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Tracer studies on dinoflagellate luciferin with [^{15}N]-glycine and [^{15}N]-L-glutamic acid in the dinoflagellate *Pyrocystis lunula*

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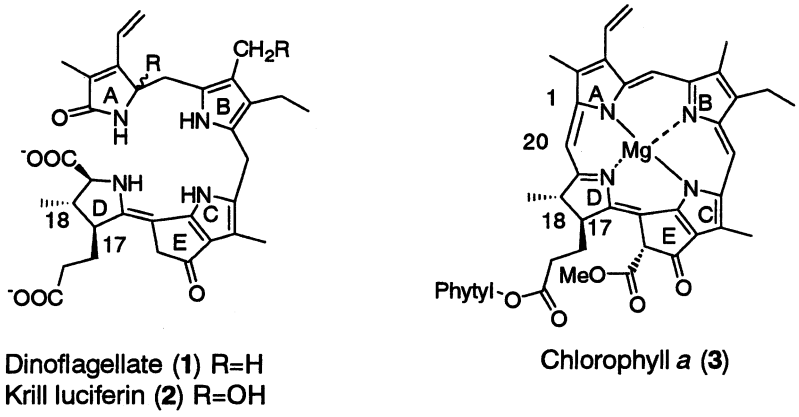
Abstract—The bioluminescence of dinoflagellate is a typical luciferin–luciferase reaction. To clarify the biosynthesis of dinoflagellate luciferin, we performed a feeding experiment with [^{15}N]-glycine and [^{15}N]-L-glutamic acid in the dinoflagellate *Pyrocystis lunula*. In a control experiment, we also examined whether or not chlorophyll *a* was incorporated with these labeled compounds. We detected by mass spectrometry the incorporation of [^{15}N]-glycine and [^{15}N]-L-glutamic acid into the four tetrapyrrole rings of the luciferin. In the control experiment, chlorophyll *a* was also incorporated with [^{15}N]-glycine and [^{15}N]-L-glutamic acid. Our results show that either glycine or glutamic acid could be the original component of dinoflagellate luciferin as well as chlorophyll *a* in the dinoflagellate *P. lunula*.

Dinoflagellates have received much attention during the past 30 years, because they are a valuable source of bioactive compounds and are related to various marine phenomena, such as red tide, bioluminescence, symbiosis and fish poisoning. The bioluminescence of dinoflagellate is a typical luciferin–luciferase reaction and the luciferin is able to cross-react not only luciferases from different luminous species of dinoflagellate but also a photoprotein of a krill bioluminescent system. The chemical structure of dinoflagellate luciferin (**1**) has been shown to be a linear tetrapyrrole structure by comparison with the spectroscopic data of the krill fluorescent compound F (**2**).[1] Interestingly, dinoflagellate luciferin (**1**) and chlorophyll *a* (**3**, Fig. 1) have the same absolute configurations at the C-17 and C-18 positions on ring D of the tetrapyrroles.[2] Recently, a photooxidation to cleave the bond at the C-1–C-20 position of a chlorophyll *a* derivative has been



Dinoflagellate (**1**) R=H
Krill luciferin (**2**) R=OH

Chlorophyll *a* (**3**)

Figure 1. Chemical structures of dinoflagellate luciferin (**1**), krill luciferin (**2**) and chlorophyll *a* (**3**).

Keywords: dinoflagellate luciferin; chlorophyll *a*; bioluminescence; biosynthesis.

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reported.[3] This experimental result strongly suggests that dinoflagellate luciferin (**1**) could be a degradation product of chlorophylls in dinoflagellate.[3] On the other hand, to clarify the biosynthesis of tetrapyrroles in vivo, feeding experiments with labeled glycine and/or glutamic acid have been studied extensively in green plants, algae and bacteria.[4a–c] In view of these observations, we decided to investigate the biosynthesis of dinoflagellate luciferin (**1**) using the feeding experiment with [^{15}N]-glycine and [^{15}N]-L-glutamic acid in the dinoflagellate *Pyrocystis lunula*. In a control experiment, we examined whether chlorophyll *a* (**3**) was incorporated with these labeled compounds.

The culture of the dinoflagellate *P. lunula* was performed according to a previous report.[1] The dinoflagellate *P. lunula* was pre-cultured in a 1 L case containing 0.5 L f/2 medium for 2 weeks under 12 h light–12 h dark cycles at 20°C. The high purity compounds [^{15}N]-glycine[5] or [^{15}N]-L-glutamic acid[5] were added to the culture medium (25 mg/L) in the presence of antibiotics (streptomycin 5 mg/L, chloroamphenicol 2.5 mg/L).[6] After the dinoflagellate was cultured for another 2 weeks, the cells were harvested by filtration. Dinoflagellate luciferin (**1**) was extracted by a boiled phosphate buffer (2 mM/pH 8.5) with 2-mercaptoethanol (5 mM). After the phosphate buffer had been centrifuged (10 min, 22,000*g*), the supernatant was collected and then concentrated until salts begin to precipitate. Two times the volume of a chilled ethanol solution was added to the crude luciferin solution and then the precipitate was removed by centrifugation (10 min, 22,000*g*).[1] To reduce the purification steps, we modified the purification method of dinoflagellate luciferin (**1**).[1] Dinoflagellate luciferin (**1**) was purified by reverse phase HPLC (ODS-SR-5, 4.6×250 mm, Nomura) with a water/ethanol linear gradient as the mobile phase (0.5 ml/min, 3°C). The HPLC unit (Waters) consisted of pumps and controller (2795), an in-line degasser, a diode-array spectrophotometer (2996) and a spectrofluorometric

