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Lambertellin system, the mechanism for fungal replacement of *Monilinia fructigena* with *Lambertella corni-maris* without competitive inhibition on agar media

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ABSTRACT

The 'Lambertellin system' was disclosed which rationally explains the fungal replacement (mycoparasitism) of *Monilinia fructigena* (*M. f.*, the host) with *Lambertella corni-maris* (*L. corni-maris*, the parasite) without competitive inhibition in the simultaneous incubations on agar media. The 'Lambertellin system' involves; (a) *L. corni-maris* secretes nontoxic lambertellols (**1**, **2**) as the diffusible precursors of the authentic responsible substance **3** regardless of existence of the host *M. f.*; (b) In the absence of the host, the environment around the parasite is kept under neutral condition, and both **1** and **2** are readily transformed into **3**; (c) Lambertellin (**3**) inhibits not only the host but also the parasite. The parasite degrades **3** for detoxification; and (d) Upon the host *M. f.* approaching closely to the area where the parasite inhabits, the environment around the parasite becomes acidic to stabilize **1** and **2**, which gives them a chance to diffuse into the host area. Then these are gradually transformed into **3** to inhibit the host without damaging the parasite. This mechanism also accords with the progress of 'Natsu-Nenju' disease on apple fruits, which is known to be a mysterious phyto-disease because of two unique stages of its lifecycle, anamorphic (asexual) and teleomorphic (sexual). The 'Lambertellin system' would be categorized as a novel class of alleropathies.

1. Introduction

In 1990, Harada, one of the present authors, found that the filamentous fungus *Lambertella corni-maris* (*L. corni-maris*) conquers the other phytopathogenic fungus *Monilinia fructigena* (*M. f.*) without remarkable competitive inhibition in the simultaneous cultivation on a PSA (potato-sucrose-agar) medium.[1] Although these two fungi were smoothly intermixed on the basis of visual observation, only *L. corni-maris* was isolated from any place of the medium after several weeks. Harada also reported that the replacement also occurred between these fungi on apple fruits. Since the symptom on the apple fruits quite resembled 'Natsu Nenju' disease, he concluded that this fungal replacement should be the identity of the disease and that these phenomena would be an example of mycoparasitism. 'Natsu Nenju' disease used to be explained by a phytopathogen *Seclerotinia phaeospora* Hori, which featured unique anamorphic (asexual) and teleomorphic (sexual) stages,[2,3] but details of this fungus have remained unknown. Harada's study consequently denied *Seclerotinia phaeospora* Hori. He suggested that Hori misunderstood the symptom with the fungal replacement.

In the course of the bioorganic investigations on this mycoparasitism, we have isolated instable metabolites lambertellols A (**1**) and B (**2**) as candidates responsible for this phenomenon (Fig. 1).[4,5] Considerable amounts of these compounds were yielded from the culture broth, when *L. corni-maris* (parasite) was incubated in the presence of *M. f.* (host). Interestingly, incubation of

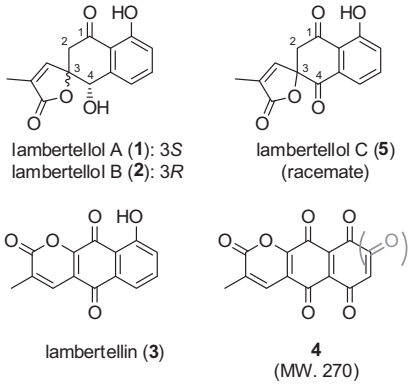

Figure 1. Structures of lambertellols A (**1**), B (**2**), lambertellin (**3**), lambertellol C (**4**) and putative degradation product **5**.

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L. corni-maris alone provided only trace amounts of these metabolites.[6] We found that acidification of the medium to pH of around 4 dramatically increased the yield of both **1** and **2** even in the absence of the host *M. f.* We also revealed that the host made its surroundings remarkably acidic, while the parasite *L. corni-maris* kept the conditions around neutral.[6] On the bases of these findings, we had proposed that acidic conditions stimulated their biosynthesis. Recently, we accomplished a total synthesis of **1** and **2**,[7] which enabled us to utilize their enantiomers and a series of stable analogs for biological experiments. These led to a different conclusion that *L. corni-maris* excretes **1** and **2** as the diffusible precursors which readily transformed into the authentic responsible lambertellin (**3**) in a non-enzymatic manner after reaching to the area of host *M. f.*

However, these were insufficient to rationally explain the progress of the fungal replacement without competitive inhibition. These are;

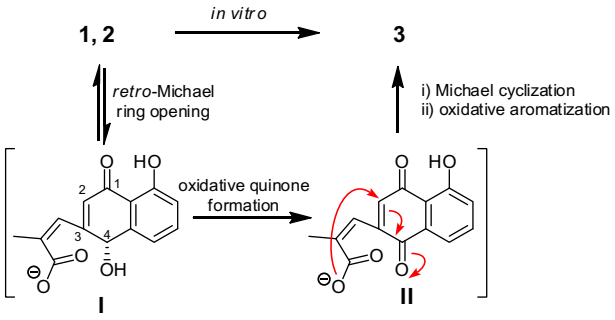
(1) The transformation of **1** and **2** should accumulate **3**, but this compound was always detected in a much lower level than **1** and **2**.
(2) The reason why acidic conditions increase the yield of **1** and **2** has remained unknown.
(3) It was unclear why *L. corni-maris* needed to produce **1** and **2** as precursors of the real toxic substance **3**.

