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Optimization of isotope-labeling conditions for lambertellin based on isotope patterns observed by mass spectrometry

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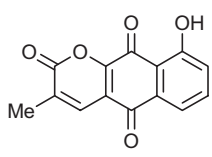
Abstract—A method for the sensitive analysis of the incorporation level of labeled acetate was developed. This method allowed for the optimization of the conditions for lambertellin with up to 48% average incorporation of labeled acetate.

The fungus *Lambertella* sp. 1346 sometimes interacts with and makes inroads into the hypha of the pathogenic fungus *Monilinia fructigena* in apple fruits. This type of interaction between these two species is referred to as mycoparasitism.[1] Recently, we have isolated lambertellin (**1**) and novel butenolides lambertellols A (**2**) and B (**3**) from *Lambertella* sp. 1346. Subsequent biological studies suggested that these natural products may be involved in this mycoparasitism.[2] From labeling experiments, we have disclosed the biosynthetic pathway by which these compounds are formed. In these studies, *Lambertella* sp. 1346 has been shown to incorporate supplemental ^{13}C-labeled acetate quite efficiently even at the hyphal growing stage.[3] In order to optimize labeling conditions, we developed a new method that simulates the mass spectral signal pattern of the molecular ions based on varying average incorporation levels. By comparing the calculated and experimental signal patterns, this simulation can distinguish a 2% difference in incorporation over the range of 15–85% total incorporation. Employing these simulations, we have succeeded in optimizing the labeling conditions for lambertellin with a 48% average incorporation level while using an economical amount of labeled acetate.



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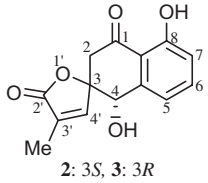

2: 3*S*, **3**: 3*R*

In our previous labeling experiments, we evaluated the incorporation on the basis of the signal increment of the ^{13}C-labeled carbons in the ^{13}C NMR (for singly labeled samples), comparison of the signal intensities between original and ^{13}C satellite signals in the ^{1}H NMR and ^{13}C NMR (for singly and doubly labeled samples, respectively), and analysis of the isotopomer patterns in the EIMS spectra (for singly labeled samples).[3] However, the incorporation levels estimated by these methods were not very accurate. For example, accurate signal intensities in the ^{13}C NMR spectra could not be measured because of the low signal/noise ratio for the non-labeled carbon signals. In the doubly labeling experiments, the $^{2}J_{CC}$ couplings, caused by sequential incorporation, also make the assignment difficult. Therefore, those methods were unable to distinguish small differences in the incorporation level. In order to optimize the labeling conditions, we have developed a method that can more accurately determine ^{13}C-labeled incorporations by analyzing the isotope patterns obtained by mass spectra.

Keywords: Mycoparasite; Labeling efficiency; Distribution of isotopomers; Simulation.
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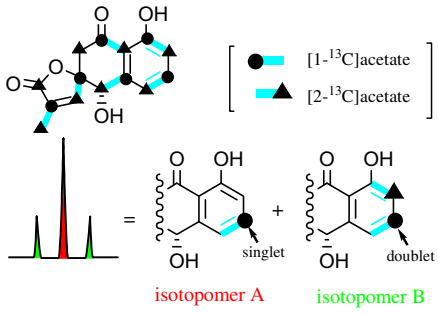

incorporation level of C7 based on C6 signal

$$= \frac{\text{population of isotopomer B}}{\text{population of (isotomoper A + isotopomer B)}}$$

$$= \frac{\text{the intensity of the flanking doublet}}{\text{the intensity of (central singlet + the flanking doublet)}}$$

Figure 1. Biosynthetic distribution of acetate in lambertellols[3] and estimation of the incorporation level at C7 based on the signal pattern of the resonance for C6.

