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Biosynthesis of Lambertellols Based on the High Specific Incorporation of the ^{13}C-Labeled Acetates and Their Biological Properties

Takanori Murakami,† Yukiko Takahashi,† Eri Fukushi,‡ Jun Kawabata,‡ Masaru Hashimoto,*,† Toshikatsu Okuno,† and Yukio Harada†

Contribution from the Faculty of Agriculture and Bioscience, Hirosaki University, 3-Bunkyo-cho, Hirosaki, 036-8561, Japan, and Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

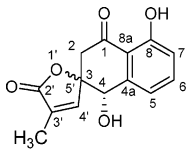
Received March 23, 2004; E-mail: hmasaru@cc.hirosaki-u.ac.jp

Abstract: Biosyntheses of lambertellols A (**1**) and B (**2**) as well as lambertellin (**3**) were investigated by isotope labeling experiments. Nearly 40% of specific incorporation of [1,2-$^{13}C_2$]acetate was achieved, and all the carbons in **1** and **2** were labeled. This high incorporation of the labeled acetate was realized by providing INADEQUATE spectra by employing only 0.4 and 0.7 mg of **1** and **2**, respectively. Our studies revealed that **1**−**3** are biogenetically synthesized via loss of two carbons from octameric acetate. A biological assay against *Monilinia fructicola* revealed those remarkably inhibited hyphal germinations. However, neither of them killed the spores immediately, even in high concentration. These conditions induced the formation of microconidia.

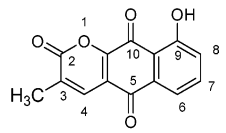
Introduction

A discomycete *Monilinia fructigena* that infects apple fruits is sometimes sectored by another discomycete *Lambertella* sp. 1346 under ambient atmosphere,[1,2] which has been recognized as mycoparasitism.[3−5] This observation led to a hypothesis that some substances produced by *Lambertella* sp. 1346 may play an important role in this phenomenon.[6] During the investigation of substances that could be responsible for mycoparasitism from *L.* sp. 1346, we have isolated novel dihydronaphthalen-1-(2*H*)-ones with spiro-1-furan-2(5′*H*)-ones, lambertellols A (**1**) and B (**2**), as potent antifungal compounds.[7] NMR studies led to structural elucidation, and the absolute stereochemistry was established on the basis of the CD analysis of derivatized natural products. In these studies, we also found that both **1** and **2** were readily transformed to lambertellin (**3**), which was first reported by Armstrong et al. as an antifungal pigment of *Lambertella corni-maris* in 1965.[8,9]

The structures of **1**, **2**, and **3** suggest that these compounds are biosynthetically obtained from acetate-derived polyketide framework.[10] However, they are somewhat unusual because of their branched framework. Armstrong et al. reported very low incorporation of radioactivity in **3** by their feeding experiments employing radio-labeled formate.[8] However, we have found that *L.* sp. 1346 incorporates ^{13}C-labeled acetates. These experiments have provided us with a plausible biosynthetic pathway for **1**−**3** that involves loss of two carbons from octa-β-ketoester. The average incorporation of [1,2-$^{13}C_2$]acetate by this fungus reached ca. 40%, which yielded 0.4 and 0.7 mg of doubly labeled samples **1f** and **2f**, respectively, thus enabling the acquisition of an INADEQUATE spectra. Analyses of MS spectra of labeled samples revealed the distributions of isotopomers. Biological observation revealed that lambertellols inhibited hyphal germination of *M. fructicora*. (Hyphae are threadlike filaments forming the mycelium of a fungus.) These metabolites resulted in hyphal swelling in low concentration and in formation of microcodinia directly from the spore in high concentration.



1: 3*S*, **2**: 3*R*



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Incorporation of ^{13}C-labeled Acetate. Stable isotope label feeding experiments[11,12] with *L.* sp. 1346 were performed according to the protocol that was used for the isolations of **1** and **2**[7], with small modification. Sodium salt of [1-^{13}C]acetate

† Hirosaki University.
‡ Hokkaido University.

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Table 1. Incorporation Rate of Labeled Lambertellol A (**1**) and B (**2**)

	lambertellol A					lambertellol B				
carbon no.	δc (ppm)[a]	**1a**[b]	**1b**[b]	**1d**[b]	**1e**[b,d]	δc (ppm)[a]	**2a**[b]	**2b**[b]	**2d**	**2e**[b,d]
1	198.79	**3.8**	1.0	**35.5**	nd[e]	199.16	**2.8**	1.1	**32.2**	nd[e]
2	43.64	1.2	**6.0**	0.9	**36.4**	43.70	1.1	**6.7**	1.1	**35.0**
3	87.37	**3.2**	1.1	**33.5**	nd[e]	86.82	**2.9**	0.9	**33.5**	nd[e]
4	70.83	1.1	**7.3**	0.9	**51.1**	71.91	1.0	**5.1**	0.8	**33.8**
4a	141.41	**3.7**	1.3	**38.2**	nd[e]	141.58	**3.1**	1.1	**37.7**	nd[e]
5	118.40	1.0	**6.7**	1.0	**49.5**	118.29	1.0	**7.1**	1.0	**39.4**
6	137.70	**3.7**	1.0	**34.0**	1.00	137.75	**2.9**	1.0	**42.5**	1.00
7	118.69	1.1	**5.3**	0.8	**30.6**	118.70	0.9	**5.4**	0.9	**29.0**
8	162.68	**4.0**	1.6	**46.8**	nd[e]	162.79	**2.8**	1.1	**45.7**	nd[e]
8a	115.02	1.3	**3.1**	0.6	**12.8**	114.72	1.0	**4.2**	nd[e]	**21.8**
2′	172.37	1.3	**4.4**	0.8	**23.9**	172.34	1.0	**3.6**	1.0	**27.6**
3′	132.32	**4.0**	1.1	**34.6**	0.8	132.54	**3.3**	1.1	**53.8**	nd[e]
4′	147.74	1.0	**4.2**	0.6	**21.7**	147.54	1.2	**6.3**	1.1	**34.3**
3′-Me	10.76	0.8	**4.8**	0.8	**23.0**	10.78	1.3	**5.6**	1.4	**30.4**
average incorporation[c]		3.8	5.2	37.1	*e*		3.0	5.5	40.9	*f*