Our continuous research revealed that the total mechanism named 'Lambertellin system' can rationally explain the above questions. The 'Lambertellin system' ingeniously controls the concentration of **3**, the authentic toxic substance not only for the host but also for the parasite. Although we had previously concluded that the host stimulates biosynthesis of **1**–**3**, the present study proved that the biosynthesis of these metabolites functions constantly regardless of the existence of the host.

2. Results and discussions

We employed two species of mycoparasites *Lambertella corni-maris* (*L. corni-maris*) and *Lambertella* sp. 1346 (*L.* sp. 1346) in the experiments. These had been isolated from apple fruits showing the similar symptom. Although the former fungus leads the fungal replacement without competitively inhibiting the host *Monilinia fructigena* (*M. f.*) in the simultaneous cultivation on agar gel, the latter simply results in competitive inhibition. Lambertellols (**1**, **2**) were first isolated from the culture broth of the latter fungus.[5] In contrast, the former yields **1** and **2** in only trace amounts under regular culture conditions. Interestingly, considerable amounts of **1** and **2** were yielded, when the former was cultured in the presence of the host *M. f.* or cultured under acidic conditions. We have also disclosed that *M. f.* makes its surroundings acidic (around pH 3.1).

In the early stage, we had excluded lambertellin (**3**) as the candidate responsible for the fungal replacement, because **3** gave only a small inhibition zone against the *Monilinia fructicola,* a model fungus of *M. f.*,[8] in the paper disk assay on agar media. However, we understood the inhibition was due to **3** after the structure–activity relationship studies employing a series of our synthetic analogs.[7] These studies suggested that lambertellols (**1**, **2**) are diffusible precursors of the authentic responsible substance **3**. Lambertellin **3** was readily crystallized even in agar gel to give rise to a low concentration of **3**, which resulted in the small inhibition zone. Transformation of lambertellols into **3** readily proceeded in vitro for example in methanol and on PSA medium.[7] That can be explained by the sequential reactions of (i) *retro*-Michael type butenolide opening affording putative intermediate **I**, (ii) autoxidation into more stable naphthoquinone **II**, (iii) Michael type re-cyclization but at C2, and (iv) oxidative aromatization, as shown in Scheme 1. This mechanism also explains the interconversion between **1** and **2** that we observed on silica gel TLC.[5]



Scheme 1. Mechanism of the transformation of **1** and **2** into **3**.

2.1. Acceleration of the transformation from lambertellols into lambertellin by increment of pH

We first investigated the conditions which contribute to the transformation of **1** and **2** into **3**. Production of **3** was monitored with its characteristic UV absorption at 430 nm (ε 6800). Since we had experienced prompt equilibration between **1** and **2**, only major **2** was employed in the experiments. Formation of **3** was slow, when **2** was dissolved in acidic aqueous solutions (200 μg/mL, pH 4.5 and 5.5) as shown in Figure 2. However, that became remarkable at pH 6.5 and 7.5. Increment of UV absorption at 430 nm seemed to be stopped at around $A = 1.5$ after 30 min in these experiments. That was due to saturation of **3**, that is, precipitation of **3** occurred. These indicated that increment of pH induced withdrawal of the proton at C2 in **2** to trigger the *retro*-Michael type ring opening and the following reactions, giving **3**. The pK_a value for 2-H is around 6.0 based on those results. Since the transformation involves irreversible steps (the quinone formation and the aromatization in Scheme 1), the reverse-transformation (**3**→**2**) does not proceed.

These accorded with our precedent results that we detected considerable amounts of **1** and **2** from *L. corni-maris* under acidic conditions such as cultivation with acids and simultaneous cultivation with the host *M. f.* The host made its surroundings remarkably acidic, while *L. corni-maris* kept the medium around pH 6 under regular culture conditions. We have already disclosed that the other parasite *L.* 1346 acidifies the circumstance and constantly provides lambertellols in practical yields.[6] Above results revealed that the acidity in the culture medium attributes to the stabilization of **1** and **2** to play an important role in the yield of them.

2.2. Biodegradation of 3 by the parasites

Prompt transformation of **1** and **2** into **3** suggested that incubation of *L. corni-maris* accumulates **3**. However, **3** was yielded less than 1/10 of **1** or **2**, as far we investigated. Furthermore, some of **3** must be artificially generated from **1** and/or **2** during isolation process by taking their lability into account. Thus, **3** should be always in trace amounts in the *L. corni-maris* culture medium. Although **1** and **2** were more stable in the culture medium of *L.* sp. 1346 because of acidic conditions, Figure 2 suggests the transformation was not blocked completely. Accumulation of **3** should occur also in *L.* sp. 1346 culture medium. But that was not observed, either.

