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Second bioluminescence-activating component in the luminous fungus *Mycena chlorophos*

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ABSTRACT – *Mycena chlorophos* is an oxygen-dependent bioluminescent fungus. The mechanisms underlying its light emission are unknown. A component that increased the bioluminescence intensity of the immature living gills of *M. chlorophos* was isolated from mature *M. chlorophos* gills and chemically characterized. The bioluminescence-activating component was found to be *trans*-3,4-dihydroxycinnamic acid and its bioluminescence activation was highly structure-specific. ^{13}C- and ^{18}O-labelling studies using the immature living gills showed that *trans*-3,4-dihydroxycinnamic acid was synthesized from *trans*-4-hydroxycinnamic acid in the gills by hydroxylation with molecular oxygen as well as by the general metabolism, and *trans*-3,4-dihydroxycinnamic acid did not produce hispidin (detection-limit concentration: 10 pmol/1 g wet gill). Addition of 0.01 mM hispidin to the immature living gills generated no bioluminescence activation. These results suggested that the prompt bioluminescence activation resulting from addition of *trans*-3,4-dihydroxycinnamic acid could not be attributed to the generation of hispidin. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: bioluminescence; fungus; metabolism; *Mycena chlorophos*; *trans*-3,4-dihydroxycinnamic acid

Introduction

Bioluminescence is the production of light by living organisms. The bioluminescence mechanisms of various organisms such as fireflies, jellyfishes, and bacteria have been elucidated previously (1). Fungal bioluminescence is also widely found on land. Chew *et al.* collected specimens from three new species of bioluminescent fungi in Malaysia, bringing the total number of documented species of luminous fungi in 2014 to 77 (2). Fungal bioluminescence, which varies among species, occurs in the basidiocarp, mycelium, and/or spores (3–5). In spite of the wide popularity of fungal bioluminescence, the molecular mechanisms underlying this phenomenon have been subjected to far less scientific investigation than bioluminescence from other sources.

Airth *et al.* have demonstrated that the cell-free light emitting system for the bioluminescence of *Armillaria mellea* required luciferase, luciferin, molecular oxygen, and reduced pyridine nucleotide (6). In 2009, Oliveira and Stevani attested that the mechanism proposed by Airth and Foerster for fungal bioluminescence is correct using four species of bioluminescent fungi (7). Thereafter, cross-reaction experiments with luciferin and luciferase extracts from bioluminescent species, performed by Oliveira *et al.*, supported the hypothesis that all known bioluminescent fungal lineages share the same type of luciferin and luciferase and that there is a single luminescence mechanism in all the bioluminescent fungi (8). However, the luciferase and luciferin were not isolated and their structures and luminescent properties were not determined. With respect to the bioluminescence system of the mycelium of the bioluminescent fungus *Neonothopanus nambi*, in 2011 and 2013, Bondar *et al.* suggested the presence of Mn-peroxidase (s) and detected a low-molecular-weight, heat-stable component, which increased the luminescence in the mycelium (9,10). However, these components have not been isolated and their chemical structures have not yet been identified. In 2014, they extracted an *N. nambi* luminescence system, which possessed the following characteristics: a stable complex comprising both protein and non-protein components, long-term light emission *in vitro*, and activation with H_2O_2 and NADPH (11). Recently, Purtov *et al.* reported that hispidin and 3-hydroxyhispidin acted as luciferin precursor and luciferin, respectively, for the mycelia bioluminescence of *N. nambi*, *Mycena citricolor*, *Panellus stipticus*, and *Armillaria borealis* (12).

Mycena chlorophos, which is primarily distributed in Southeast Asia, is a species of oxygen-dependent bioluminescent fungus (13). The fungus emits light spontaneously and continuously from the mycelium and the pileus. In our cultivation system, the light emitted by the mycelium, when grown on a solid medium, is so weak that a few minutes of dark adaptation is required to see it. When young fruiting bodies grew, the pileus contracted, the stipe began to elongate at 20°C, and at approximately 90% relative humidity, bioluminescence was weak (Fig. 1, stage 1). Subsequently, the pileus expanded horizontally; bright green light was emitted from the whole pileus and was continuously emitted for approximately 1 day at 20°C and at approximately 90% relative humidity (Fig. 1, stage 2). Few chemical and biochemical studies on *M. chlorophos* bioluminescence have been reported to date (12,14–17). Mori *et al.* suggested that the bioluminescence of *M. chlorophos* involves enzymatic reaction (s) in the cell membrane (14). Purtov *et al.* reported that adding hispidin to a mixture of NADPH and a crude cold water-extract solution prepared from the bioluminescent fruiting body of *M. chlorophos* enhanced the *in vitro* luminescence (12). However, they did not state whether hispidin acted as luciferin precursor or luciferin for the biolumines-

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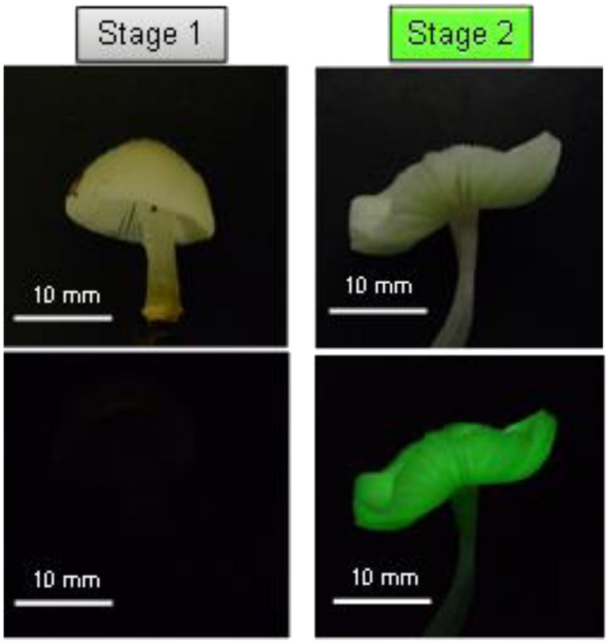