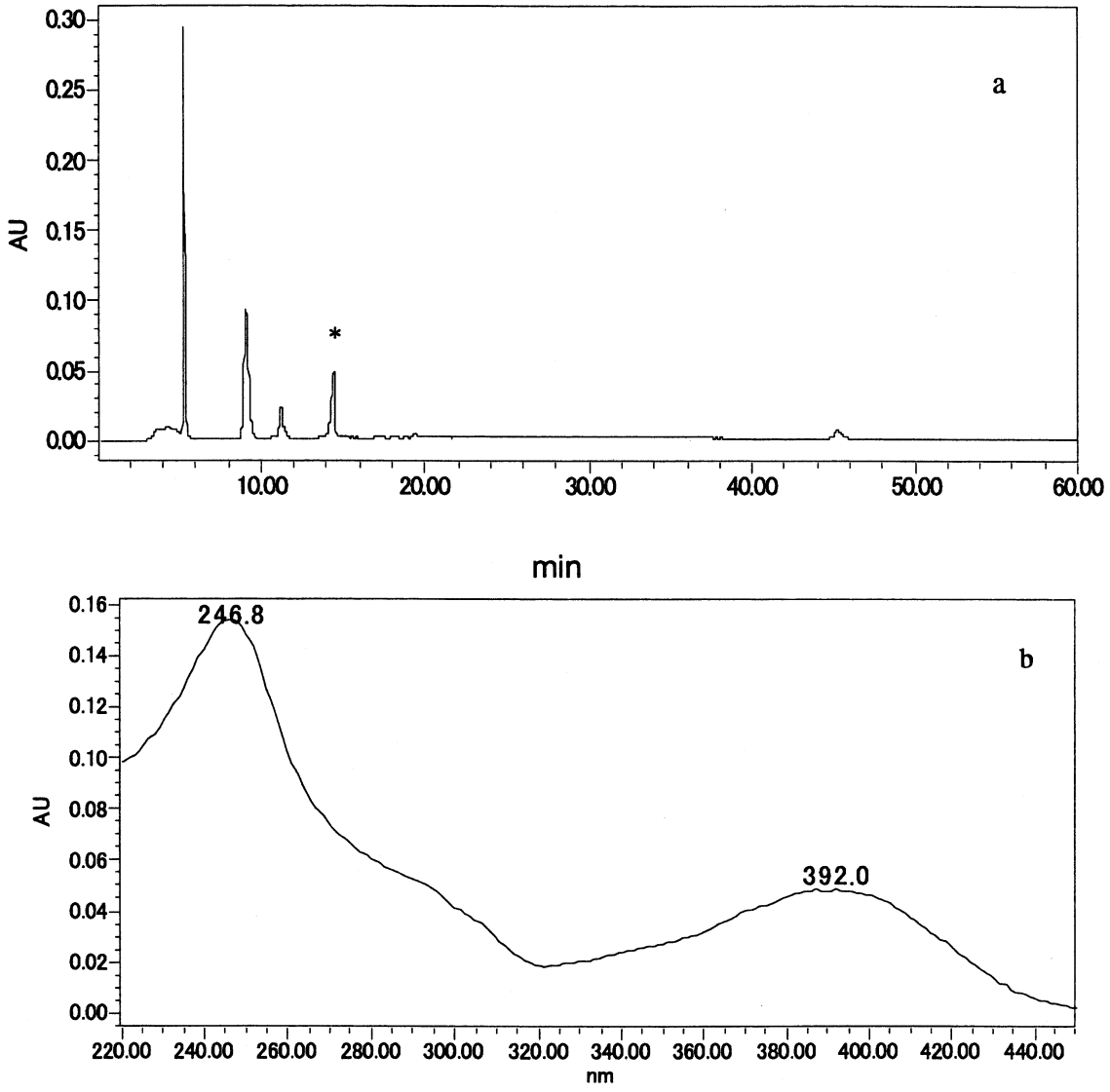

Figure 2. (a,b) The peak with an asterisk represents dinoflagellate luciferin (**1**) and exhibited an absorption peak at 247 nm and a shoulder at 392 nm.

detector (2475). The effluent was monitored by UV absorbance at 380 nm (Fig. 2a). The peak with an asterisk had an absorbance spectrum identical to that of dinoflagellate luciferin (**1**, Fig. 2b).[1] The collected HPLC fraction, whose mass spectral analysis was consistent with dinoflagellate luciferin (Table 1),[7] showed a strong bioluminescent activity in the presence of the dinoflagellate luciferase.