[a] The proton-decoupled ^{13}C NMR spectra (100 MHz) were measured in $CDCl_3$. [b] Intensity ratios of each peak in the labeled **1** divided by that of the corresponding signal in the unlabeled **1**, respectively, normalized to give a ratio of **1** for the nonenriched peak (C-5 for [1-^{13}C]acetate labeling and C-6 for [2-^{13}C]-acetate labeling). [c] Average incorporations were calculated by the arithmetic means of incorporation rates of enriched carbons (bold face). [d] Because of low signal-to-noise ratio, the relevant average incorporation could not be estimated. [e] nd = not detected. [f] The average incorporation rates were not determined because of the disappearance of almost of all nonlabeled signals.

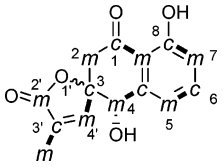

Figure 1. Distribution of ^{13}C atoms in labertellos A (**1**) and B (**2**) incorporated by the labeled acetates. c = carbons labeled by [1-^{13}C]acetate; m = carbons labeled by [2-^{13}C]acetate; and − = intact acetate unit.

(20 mg/200 mL of media × 10 flasks) was added to potato sucrose medium at the same time as the *L.* sp. 1346 was inoculated. Isolation after 5 days yielded the labeled lambertellols A (**1a**, 2.5 mg) and B (**2a**, 3.5 mg). A similar experiment employing sodium [2-^{13}C]acetate gave the differentially labeled pair of lambertellols A (**1b**, 1.9 mg) and B (**2b**, 2.3 mg).

There was not a remarkable alteration in the isolated yields between labeled lambertellols (**1a**, **1b**, **2a**, and **2b**) and nonlabeled compounds. This suggested that the addition of exogenous acetates did not affect their biogenetic production.[13−15] The proton-decoupled ^{13}C NMR spectra of the labeled samples revealed that ^{13}C atoms were incorporated as listed in Table 1.[7] Addition of sodium [1-^{13}C]acetate induced enrichments of ^{13}C atoms at C1, C3, C4a, C6, C8, and C3′ of both **1a** and **2a**. In contrast, the addition of [2-^{13}C]acetate increased relative intensities of signals for C2, C4, C5, C7, C8a, C2′, C4′, and the methyl carbon attached to C3′ for both **1b** and **2b**. These experiments revealed that all the carbons in **1** and **2** were labeled by either [1-^{13}C]acetate and [2-^{13}C]acetate as shown in Figure 1.

Acetate incorporation rates for **1a** and **2a** were estimated by ratio of the signal heights of labeled lambertellols to that of corresponding native compounds using C5 as an internal standard. On the other hand, intensity of C6 was employed as the internal standard for **1b** and **2b**. We used the corresponding peak heights in place of peak areas for the estimation of intensities, while taking into account the digital resolution (0.8 Hz) as well as signal-to-noise ratio in the ^{13}C NMR spectra. However, the estimation was less accurate for the highly labeled samples because of signal broadening due to the contribution of long-range coupling and also because of the deteriorating signal-to-noise ratio of the nonlabeled signal used as the internal standard. The average incorporations for **1b** and **2b** (labeled by [2-^{13}C]acetate) were slightly higher than those for **1a** and **2a** (labeled by [1-^{13}C]acetate). In contrast, incorporation rates employing [2-^{13}C]acetate are usually lower than those that employed [1-^{13}C]acetate because of the Krebs cycle.[16] The reason for this inconsistency remains unclear.

The feeding experiment employing [1,2-$^{13}C_2$]acetate was performed next to determine the distribution of the acetate units. The doubly labeled acetate was also successfully introduced to yield the third labeled samples of lambertellols A (**1c**) and B (**2c**) under similar conditions discussed above. The proton-decoupled ^{13}C NMR spectra of **1c** and **2c** displayed signals with complex splitting,[17] as illustrated in Figure 2. The resonances for the C2 and C2′ positions (highlighted in boxes) appeared as singlets with clearly visible minor satellites (C2: $J = 37$ Hz, C2′: $J = 63$ Hz). The other split resonances consist of the central singlet peaks due to natural acetates, the main flanking doublets, and the minor triplet-like double of doublet signals, as shown in Figure 3, which demonstrates the resolution of the C4 signal in **2c**. The main doublets ($J = 35-67$ Hz, Table 2) are due to $^1J_{CC}$ coupling corresponding to the intact acetate units. The minor triplet-like peaks suggest the sequential incorporations of [1,2-$^{13}C_2$]acetates with neighboring unit (vide infra). The spectrum of **1c** gave similar results.