Figure 1. Growth and bioluminescence of *M. chlorophos*. The upper and lower photographs were taken under white light and in the dark, respectively, using a digital camera (Lumix; Panasonic, Osaka, Japan) at 20°C and approximately 90% relative humidity using the following settings: ISO, 400; aperture, f2.8; and exposure time, 1.3 sec as a representative of many fruiting bodies.

cence of the *M. chlorophos* fruiting body or whether hispidin was detected in the fruiting bodies of *M. chlorophos*. Recently, Teranishi indicated that *trans*-4-hydroxycinnamic acid, in the gills of *M. chlorophos* structure specifically, increased the bioluminescence activity in the living gills (17). At present, little chemical information is available concerning the bioluminescence of *M. chlorophos*, and the chemical mechanism underlying this phenomenon is unknown.

In the present study, we discovered a second bioluminescence-activating component, which played a more significant role in activating bioluminescence in living *M. chlorophos* gills at stage 1 than did *trans*-4-hydroxycinnamic acid, from the mature *M. chlorophos* gills, at stage 2. This paper describes the isolation, chemical structure, and metabolism of the second bioluminescence-activating component.

Experimental

Chemicals

trans-4-Hydroxycinnamic acid; *trans*-3,4-dihydroxycinnamic acid; and *trans*-ferulic acid were purchased from TCI chemicals (Tokyo, Japan). *trans*-2,4-Dihydroxycinnamic acid; *trans*-2,3-dimethoxycinnamic acid; *trans*-3,5-dimethoxycinnamic acid; 3,4-dihydroxyhydrocinnamic acid; *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid (99 atom % ^{13}C); hispidin; and 1 M BBr_3 in CH_2Cl_2 were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). *trans*-3,4-Dihydroxycinnamamide was purchased from Ark Pharm Inc. (Libertyville, IL, USA). *1,2,3–*$^{13}C_3$*-trans*-3,4-dihydroxycinnamic acid (98.7 atom % ^{13}C) was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). $^{18}O_2$ (97 atom % ^{18}O) was purchased from Taiyo Nippon Sanso Co. (Tokyo, Japan). *trans*-3,4,5-Trimethoxycinnamic acid and other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *trans*-3,4,5-Trihydroxycinnamic acid was synthesized from *trans*-3,4,5-trimethoxycinnamic acid according to a literature method (18).

Fruiting bodies of *M. chlorophos*. A mycelium strain of *M. chlorophos* (Berkeley & M.A. Curtis) Saccardo was purchased from the National Institute of Technology and Evaluation's Biological Resource Center in Japan. Fruiting bodies of *M. chlorophos* were cultivated from the mycelium at 20°C and at approximately 90% relative humidity in the dark, as previously described (14), and were then carefully harvested at stages 1 and 2. The fruiting bodies were immediately used for bioluminescence assay after harvesting or were stored in liquid nitrogen for extraction.

Extraction. The pilei at stage 2 (10 g) stored in liquid nitrogen were homogenized with 10 mL water using a Vibracell ultrasonic disintegrator (Sonics & Materials Inc, Newtown, CT, USA) in an ice bath for 30 sec, then centrifuged at 12,000 rpm at 5°C for 10 min. After separating the supernatant, the pellet was extracted twice, using 10 mL water each time. All the supernatants were combined and 10 mL methanol was added; the resulting mixture was stored in an ice bath for 10 min and centrifuged at 12,000 rpm at 5°C for 10 min. The supernatant was evaporated under reduced pressure to approximately 5 mL. The concentrate was dissolved in water to 10 mL and centrifuged at 12,000 rpm at 5°C for 10 min. The supernatant was stored at −80°C until it was used for isolation of the bioluminescence-activating component. This extraction process was also performed with 5 kg of wet pileus.

High-performance liquid chromatography photo-diode array detection (HPLC-PDA)-MS analysis of the extract was performed using the following procedure. A mixture of fresh gills (2.0 g) at stage 2 and 10 mM phosphate buffer at pH 7.0 (8 mL) were homogenized using a Vibracell ultrasonic disintegrator in an ice bath for 30 sec, then centrifuged at 12,000 rpm at 5°C for 10 min. After separating the supernatant, the pellet was extracted one more time using 10 mL water. All the supernatants were combined and 20 mL methanol was added. The resulting mixture was stored in an ice bath for 10 min and centrifuged at 12,000 rpm at 5 °C for 10 min. The supernatant was evaporated under reduced pressure to approximately 2 mL. The concentrate was centrifuged at 12,000 rpm at 5°C for 10 min. The supernatant was evaporated under reduced pressure to dryness, and the concentrate was dissolved in water to 0.2 mL and centrifuged at 12,000 rpm at 5°C for 10 min. The resulting supernatant (0.02 mL) was analyzed using the HPLC–PDA-MS system equipped with an MD-910 detector (JASCO Corp, Tokyo, Japan), a COSMOSIL 5PBr column (4.6 mm × 250 mm; Nacalai Tesque, Inc, Kyoto, Japan), and a ZQ 4000 MS spectrometer (Waters Corporation, Milford, MA, USA) using a subsequent mobile phase system-1. The HPLC mobile phase was a mixture of 10 mM aqueous formic acid (A) and CH_3CN (B). A linear gradient was achieved starting with 0% B, reaching 50% B in 30 min, and subsequently reaching 100% B in 10 min at a flow rate of 0.8 mL/min (see Fig. 2). MS analysis was carried out using electrospray ionization (ESI) in negative mode.