Chlorophyll *a* (**3**) was extracted with an ethanol solution (2×1 mL) from the precipitate that was obtained by centrifuging of the phosphate buffer. After centrifuging (10 min, 22,000*g*), the supernatant was collected and then concentrated. Chlorophyll *a* (**3**) was purified by reversed phase HPLC using the same column with an ethanol solution as the mobile phase (0.5 mL/min, 60°C). The effluent absorbance was monitored at 440 and 660 nm. The peak with a retention time of 9 min was identical to that of a standard chlorophyll *a* sample (data not shown).[8]

Mass spectral analysis was used to detect isotopic incorporation of dinoflagellate luciferin (**1**). Mass spectra were obtained on the LC/ESI-MS mass spectrometer.[7] Mass spectral analyses showed that the nitrogen at all four tetrapyrrole ring positions of **1** was labeled with [^{15}N]-glycine and [^{15}N]-L-glutamic acid (Table 1). The labeling efficiency of [^{15}N]-L-glutamic acid was estimated to be about 0.57 from the calculation of excess abundance ($M_{593}/(M_{589}+M_{593})$). Similarly, the labeling efficiency of [^{15}N]-glycine acid was estimated to be about 0.74 (Table 1).

Mass spectral analyses were also used to detect isotopic incorporation of chlorophyll *a* (**3**). Electron spray ionization (ESI) mass spectrometry showed base peak corresponding to the sodium additive of **3** (Table 2).[7,8] Mass spectral analyses showed that chlorophyll *a* was also incorporated with [^{15}N]-glycine and [^{15}N]-L-glutamic acid (Table 2). The labeling efficiency of [^{15}N]-L-glutamic acid was estimated to be about 0.81 from the calculation of excess abundance ($M_{919}/(M_{915}+M_{919})$) and the labeling efficiency of [^{15}N]-glycine was similarly estimated to be about 0.86 (Table 2).

Table 1. Relative abundance in the mass of dinoflagellate luciferin and the incorporated ^{15}N-luciferins

m/z	Luciferin*	^{15}N Glu	^{15}N Gly
589*	100	74	34
590	34	44	23
591	6	53	37
592	2	93	74
593	3	100	100
594	4	32	31
595	2	7	6
Excess abundance $M_{593}/(M_{589}+M_{593})$	–	0.57	0.74

* $m/z=589$ (M+H); $m/z==593$ (M+H+4).

Table 2. Relative abundance in the mass of chlorophyll *a* and the incorporated ^{15}N-chlorophyll *a*

m/z	Chlorophyll *a*	^{15}N Glu	^{15}N Gly
915*	100	22	16
916	64	30	14
917	38	60	12
918	15	84	42
919	4	100	100
920	5	59	61
921	2	24	44
Excess abundance $M_{919}/(M_{915}+M_{919})$	–	0.81	0.86

* $m/z=915$ (M+Na); $m/z==919$ (M+Na+4).

Our result of this experiment showed that either glycine or glutamic acid was incorporated into dinoflagellate luciferin as well as chlorophyll *a* in the cultured dinoflagellate. However, the labeling efficiency of chlorophyll *a* (**3**) was higher than that of dinoflagellate luciferin (**1**). This result might suggest that the biosynthetic steps of the luciferin from glycine or glutamic acid was longer than that of chlorophyll *a*. It has been proposed that dinoflagellate luciferin (**1**) could be a degradation product of chlorophylls in dinoflagellate.[3] The difference in the labeling efficiency between chlorophyll *a* (**3**) and dinoflagellate luciferin (**1**) might be explained by this suggestion. The luciferin–luciferase reactions are widely observed in the marine bioluminescent organisms. However, the biosynthesis of luciferin in the marine organisms is not clear, because it is difficult to exclude the possibility that the luminous organisms obtain luciferin from a food chain or symbiosis. Our result could provide the first experimental evidence that dinoflagellates synthesized dinoflagellate luciferin as well as chlorophyll *a* by themselves. It should be mentioned here that the cultured *P. lunula* had been separated from other dinoflagellate species or marine organisms and grew in a sterilized sea water.

In conclusion, we detected by mass spectrometry the incorporation of [^{15}N]-glycine and [^{15}N]-L-glutamic acid into the four tetrapyrrole rings of the luciferin. In the control experiment, chlorophyll *a* was also incorporated with [^{15}N]-glycine and [^{15}N]-L-glutamic acid. Our results show that either glycine or glutamic acid could be the original component of dinoflagellate luciferin as well as chlorophyll *a* in the dinoflagellate. A study of whether chlorophyll *a* is the precursor of dinoflagellate luciferin is still in progress.

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6. The feeding experiment with antibiotics was reported by: Nakamura, H.; Jin, T.; Funahashi, M.; Fujimaki, K.; Sampei, O.; Murai, A. *Tetrahedron* **1997**, *53*, 9067–9074.
7. Mass spectra were recorded on a LC/ESI-MS (LCQ, thermoqust) spectrometer at Sumika Chemical Analysis Service, Ltd.
8. Chlorophyll *a* was purchased from Wako Co.