]), 149.7 (s, C.14 or C.3), 88.1 (s, C.15), 160.8 (s, C.16), 52.1 (d, C.17), 29.9 (t, C.17[1]), 34.9 (t, C.17[2]), 182.0 (s, C.17[3]), 42.2 (d, C.18), 23.5 (q, C.18[1]), 69.6 (d, C.19), and 182.5 (s, C.20) ppm.

(17) Complete deuterium exchange required 10 *h* at 0 °C.

(18) The relative peak intensities of 633 over 649 and of 655 over 671 varied in each experiment.

(19) For a recent review, see: Leeper, F. J. *Natl. Prod. Rep.* **1987**, *4*, 441.

(20) Dunlap, J. C.; Hastings, J. W. *J. Biol. Chem.* **1981**, *256*, 10509.

(21) A modification was made for purification of luciferase (see Experimental Section), since pseudoaffinity chromatography using Affi-Gel Blue (Bio-Rad) was not as effective as previously described.[20]

(22) An isolation of a peptidic luciferin and its precursor with an absorption at 630 nm from dinoflagellate *Pyrocystis lunula* was reported: see (a) Fresneau, C.; Hill, M.; Lescure, N.; Arrio, B.; Dupaix, A.; Volfin, P. *Arch. Biochem. Biophys.* **1986**, *251*, 495. (b) Fresneau, C.; Blanot, D.; Hill, M.; Dupaix, A.; Arrio, B. *Int. J. Pept. Protein Res.* **1988**, *31*, 126. (c) Fresneau, C.; Arrio, B. *Arch. Biochem. Biophys.* **1988**, *265*, 22. However, the total amount of isolated, highly purified luciferin (**1**) can account for almost all of the capacity of light emission observed from our crude extracts.

(23) The simple first-order kinetic observed at a low concentration of luciferin did not hold when the optical density of the solution exceeded 0.1.

Table I. ^1H (500 MHz) and ^{13}C (125 MHz) NMR Data for Dinoflagellate Luciferin (**1**), Nonenzymatic Air-Oxidation Product **2**, Blue Air-Oxidation Product **3**, and Enzymatic Air-Oxidation Product **4** in CD_3OD at −5 °C[a]