Distribution of the intact acetate linkages was further confirmed by INADEQUATE spectra of labeled **1f** and **2f** (ca. 30% average incorporation for each), which were obtained by reducing the time for culturing as described below. Figure 4 depicts the INADEQUATE spectrum[18−23] of **1f** (0.4 mg, 62 h

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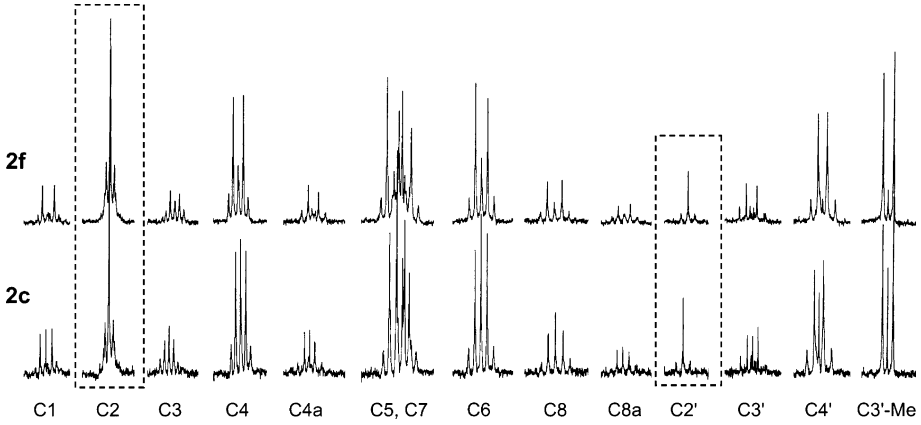

Figure 2. Signals of the proton-decoupled ^{13}C NMR spectra of the labeled samples of lambertellol B (**2**) in $CDCl_3$. Lower line: the signals of **2c** obtained after culturing L. sp. 1346 for 5 days. Upper line: the signals of **2f** obtained after culturing for 2 days.

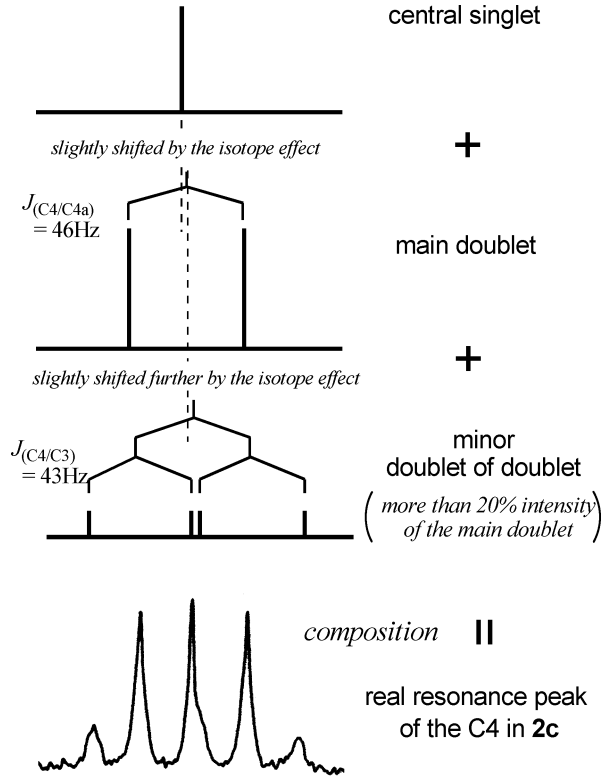

Figure 3. Composition of the resonance for C4 in **2c**.

Table 2. $^1J_{CC}$ Values (Hz) Revealed by a Feeding Experiment with [1,2-$^{13}C_2$]Acetate

compds	J_{C1C8a}	$J_{C3C4'}$	J_{C4C4a}	J_{C5C6}	J_{C7C8}	$J_{C2'C3'-Me}$
1	54	46	45	55	66	48
2	54	46	46	54	66	48

of accumulation). The spectrum clearly indicates the distribution of the acetate unit introduced, as shown in Figure 1. However, the cross-peaks showing the correlation between the acetate units were not observed. The sample **2f** also gave the INADEQUATE spectrum, revealing the same distribution.

Biosynthetic Pathway of 1 and 2. Biosynthesis of lambertellols is discussed next on the basis of results discussed above.

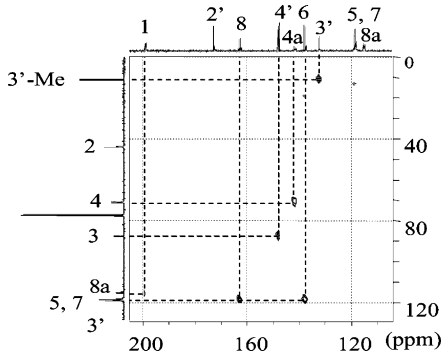

Figure 4. Part of the 2D INADEQUATE spectrum of **2f** (0.4 mg) in $CDCl_3$ (60 μL) by 384 scans (62 hours) employing 256 (F_2) × 64 (F_1).