To overcome the above inconsistency, we assumed a biodegradation of **3** by the responsible fungi, *L. corni-maris* and *L.* sp. 1346 themselves. In this series of experiments, we first used *L.* sp. 1346

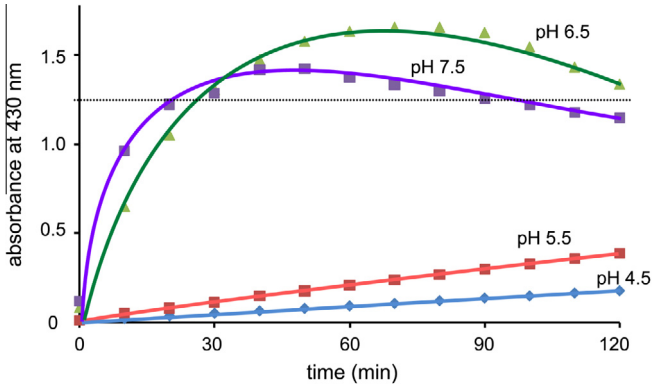


Figure 2. Production of lambertellin (**3**) from lambertellol B (**2**) monitored with UV 430 nm.

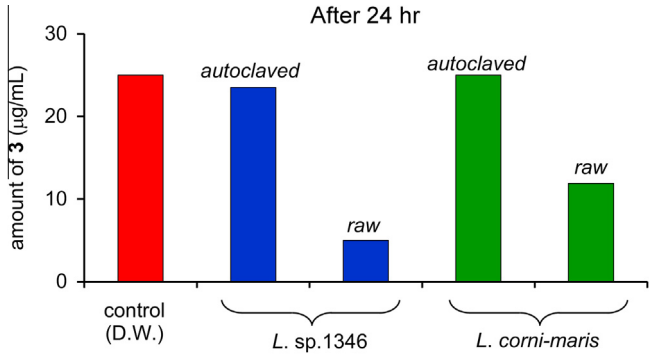


Figure 3. Biodegradation of **3** (initially 25 μg/mL) by *Lambertella* spp.

because this fungus makes the surroundings acidic to minimize *denovo* **3**. The remaining **3** was quantified with UV at 430 nm. Although **3** is a chemically inert molecule, the amount of **3** dramatically decreased after 24 h, as shown in Figure 3, when **3** (25 μg/mL) was incubated with *L.* sp. 1346 at room temperature. The amount of **3** was constant within experimental error, when that was incubated with an autoclaved *L.* sp. 1346 culture medium. A similar tendency was observed by employing *L. corni-maris*. The rate of decrement depended on the amount and the conditions of the fungi, and it was technically difficult to control them completely. Thus, these experiments were insufficient for detailed quantitative discussions. But, the decrement by *L. corni-maris* was slower than that by *L.* sp. 1346 with reproducibility. We assume that *denovo* **3** in the case of *L. corni-maris* might be one of the reasons. As described, these experiments revealed that *L.* sp. 1346 and *L. corni-maris* biologically degrade **3**. As a result of biodegradation, **3** becomes nearly invisible in the culture broths.

2.3. Toxicity of lambertellin against the producer *L. corni-maris*

The next query arose as to why the parasites needed to decompose **3** although that was synthesized by themselves. Our previous studies disclosed that **3** is the authentic toxic substance for the host *M. f.*[7] We assumed that **3** is also toxic for the parasites *Lambertella* spp.

With the above assumption, we investigated the growth rate of *L. corni-maris* in the presence of **3**. Technically, it was difficult to estimate the growth curves of filamentous fungus *Lambertella* spp. by way of optical manners. Thus we investigated that by estimating the relative colony volumes by the following approximation;

$$\text{relative colony volume} = \{\sqrt{(\text{rel.}diameter_{major}) \times (\text{rel.}diameter_{minor})}\}^3$$

The $diameter_{major}$ and $diameter_{minor}$ were obtained by measuring the oval shaped colonies with a ruler through the bottom of the culture flasks. Since *Lambertella* species degrade **3** as described in the preceding section, **3** was supplemented every 24 h as the DMSO solution (run 3, run 4) to maintain concentration. Blank DMSO was added into a set of control flasks every 24 h. Quantity of supplemental **3** (0.6 mg for 100 mL culture medium) was settled based on the preliminary experiments. The total amount of **3** in test flasks decreased because of the biodegradation, and *denovo* **3** slightly increased in the control flasks as the culture proceeded. However, supplement **3** made its concentrations more than 500 times of those in the control flasks throughout the experiments. Even just before boosting, concentrations of **3** in test flasks were 25–200 times larger than those in the controls.