	1		2		3	4	
	δ_H	δ_C[b,c]	δ_H	δ_C[b,c]	δ_H	δ_H	δ_C[b,c]
1		176.0 (s)		176.1 (s)			175.9 (s)
2		130.8 (s)		130.9 (s)			130.8 (s)
2^1	1.75 (br s)	8.6 (q)	1.76 (br s)	8.6 (q)	1.74 (br s)	1.76 (br s)	8.6 (q)
3		152.8 (s)[e]		152.8 (s)[e]			152.8 (s)
3^1	6.62 (dd, J = 18, 11 Hz)	128.9 (d)	6.70 (dd, J = 18, 11 Hz)	129.1 (d)	6.80 (dd, J = 18, 11 Hz)	6.69 (dd, J = 18, 11 Hz)	128.9 (d)
3^2	5.41 (d, J = 11 Hz)	121.0 (t)	5.45 (d, J = 11 Hz)	120.8 (t)	5.55 (d, J = 11 Hz)	5.51 (d, J = 11 Hz)	121.1 (t)
	5.49 (d, J = 18 Hz)		5.53 (d, J = 18 Hz)		5.68 (d, J = 18 Hz)	5.61 (d, J = 18 Hz)	
4	4.54 (m)	59.1 (d)	4.54 (m)	58.9 (d)[d]	4.76 (m)	4.48 (m)	59.0 (d)
5	2.76 (dd, J = 14, 6 Hz)	31.5 (t)	2.71 (dd, J = 15, 6 Hz)	31.5 (t)	3.33 (dd, J = 14, 4 Hz)	2.55 (dd, J = 15, 8 Hz)	32.0 (t)
	3.03 (dd, J = 14, 5 Hz)		3.02 (dd, J = 15, 5 Hz)		3.56 (dd, J = 14, 6 Hz)	3.06 (dd, J = 15, 4 Hz)	
6		121.9 (s)[f]		122.0 (s)[f]			123.6 (s)[f]
7		114.7 (s)		115.0 (s)			115.8 (s)
7^1	1.78 (s)	9.7 (q)	1.76 (s)	9.9 (q)[d]	1.85 (s)	1.87 (s)	9.6 (q)
8		120.1 (s)[f]		120.3 (s)[f]			118.9 (s)[f]
8^1	2.39 (m)	18.7 (t)	2.38 (m)	18.8 (t)	2.66 (q, J = 7.5 Hz)	2.39 (m)	18.7 (t)
8^2	0.92 (t, J = 7 Hz)	16.6 (q)	0.96 (t, J = 7 Hz)	16.7 (q)	1.10 (t, J = 7.5 Hz)	0.98 (t, J = 7 Hz)	16.5 (q)
9		124.9 (s)[f]		124.2 (s)[f]			123.9 (s)[f]
10	3.65 (d, J = 16 Hz)	22.9 (t)	3.78 (d, J = 16 Hz)	22.5 (t)[d]	6.88 (s)	4.09 (d, J = 16.5 Hz)	33.1 (t)[d]
	3.73 (d, J = 16 Hz)		3.81 (d, J = 16 Hz)			4.16 (d, J = 16.5 Hz)	
11		137.5 (s)		139.4 (s)			152.0 (s)
12		123.5 (s)[f]		128.1 (s)			*f, h*
12^1	2.12 (s)	9.4 (q)	2.13 (s)	9.5 (q)	2.48 (s)	2.45 (s)	30.5 (q)
13		108.5 (s)		109.4 (s)			*g, h*
13^1		194.4 (s)		197.8 (s)			195.9 (s)
13^2	3.11 (d, J = 18 Hz)	45.4 (t)	2.83 (d, J = 18 Hz)	57.4 (t)	3.37 (d, J = 20 Hz)		200.5 (s)
	3.29 (d, J = 18 Hz)		3.81 (d, J = 18 Hz)		3.56 (d, J = 20 Hz)		
14		149.7 (s)[e]		156.3 (s)[e]			162.0 (s)
15		88.2 (s)		74.2 (s)			113.3 (s)[g]
16		160.7 (s)		181.0 (s)[g]			174.8 (s)
17	2.57 (br d, J = 13 Hz)	50.0 (d)	2.74 (dd, J = 13, 2 Hz)	58.1 (d)	3.25 (br d, J = 13 Hz)	2.94 (dd, J = 11, 3.5 Hz)	50.0 (d)
17^1	1.44 (br t, J = 13 Hz)	30.2 (t)	1.44 (br t, J = 13 Hz)	30.8 (t)	1.69 (br t, J = 13 Hz)	1.75 (m, large J with 17 H)	29.9 (t)
	2.38		2.76 (br t, J = 13 Hz)		2.03 (br t, J = 13 Hz)	2.07 (m)	
17^2	2.03	34.6 (t)	2.29 (ddd, J = 17, 13, 2 Hz)	35.4 (t)	2.43 (br dd, J = 16, 13 Hz)	2.26 (m)	36.4 (t)
	2.38		2.43 (br dt, J = 17, 2 Hz)		2.54 (dt, J = 16, 3 Hz)	2.29 (m)	
17^3		181.6 s)[g]		181.6 (s)[g]			181.0 (s)
18	2.47 (br q, J = 7 Hz)	42.3 (d)	2.20 (m)	44.4 (d)	2.60 (q, J = 7 Hz)	2.74 (q, J = 7 Hz)	41.1 (d)
18^1	1.16 (d, J = 7 Hz)	23.4 (q)	1.09 (d, J = 7 Hz)	23.5 (q)	1.24 (d, J = 7 Hz)	1.07 (d, J = 7 Hz)	22.2 (q)
19	3.58 (d, J = 1 Hz)	69.6 (d)	4.10 (d, J = 4 Hz)	83.7 (d)	3.87 (d, J = 1 Hz)	4.52 (d, J = 1 Hz)	75.0 (d)
20		182.4 (s)[g]		181.3 (s)[g]			177.1 (s)
NH	10.7 (s), 11.9 (s)						

[a] δ in ppm. [b] Multiplicity of carbon signals was determined by DEPT. [c] Assignments were based on comparison with related compounds. [d] Signals were assigned by proton selective decoupling experiment. [e] Signals were interchangeable within a column. [f] See footnote *e*. [g] See footnote *e*. [h] Two aromatic carbons could not be assigned because of signal overlapping.

proton(s) by DEPT experiments. There were five methyls, five methylenes including one isolated methylene, four methines, and one vinyl group. From these NMR data, it was evident that the air oxidation did not take place at either the C.5 or the C.10 positions. The ^{13}C NMR spectrum demonstrated the presence of one additional carbonyl group [δ_C 200.5 (s)] at the C.13^2 position, which appears to explain well the significant low-field shift of the resonances for C.12^1 [δ_H 2.45 (s), δ_C 30.5 (q)][24] and C.19 [δ_H 4.52 (d, J = 1 Hz), δ_C 75.0 (d)].[24] On the basis of the evidence presented, structure **4**[15] was assigned for the enzymatic air-oxidation product of dinoflagellate luciferin (**1**).

