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7,8-Dihydropterin-6-carboxylic Acid as Light Emitter of Luminous Millipede, *Luminodesmus sequoiae*

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Abstract—A luminous millipede, *Luminodesmus sequoiae*, emits light centered at a wavelength of 500 nm. To determine the light emitter of this bioluminescent system, fluorescent compounds were isolated from pulverized cuticles. NMR and MS spectra of these compounds showed them to be pterin derivatives. Furthermore, proton/deuterium (H/D) exchange experiments by ESI-Q-TOF-MS and -MS/MS measurements have proved to be a powerful tool for elucidating these heteroaromatic compounds. Finally, we have concluded that 7,8-dihydropterin-6-carboxylic acid, a new natural product, is the light emitter of *Luminodesmus* bioluminescence.

Introduction

A luminous millipede, *Luminodesmus sequoiae*, emits light when four components get together such as a photoprotein, magnesium ion, molecular oxygen, and ATP.[1,2] No additional organic substrate such as the firefly luciferins[3] was required for this bioluminescence. The *Luminodesmus* photoprotein was isolated in 1981,[4] but the bioluminescence mechanism of this millipede has not been fully understood yet.[5] In this report, we describe that 7,8-dihydropterin-6-carboxylic acid (**2**) is the light emitter in the *Luminodesmus* bioluminescence.

Results and Discussion

A cuticle of the millipede shows a strong blue-green fluorescence [emission maximum at 500 nm upon irradiation of UV (350 nm)] as shown in Figure 1. This emission spectrum is superimposable with the

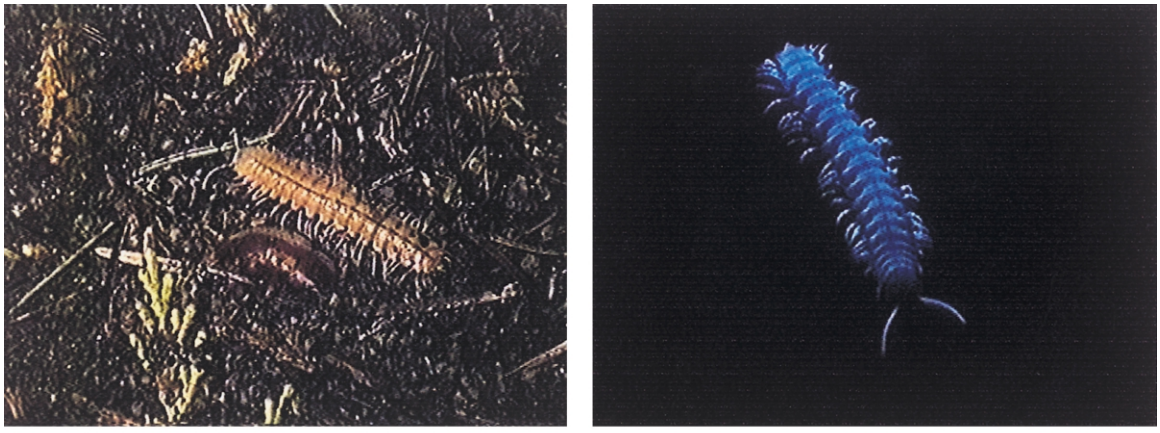

Figure 1. Photographs of *Luminodesmus sequoiae*: (a) live specimen; (b) fluorescence of the body under UV (350 nm).

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bioluminescence spectrum. Two fluorescent compounds were extracted from the pulverized cuticles with acidic methanol and purified in accordance to Chart 1.

Two peaks were detected as the fluorescent compounds in the extract of the cuticle. One of the peaks showing 450 nm emission was named as Fraction 1 (Fr-1), and the major peak giving 500 nm emission was named as Fraction 2 (Fr-2). As shown in Chart 1, three step purification by reversed phase column chromatography, Cosmosil 75C_{18}-OPN, ODS-UG5, and C30-UG5, gave pure Fr-2 in about 50 μg yield and almost pure Fr-1 (~20 μg) from 6 g of original cuticles which corresponded to 20 specimens of the millipede (Fig. 2).