As shown in Figure 4, *L. corni-maris* grew well without supplemental **3**. Addition of blank DMSO (100 μL/100 mL medium, every 24 h) did not affect the colony growth (run 2). On the other hand, supplementation of **3** significantly inhibited the growth (run 3, run 4). Interestingly, *L. corni-maris* resumed growing, when the media were refreshed with the regular one after 48 h (run 4). We also performed similar experiments under acidic conditions. However, acidification led to scattering results probably due to partial colony collapses. Experiments with *L.* sp. 1346 suggested that this fungus was found to be less susceptible for **3** to lead the results less remarkable. However, the experiments at higher concentrations (5.0 mg in 100 mL medium) exhibited obvious inhibition. The results are shown in Supplemental data. These findings indicated that **3** is toxic not only against the host *M. f.* but also the *Lambertella* spp. even those are the responsible fungi. Thus, we concluded that *Lambertella* spp. need to degrade toxic **3** to keep its concentration at low level.

2.4. Constant production of 3 by the parasite *L. cornimaris*

As described, the mycoparasitism between the parasites *Lambertella* spp. and the host *M. f.* involves sequential processes of; (i) biosynthesis of precursors **1** and **2**, (ii) transformation into the authentic active substance **3**, and (iii) degradative detoxification of **3** by the producer *Lambertella* spp.

We next investigated whether *L. corni-maris* operates the processes constantly (constant production hypothesis) or only temporally when the host *M. f.* co-inhabits (temporal production hypothesis). We have experienced remarkable increments of **1**

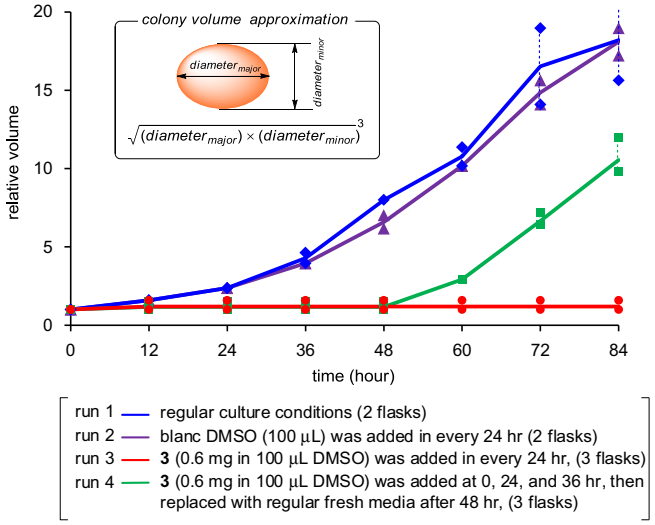


Figure 4. Influence of external lambertellin (**3**) to *L. corni-maris*.

and **2**, when *L. corni-maris* was simultaneously cultured with the host *M. f.* This might support the temporal operation hypothesis, that is, the host might stimulate their productions. However, that can be also explained by the constant operation hypothesis by supposing that the rate of the process (iii) is larger than that of both processes (i) and (ii).

We studied this subject with mass spectral analyses by utilizing ^{13}C isotope labeled **3**. We have already developed a method preparing ^{13}C labeled **1** and **2** with a high incorporation level (>40% average incorporation).[9] The labeled samples thus prepared afforded characteristic isotopologue distribution patterns of molecular (and fragment) ions in the mass spectra. We expected that biodegradation products should preserve the isotopologue distribution, when the parasite was cultured with ^{13}C labeled **3** in such a high incorporation level. We planned to explore the biodegradation substances with a guidance of the isotopologue distribution patterns after incubation with ^{13}C labeled **3**.

The ^{13}C labeled **3** was successfully prepared from ^{13}C labeled **1** and **2** on silica gel, and was added into the culture broth of *L.* sp. 1346. This fungus was chosen to minimize dilution with *denovo* non-labeled **3**. After incubation for 40 min, the GC total ion chromatogram (TIC) of the broth provided several small signals along with a large one due to **3**. A detailed analysis revealed that a signal appearing at 13.5 min reflected the isotopologue distribution pattern of the ^{13}C labeled **3** as shown in Figure 5, although there was some dilution with non-labeled *denovo* **3**. The experiment with non-labeled **3** gave the signal with a simple isotopologue profile. Thus, we assigned that the GC signal at 13.5 min was one of biodegradation products derived from **3**. The mass number *m*/*z* 270 suggested that this compound would be *o*- or *p*-quinone **4**, an oxidized product form of **3**. The SIC at *m*/*z* 270 gave another signal at 15.4 min. However that did not correspond to the biodegradation products, because the isotopologue distribution was identical between those obtained after incubations with the labeled and non-labeled **3**.

