Identification of a Fluorescent Compound in the Cuticle of the Train Millipede *Parafontaria laminata armigera*

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The train millipede (*Parafontaria laminata armigera*) emits a blue fluorescence (λ_max = 455 nm) under black light (350 nm). The isolated fluorescent compound from the cuticle of *P. laminata armigera* was identified as pterin-6-carboxylic acid. The structure of this compound was identified by fluorescent, HPLC, and mass spectrometric (ESI-ion trap MS) analyses, and then compared with an authentic sample.

Key words: fluorescence; ESI-MS; pterin; millipede

An outbreak of the millipede, *Parafontaria laminata* group, occurs in the autumn every eight years in the mountainous area of central Japan, resulting in stopping the local trains.¹,² An outbreak of *P. laminata armigera* (kiyasya-yasude in Japanese) in 2008 occurred in Tateshina-kogen which is located in the mountainous area of central Japan.³ We found at that time that the outer shell of the millipede emitted a blue fluorescence when it was illuminated by a UV lamp (λ_max = 350 nm) (Fig. 1A and B).

Another fluorescent millipede, *Luminodesmus sequoiae*, which is bioluminescent, lives in the mountainous area of northern California.⁴,⁵ Shimomura has extensively studied the bioluminescent mechanism for *L. sequoiae* and isolated the *Luminodesmus* photoprotein.⁶,⁷ He reported that the photoprotein needed ATP, magnesium ions, and oxygen for emitting light.

The components of the *Luminodesmus* luminescence were similar to those of the flyfire luminescence which needs ATP, magnesium ions, oxygen, and firefly luciferin as an organic substance for light emission. The characteristic difference is that the *Luminodesmus* photoprotein has a porphyrin chromophore which is responsible for light emission inside the protein, and therefore does not require any additional organic substance such as luciferin. The fluorescent compounds of *L. sequoiae* were identified as 7,8-dihydropterin-6-carboxylic acid (1) and pterin-6-carboxylic acid (2) (Fig. 1C and D).⁸

We assumed that the pterins (1 and 2) might be present in the cuticle of *P. laminata armigera*, similar to the case of *L. sequoiae*. An attempt was therefore made to identify the fluorescent compound in *P. laminata armigera*.

The cuticle of *P. laminata armigera* afforded the fluorescent spectrum shown in Fig. 2A when it was irradiated by UV light (λ_max = 350 nm). After removing the guts from the body of *P. laminata armigera* (10 specimens), the remaining cuticles (about 200 mg per specimen) were homogenized in methanol (4.0 ml) at 0 °C. The non-fluorescent materials were separated into the supernatant by centrifugation, and the remaining precipitate was homogenized in acidified methanol (4.0 ml) containing 0.1% trifluoroacetic acid. The homogenate was again centrifuged to afford a fluorescent supernatant. This extraction method with acidified methanol was conducted twice. The combined fluorescent supernatant (12 ml) was evaporated, and then the residue was separated in a column of Cosmosil®-75C₁₈ (10 mm i.d. × 100 mm), eluting with a mixture of methanol and water. A blue fluorescent fraction was collected and evaporated.

The fluorescent fraction was analyzed by HPLC in a column of Cosmosil®-5C₁₈-MS-II (4.6 mm i.d. × 250 mm). The chromatogram was monitored by using a fluorescence detector (light emission at 460 nm and excitation at 350 nm) (Fig. 2B). The strongest fluorescence peak was observed at 38 min, this being the same retention time as that of authentic compound 2 (Fig. 2B, dotted line). This suggested the presence of 2 in the fluorescent fraction which had been extracted from the cuticle of *P. laminata armigera*.

The fluorescent compound (observed at 38 min on the chromatogram) was purified by HPLC, and the resulting purified fluorescent compound was also analyzed by HPLC (Fig. 2C). The single peak of the fluorescent compound was observed at 38 min with the same retention time as that of 2. The fluorescence spectra of the isolated fluorescent compound and of compound 2 were then analyzed (Fig. 2D). Both compounds showed the same emission spectrum (λ_max = 455 nm) when excited by UV light (λ_max = 350 nm).

To confirm that compound 2 was the fluorescent component in the cuticle of *P. laminata armigera*, an ESI-ion trap MS analysis was conducted.⁸,¹⁰,¹¹ The isolated fluorescent compound (Fig. 2C) and compound 2 were analyzed by ESI-MS. Both the isolated fluorescent compound and authentic pterin-6-carboxylic acid (2) afforded a peak at m/z 205.9 [M – H]⁻ in the negative ion mode (Fig. 3a and c).¹² An MS/MS analysis of each ion (m/z 205.9 [M – H]⁻) afforded a product ion at m/z 161.9 which was derived from the loss of carbon dioxide (Fig. 3b and d).

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Further proof that the isolated fluorescent component was compound 2 was provided by the results of a hydrogen-deuterium exchange experiment (Fig. 3e–h). This method would afford the exact number of exchangeable protons (N–H, O–H) of the compounds, because this number is characteristic of the structure of the compounds.

The authentic and isolated compounds were dissolved in 50% acetonitrile in deuterium oxide to convert the exchangeable protons into deuteriums. Each resulting solution was directly analyzed by ESI-MS in the negative ion mode. The ion of the compounds was observed at \( m/z = 208.9 \) due to the loss of carbon dioxide (Fig. 3f and h). Since these product ions still had three exchangeable protons (Fig. 3b and d), they were observed at \( m/z = 164.9 \) after being deuterated, this being an increase of +3.0 from \( m/z = 161.9 \). We confirmed from these analyses that the isolated fluorescent compound and compound 2 presented the same mass spectrum, and that they had three exchangeable protons in the anion state.

The fluorescent compound isolated from \( P. \text{ laminata armigera} \) and authentic compound 2 afforded the same retention time on the chromatogram from an HPLC analysis (Fig. 2B and C), the same fluorescence spectrum (Fig. 2D), and afforded the same mass spectrum, the same fragmentation pattern and had the same number of exchangeable protons as shown in Fig. 3. We therefore concluded that the fluorescent compound isolated from \( P. \text{ laminata armigera} \) was indeed pterin-6-carboxylic acid (2).
It was estimated from the HPLC analysis results that *P. laminata armigera* had more than 2–3 mg of 2 per specimen. The role of 2 in the cuticle of *P. laminata armigera* is still unclear. *Parafontaria laminata armigera* and *Luminodesmus sequoiae* belong to the family Xystodesmidae, and only *L. sequoiae* is known as a bioluminescent millipede. *P. laminata armigera* does not in fact show any luminescence in the dark. Preliminary data showed that a homogenate of the cuticle of *P. laminata armigera* afforded weak luminescence when mixed with hydrogen peroxide. This weak luminescence was not related to ATP and the magnesium ion that are both essential for *Luminodesmus* luminescence. The presence of 2 was not responsible for the weak luminescence either. We are now trying to determine the role of the weak luminescence of *P. laminata armigera*.

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**References and Notes**

9) In this extract, we could not observe the fluorescent peak of compound 1 during the HPLC analysis. However, this fact cannot exclude the possible existence of compound 1 in *P. laminata armigera*.
12) Molecular ions were not observed in the positive ion mode, probably because these compounds are acidic, which are always measured in the negative ion mode of the MS analysis.