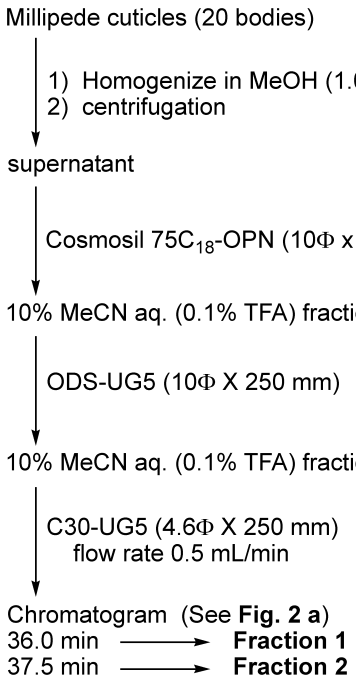


Chart 1. Protocols of isolation of fluorescent compounds from *Luminodesmus* extracts.

Table 1. The NMR and MS data of Fr-1 and Fr-2

	Fr-1 pterin-6-carboxylic acid (**1**)		Fr-2 7,8-dihydropterin-6-carboxylic acid (**2**)	
Assignment	NMR chemical shifts[a]			
	δ_H	δ_C	δ_H	δ_C
2		160.5		158.2
3	7.20 (br)		7.21 (br)	
4		165.0		165.2
4a		128.8		103.0
6		137.6		134.3
7	9.09	150.5	4.08	40.5
8			7.21	
8a		155.3		155.4
6a (COOH)	11.6	159.1	10.0	156.2
2a (NH2)	12.0 (br)		6.65 (br)	
	Mass spectra[b]			
	m/z		*m/z*	
M + 1	208.042 (calcd 208.047)		210.060 (calcd 210.063)	
Composition	$C_7H_6N_5O_3$		$C_7H_8N_5O_3$	
Exchangeable H's	5		6	

[a]All chemical shifts are written in ppm as referenced values at DMSO-d_6; 2.49 for ^{1}H and 39.7 for ^{13}C.
[b]ESI-MS by a Q-TOF-MS spectrometer (Micro Mass Co. Ltd., Manchester, UK).

Each of the isolated Fr-1 and Fr-2 was analyzed with a 600 MHz NMR in a micro-cell tubing[6] as DMSO-d_6 solution. Table 1 summarizes the chemical shifts of these compounds. Only one proton at 9.09 ppm was found to correspond to a C-H proton for Fr-1 from the HSQC spectrum.[6] Two C-H protons at 4.08 ppm were found in Fr-2. The other protons were assigned to NH or OH protons. From ^{13}C NMR data of these compounds, we assumed that Fr-1 and Fr-2 might be highly unsaturated compounds. However, little information was obtained from NMR spectra, since limited correlation of signals was observed by 2D-NMR. Therefore, there were some limitations to elucidate the structure of these compounds in detail. ESI-MS measurements of these compounds with a Q-TOF-MS spectrometer (Micro Mass Co. Ltd., Manchester, UK) suggested that the composition of Fr-1 is $C_7H_5N_5O_3$ and that of Fr-2 is $C_7H_7N_5O_3$, respectively. A high resolution FAB-MS of

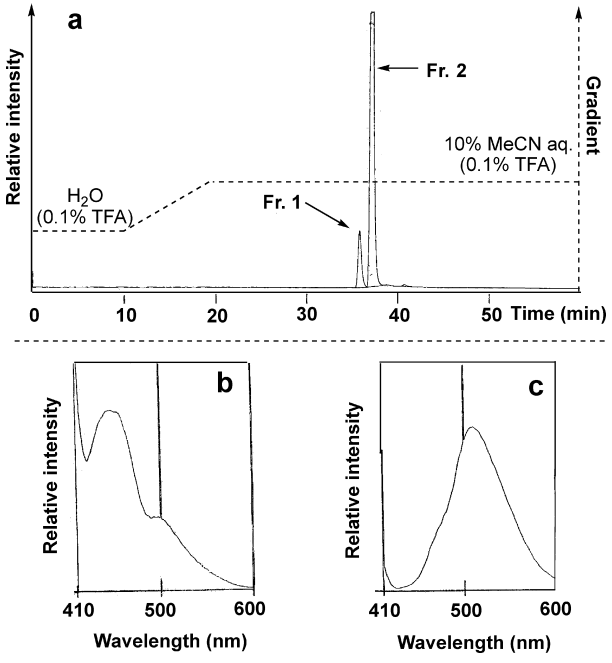
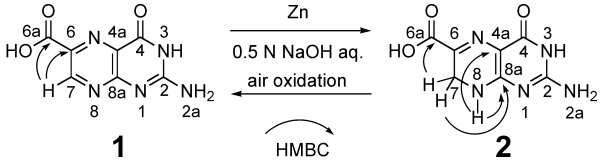