Next, the GC signal at 13.5 min was explored in the culture broth of *L. corni-maris* without supplemental **3**. This parasite gives **3** only in trace amounts under the condition, the signal of **3** was, however, the biggest GC signal in the TIC. Although the target signal was hard to recognize in the TIC, the SIC at *m*/*z* = 270 showed that clearly. Lambertellols A and B (**1**, **2**) also existed in the broth, but these were decomposed into **3** in the GC instrument. Thus, those were not seen in the GCMS.[10] These results indicated that **3** is produced constantly, even it is almost invisible. Metabolites

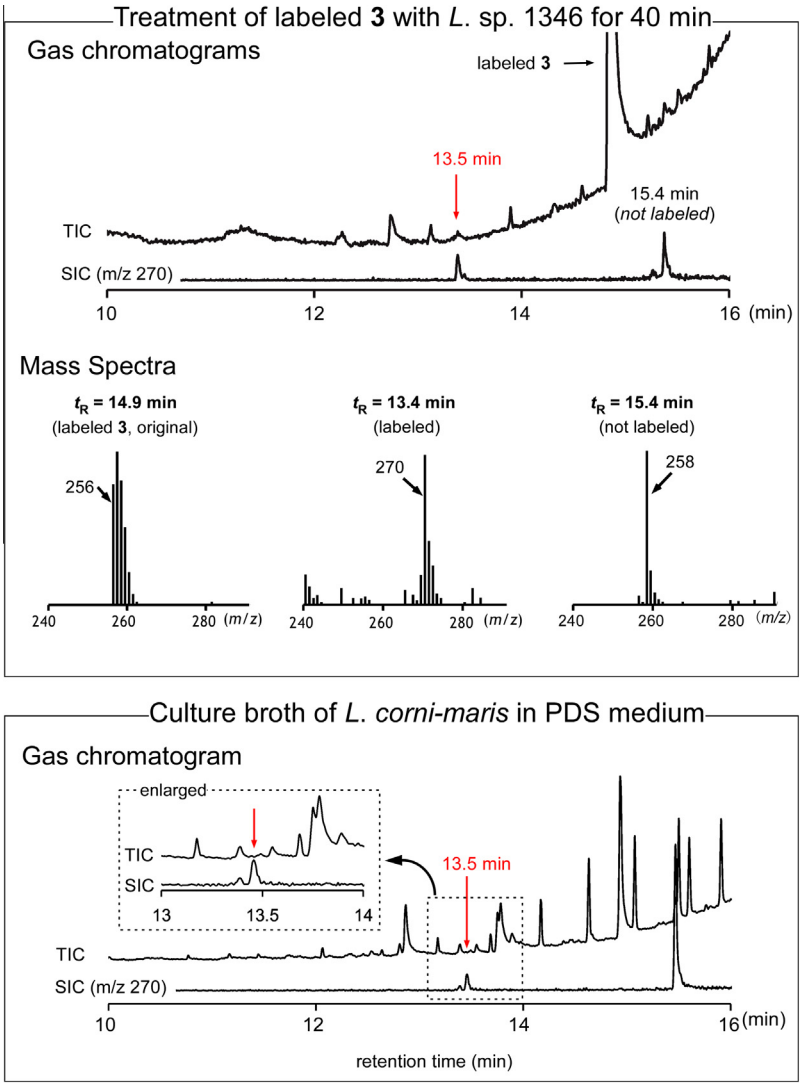


Figure 5. TIC and SIC (*m*/*z* = 270) chromatograms of *L.* sp. 1346 culture medium after treatment of labeled **3**, the molecular ions (t_R = 13.4, 14.9, and 15.4 min), and those of *L. corni-maris* prepared by regular cultivation.

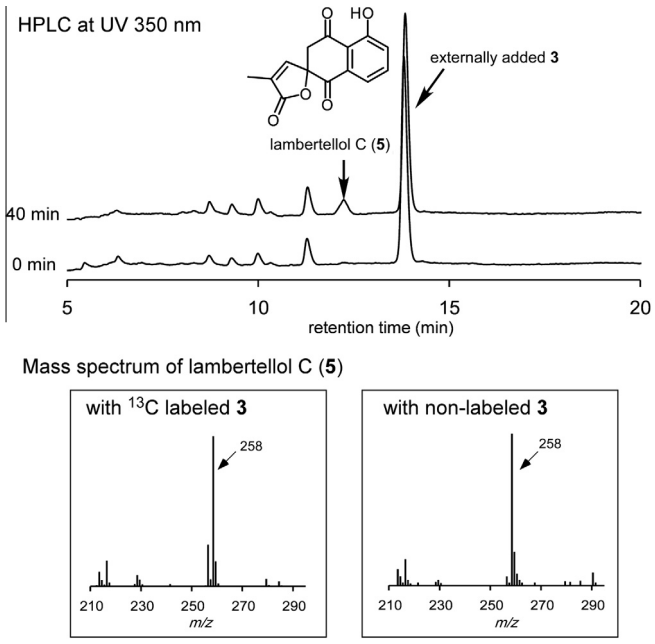


Figure 6. Increment of lambertellol C (**4**) by incubation with external **3** for 40 min and its mass spectra under labeled and non-labeled conditions.

1-**3** are in trace amounts as a result of dynamic equilibrium among the processes (i), (ii), and (iii). Thus, the temporal production hypothesis was experimentally denied.