Detectable incorporation of L-methionine-*methyl*-^{13}C was not observed. Thus, other biosynthetic routes involving methylation steps are unlikely. The feeding experiments employing the [1-^{13}C] and [2-^{13}C]acetate introduced isotope labels for six and eight carbons, respectively, in both **1b** and **2b**. Taking into account the similar incorporation rates of labeled acetate units, our results suggest an octaketide such as **4** as the biosynthetic precursors for **1** and **2**. If there are pathways that would introduce one carbon of the acetate, specific incorporation for the corresponding carbons should be lower than those for the others. This is because the process requires complex biosynthetic steps that would bring about dilution with nonlabeled acetate produced from supplemented sucrose. Cyclization of **4** could produce the tricyclic intermediate **5** or its equivalent in the biosynthetic cascade as shown in Scheme 1. Labeling experiments employing [1-^{13}C]acetate introduced ^{13}C atoms in six positions. Thus, elimination of two carbons from **4** should be considered for the generation of lambertellols. A decarboxylation followed by an oxidative aromatization (or different order, path A) may provide chrysophanol (**6**), which has been isolated along with lambertellin (**3**) from another fungus *Pseudospopes simplex* by van Eijk et al. in 1978.[4,24] They reported that **6** was a candidate as the biosynthetic precursor of **3** because of the occurrence of **6** together with **3**.

Our investigation can suggest an alternative route (path B). The tricyclic intermediate **5** can be transformed into a carboxylic acid **7** via the oxidative eliminations of two carbonyl groups. This process might involve an oxidation at the C4 position

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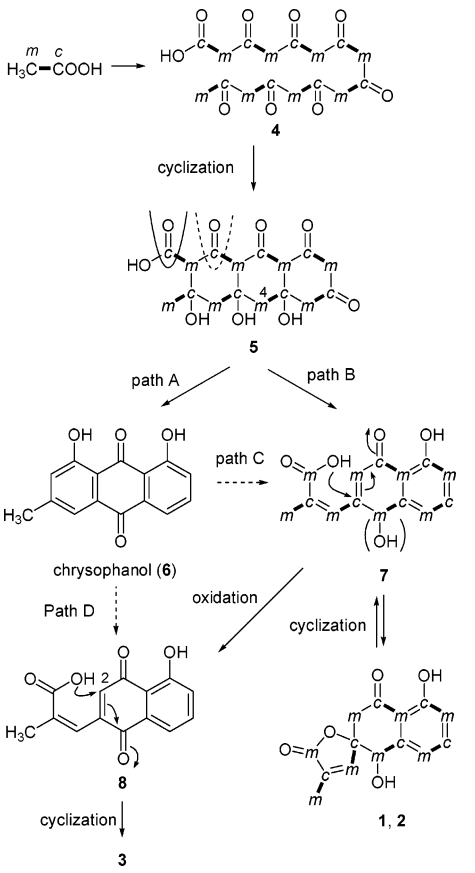
Scheme 1



(lambertellol numbering). Enzymatic or nonenzymatic Michael-type spirocyclization of **7** could produce lambertellols **1** and **2**. The nonenzymatic process might be more plausible, since *L.* sp. 1346 produces both diastereomers **1** and **2** simultaneously in similar amounts.[7] Retro-Michael ring opening regenerates **7**, which upon a second Michael addition from the opposite side of the cyclohexane ring results in the isomerization between **1** and **2**. These reactions can take place under nonenzymatic conditions. In fact, we have observed the interconversion between **1** and **2** on silica gel. Thus, we would propose the possibility that **7** might be a real biosynthetic product.

Oxidative aromatization of **7** in an enzymatic or nonenzymatic manner plausibly occurs to give naphthoquinone **8**. The quinone ring of **8** can undergo another Michael-type addition at the C2 position (lambertellol numbering), giving lambertellin (**3**). Pathways from **6** to **7** (path C) or **6** to **8** (path D) are also possible; however, at this stage, it is difficult to discern which pathways contribute predominantly to the biosynthesis of lambertellols (**1**, **2**) and lambertellin (**3**), since we have not succeeded to detect **6** or **8** from the culture broth of *L.* sp. 1346.

We have found that the decomposition of **1** and **2** gives rise to **3** in ambient conditions.[7] These processes probably involve **7** as the common intermediate. For example, dissolving in methanol gradually decomposed both **1** and **2** to give **3** in high yield. This observation suggested that **3** was an artifact derived from lambertellols during or after isolation. However, as described in our previous communication, we excluded this possibility by the following observations. The congeneric fungi, *L. corni-maris*,[25] definitely produced **3** as the major metabolite along with trace amounts of **1** and **2**[7] in our own experiments. In contrast, cultivation of *L.* sp. 1346 under the similar conditions was found to produce both **1** and **2** in high levels, but **3** was present as a minor component.