Figure 2. Chromatogram of crude extracts (detected by fluorescence). (a) Fluorescent chromatogram (λ_{em} = 500 nm, λ_{ex} = 390 nm); (b) emission spectrum of Fr-1; (c) emission spectrum of Fr-2 (excitation at 390 nm).



Scheme 1. Zinc reduction of **1** to give **2** (arrows indicate HMBC correlation).

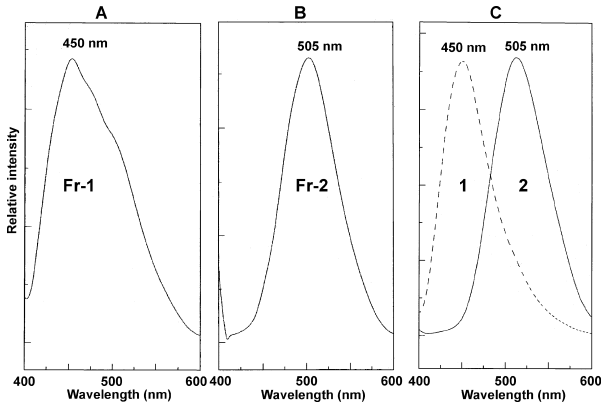


Figure 3. Fluorescence emission spectra (excitation at 390 nm): (A) Fr-1; (B) Fr-2; (C) authentic **1** and synthetic **2**.

Fr-2 gave m/z 210.0584 (M+H) [calcd $C_7H_8N_5O_3$ 210.0627]. From NMR and MS data, we assigned that Fr-1 is pterin-6-carboxylic acid (**1**) and that Fr-2 is 7,8-dihydropterin-6-carboxylic acid (**2**) as a new natural product (Table 1).[7]

Fr-1 was identical with the authentic sample **1** (Aldrich® 10,008-0, CAS No. [948-60-7]). Zinc reduction[8] of **1** gave **2**, which was also spectrometrically identical with Fr-2 as shown in Scheme 1. Figure 3 shows the fluorescence emission spectra of the isolated Fr-1, Fr-2, and authentic **1** and synthetic **2**.

To confirm the numbers of exchangeable protons in Fr-1 and Fr-2, we performed proton/deuterium (H/D) exchange experiments with ESI-Q-TOF-MS and -MS/MS. These