Isolation. The extract solution stored at −80°C was centrifuged at 12,000 rpm at 5°C for 10 min. After separating the supernatant, a mixture of the supernatant (1 L) from pilei (1 kg) and 0.1 M aqueous phosphoric acid (100 mL) was adsorbed on a column (45 mm × 170 mm) containing Chromatorex DM1020T ODS gel (Fuji Silysia, Aichi, Japan) prepared with 10 mM aqueous phosphoric acid. The elution was conducted by adding, stepwise, 0, 10, 20, 30, 40, 50 and 100% of 10 mM phosphoric acid/methanol to the mixture (each volume = 1 L). The bioluminescence-activating

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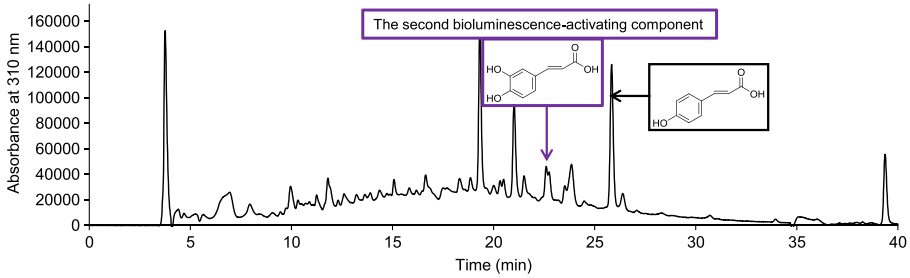

Figure 2. HPLC chromatogram of an aqueous extract (0.1 mL/1 g wet gill) from *M. chlorophos* gills at stage 2.

component existed in 20% of a 10 mM phosphoric acid/methanol eluent. The eluent was neutralized to pH = 7.0 with aqueous NaOH and evaporated to 100 mL under reduced pressure in the dark. This chromatography process was also performed for 5 kg of pileus.

A second purification of the fraction of the component obtained in the first chromatography experiment (for 50 g of pileus) was conducted using the HPLC–PDA system and a COSMOSIL 5C18-PAQ column (20 mm × 250 mm; Nacalai Tesque, Inc.) with a 10 mM aqueous sodium phosphate buffer (pH = 7.0) as the mobile phase at a flow rate of 5 mL/min. The bioluminescence-activating component was eluted after 35 and 40 min (Fig. S1A), and the fraction was evaporated under reduced pressure to near dryness in the dark. This second HPLC purification was also performed for all 20% of 10 mM phosphoric acid/methanol fractions obtained in the first purification.

The final purification of the component fraction derived from 500 g of pileus in the second chromatography run was obtained using the HPLC–PDA system and a COSMOSIL 5PBr column (20 mm × 250 mm; Nacalai Tesque, Inc.) with a 1 mM sodium phosphate buffer (pH = 7.0). The flow rate was set to 5 mL/min to decrease the phosphate salt concentration. The bioluminescence-activating component eluted between 33 and 42 min (Fig. S1B), and the fraction containing the bioluminescence-activating component was evaporated until dry under reduced pressure in the dark. This third HPLC purification was also performed for all fractions obtained in the second purification. Approximately 0.5 mg of the bioluminescence-activating component was obtained from 5 kg of wet pileus. The purity of the isolated bioluminescence-activating component was analyzed using the HPLC–PDA system and a COSMOSIL 5PBr column (4.6 mm × 250 mm) with the mobile phase system-1 (Fig. S1C).

Chemical structure

HPLC–PDA-MS spectrometry analyses of the bioluminescence-activating component and commercially available *trans*-3,4-dihydroxycinnamic acid were performed at 25°C on an HPLC system equipped with a COSMOSIL 5PBr column (4.6 mm × 250 mm). The HPLC mobile phase system-1 was used for the analysis. MS analysis was carried out using ESI in negative mode. A UV–vis absorption spectrum was obtained from the HPLC–PDA analysis of the bioluminescence-activating component (Fig. S4). The negative ESI-MS spectrum showed 178.98 $[M-1]^-$ as a molecular ion (Fig. S2). ^{1}H and ^{13}C NMR spectra of the bioluminescence-activating component and commercially available *trans*-3,4-dihydroxycinnamic acid were measured with a JNM-A500 spectrometer (JEOL, Tokyo, Japan) at 21°C operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Acetone was used as an internal standard in approximately 0.2 M sodium phosphate buffer (pD = 7.0)/D_2O; ^{1}H of acetone: 2.04 ppm, ^{13}C of acetone CH_3: 31.6 ppm. Chemical shifts and coupling constants are reported as δ/(ppm) and J (Hz), respectively; ^{1}H NMR: δ = 6.16 (1H, d, J = 15.9 Hz), 6.74 (1H, d, J = 7.9 Hz), 6.88 (1H, dd, J = 1.8 and 7.9 Hz), 6.97 (1H, d, J = 1.8 Hz), 7.10 (1H, d, J = 15.9 Hz) (Fig. S3A), ^{13}C NMR: δ115.19, 116.93, 122.15, 122.36, 128.73, 141.36, 144.90, 146.52, 176.69 (Fig. S3B).