Attempts To Achieve Higher Specific Incorporation in the Labeling Experiments. The specific incorporation of the doubly labeled acetate for 3′-Me in lambertellol B (**2c**) was estimated as 7.1% on the basis of the comparison of the peak intensities between the central singlet and the flanking doublet peaks, as shown in Figure 2 (central singlet:flanking doublet = 1:6.5). The levels of intensities for other carbons were not assignable because of overlapping signals. However, they could be estimated roughly as 7%, because the ^{13}C NMR spectra of **2a** and **2b** suggested that the incorporation of the exogenous acetate occurs at a similar level for each unit. The intensities of the minor doublet of doublet (minor doublets for C2 and C2′) signals are calculated to be about 7% $\{(0.065 \times 0.065)/[0.065 \times (1 - 0.065)] = 0.07\}$ of the major doublets (major singlets in the cases for C2H and C2′).[26] However, the intensities of the observed minor doublet of doublets (minor doublets in the cases for C2 and C2′) of **2c** in the actual spectrum were much larger than those expected by the calculation. For example, intensity of the doublet of doublet signal for C4 in Figure 2 seems greater than 20% of the main doublet.[27]

This suggested that *L.* sp. 1346 produced considerable amounts of **1** and **2** by only using newly generated nonlabeled acetate after running out of the labeled acetates. These were probably consumed rapidly. Before consumption of the exogenous acetate, the fungus seemed to incorporate them more efficiently. To confirm this hypothesis, we performed the labeling experiments by reducing the time for cultivations. Isolation after culturing *L.* sp. 1346 with sodium [1-^{13}C]acetate (20 mg/200 mL of media × six flasks) in the standard potato sucrose media at 25 °C for 48 h (originally 5 days) afforded 1.2 and 1.4 mg of labeled lambertellols A (**1d**) and B (**2d**), respectively. The yield of **1d** and **2d** decreased as compared to the metabolites obtained after 5 days of culture.[28] Despite the smaller amounts, the samples produced high-quality ^{13}C NMR spectra after 3 h of accumulation. The proton-decoupled ^{13}C NMR spectrum of **2d**, as shown in Figure 5, indicates remarkably higher incorporation of ^{13}C atoms. Indeed, such high incorporation was observed also in the spectrum of **1d**. The average incorporation of **2d** was estimated to be 40.9%.

This method was also effective for incorporation of [2-^{13}C]-acetate, affording labeled lambertellols A (**1e**, 0.3 mg) and B (**2e**, 0.4 mg) after 2 days of culturing. In the cases of **1e** and **2e**, only the signals for C6 were detectable in their ^{13}C NMR spectra because of high incorporation. Thus, the average

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(26) The expected ratio was estimated as follows:

$$\frac{\text{intensity of minor double} - \text{doublet}}{\text{intensity of main doublet}} \times 100 = \frac{\text{probability of sequentially labeled acetate unit}}{\text{probability of isolately labeled acetate unit}} \times 100 = \frac{0.07 \times 0.07}{0.07 \times (1-0.07)} \times 100 \cong 7\ (\%)$$

(27) The intensity of the minor double doublet can be estimated roughly as 4 times intensity of the rightmost (or leftmost) small peak because of the double doublet.

(28) Since isolation was performed before fully growing the number of fungi, their yields depended much on the number of hyphae inoculated. Their yield varied because of the difficulty of implanting the same number of hyphae.

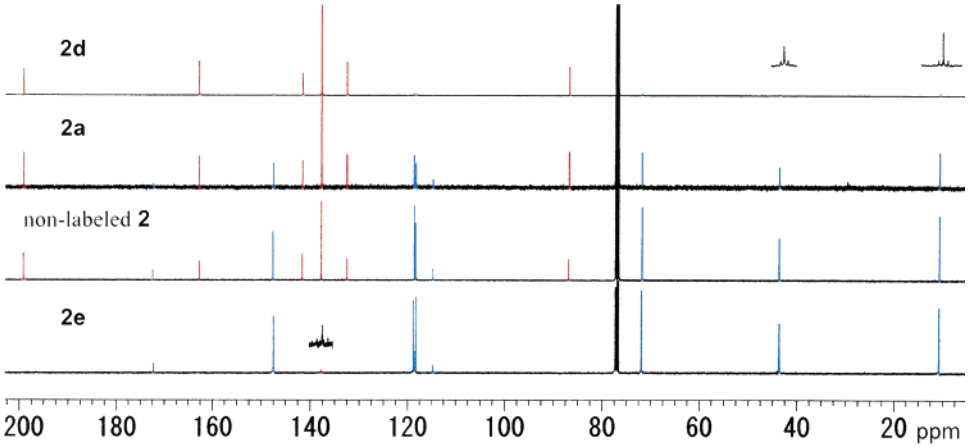

Figure 5. The proton-decoupled ^{13}C NMR (100 MHz) of nonlabeled **2** and labeled samples **2a**, **2d**, and **2e** in $CDCl_3$. The signals labeled by [1-^{13}C]acetate and [2-^{13}C]acetate are highlighted with red and blue, respectively. Some signals not labeled were enlarged (expanded to 5 times also for the *x*-axis).

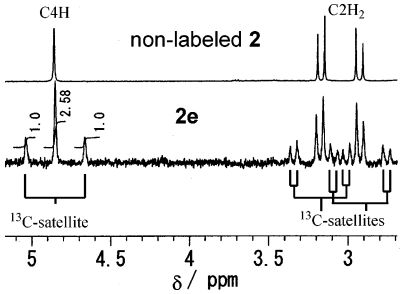

Figure 6. Region of the ^1H NMR spectra of nonlabeled **2** and labeled **2e** in $CDCl_3$ corresponding to the C2H_2 and the C4*H*.