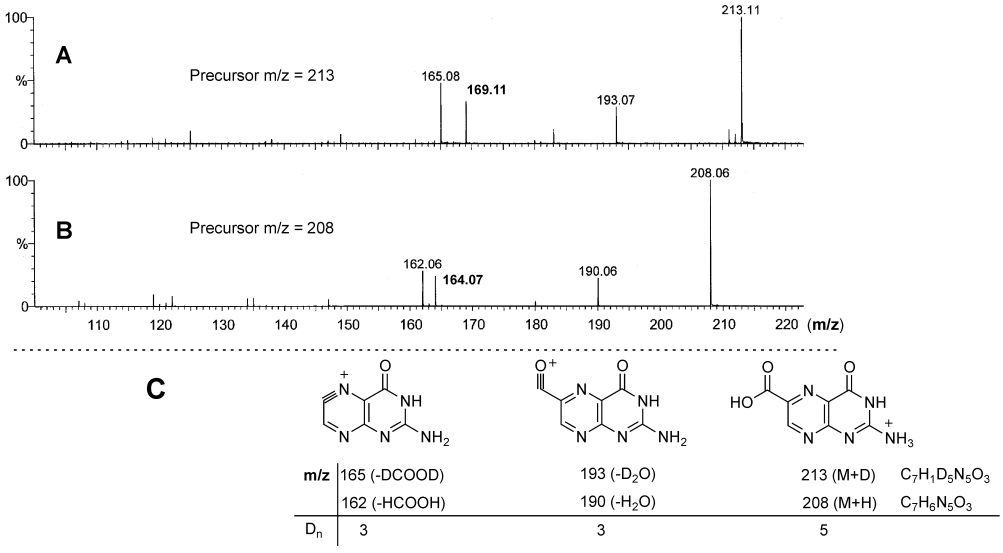


Figure 4. MS/MS spectra of Fr-1: (A) deuterated sample of the exchangeable protons of Fr-1 precursor m/z 213 (Fr-1+D); (B) isolated natural abundance precursor m/z 208 (Fr-1+H); (C) assignment of precursor and fragment ions. Dn: numbers of the exchangeable protons.

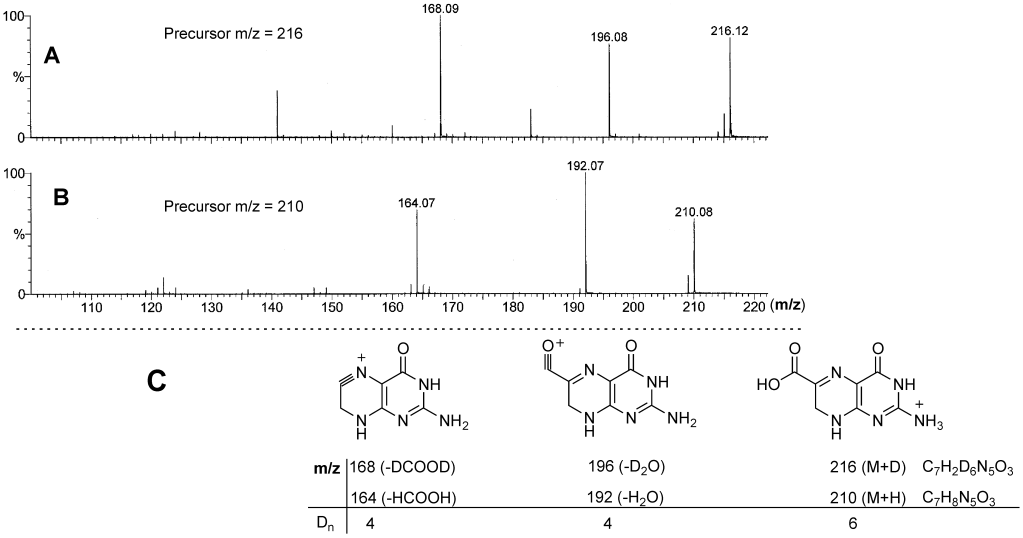


Figure 5. MS/MS spectra of Fr-2: (A) deuterated sample of the exchangeable protons of Fr-2 precursor m/z 216 (Fr-2+D); (B) isolated natural abundance precursor m/z 210 (Fr-2+H); (C) assignment of precursor and fragment ions. Dn: numbers of the exchangeable protons.

experiments readily provided the numbers of exchangeable protons which connected to N or O atoms.[9] The numbers of exchangeable protons came out simply by comparing the mass numbers of the precursor ions and fragment ions measured in protic media with that in deuterated media.

The samples for proton/deuterium (H/D) exchange experiments were prepared as such that Fr-1 and Fr-2 were completely dried in vacuo, then redissolved with 99% CH_3OD:1% CH_3COOD for ESI-Q-TOF-MS with deuterium measurement or with 99% CH_3OH:1% CH_3COOH for MS with proton measurement. The resultant spectra are illustrated in Figures 4(A,B) and 5(A,B), respectively.

