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Lambertellol C, a labile and novel biosynthetic congener of lambertellols A and B

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Abstract—Lambertellol C (**1**), a labile biosynthetic congener of lambertellols A and B, was isolated from a fermentation broth of *Lambertella* sp. 1346 based on the diagnostic isotope patterns in the mass spectrum of a highly labeled sample. The CD spectrum of **1** indicated that this lambertellol analogue exists as a racemate. The mechanism for this racemization is discussed.

The filamentous fungi *Lambertella* sp. are sometimes isolated from apples that have been infected by another pathogenic fungus, *Monilinia fructigena*.[1,2] Microscopic observations have indicated that the interaction of these two fungi is a mycoparasitism since hyphae of *Lambertella* make inroads into those of the host *Monilinia*.[3]

We have isolated lambertellols A (**2**) and B (**3**), novel polyketides containing a spiro-butenolide moiety, along with the previously identified lambertellin (**4**)[4] and suggested that these natural products may be mediators of the mycoparasitism.[5] Further, we have achieved extremely high incorporation of exogenous ^{13}C-labeled acetate in up to 40% average incorporation into lambertellols, allowing for the accumulation of a 2D-INADEQUATE NMR spectrum using sub-milligram quantities of **2** and **3**.[6] In this communication, we report the isolation and structure determination of lambertellol C (**1**), taking advantage of the characteristic MS signal profile of the labeled sample. Since the CD spectrum demonstrated that **1** exists as a racemate, the biosynthetic mechanism for this racemization is discussed.

As reported earlier, a remarkable amount of exogenous ^{13}C-labeled acetate was incorporated into **2** and **3** by *Lambertella* sp. 1346.[6] This incorporation reached up to 40% by the addition of small amounts of isotope labeled sodium acetate into potato-sucrose medium.[7] As shown in Figure 2, labeled samples of lambertellol B (**3**) displayed a characteristic isotope pattern in EIMS due to mixture of isotopomers. It was expected that biogenetically related metabolite should also incorporate the labeled acetate in the similar level showing resemble isotope pattern. Thus, LC–ESIMS analysis of crude extracts that were prepared under these labeling conditions was performed. After fermentation of *Lambertella* sp. 1346 for two days in the presence of sodium [1-^{13}C]acetate, the supernatant of the culture broth was extracted with $CHCl_3$. The crude extracts thus obtained were subjected to HPLC [Merck Lichrosphere RP18e 120 Å, 4 mm (I.D.) × 150 mm, H_2O/CH_3CN (without TFA, 1.0 mL/min flow, 20–100% linear gradient for 30 min)] and a minor peak at 14.7 min provided a similar isotope pattern by LC–ESIMS analysis, suggesting this peak as lambertellol related compound.

The structure of this component was elucidated from a nonlabeled sample isolated from a large scale fermentation (4.0 L medium). Since the photo-diode array spectrum of this fraction (λ_{max}: 280 and 350 nm) was similar to those of **2** and **3** but slightly red-shifted (λ_{max}: 260 and 339 nm for both **2** and **3**), this compound was named lambertellol C (**1**). Upon elution from the HPLC column, this component was colorless, but rapidly became pale yellow indicating the likely conversion to lambertellin (**4**). After standing at room temperature for

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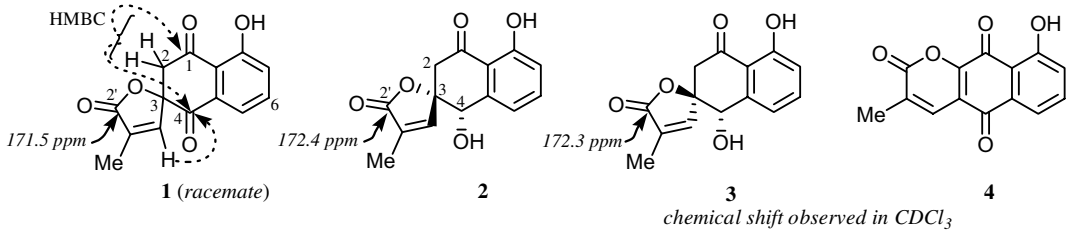


Figure 1. Structures of lambertellol C (**1**), A (**2**), B (**3**), and lambertellin (**4**).