Measurement of bioluminescence activity. The gill bioluminescence-activating capacities of each fraction in the three-step chromatography processes for the purification of the bioluminescence-activating component, the isolated bioluminescence-activating component, and the commercially available *trans*-3,4-dihydroxycinnamic acid and its analogues were measured by the following method. Phosphate buffer (10 mM, pH = 7.0; 1.5 μL) and a gill section (2 mm × 2 mm) of *M. chlorophos* at stage 1 were placed in a black 96-well plate (96/V-PP, Eppendorf AG, Hamburg, Germany). The bioluminescence intensity was measured every 1 min for approximately 20 min at 25°C using a Microplate Luminometer Centro (Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany) to confirm the steadfastness of the bioluminescence intensity. Then, 10 μL of the extract solution, each fraction solution, the solution of the isolated bioluminescence-activating component in 10 mM phosphate buffer (pH = 7.0), *trans*-3,4-dihydroxycinnamic acid solution in 10 mM phosphate buffer (pH = 7.0) or its analogue solution in 10 mM phosphate buffer (pH = 7.0) was added to the wells, and the bioluminescence intensity was measured every 1 min for more than 20 min at 25°C using a Microplate Luminometer Centro. At each time point, the intensities were accumulated for 1 sec. The bioluminescence intensities are provided as the mean of independent measurements.

Measurement of bioluminescence spectra. A gill section at stage 1 was placed in a tube, 1.5 μL of 10 mM phosphate buffer (pH = 7.0) was added, and the bioluminescence spectrum was measured at 25°C using a LumiFL-Spectrocapture (Atto Co., Ltd, Tokyo, Japan). Then, 0.01 mL of the 0.3 mM *trans*-3,4-dihydroxycinnamic acid in 10 mM phosphate buffer (pH = 7.0) was added to the tube before recording luminescence spectra. The intensities were accumulated for 30 sec.

Metabolism analysis. A pileus at stage 1, was divided into equal parts. Approximately 20 parts (2.0 g) were then incubated in 0.23 mM *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid in 10 mM phosphate buffer at pH 7.0 (8.0 mL) under $^{18}O_2$ at 25°C in the dark for 1.5 h and then the mixture was kept at −80°C. The mixture was homogenized using a Vibracell ultrasonic disintegrator in an ice bath for 30 sec, then centrifuged at 12,000 rpm at 5 °C for 10 min. After separating the supernatant, the pellet was extracted one more

time using 10 mL water. All supernatants were combined and 20 mL methanol was added. The resulting mixture was stored in an ice bath for 10 min and centrifuged at 12,000 rpm at 5°C for 10 min. The supernatant was evaporated under reduced pressure to approximately 2 mL. The concentrate was centrifuged at 12,000 rpm at 5°C for 10 min. The supernatant was evaporated under reduced pressure to dryness, and the concentrate was dissolved in water to 0.2 mL and centrifuged at 12,000 rpm at 5°C for 10 min. The resulting supernatant (0.02 mL) was analyzed using the HPLC–PDA–MS system equipped with a COSMOSIL 5PBr column (4.6 mm × 250 mm). The HPLC mobile phase system-1 was used for the analysis (see Fig. 6). MS analysis was carried out using ESI in negative mode. A mixture of the residual pileus (2.0 g) and 10 mM phosphate buffer at pH 7.0 (8.0 mL) was kept at −80 °C, and its extract solution was prepared and analyzed using the HPLC–PDA-MS system in the same manner as mentioned above (see Fig. 6). For identification of *trans*-3,4-dihydroxycinnamic acid in HPLC–PDA–MS analysis, a mixture of extract (10 μL), 0.1 mM *trans*-3,4-dihydroxycinnamic acid (5 μL), and 0.1 mM *trans*-2,4-dihydroxycinnamic acid (2.5 μL) was analyzed.

A pileus at stage 1, was divided into three equal parts. Approximately 30 parts (2.0 g) were then incubated in 0.23 mM *1,2,3–*$^{13}C_3$*-trans*-3,4-dihydroxycinnamic acid in 10 mM phosphate buffer at pH 7.0 (8.0 mL) under $^{18}O_2$ at 25 °C in the dark for 1.5 h and then the mixture was kept at −80 °C. The residual parts (2.0 g) were kept in 0.23 mM *1,2,3–*$^{13}C_3$*-trans*-3,4-dihydroxycinnamic acid in 10 mM phosphate buffer at pH 7.0 (8.0 mL) under nitrogen at 25°C in the dark for 1.5 h and then the mixture was kept at −80 °C. In addition, the residual parts (2.0 g) were kept in 8 mL 10 mM phosphate buffer (pH 7.0) at −80°C. Extract solutions for these mixtures were prepared and analyzed in the same manner as mentioned above (FigS5).

trans-4-Hydroxycinnamic acid and *trans*-3,4-dihydroxycinnamic acid were detected at 310 nm and hispidin was detected at 370 nm during HPLC-PDA analysis. The retention time of hispidin in the HPLC analytical system was approximately 27 min, and the detection-limit concentrations of hispidin in HPLC–PDA and HPLC–MS analyses in our analysis method were 100 pmol/1 g wet gill and 10 pmol/1 g wet gill, respectively.