Pseudo molecular ions of protonated Fr-1 and Fr-2 were observed at *m/z* 208 (M + H) and 211 (M + H); each of which was employed as the precursor ion for MS/MS measurement (sample cone 20 V, collision 12 V), and the results are shown in Figure 4(B) with structure Fr-1 and in Figure 5(B) with structure Fr-2, respectively.[10] The product ions are seen at *m/z* 190 and 162 from the *m/z* 208 precursor, and at 192 and 164 from the *m/z* 210 one. Figures 4(C) and 5(C) show the assignment of these fragments. For the validation of the above product ions generated under the MS/MS collision process, deuteration of all the exchangeable protons, as shown in Figures 4(A) and 5(A), made mass increase of mass numbers *m/z* 208 and 210, to *m/z* 213 and 216, respectively. The increased 5 and 3 mass units to the product ions for Fr-1 and the increased 6 and 4 mass units to the product ions for Fr-2, also provide strong support for their structures in detail as shown in Figures 4(C) and 5(C). It has been concluded that the structures **1** and **2** are elucidated as pterin-6-carboxylic acid and 7,8-dihydropterin-6-carboxylic acid to the compounds of Fr-1 and Fr-2, respectively.

Conclusion and Summary

Proton/deuterium (H/D) exchange experiments by ESI-Q-TOF-MS and -MS/MS measurements have proved to be a powerful tool for elucidating these heteroaromatic compound in small amounts.

Compound **2** (7,8-dihydropterin-6-carboxylic acid) was easily air-oxidized into **1** (pterin-6-carboxylic acid), though **2** was quite stable in the cuticle even after kept for years in a refrigerator. It is suggested that 7,8-dihydropterin-6-carboxylic acid (**2**; a new natural product) is well protected in the tissue. The protein extracts with a buffer[5] (NaOAc buffer, pH 4.75) and the fraction showed strong fluorescence with a GPC column (Shodex PROTEIN KW-300, 8×300 mm; SHOKO Co., Ltd. Japan) chromatography as well as the repeating precipitates with $(NH_4)_2SO_4$. This binding nature and the strong fluorescence at 505 nm of the protein fraction suggests that the compound **2** should be the light emitter in this bioluminescence system.

The correlation of compound **2** with the bioluminescence of *Luminodesmus* photoprotein has yet to be observed, however, further studies on the structure and function of the *Luminodesmus* photoprotein are now underway.

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6. (a) The Micro-Cell tubing is commercially available from Shigemi Glass Co. Ltd., Japan. The isolated samples were dissolved in 200 μL of DMSO-d_6 for NMR measurement. (b) Braun, S.; Kalinowski, H.-O.; Berger, S. *100 and More Basic NMR Experiments*; VCH: Weinheim, 1996; pp 318–321.
7. Similar compounds are seen in the following text: Pretsch, E.; Simom, W.; Seibl, J.; Clerc, J. In *Tables of Spectral Data for Structure Determination of Organic Compounds*, 2nd ed.; Fresenius, W. et al., Eds.; Springer-Verlag: Berlin, 1989; pp C5–H370.
8. Compound **2** was prepared from **1** in one step as follows. A solution of **1** (2.0 mg, 9.7 μmol) in 0.8 mL of 0.5 N NaOH aq was stirred with zinc powder (10 mg, 153 μmol) for 4 h under Ar atmosphere. The reaction mixture was centrifuged to remove zinc and then mixed with 1 N TFA aq (1.6 mL). The resultant solution was purified by chromatography [ODS-UG5 (10×250 mm)] to give **2** (1.0 mg, 4.8 μmol) in 49% yield. A similar procedure was reported in the following references: Viscontini, M. In *Methods in Enzymology*; McCormick, D. B., Wright, L. D., Eds.; Academic Press: New York, 1971; Vol. 18B, pp 678–705.
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10. In-source reduction of pterin-6-carboxylic acid (Fr-1) to dihydro forms on the product ions was observed as shown in Figures 5(A) and (B): *m/z* 169 (D_2 addition) from 165 in Figure 5(A), and *m/z* 164 (H_2 addition) from 162 in Figure 5(B) were assigned as the reduced product ions.