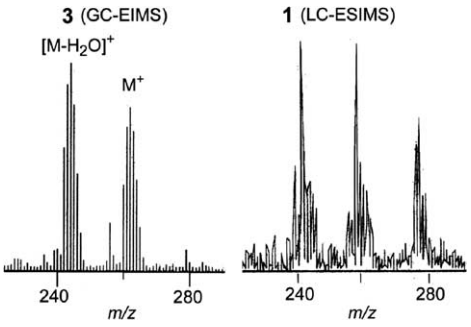


Figure 2. Regions of labeled lambertellol B (**3**) (left, EIMS) and lambertellol C (**1**) (right, LC–ESIMS) from the extracts prepared under labeling conditions.

30 min, HPLC analysis indicated that the sample had been converted to a ca. 2:1 mixture of **1** and **4** (UV = 350 nm). Interestingly, addition of 0.1% TFA into the eluent prevented **1** from any significant decomposition.

The solution thus obtained was immediately analyzed by EIMS, indicating *m*/*z* = 258.0559. Since EIMS may provide only fragments and/or dehydrated ion signals, this sample was further analyzed by LC–ESIMS. ESIMS showed a series of signals for **1** at *m*/*z* = 241, 259, 277, 300, and 531 (Fig. 3), corresponding to $[MH-H_2O]^+$, $[MH]^+$, $[MH+H_2O]^+$, $[MH+CH_3CN]^+$, and $[2M+H_2O+H]^+$, respectively.[8] These data were consistent with a molecular formula for **1** of $C_{14}H_{10}O_5$.

Although conventional concentration of the HPLC fraction decomposed **1** into polar materials, solutions of this compound could be concentrated without decomposition by the following operations: (1) removal of CH_3CN in vacuo at low temperature, (2) extraction with $CHCl_3$ from the resulting aqueous solution, and (3) concentration of the $CHCl_3$ layer in vacuo. A solution of **1** in $CDCl_3$ was stable for several hours. The ^1H NMR spectrum of **1** resembled those of **2** and **3**, except that the C4 methine proton (*lambertellol numbering*) was not observed.[9] This position in **1** was assigned as a carbonyl group based on the HMBC correlations between C4 (197.6 ppm) and both C2-methylene protons (3.28, 3.49 ppm). The C2-methylene protons also showed an HMBC cross-peak with C1 (188.4 ppm). The spiro-butenolide moiety was confirmed by comparing the chemical shift for C2′ (171.5 ppm) with those of **2** (172.4 ppm) and **3** (172.3 ppm).[10] Accordingly, the structure of lambertellol C (**1**) was consistent with that depicted in Figure 1. In fact, methanolic solutions of both **2** and **3** afforded **1** after four days even in trace amount.

Next, we focused on the stereochemistry of **1**, since both **2** and **3** have been isolated as optically active diastereomers with opposite stereochemistry at the C3 spiro-carbon.[5] Due to the instability of **1**, the isolated HPLC fraction (conditions containing 0.1% TFA in the eluent, 0.8–1.2 AU at 350 nm) was directly subjected to circular dichroism (CD) measurement. The CD spectrum of **1** thus obtained was flat in the entire wavelength range from 250 to 400 nm as shown in Figure 4. In contrast, the CD spectra of **2** and **3** prepared by similar methods showed Cotton effects around their UV absorption maxima (ca. 260 and 340 nm). From these experiments, it can be concluded that **1** was isolated as a racemate.