Synthesis

***trans*-2,3-Dihydroxycinnamic acid (1) and *trans*-3,5-dihydroxycinnamic acid (2).** *trans*-2,3-Dimethoxycinnamic acid (100 mg, 0.56 mmol) or *trans*-3,5-dimethoxycinnamic acid (100 mg, 0.56 mmol) was added to 1 M BBr_3 in CH_2Cl_2 (20 mL, 20 mmol) at −80°C. The reaction mixture was kept at 20°C for 3 h; it was then poured into water and CH_2Cl_2 (20 mL) was added to it. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was crystallized with AcOEt to afford the desired demethylated products as a white solid. Melting points were determined using an ASONE ATM-01 and are uncorrected. UV–vis absorption and IR spectra were obtained using a V-530 UV-Vis spectrometer (JASCO Corp., Tokyo, Japan) and an FT/IR 410 spectrometer (JASCO Corp, Tokyo, Japan), respectively. ^{1}H and ^{13}C NMR spectra were measured with a JNM-A500 spectrometer. MS analyses were carried out using a ZQ 4000 MS spectrometer in ESI negative mode. Elemental analyses were performed with a Yanaco CHN CORDER MT-3 instrument.

Compound 1. 82% yield; mp. 210–212°C; λ_{max} /nm (ε) (10 mM phosphate buffer pH 7.0) 218 (17300), 278(15900); IR (KBr) ν_{max} 3459, 3282, 1678, 1624, 1588, 1484, 1373, 1333, 1254, 1193 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 20°C) δ/ppm 6.46 (1H, d, J = 15.9 Hz), 6.65 (1H, t, J = 7.9 Hz), 6.82 (1H, d, J = 7.9 Hz), 7.03 (1H, d, J = 7.9 Hz), 7.83(1H, d, J = 15.9 Hz), 9.07 (1H, br. s), 9.67 (1H, br.s), 12.20 (1H, br.s) (Fig. S6A); ^{13}C NMR (125 MHz, DMSO-d_6, 21°C) δ/ppm 116.63, 118.12, 118.62, 119.15, 121.49, 139.83, 145.23, 145.60, 168.10(Fig. S6B); ESI-MS *m/z*: [M – 1]$^-$ found 178.92 (calculated M for $C_9H_8O_4$ - H: 179.03); *Anal.* Found: C, 60.39; H, 5.03%, Calculated for $C_9H_8O_4$: C, 60.00; H, 4.48%.

Compound 2. 89% yield; mp. 220°C decomposition; λ_{max} /nm (ε) (10 mM phosphate buffer pH 7.0) 220 (24500), 280 (18600); IR (KBr) ν_{max} 3349, 1659, 1616, 1483, 1433, 1348, 1290, 1253, 1167, 1013 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 20°C) δ/ppm 6.28 (1H, d, J = 15.9 Hz), 6.29 (1H, d, J = 2.4 Hz), 6.46 (2H, d, J = 2.4 Hz), 7.38 (1H, d, J = 15.9 Hz), 9.46 (2H, br.s), 12.36 (1H, br.s) (Fig. S7A); ^{13}C NMR (125 MHz, DMSO-d_6, 27 °C) δ/ppm 104.64, 106.11, 118.62, 135.81, 144.45, 158.68, 167.55 (Fig. S7B); ESI-MS *m/z*: [M – 1]$^-$ found

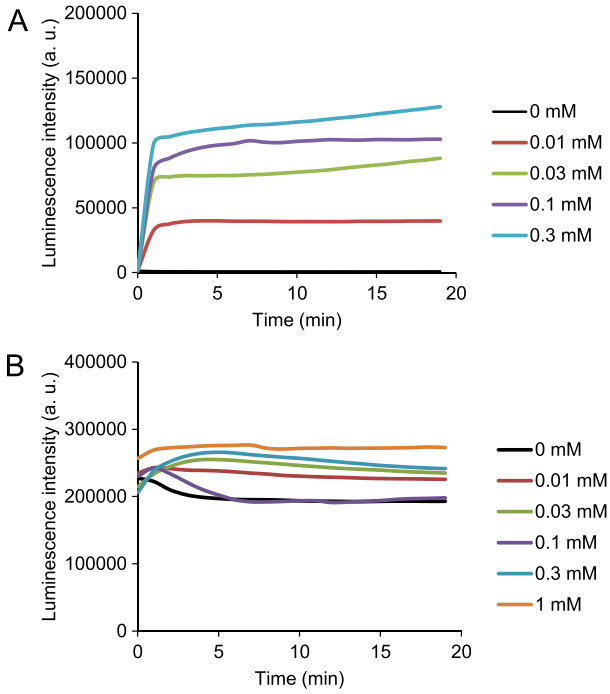

Figure 3. Effect of *trans*-3,4-dihydroxycinnamic acid on gill bioluminescence of *M. chlorophos* at stages 1 (A) and 2 (B). Gills in which the original bioluminescence intensity was approximately 5000 were used for (A) (gills used: $n > 16$).

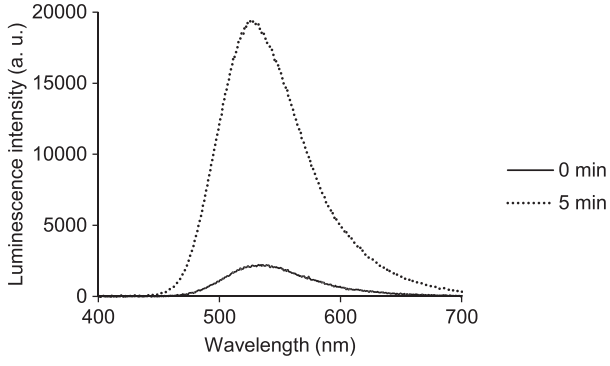

Figure 4. Bioluminescence spectra after the addition of 0.3 mM *trans*-3,4-dihydroxycinnamic acid to *M. chlorophos* gills at stage 1.