Prior to this study, we confirmed the Krebs cycle does not contribute to biosynthesis of **1**–**3** under the labeling conditions, because Krebs cycle transforms [2-^{13}C]acetate into [1-^{13}C]acetate to result in randomization of the labeling carbon.[4] This makes the analysis so difficult. We determined that by a labeling experiment employing 1:1 mixture of sodium [1-^{13}C]acetate and [2-^{13}C]acetate the so-called alternate labeling method.[5,6] Each signal in the proton decoupled ^{13}C NMR spectrum of **2** consists of a central singlet and flanking doublet due to two species of isotopomers except for C3′-methyl group (10.8 ppm). Figure 1 explains the assignment of the signal for C6 (137.7 ppm) as an example. The major singlet signal comes from a species caused by introduction of [1-^{13}C]acetate into the C5C6 position (isotopomer A). The C5 position in this isotopomer should be ^{12}C. The flanking doublet is caused by sequential labeling with [1-^{13}C]acetate into the C5C6 unit and [2-^{13}C]acetate into the C7C8 unit (isotopomer B). Accordingly the ratio between the central singlet and the flanking doublet at the C6 position is due to a ratio (isotopomer A)/(isotopomer B), which provides information about incorporation level of the C7 position as shown in Figure 1. A similar analyses of the signals due to C1, C4, C5, C6, C7, C8, C2′, and C4′ positions provide the incorporation rate for C2, C3, C4a, C7, C6, C8a, C3′, and C3′ positions, respectively.[7] The juncture carbons cannot be used for the evaluations in this method.

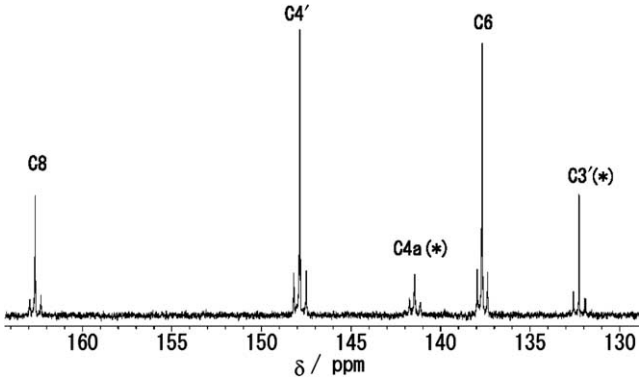

Figure 2. A part of proton decoupled ^{13}C NMR spectra of **2** prepared by cultivation with 1:1 mixture of [1-^{13}C]- and [2-^{13}C]acetate. The signal for C4a and C3′ should be eliminated from the consideration. See text.

A portion of the ^{13}C NMR spectrum of **2** obtained by this experiment is shown in Figure 2. The ratios between the central singlets and the flanking small doublets for C6, C8, and C4′ were in a ratio of ca. 100:35 regardless of the origin of the acetate unit. The Krebs cycle suggests a possibility transforming the C2 atom of acetate into C1 through gluconeogenesis.[4] If this cycle contributes to any significant extent in the biosynthesis of lambertellols, the signal ratio for C4′ (derived from C2 of acetate) should be smaller than those of C6 and C8 (derived from C1 of acetate). Assuming that the isotope effect in the biosynthesis can be ignored, randomization of the labeling position due to the Krebs cycle does not occur.

Thus, we can simply discuss the incorporation levels based on the profiles of the isotopomers. Stochastic calculations for **1**–**3** can predict the intensities of each monoisotopic signal [I_M, $I_{(M+1)}$, and $I_{(M+k)}$] from the equations shown in Table 1. As an example, we determined the incorporation of a labeled sample of **1** from the mass spectrum shown in Figure 3. Distribution of the isotopomers closely resembles the calculated pattern employing 38% as the incorporation level. This profile is clearly different from that employing 40% as the incorporation level (Fig. 3). Thus, the incorporation level of this sample was assigned to be 38%. This simulation is found to be quite sensitive, distinguishing 2% differences in the incorporation. However, this method cannot be applied for samples with low levels of incorporation. For example, there is no significant difference in the signal patterns between 5% and 7% incorporation, as shown in Figure 4.