In early stage, we performed the experiments with UV detected HPLC to observe a remarkable increment of lambertellol C (**5**)[11] upon the addition of **3** into the *L.* sp. 1346 culture medium, as shown in Figure 6. This peak was confirmed with the retention time as well as the ^{1}H NMR spectrum after isolation. These results might imply a biological transformation of **3** into **5**. However, that was the reverse conversion of that we observed in vitro as shown in Scheme 1, and hard to understand based on chemistry, because that transformation involves both enthalpically and entropically disfavored reductive dearomatization step. This transformation (**3** → **5**) was denied experimentally because incubation of *L.* sp. 1346 with ^{13}C labeled **3** did not affect the isotopologue distribution of **5** in the EI mass spectra. The mass spectruma of **5** was obtained by the direct ionization method after isolation. The GCMS conditions totally decomposed **5** to give **3**. Although HPLC provides the signal of **5** without remarkable decomposition, unfortunately the LC-ESI and LC-APCI methods were less sensitive for **1**–**3** and **5**, as far we investigated.

Formation of **4** can be explained by an oxidation of **1** and/or **2**. We presumed that the addition of toxic **3** stimulated secretions of oxidative enzymes in order to detoxify it, and one of those gave rise to the oxidation of *denovo* **1** and/or **2** to provide **5**. This accorded with the putative structure **4**, because that is also an oxidant form of **3**.

2.5. The 'Lambertellin system'

Comprehensive analyses of these results revealed a total mechanism of this fungal replacement on the agar media without competitive inhibition as described below. This phenomenon cannot be explained simply by secretions of antibiotics Hence we named the "Lambertellin system" which is comprised of;

(a) *L. corni-maris* secretes nontoxic lambertellols (**1**, **2**) as the diffusible precursors of the authentic responsible substance **3** regardless of existence of the host *M. f.*

(b) In the absence of the host, the environment around the parasite *L. corni-maris* is kept under neutral condition, and both **1** and **2** are readily transformed into **3**.

(c) Lambertellin (**3**) inhibits not only the host but also the parasite. The parasite decomposes **3** for detoxification.

(d) Upon the host *M. f.* approaching closely to the area where the parasite inhabits, the environment around the parasite becomes acidic to stabilize **1** and **2**, which gives them a chance to diffuse to the host area. Then these are gradually transformed into **3** to inhibit the host.

The 'Lambertellin system' makes **1**–**3** nearly invisible in the regular *L. corni-maris* culture broth, while that works constantly. In the case, the host is far enough from the parasite, **3** is kept at a low concentration around host because of its less diffusible property as well as prompt degradative detoxification by the parasite itself. Thus, the host can grow freely. In personification, the host cannot be aware of the parasite as shown in Figure 7. When the host approaches closely, the environment around the parasite becomes acidic to make **1** and **2** stable, which gives them a chance to diffuse into the host area. After reaching, these are gradually decomposed into **3** to inhibit the host. Since the diffusion rate of the acidic area is nearly equal to that of the host growth on agar gel,[6] we do not observe competitive inhibition in the simultaneous cultivations. The mechanism as described explains the fungal replacement on agar media in all respects. The 'Lambertellin system' enables to keep the authentically toxic **3** at a low concentration, which minimizes damage of the parasite.

2.6. Discussions about the fungal replacement on apple fruit

L. corni-maris conquers *M. f.* also on the apple fruits in the fields. Harada, one of the present authors, reported that this would be the real progress of 'Natsu-Nenju' disease which had been reported by Hori in 1912.[1–3] Finally, we discuss this phenomenon based on the results we obtained. The skin of apple fruits must have tolerance against pathogenic microorganisms. Thus, *Lambertella* spp. cannot get into healthy apple fruits. Since the host *M. f.* is also known as the pathogen of Monilia disease, a serious one in apple production in the early spring, *M. f.* might be somehow more infective to the fruits and it can inroad solely while the detailed infection route has remained unknown. Propagation of *M. f.* in the apple fruits reduces the defense system of the skin, which allows intrusion by the parasite. Then the intruder *Lambertella* spp. exterminates the former occupant *M. f.* by using **3**.

Mycoparasitisms are usually observed in soil.[12] It is not necessary for *Lambertella* spp. to utilize chemicals of the host *M. f.* because of the phenomenon in nutritious fruits. We assume that co-inhabitation with *M. f.* is inconvenient for *Lambertella* spp. due to some reason. So, they need to wipe out the former occupant *M. f.* Both *Monilinia* and *Lambertella* species belong to sclerotiniaceae family, that is, they are genetically close to each other. Due to their resembling biological system, *Lambertella* spp., probably, could not obtain substances giving damage specifically to *M. f.* *Lambertella* spp. overcame this inconvenience by acquiring such ingenious 'Lambertellin system' in their revolution. This system enables *Lambertella* spp. to inhibit the host *M. f.* with minimum damage for themselves.