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178.92 (calculated M for $C_9H_8O_4$ - H: 179.03); *Anal.* Found: C, 60.27; H, 4.72%, Calculated for $C_9H_8O_4$: C, 60.00; H, 4.48%.

Results and discussion

Isolation

Recently, we reported a bioluminescence assay of living *M. chlorophos* gills at stage 1 in the research of bioluminescence-activating components (17). In the present study, we found the presence of a second bioluminescence-activating component in *M. chlorophos* gills at stage 2, using the bioluminescence assay method (see Experimental). HPLC-PDA analysis of an aqueous extract from the gills at stage 2 showed that the amount of the second bioluminescence-activating component was lower than that (4.2 nmol/1 g wet gill used in the present study) of *trans*-4-hydroxycinnamic acid (Fig. 2); the amount of the second bioluminescence-activating component in the wet gill is mentioned in the next section. The second bioluminescence-activating component (approximately 0.5 mg) was isolated from an aqueous extract from 5 kg of wet pilei at stage 2 using three-step chromatography (see Experimental and Fig. S1A, B). The high purity of the isolated bioluminescence-activating component was confirmed by analytical HPLC as shown in Fig. S1C.

Chemical structure

Negative ESI-MS spectrometry of the second bioluminescence-activating component indicated a molecular weight of 178.98 [M – 1]$^-$ (Fig. S2). ^{1}H and ^{13}C NMR (Fig. S3) measurements revealed *trans*-3,4-dihydroxycinnamic acid and the HPLC–PDA

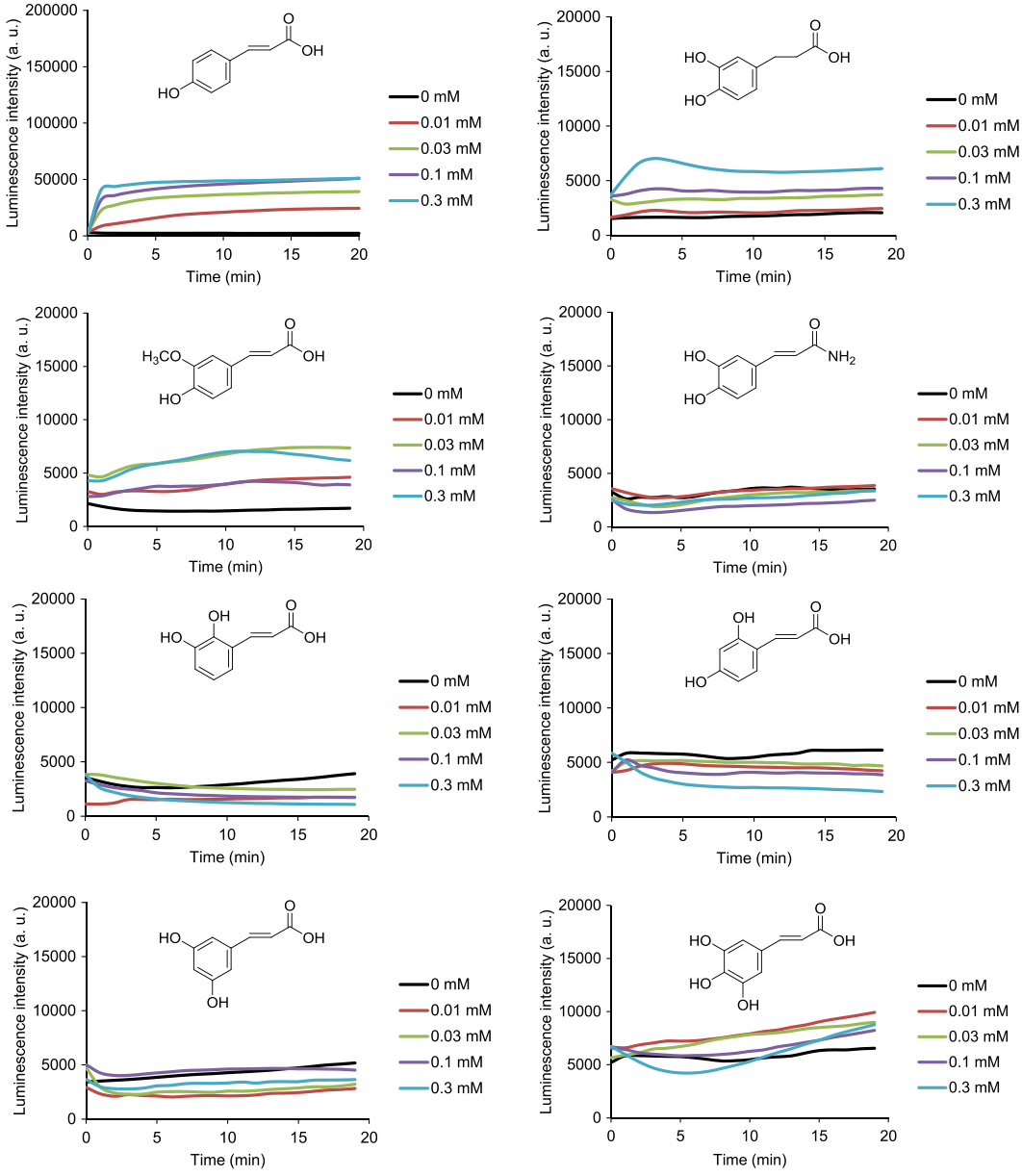

Figure 5. Effect of *trans*-3,4-dihydroxycinnamic acid analogues on the gill bioluminescence of *M. chlorophos* at stage 1. Gills in which the original bioluminescence intensity was approximately 5000 were used for this assay (gills used: $n > 8$).