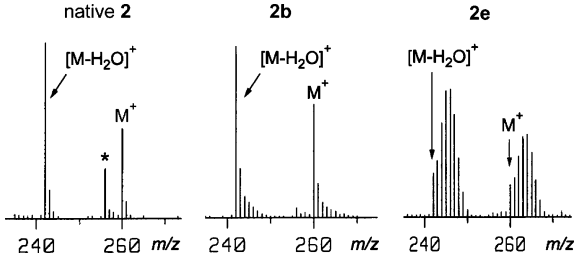

Figure 7. Part of EI-MS spectra of lambertellol B (native **2**, **2b**, and **2e**). * indicates impurity (lambertellin caused by decomposition during storing).

incorporation for those samples could not be determined. The specific incorporation at C4 of **2e** was estimated to be 44% from the ^1H NMR spectra, as shown in Figure 6.

The latter conditions were applied for feeding experiments with doubly labeled acetate. As expected, *L*. sp. 1346 incorporated sodium [1,2-$^{13}C_2$]acetate well, giving doubly labeled samples of lambertellols A (**1f**, 0.4 mg) and B (**2f**, 0.7 mg), after 2 days of cultivation. The high incorporation led practically to elimination of the native central signals in their ^{13}C NMR spectra. As shown in Figure 2, the original signal for the C3′-Me (10.78 ppm) almost disappeared. The specific incorporation of the [1,2-$^{13}C_2$]acetate for C3′-Me of **2f** was estimated to be 30% on the basis of the comparison of the signal intensities between the original singlet and the newly observed flanking doublet (1:27). This high incorporation made other signals simpler. For example, the doublet of doublet signal corresponding to C4 of **2f** became assignable (J = 43 and 46 Hz, Figure 2). The coupling (J = 46 Hz) for this signal was already detected using **1c**. The newly detected splitting (J = 43.0 Hz) is a result of the C3−C4 bond, indicating a continuous introduction of labeled acetate units. The ratio of the peak intensities between the minor doublet of doublet and the main doublet was estimated to be ca. 1:2, which is comparable with the ratio expected by the similar calculations [(0.3 × 0.3)/{0.3 × (1 − 0.3)} ≈ 0.5]. As described above, by taking advantage of a 2.5 mmϕ microprobe and SHIGEMI-Tube,[29] the high incorporation allowed us to obtain 2D INADEQUATE spectra using only 0.4 and 0.7 mg of **1f** and **2f**, respectively. Taking the poor solubility especially of **1** into consideration,[30] only these highly labeled lambertellols can practically provide assignable cross-peaks in the INADEQUATE spectra.

Mass spectral analyses of highly labeled lambertellols were performed to investigate the number of ^{13}C atoms that was introduced in one molecule. The EI-MS spectra of native **2** indicated typical patterns of the molecular ion signals. In the case of **2b** (10.9% average incorporation based on the NMR analysis), the signals corresponding to isotopomers were slightly increased, as shown in Figure 7. Higher incorporated sample **2e** (the average incorporation could not be determined because of the disappearance of the internal standard in the NMR spectrum) displayed a significant increment of population of isotopomers. Notably, this sample involves an isotopomer that consists of only exogenous acetate (m/z = 268) as the detectable population. Subtracting the theoretical distribution in natural abundance for the isotopomers led to the populations of each isotopomer that resulted by incorporation of the labeled acetate (Table 3; see also Supporting Information).[31,32] The population of isotopomers and calculated average incorporation rates are

(29) A 2.5-mm symmetrical microtube matched with $CDCL_3$ (cat no. CMS-0025) was employed. Shigemi, Inc. Home Page. http://www.geocities.com/~shigemi/index.html.

(30) Actually, **1c** and **2c** gave correlation peaks in the INADEQUATE spectra. However, in the case of **1c**, some peaks were missing because of poor signal-to-noise ratio, and precipitations of samples occurred in the sample tube from $CDCl_3$ solution during measurement of the spectrum. Lambertellol A (**1**) was gradually decomposed in methanol, which dissolves **1** well to lambertellin (**3**) after 12 h.

(31) Protonation during ionization in EI-mass spectra was ignorable, because the theoretical intensities for the M + 1 and M + 2 ions resembled those of the observed. The weighted average of the population as shown in the equation below can express the average incorporation:

$$\text{average incorporation} = \frac{\sum[(\text{population of isotopomer}) \times (\text{no. } ^{13}\text{C in corresponding isotopomer})]}{\text{total number of isotopomers}}$$

(32) Pretsch, E.; Buhlmann, P.; Affolter, C. *Structure Determination of Organic Compounds: Tables of Spectral Data*; Springer-Verlag: Berlin, 2000.