3. Conclusion

As described, we succeeded in disclosing the 'Lambertellin system' which rationally explains the fungal replacement of *M. f.* with *L. corni-maris* on agar media. This mechanism also accords with the fungal replacement on apple fruits. 'Lambertellin system'

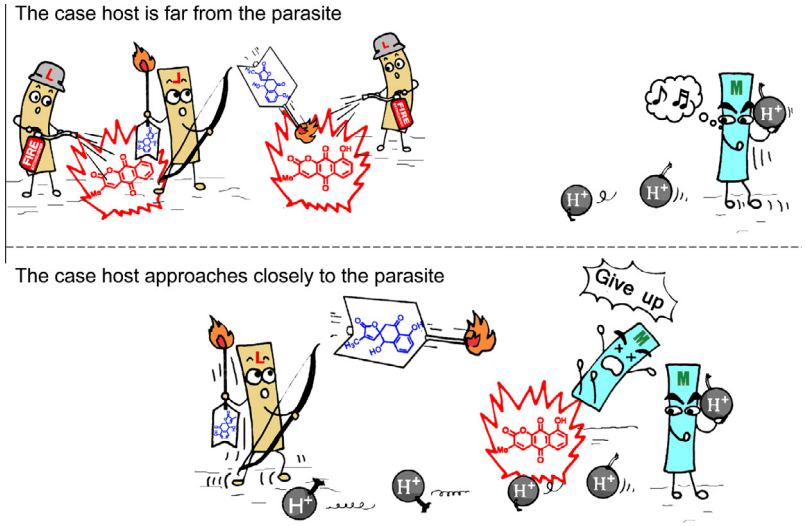


Figure 7. The lambertellin system disclosed in the present study.

would be categorized as a novel class of alleropathies. Although productions of **1** and **2** should be coded in gene, the authentic responsible **3** is not controlled by the genetic system. The acidity around the parasite governs its production. Thus, only bioorganic investigations could dissolve the mechanism in this particular phenomenon.

4. Experimental

4.1. General

L. corni-maris, *L.* sp. 1346, and *M. f.* were provided from Yukio Harada of Hirosaki University. Isotope labeled and non-labeled lambertellols A (**1**) and B (**2**) were prepared with established procedure.[4,5,9] HPLCs were performed with SunFire C_{18}® (3.5 μm 4.6 × 100 mm) employing a Waters 1525 Binary Pump equipped with a Waters 996 Photodiode Array Detector. Measurements of GCMS were performed with a RESTREK Rtx®-1 (0.25 mm × 30 m) using SIMADZU GCMS-QP2010 gas chromatography mass spectrometer.

4.2. Non-labeled lambertellin (3)

Lambertella sp. 1346 extracts (51 mg) were prepared by the established procedure employing PS medium (1.0 L) and the following extraction with EtOAc.[4] The crude extracts were loaded onto a silica gel (Merck 7734) column. Slow development with EtOAc/hexane (70:30) gave non-labeled lambertellin (**3**, 6.0 mg). The ^{1}H NMR spectrum of the sample was identical to that of authentic sample. These operations were repeated when we needed.

4.3. In vitro transformation of 2 into 3

Lambertellol B (**2**, 300 μg) in DMSO (50 μL) was added into pH 5.5 acetate buffer solution (3.0 mL) and pH 6.5 phosphate buffer solutions. After each solution was kept at room temperature for 24 h, the absorbance at UV 430 nm was measured. The similar experiments were performed with pH 4.5 acetate buffer and pH 7.5 phosphate buffer solutions. Obvious yellow colorization was observed in pH 6.5 and 7.5 solution after the experiments. Decomposition of **2** with 330 nm was also examined, but to give unclear curves, because both **2** and **3** absorb the UV light at 330 nm.

4.4. Decomposition of 3 by the parasites

Five media (200 mL) in 500 mL baffled flasks were prepared; those contained (i) culture broth that *Lambertella.* sp. 1346 was cultured for 4 days (×3), (ii) the culture broth that *Lambertella.* sp. 1346 was cultured for 4 days and then autoclaved (×1), and (iii) distilled water (×1). Lambertellin (**3**, 5.0 mg) in acetone (2.0 mL) was added into each flask. After stirring (100 rpm) at room temperature for 24 h, each culture broth was filtered, and extracted with AcOEt. Each extract was independently dried over $MgSO_4$, filtered and then concentrated in vacuo. Then each residue was diluted again with acetone (2.0 mL) and subjected to HPLC. Recovered **3** was quantified by comparing the peak area at UV 430 nm with that of the standard solution (5.0 mg of **3** in 2.0 acetone). Experiments with *L. corni-maris* were also performed.

4.5. Inhibition of *L. corni-maris* by addition of external 3

Colonies of *L. corni-maris* were prepared in a 200 mL Erlenmeyer flask with established procedure but stirred at 60 rpm. Each colony (ca. 0.7 cm) was transferred into a 100 mL flask and filled with potato-sucrose medium (100 mL). Totally 10 flasks were prepared. These flasks were gently shaken (50 rpm) at 24 °C. For the first two flasks, nothing was added (run 1). Into the second two flasks, blank DMSO (100 μL) was added every 24 h (run 2). Into the third three flasks, **3** (0.6 mg in 100 μL DMSO) was added at 0, 24, 36, and 60 h. Into the last three flasks, **3** (0.6 mg in 100 μL DMSO) was added at 0 and 24 h. For the last three flasks, culture media in these flasks were removed by decantation after 48 h and then filled with fresh regular PS medium (100 mL).