Air oxidation of dinoflagellate luciferin (**1**) took place along different routes depending on the conditions. Enzymatic air oxidation in water occurred at the C.13^2 carbon with emission of light while nonenzymatic air oxidation in methanol occurred at the C.15 position without emission of light. The former might involve a series of reactions including anion formation of the enamine system, electron transfer to molecular oxygen, and then allylic radical formation at the C.13^2 position to yield a C.13^2 hydroperoxide, which could yield product **4** by heterolytic cleavage of the peroxide linkage. In this connection, it is interesting to note that a heterolytic cleavage of 1-phenylethyl peroxyacetate in the presence of an easily oxidized substance is known to be accompanied with light emission at the wavelength matching the fluorescence spectrum of the easily oxidized substance.[25] It is intriguing to point out the fact that product **4** is not fluorescent in either a protic solvent (ethanol) or an aprotic solvent (dimethyl sulfoxide) under both neutral and basic conditions, but dinoflagellate luciferin (**1**) itself is strongly fluorescent under the in vitro bioluminescence conditions with an emission spectrum almost identical with the bioluminescence emission.[26] Clearly, the bioluminescence of dinoflagellate represents a new type of bioluminescent system, and further studies on dinoflagellate and krill systems are in progress in our laboratories.

Experimental Section

General. Acetonitrile, methanol, 1-butanol, and ethanol were used as supplied (HPLC grades). Reagents were used without purification. Purification of **1**–**4** was conducted under Ar at ca. 5 °C. Phase separation in the extraction of luciferin was performed by centrifugation at ca. 0 °C, and solvents were removed under reduced pressure (oil pump). HPLC was equipped with a degasser (Erma ERC-3000). UV–visible and fluorescence spectra were recorded on Shimadzu UV-240 and Perkin-Elmer MPF-44B spectrophotometers, respectively. NMR spectra were run at −5 °C on a Brucker AM 500 spectrometer. A sample was dissolved in CD_3OD and sealed in a NMR tube after degassing. 2D COSY spectra were recorded with Brucker's standard programs. The FAB mass spectrum was taken with a JEOL JMS-DX303 spectrometer. Glycerol

(24) The C–H correlation was established by single-frequency proton selective decoupling experiments.

(25) (a) Koo, J.-K.; Schmidt, S. P.; Schuster, G. B. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 30. (b) Dixon, B. G.; Schuster, G. B. *J. Am. Chem. Soc.* **1981**, *103*, 3068.

(26) The emission spectrum of bioluminescence matched well with the fluorescence spectrum of **1** except that the width of the bioluminescence emission was slightly narrower than that of the fluorescence emission.

and PEG600 were used as a matrix and an internal standard for the high-resolution spectrum, respectively.

Culture and Extraction of *Pyrocystis lunula*.[9] Unialgal but not axenic cultures of the dinoflagellate *P. lunula* (clone T37) were grown at 20 ± 2 °C in 2.8-L Fernbach flasks with 1500 mL of f/2 medium, without silicate but with 0.5% (v/v) soil extract, under a 12:12 light/dark cycle for about 40 days after innoculation. Day- (light-) phase cells of 19 flasks (ca. 27 g wet weight) were harvested by filtration onto Whatman 541 filter paper. The cells, scraped off the filter paper, were dispersed into boiling extraction buffer (2 mM potassium phosphate containing 5 mM 2-mercaptoethanol, pH 8.5, 5 mL/L culture), heated for 60 s, and immediately cooled with an ice–water bath. The solution was saturated with Ar while being cooled and readjusted to pH 8.0 with 0.1 N NaOH. The slurry, obtained from four extractions (4 × 19 flasks), was centrifuged for 20 min at 27000*g* in a Sorvall RC2B refrigerated centrifuge at 0 °C. The supernatant was diluted by 4–5 volumes of chilled water containing 5 mM 2-mercaptoethanol, to give conductivity lower than 0.7 $m\Omega^{-1}$. The crude luciferin was applied to a 5 × 15 cm DEAE-cellulose column (coarse grade, Sigma), equilibrated in 2 mM potassium phosphate containing 5 mM 2-mercaptoethanol before use. The column was washed with 200 mL of the equilibration buffer and then eluted first with a linear gradient of potassium phosphate from 2 mM to 0.3 M (400 mL) at pH 8.0 in the presence of 5 mM 2-mercaptoethanol and followed by 600 mL of 0.3 M potassium phosphate containing 5 mM 2-mercaptoethanol. Fractions containing luciferin were detected by fluorescence and UV absorption at 388 nm and combined (400–700 mL). After addition of 2-mercaptoethanol up to 50 mM, the luciferin solution was frozen by liquid nitrogen and stored under Ar at −80 °C until use.