Table 3. Population of Labeled **1** and **2** (%) Obtained after 2 Days of Cultivation[a]

	lambertellol A					lambertellol B					lambertellin
no. ^{13}C atoms incorporated	**1a**	**1b**	**1d**	**1e**	**1g**	**2a**	**2b**	**2d**	**2e**	**2g**	**3g**
$^{13}C \times 0$	87.5	68.7	29.4	10.2	19.1	87.2	69.8	30.3	8.5	10.9	10.9
$^{13}C \times 1$	7.9	11.1	17.1	11.1	19.2	8.0	9.5	16.4	9.2	22.5	22.5
$^{13}C \times 2$	3.3	6.7	20.8	15.8	32.4	2.8	6.1	20.9	15.0	27.7	27.7
$^{13}C \times 3$	0.4	5.0	18.5	18.6	20.5	1.2	5.0	17.9	19.7	22.1	22.1
$^{13}C \times 4$	nd[b]	3.6	9.6	18.3	11.9	0.6	3.4	10.4	19.8	11.8	11.8
$^{13}C \times 5$	nd[b]	2.3	3.8	14.2	4.7	0.2	2.9	3.4	15.3	4.2	4.2
$^{13}C \times 6$	nd[b]	0.9	1.0	7.6	1.2	nd[b]	1.1	0.7	8.3	0.8	0.8
$^{13}C \times 7$	—[c]	0.8	—[c]	3.4	—[c]	—[c]	1.6	—[c]	3.4	—[c]	—[c]
$^{13}C \times 8$	—[c]	nd	—[c]	0.7	—[c]	—[c]	0.6	—[c]	0.8	—[c]	—[c]
average incorporation (%)	3.1	10.4	29.5	39.7	34.3	3.4	10.9	29.1	41.7	36.2	36.2
those by NMR	3.8	5.2	37.1	—[d]	—[d]	3.0	5.5	40.9	—[d]	—[d]	—[d]

[a] For detail, see Supporting Information. [b] nd = not detected. [c] The ^{13}C atoms were theoretically not introduced. [d] The average incorporation rates by ^{13}C NMR could not be obtained because of very low signal-to-noise ratios for the nonlabeled signals.

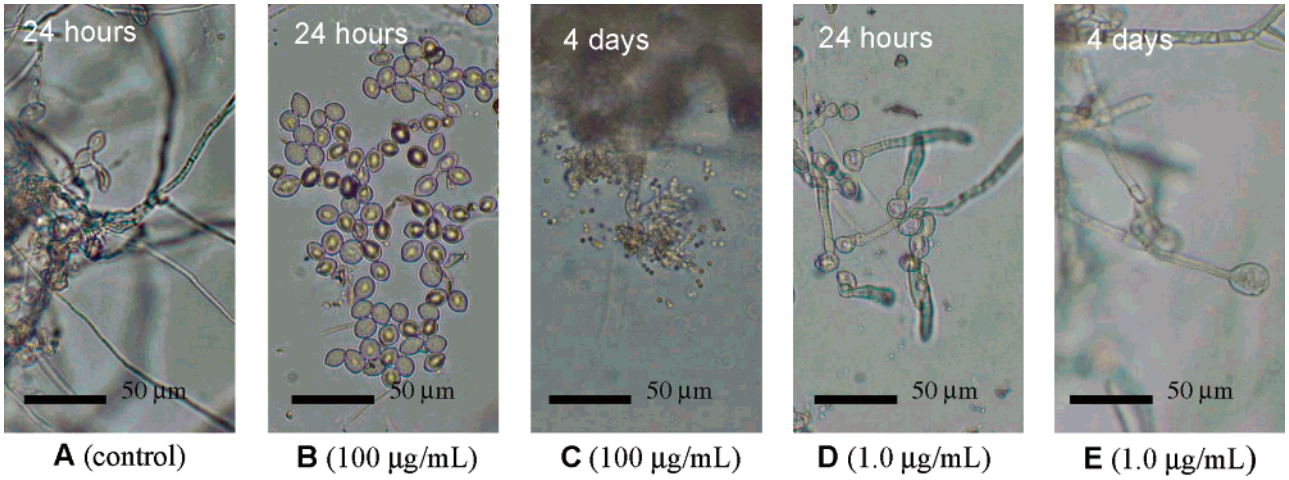

Figure 8. Inhibitory effects of lambertellol A, on condial germination of *M. fructicola*, incubated for 24 hours at 20 °C in dark. (A) control after 24 hr. (B) 100 μg/mL after 24 hr. (C) 100 μg/mL after 7 days. (D) 1.0 μg/mL after 24 hr. (E) 100 μg/mL after 4 days.

summarized in Table 3. The average incorporation rates obtained by this analysis are slightly different from those obtained by ^{13}C NMR spectra. In cases of highly labeled samples, the signals for nonlabeled carbons in the ^{13}C NMR spectra became relatively smaller. For example, some signals of **2d** almost disappeared, as shown in Figure 5. Taking the signal-to-noise ratio into account, the value obtained by MS spectra rather than ^{13}C NMR spectra is more accurate for the highly labeled samples. In contrast, the signals for isotopomers become salient only in highly labeled samples (see Figure 7). Thus, ^{13}C NMR spectra provide the reliable rates for samples with low level of incorporation. The fragment signals corresponding to $[M - H_2O]^+$ of labeled lambertellols were found to show similar patterns.