For every 12 h, the major and minor diameters of colonies were measured from the bottom of the flasks with a ruler. The relative volumes were estimated by the following approximation;

$$\text{relative colony volume} \fallingdotseq \frac{\sqrt{\{(diameter_{major}) \times (diameter_{minor})\}}^2}{\sqrt{\{(\text{initial}\, diameter_{major}) \times (\text{initial}\, diameter_{minor})\}}^3}$$

When considerable colony collapses were observed, the experiment was stopped. The similar experiments employing *L.* sp. 1346 with **3** (0.6 and 5.0 mg in 100 mL media) were also performed. The results are shown in the Supplemental data.

4.6. ^{13}C Labeled 3

Colonies of *Lambertella* sp.1346 (ca. 5 × 5 mm ca. 20 colonies) were suspended with distilled water (15 mL) and the fungus was recovered after centrifugation (4000 rpm, 10 min). The recovered hyphae were filled again with distilled water (15 mL), suspended, centrifuged (4000 rpm, 10 min), and then recovered. The recovered hyphae were suspended with PS medium (200 mL) containing sodium [1-^{13}C]acetate (20 mg). After the resulting broth was cultured for 2 days at 24 °C, MeOH (200 mL) was added. The resulting suspension was filtered in suction and the filtrate was concentrated in vacuo until the whole volume became 150 mL. The resulting solution was extracted with AcOEt, washed with brine, dried over $MgSO_4$ and concentrated in vacuo to give the crude residue (9.8 mg). The residue was loaded onto a relatively large amount of silica gel column (20 g). Slow development with EtOAc/hexane (70:30) gave **3** (5.8 mg) in almost pure state based on the ^{1}H NMR spectrum. Since, GC analysis of the sample suggested some small impurities, a part of sample was further purified with HPLC under the established procedure to afford pure **3** (0.7 mg). The incorporation rate (ca. 40%) of the sample was checked with GCMS.[9]

4.7. Exploration of the degradation products from 3

The ^{13}C labeled **3** (1.0 mg in 0.5 mL, DMSO, ca. 40% incorporation based on the isotopolgue distribution) was added into *L.* sp. 1346 culture medium (200 mL) and the medium was incubated for 40 min. Culture broth (2.0 mL) was sampled and extracted with ethyl acetate (20 mL). After concentration, the residue was diluted with MeOH (1.0 mL) and then subjected to GCMS (conditions: Rtx®-1, 0.25 mm × 30 m, 100 → 320 °C, 20 °C/min and then 320 °C for 10 min). The extracts were also analyzed with HPLC (UV 350 nm). The signal for lambertellol C (**4**) was confirmed with EIMS (direct insertion) and ^{1}H NMR spectrum after preparative HPLC. The similar experiment was performed with non-labeled **3**. Experiment with *L. corni-maris* in place of L. sp. 1346 was also performed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.051.

References and notes

1. Harada, Y.; Sasaki, M. *Rep. Tottori Mycol. Inst.* **1990**, *28*, 275.
2. Hori, S. *SHOKUBUTU BYOUGAI KOUWA (in Japanese)* **1916**, *II*, 97.
3. Hori, S. *ENGEINO TOMO (in Japanese)* **1912**, *8*, 949.
4. Murakami, T.; Takahashi, Y.; Fukushi, E.; Kawabata, J.; Hashimoto, M.; Okuno, T.; Harada, Y. *J. Am. Chem. Soc.* **2004**, *126*, 9214.
5. Murakami, T.; Morikawa, Y.; Hashimoto, M.; Okuno, T.; Harada, Y. *Org. Lett.* **2004**, *6*, 157.
6. Murakami, T.; Takada, N.; Harada, Y.; Okuno, T.; Hashimoto, M. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 1230.
7. Nomiya, M.; Murakami, T.; Takada, N.; Okuno, T.; Harada, Y.; Hashimoto, M. *J. Org. Chem.* **2008**, *73*, 5039.
8. *Monilinia fructigena* makes the spores only limited conditions. *Monilinia fructicola* is isolated from peach fruit which also belongs to Rosaceae family.
9. Murakami, T.; Sasaki, A.; Fukushi, E.; Kawabata, J.; Hashimoto, M.; Okuno, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2591.
10. Lambertellols A and B (**1**, **2**) could be ionized without decomposition when these were introduced into the instrument directly (EI method).
11. Murakami, T.; Sasaki, A.; Fukushi, E.; Kawabata, J.; Hashimoto, M.; Okuno, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2587.
12. Jeffries, P.; Young, T. W. K. *Interfungal parasitic relationships*; CABI International: Wallingford, 1994.