Purification of Luciferin. The frozen solution was thawed and concentrated down to approximately 30 mL. The solution was diluted with 50 mL of ethanol and extracted with 90 mL of 1-butanol. The 1-butanol extract was applied to an alumina column (16 × 90 mm, Pharmacia column, Woelm basic alumina, grade I) under Ar pressure and eluted with 50% ethanol containing 0.6% ammonium hydroxide. The fluorescent fractions, exhibiting a UV absorption band at 388 nm, were combined and concentrated to near dryness after addition of a small amount of tris(hydroxymethyl)aminomethane (solid). The residue was extracted with 15 mL of ethanol. A small amount of precipitated salts was removed by centrifugation, and the supernatant was concentrated to dryness. The crude extract was dissolved into 2–3 mL of 30% aqueous acetonitrile and purified by HPLC on TSK DEAE-5PW (7.5 × 75 mm) with 85 mM NaCl and 3 mM $NaHCO_3$ in 40% aqueous acetonitrile as a mobile phase. The eluent exhibiting a UV absorption band at 388 nm was collected (total volume ca. 8.8 mL) and concentrated until just before salts began to precipitate. The residual solution was diluted with approximately 10 mL of ethanol, and the precipitated salts were removed by centrifugation. The supernatant was concentrated to dryness, to give luciferin (**1**). The purity of **1** thus obtained was usually 60–70%. When a small amount of 2-mercaptoethanol was added at the steps of concentration and desalting, the yield and purity of luciferin were greatly improved (purity, >90%; approximately 0.003% yield based on the wet cell weight): UV–visible spectrum (40% acetonitrile containing 85 mM NaCl and 3 mM $NaHCO_3$) λ_{max} 241 and 388 nm; ^1H and ^{13}C NMR, Table I; FAB mass spectrum (glycerol/2-mercaptoethanol) m/z 633 $[(M + H)^+]$, 655 $[(M + Na)^+]$, and 677 $[(M + 2Na - H)^+]$ and m/z 649 $[(M + 16 + H)^+]$, 671 $[(M + 16 + Na)^+]$, and 693 $[(M + 16 + 2Na - H)^+]$.

Purification of Blue Compound (3). The fractions containing crude blue compound (total volume 4.1 mL) were collected at the second step of purification of the crude luciferin extracted from 380 culture flasks. The salts were removed by two precipitations with ethanol. The crude material was dissolved in 50% methanol and purified by HPLC on a Hamilton PRP-1 (7 × 300 mm) with 0.1% ammonium acetate in 80% aqueous methanol as a mobile phase. The fractions exhibiting an absorption band at 625 nm (total volume ca. 27.5 mL) were collected and concentrated to dryness. The salts in the residue were removed under vacuum with phosphorous pentoxide and potassium hydroxide for 3 h at room temperature to give pure blue compound (**3**): UV–visible spectrum (80% methanol containing 0.1% NH_4OAc) λ_{max} 234, 254, 315, 370, 410, 590 (shoulder), and 633 nm; ^1H and ^{13}C NMR, Table I; FAB mass spectrum (glycerol) m/z 587 $[(M - 2Na + 3H)^+]$, 609 $[(M - Na + H)^+]$, and 631 $[(M + H)^+]$; exact mass calcd for $C_{33}H_{38}O_6N_4Na$ 609.2689, found 609.2672, and for $C_{33}H_{37}O_6N_4Na_2$ 631.2509, found 631.2524.

Nonenzymatic Air Oxidation of Luciferin (1). A CD_3OD solution of luciferin in an NMR tube was kept at −20 °C in the presence of air, and oxidation was monitored by ^1H NMR. After 3 days, most of the luciferin was consumed. The reaction mixture was directly subjected to HPLC on TSK DEAE-5PW with 85 mM NaCl and 3 mM $NaHCO_3$ in 35% aqueous acetonitrile. The major peak (total volume ca. 8.2 mL) was collected, concentrated, and desalted by precipitation with ethanol to give pure air-oxidation product **2**: UV–visible spectrum (35% acetonitrile containing 85 mM NaCl and 3 mM $NaHCO_3$) λ_{max} 237 and 300 (shoulder) nm; ^1H and ^{13}C NMR, Table I; FAB mass spectrum m/z 627 $[(M + 2H - Na)^+]$, 649 $[(M + H)^+]$, and 671 $[(M + Na)^+]$; exact mass calcd for $C_{33}H_{39}O_7N_4Na_2$ 649.2614, found 649.2620, and for $C_{33}H_{38}O_7N_4Na_3$ 671.2433, found 671.2484.