Finally, labeling experiment with sodium [1-^{13}C]acetate was carried out without the addition of sucrose into the potato media, affording the labeled labmertellols A (**1g**) and B (**2g**) after culturing for 2 days. This experiment aimed to achieve higher incorporation by preventing the dilution of labeled acetate with nonlabeled acetate, which is newly produced from carbohydrates. However, the incorporation rates for both **1g** and **2g** were almost the same as those for **1f** and **2f**.

Interestingly, the starvation conditions afforded the labeled lambertellin **3g** as the major metabolite. The mass spectrum of **3g** also indicated that the incorporation of exogenous acetate occurred at a level (36.2%) similar to that of **1g** and **2g**. These results might be further evidence that **1**, **2**, and **3** are synthesized via the same biosynthetic cascade. However, the reason starvation conditions afford **3** remains unclear.

Biological Observation. As described above, our studies revealed *L*. sp. 1346 produced measurable amount of lambertellols only after 2 days. These results indicate that the biosynthetic system for lambertellols already works at the hyphal growing stage. Accordingly, the fungus might produce these metabolites for its propagation. Finally, inhibition assay of hypal germination against *Monilinia fructicola* Honey[33] was performed. This fungi is closely related to the host for *M. fructigena*,[25] but is not the major host parasite in nature. However, we employed this as the test fungi because of its experimental feasibility.[34]

It was found that **1** and **2** inhibited hyphal germination against *M. fructicola* with an IC_{50} value roughly estimated as ca. 1.0 μg/mL for both compounds. Microscopic observation suggested the hyphal behavior is the same with both **1** and **2**. Figure 8 shows the results obtained by employing **1**. Lambertellol B (**2**) gave similar results. Neither **1** nor **2** killed the spores immediately, even at high concentrations. These metabolites did result in the formation of microconidia[35] directly from the spore after 4 days at high concentration (100 μg/mL) as shown. (Microconidia are generally unicellar small conidium. Micro-

(33) Terui, M.; Harada, Y. *Ann. Phytopathol. Soc. Jpn.* **1966**, *32*, 291−294.
(34) *Monilinia fructigana* produces spores in limited conditions. It requires a rather long time cultivation in vitro.
(35) Ulloa, M.; Hanlin, R. *Illustrated Dictionary of Mycology*; APS Press: St. Paul, MN, 2000.

conidia in some conidial fungi function as asexual spores. However, the role in *Lambertella sp*. has not been well studied.) Lambertellols also induced hyphal swelling of *M. fructicola* at low concentration (both 1.0 μg/mL). Hyphal swellings in fungi are sometimes induced by antibiotics.[36]

Lambertella sp. 1346 produces **3** as the major metabolite under starvation conditions. Thus, **3** can also be a candidate for the real antibiotic for *L.* sp. 1346. Lambertellin (**3**) also exhibits hyphal germination against *M. fructicola*, but it required higher concentration (100 μg/mL).

Conclusion

These studies disclose the biosynthetic pathway for lambertellols A (**1**) and B (**2**) as well as lambertellin (**3**) and chrysophanol (**6**), which includes loss of two carbons from octaacetate of ketide during the biosynthesis. Reducing the period for the cultivation resulted in increasing the average incorporation of the exogenous acetate inasmuch as 40% by a single feeding of labeled acetates, although pulse feeding technique is required for high incorporation in many cases.[21] Our result might be an example with the highest incorporation of exogenous acetates.[37] These highly labeled samples made it possible to acquire INADEQUATE spectra using less than 1 mg of sample. Biologically related other compounds, obtained by labeling experiments, are also expected to indicate the characteristic signal patterns of molecular ion signals similar to those of **1d**,**e** and **2d**,**e**. Taking advantage of the signal patterns, GC or LC mass analyses of the extracts obtained in these experiments might allow us to discover unknown biosynthetic congeners.

Our biological observations revealed remarkable inhibition of hyphal germination of **1**, **2**, and **3** against *M. fructicola*, a substitutive fungus of host *M. fructigena*. Our results suggest that either **1**, **2**, or **3** play a role in mycoparasitism of *Lamberlla* against *Monilinia.* Taking the isomerization between **1** and **2**, as well as the transformation from both **1** → **3** and **2** → **3** in vitro, it is difficult to judge the metabolite which is responsible for mycoparasitisizm. Thus, stable analogues of these compounds are required for further biological studies. Preparation of them as well as total synthesis of **1** and **2** are under way in our laboratories.

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Note Added after ASAP Publication: There were errors in Scheme 1 in the version published on the Web 7/10/2004. The final version published 7/15/2004 and the print version are correct.

Supporting Information Available: Complete experimental details, the ^{13}C NMR spectra of labeled **1a**−**g**, **2a**−**g**, and **3g**, and 2D INADEQUATE spectra of **1f** and **2f**. This material is available free of charge via the Internet at http://pubs.acs.org.

JA048345V

(36) Nihei, K.; Itoh, H.; Hashimoto, K.; Miyairi, K.; Okuno, T. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 852−857.

(37) Moore, B. S.; Walker, K.; Tomus, I.; Handa, S.; Poralla, K.; Floss, H. G. *J. Org. Chem.* **1997**, *62*, 2173-2185. Moore et al. reported high specific incorporation employing sodium [1-^{13}C]acetate (32.3%). However, they used exogenous acetate in much higher concentration (450 mg/900 mL of media). We kept a low concentration of the acetate to not disturb the biosynthetic system of *L*. 1346.