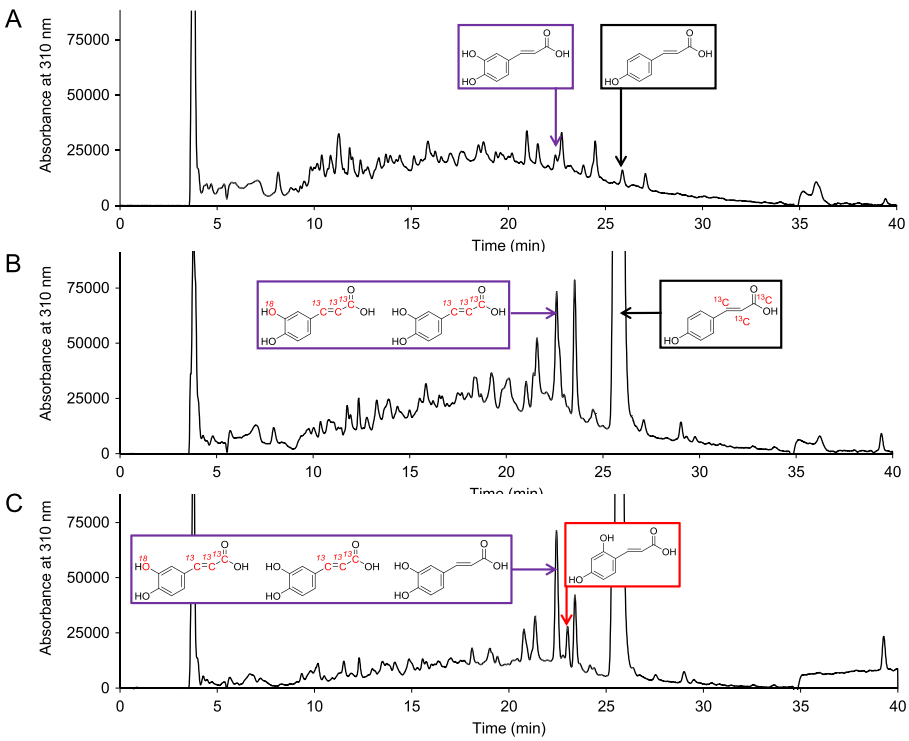

Figure 6. HPLC chromatograms of the extracts. (A) Extract (0.1 mL/1 g wet gill, 20 μL) from fresh gills at stage 1. (B) Extract (0.1 mL/1 g wet gill, 20 μL) from gills incubated with *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid under $^{18}O_2$. (C) Mixture of the extract shown in B (10 μL); 0.1 mM *trans*-3,4-dihydroxycinnamic acid (5 μL); and 0.1 mM *trans*-2,4-dihydroxycinnamic acid (2.5 μL).

chromatogram and PDA spectrum (Fig. S4) of the component were consistent with those of commercially available *trans*-3,4-dihydroxycinnamic acid, indicating that the second bioluminescence-activating component was *trans*-3,4-dihydroxycinnamic acid. The amount of bioluminescence-activating component in the aqueous extract from the gills at stage 2 used in the present study was approximately 1 nmol/1 g wet gill based on the HPLC–PDA analysis using commercially available *trans*-3,4-dihydroxycinnamic acid.

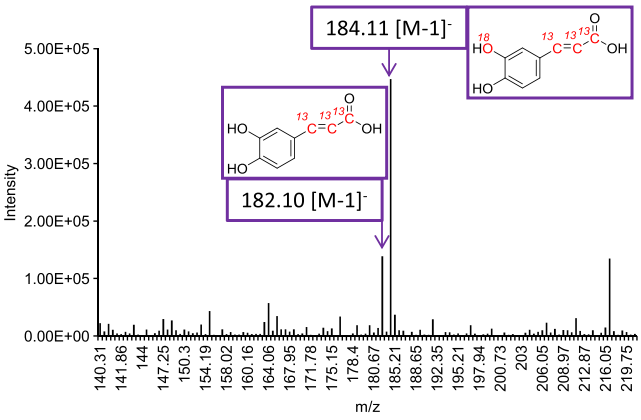

Figure 7. Negative ESI-MS spectrum of *trans*-3,4-dihydroxycinnamic acid in the extract from the gills incubated with *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid under $^{18}O_2$.

Bioluminescence activation

The addition of 0.01, 0.03, 0.1, and 0.3 mM *trans*-3,4-dihydroxycinnamic acid to the living gills at stage 1 promptly increased the bioluminescence intensity in a concentration-dependent manner (Fig. 3). The intensity reached a maximum value in 1 min after the addition. The bioluminescence spectrum, after activation by the addition of *trans*-3,4-dihydroxycinnamic acid, showed a light emission maximum wavelength at approximately 525 nm, coinciding with the original bioluminescence spectrum (Fig. 4). The addition of even 1 mM *trans*-3,4-dihydroxycinnamic acid to the living gills at stage 2 did not increase the bioluminescence intensity as well as did *trans*-4-hydroxycinnamic acid, as shown in our previous report (17). Because the contents of *trans*-4-hydroxycinnamic acid and *trans*-3,4-dihydroxycinnamic acid in gills at stage 2 were significantly higher than those at stage 1, as shown in Figs 2 and 6(A), no influence of *trans*-3,4-dihydroxycinnamic acid to the bioluminescence activities of the gills at stage 2 was attributed to the original presence of sufficient amount of the bioluminescence-activating components in the gills.