Enzymatic Air Oxidation of Luciferin (1). Luciferin, stored in methanol in the presence of 2-mercaptoethanol or freshly prepared (HPLC eluents), was diluted to a concentration of less than 0.1 optical density (OD) at 388 nm (10-mm cell) with the assay buffer [0.2 M phosphate buffer, pH 6.3, containing 0.1 mM EDTA and 0.1 mg/mL bovine serum albumin (Sigma)] at room temperature. To the luciferin solution was added luciferase solution, which had been passed through a Bio-Gel P-10 column (Bio-Rad; 10 mM Tris buffer, pH 8.5, containing 100 mM NaCl, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol) to remove glycerol before use. The reaction was monitored by light emission and UV spectroscopy (see Figure 1). At a low concentration (OD <0.01), at least 50% of luciferin was consumed within 30 s. However, at a high concentration (OD 0.05–0.1), luciferin was not consumed at a sufficiently fast rate so that a larger amount of luciferase was used.[23] The enzymatic air oxidation was carried out in preparative scale under a high concentration; a total volume of approximately 40 mL of luciferase solution (ca. 3–5 μg of luciferase/mL), added in three aliquots, was required to consume completely 1000 mL of the luciferin solution with OD 0.08–0.1 at 388 nm. After the light emission ceased, the solution (ca. 1050 mL, ca. 150 total OD at 350 nm) was concentrated down to 150 mL, diluted with 40 mL of ethanol, and extracted with 150 mL of 1-BuOH. The organic layer (180 mL, 120 total OD at 350 nm) was concentrated, and the residue was extracted with 40 mL of ethanol (110 total OD at 350 nm). The ethanol solution was concentrated, and the crude material was dissolved in 6 mL of 20% acetonitrile. The solution was subjected to HPLC (TSK DEAE-5PW, 30% acetonitrile containing 50 mM NaCl and 3 mM $NaHCO_3$) to give air-oxidation product **4**. The eluent (75 total OD at 350 nm) was concentrated down until just before salts began to precipitate and extracted with ethanol (ca. 5 mL) in order to remove salts. After centrifugation, the ethanol extract was concentrated to dryness and dissolved in CD_3OD (50 total OD at 350 nm); UV–visible spectrum (30% acetonitrile containing 50 mM NaCl and 3 mM $NaHCO_3$) λ_{max} 243, 262, and 348 nm; ^1H and ^{13}C NMR, Table I; FAB mass spectrum (glycerol) m/z 647 $[(M + H)^+]$ and 669 $[(M + Na)^+]$; exact mass calcd for $C_{33}H_{37}O_7N_4Na_2$ 647.2457, found 647.2463, and for $C_{33}H_{36}O_7N_4Na_3$ 669.2277, found 669.2252.

Culture and Purification of Luciferase from *Gonyaulax polyedra*.[20] Purification of luciferase was conducted at 0–5 °C. Unialgal (not axenic) cultures of *G. polyedra* (strain E5) were grown for 3 weeks under the same conditions as those used for *P. lunula*. Night- (dark-) phase cells were collected onto Whatman 541 paper and suspended in 100 mM Tris buffer, pH 8.5, containing 10 mM EDTA and 3 mM dithiothreitol (2 mL/0.5 g of cells). The cell suspension was passed once through a French press and centrifuged at 13 000 rpm for 15 min. To the supernatant was added $^1/_3$ volume of saturated ammonium sulfate solution in the same buffer (25% saturation) and centrifuged at 13 000 rpm for 10 min. The volume was remeasured, $^1/_2$ volume of a saturated ammonium sulfate solution was added (50% saturation), and the precipitate containing luciferase was pelleted. The activity during purification was followed by monitoring bioluminescence. The pellet was dissolved in 1 mM Tris buffer, pH 8.5, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol, and the solution was applied to a Bio-Gel P-10 (Bio-Rad) column and eluted with the same buffer. The active fraction (conductivity must be lower than 0.7 $m\Omega^{-1}$) was chromatographed on DEAE Bio-Gel A (Bio-Rad) and eluted by a linear gradient of NaCl from 0 to 100 mM. The activity was eluted at an ion conductivity of 0.6–1.2 $m\Omega^{-1}$. The active fraction was stored at −80 °C in the presence of 25% (v/v) glycerol.

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Supplementary Material Available: ^1H NMR, ^{13}C NMR, FAB mass, and UV–visible spectra of **1–4** (16 pages). Ordering information is given on any current masthead page.