Table 1. The intensities of signals in mass spectra predicted by the stochastic method

I_M	I_{M+1}	I_{M+k} $(2 \leqslant k \leqslant n)$
$(1-x)^n$	$(1-x)^n \times 0.011 \times m + [x(1-x)^{(n-1)} \times {}_nC_1]$	$\left[x^{(k-2)}(1-x)^{(n-k+2)} \times {}_nC_{(k-2)} \times \left\{\frac{(0.011 \times (m-k+2))^2}{200} + 0.005 \times o\right\}\right]$ $+ \{x^{(k-1)}(1-x)^{(n-k+1)} \times {}_nC_{(k-1)} \times 0.011 \times (m-k+1)\}$ $+ x^k(1-x)^{(n-k)} \times {}_nC_k$

M = molecular number, I_M = intensity of the ion ($m/z = M$), x = average incorporation rate, n = maximum number of the labeled carbons, m = number of carbons in the molecule, o = number of oxygen in the molecule, ${}_xC_y$ = combination which chooses y from x (x, y: integer).

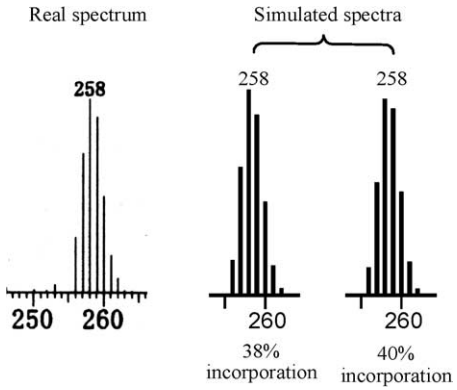

Figure 3. Observed mass spectrum obtained in run 4 and predicted spectra.

With this sensitive evaluation method in hand, optimization of the labeling conditions for **1** was performed (Table 2). Labeled **1**–**3** that have been highly enriched with an isotopic label can be useful probes for biological investigations. The original fermentation conditions for *Lambertella* sp. 1346 employed a potato–sucrose media consisting of potato extracts (from 40 g of potato) and sucrose (4.0 g) in water (200 mL) and supplemental labeled sodium acetate (20 mg) (run 1). Although it is well established that excess labeled acetate results in higher incorporation,[8] we fixed the amount of the supplemental acetate (20 mg) used in each experiment due to financial constraints. After extraction of the broth (5.0 mL) with $CHCl_3$, each extract was immediately subjected to GC–MS. Only compound **1** was detectable by GC–MS, since these conditions decomposed both lambertellols **2** and **3**. However, we have already proven that the incorporation levels for **2** and **3** are almost identical with that for **1** since all three compounds are derived from the same biosynthetic cascade.[3]

As described in our previous report, a longer cultivation time decreased the level of incorporation due to the consumption of the labeled acetate as well as the dilution by denovo acetate (runs 2 and 3).[3] When sucrose was removed from the medium, the expectation was that

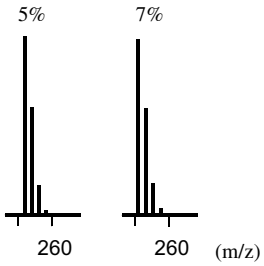

Figure 4. Simulated spectra for lower incorporations.

this dilution would be minimized. However, an increase of the incorporation rate was not observed in the absence of sucrose even when **1**–**3** were still being produced after 2 days (run 4). This result suggested that the non-labeled acetate ion and/or its congeners might already be present in the potato extract. Potato extract is required in the media since it provides many essential ingredients such as vitamins and minerals. We then removed any acetic acid present in the extracts by boiling under acidic conditions. After neutralization to pH 5.8, *Lambertella* sp. 1346 was inoculated into the medium thus prepared. This experiment resulted in a slight increase in the incorporation to 42% (run 5). Then, the fermentation was performed while reducing the potato extracts to one-tenth its previous concentration. This condition still produced **1**–**3**, but only very slowly. The GC–MS analysis after a 24 h fermentation indicated that the incorporation into **1** was 48% (run 6 and Fig. 5). These optimized conditions have improved the incorporation of the labeled acetate by 10% as compared with the original conditions. Longer fermentation times did result in the supplemental acetate being diluted by denovo acetate. When the culture broth was analyzed after 48 h, the mass spectrum indicated that the incorporation had decreased to 42% (run 7). For this high level of incorporation, the hyphae must be washed with sterile water prior to inoculation[9] to prevent contamination with non-labeled **1** that was present in the original preculture media. This contaminant increases the ion signal at $m/z = 256$ considerably, making further analysis difficult (spectrum A in Fig. 5).