Of the various *trans*-3,4-dihydroxycinnamic acid analogues tested for bioluminescence-activating capacity, using the gills at stage 1 (Fig. 5), there was no compound that possessed bioluminescence-activating capacity. Based on the results presented in Fig. 5 and the previous result, showing that *trans*-3-hydroxycinnamic acid had no bioluminescence-activating capacity (17), *trans*-alkene and 4-hydroxy groups were necessary for the activation of gill bioluminescence, and the 3-hydroxy group in *trans*-3,4-dihydroxycinnamic acid reinforced the bioluminescence-activating capacity of the *trans*-4-hydroxycinnamic acid molecule.

3-Methoxy; 3,5-dihydroxy; and 2-hydroxy groups substituted in *trans*-4-hydroxycinnamic acid completely inhibited its activating capacity, suggesting that the hydroxyl groups substituted on the phenyl skeleton of *trans*-cinnamic acid are strongly recognized by the bioluminescence system and that *trans*-4-hydroxycinnamic acid and *trans*-3,4-dihydroxycinnamic acid affect the bioluminescence system in a highly structure-specific way.

Metabolism

Hispidin is known to be biosynthesized from *trans*-3,4-dihydroxycinnamic acid, which is generated from *trans*-4-hydroxycinnamic acid in the fungus, *Polyporus hispidus* (19–21). In the present study, biosynthesis of *trans*-3,4-dihydroxycinnamic acid in *M. chlorophos* was investigated using the fresh gills at stage 1 with labelled *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid (found *m/z* 166.13 $[M-1]^-$) and $^{18}O_2$. As shown in Fig. 6(A), there was a trace of *trans*-4-hydroxycinnamic acid and *trans*-3,4-dihydroxycinnamic acid in the gills at stage 1. The gills were incubated with 0.23 mM *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid under $^{18}O_2$ at 25 °C for 1.5 h in the dark, and the labeled products were analyzed by HPLC–PDA–MS spectrometry. It was found that the addition of ^{13}C labelled *trans*-4-hydroxycinnamic acid to the gills at stage 1 increased the detected amount of *trans*-3,4-dihydroxycinnamic acid at first (Fig. 6B) and that it carried a significant amount of the ^{18}O label (*m/z* 182.10 $[M-1]^-$/184.11 $[M-1]^-$ = 1.4:4.5) (Fig. 7). These results indicated that *trans*-3,4-dihydroxycinnamic acid was synthesized from *1,2,3–*$^{13}C_3$*-trans*-4-hydroxycinnamic acid with molecular oxygen. In contrast, *trans*-2,4-dihydroxycinnamic acid, which was expected as an oxidation product of *trans*-4-hydroxycinnamic acid, was not generated (Fig. 6B, C). This result was not inconsistent with the result that *trans*-2,4-dihydroxycinnamic acid was not detected in the extract mixture analyzed by HPLC shown in Fig. 2. In addition, hispidin was not detected in the extract of the incubated gills: detection-limit concentrations of hispidin in HPLC–PDA and HPLC–MS analyses in our analysis method were 100 pmol/1 g wet gill and 10 pmol/1 g wet gill, respectively.

In addition, the biosynthesis of hispidin from *trans*-3,4-dihydroxycinnamic acid was investigated by incubation of the fresh gills at stage 1 with 0.23 mM labelled *1,2,3–*$^{13}C_3$*-trans*-3,4-dihydroxycinnamic acid under $^{18}O_2$ at 25°C in the dark for 1.5 h. Hispidin was not observed in the extract of incubated gills (Fig. S5B). According to Purtov's report, hispidin is used in the hydroxylation and oxidation metabolism in the presence of molecular oxygen. Thus, to accumulate hispidin (if hispidin could be biosynthesized), incubation under nitrogen was performed. Now, hispidin was not detected in HPLC–PDA-MS analysis (Fig. S5C). Because the detection-limit concentration of hispidin was 10 pmol/1 g wet gill in our HPLC–MS analysis method, these results indicated that more than 10 pmol hispidin/1 g wet gill was not accumulated via the biosynthesis from *trans*-3,4-dihydroxycinnamic acid in the gills at stage 1. Moreover, it was shown that the addition of 0.01 mM hispidin to the living gills at stage 1 generated no bioluminescence activation as well as demonstrated in our previous report (17). Thus, these result indicated that if less than 10 pmol hispidin/1 g wet gill would be accumulated via the biosynthesis from *trans*-3,4-dihydroxycinnamic acid, the prompt bioluminescence activation resulting from the addition of *trans*-3,4-dihydroxycinnamic acid should not be attributed to the generation of hispidin.

Conclusion

We found a second bioluminescence-activating component, *trans*-3,4-dihydroxycinnamic acid in *M. chlorophos* gills at stage 2. This component promptly and highly structure-specifically increased the bioluminescence intensity of the living gills at stage 1. Moreover, incubation experiments revealed that this component was synthesized from *trans*-4-hydroxycinnamic acid in the gills as well as by the general metabolism and that hispidin (detection-limit concentration: 10 pmol/1 g wet gill) was not accumulated via the biosynthesis from *trans*-3,4-dihydroxycinnamic acid in the gills in the presence or absence of molecular oxygen. Addition of 0.01 mM hispidin to the living gills at stage 1 generated no bioluminescence activation. These results suggested that the prompt bioluminescence activation resulting from addition of *trans*-3,4-dihydroxycinnamic acid could not be attributed to the generation of hispidin. We are now aiming to elucidate the mechanisms of bioluminescence activation by *trans*-3,4-dihydroxycinnamic acid and *trans*-4-hydroxycinnamic acid to chemically understand the bioluminescence phenomenon.

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