Table 2. Incorporation of labeled acetate

Run	Labeled acetate[a]	Potato extracts[b] (g)	Sucrose (g)	Cultivation time	Incorporation rate (%)	Special treatment
1	Na[1-^{13}C]acetate	40	4.0	48 h	38	The hyphae were used without washing with water
2	Na[1-^{13}C]acetate	40	4.0	5 days	7[c]	The hyphae were used without washing with water
3	Na[2-^{13}C]acetate	40	4.0	5 days	7[c]	The hyphae were used without washing with water
4	Na[1-^{13}C]acetate	40	0	48 h	38	The hyphae were used without washing with water
5	Na[1-^{13}C]acetate	40	4.0	48 h	42	The solution of potato extracts was boiled at pH 2.0 in order to remove trace acetic acid in the extracts. The solution was neutralized before inoculation
6	Na[1-^{13}C]acetate	4.0	4.0	24 h	48	The hyphae were washed with sterilized water before inoculation
7	Na[1-^{13}C]acetate	4.0	4.0	48 h	42	The hyphae were washed with sterilized water before inoculation

[a] 20 mg of the labeled acetate was employed for each experiment.
[b] Commuted by the amount of the potato employed.
[c] Incorporation levels were obtained based on ^{13}C NMR spectra.

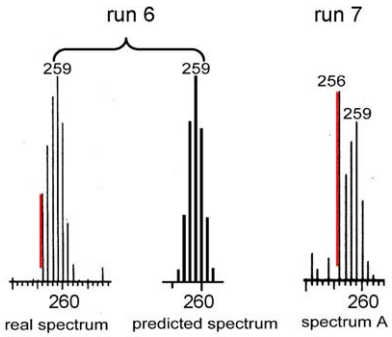

Figure 5. Observed mass spectra obtained in runs 6 and 7, and predicted spectra for 48% incorporation. Portions highlighted with red lines come from the original precultured media.

Unfortunately, further attempts to improve the level of incorporation have failed while maintaining the amount of supplemental labeled acetate at 20 mg. It is known that supplemental acetate is utilized for polyketide synthesis after the conversion into acetyl coenzyme A. In contrast, glycolysis in vivo produces acetyl coenzyme A directly through pyruvic acid. It is likely that the ratio between the reaction velocities of these two processes would limit the incorporation level when employing non-labeled carbohydrate.

As described, we have developed a sensitive simulation for the isotope profiles of labeled compounds and this method can distinguish small differences in the incorporation level. By utilizing this method, the incorporation of labeled acetate into **1** was optimized to a 48% average, an increase of 10% compared with the original protocol. Taking advantage of the sensitivity of GC–MS, the analysis could be performed on small scale experiments. For example, 5.0 mL of culture broth after only a 12 h fermentation was sufficient for the analysis. This allows for the determination of a detailed time course of labeling incorporation while using a single culture batch. In this manner, this method is applicable for the direct determination of optimal labeling conditions.

Generally, isotope-labeled biologically active compounds, including radiolabeled compounds, are useful probes for a wide range of biological investigations. Typically these compounds are prepared by chemical synthesis.[10] However, synthesis may require many chemical transformations. The ability to optimize the labeling conditions during fermentation should provide a practical biosynthetic method for the preparation of radioactive lambertellols.[11] Since the optimized labeling condition for lambertellols requires only a small amount of supplemental acetate, these conditions can also be applied to the preparation of radioisotope-labeled lambertellols by employing ^{14}C- or ^{3}H-labeled acetates while minimizing the amount of radiolabeled precursor required.

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