We propose that **1** is generated through planar intermediate **6** as shown in Scheme 1. The major pathway of this scheme has been used to explain the mechanism of isomerization between **2** and **3** as well as the in vitro formation of **4** from both **2** and **3**.[6] Retro-Michael reactions of both **2** and **3** produce the identical α,β-unsaturated ketone **5**. Nonenzymatic re-Michael cyclization of **5** may provide a mixture of **1** and **2**. An oxidative

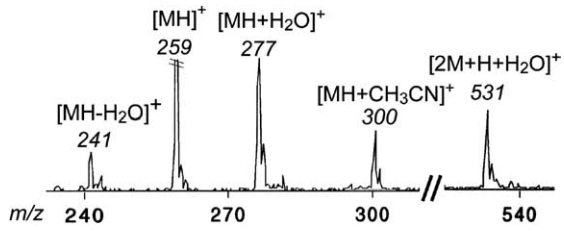


Figure 3. Region of ESI-MS spectrum of nonlabeled lambertellol C (**1**) and assignments for these ions.

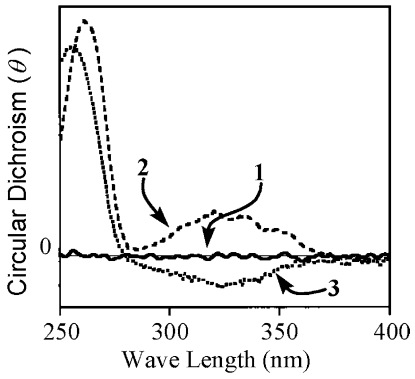
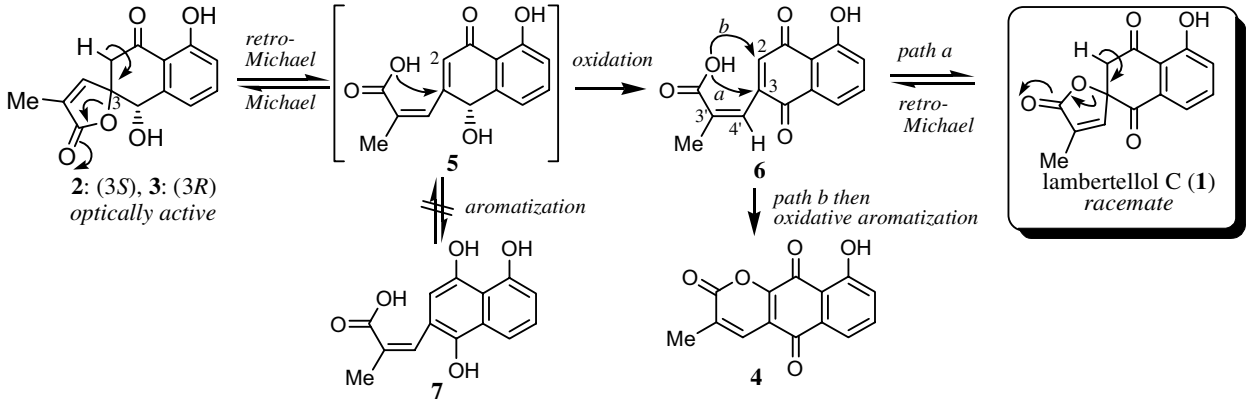


Figure 4. The CD spectra of lambertellols A (**2**, dashed line), B (**3**, dotted line), and C (**1**, solid line).



Scheme 1.

aromatization of **5** likely occurs, giving **6**. Lambertellol C (**1**) can be generated from **6** by the Michael cyclization at the C3 position (path a). Since **6** is an achiral compound, this nonenzymatic cyclization provides a racemic product. Although oxidation of the C4-hydroxyl in an optically active mixture of **2** and **3** would provide racemate **1**, this direct pathway is unlikely to occur. The oxidation rate of **2** and **3** would not be identical. In addition, the other Michael cyclization at the C2 position (path b) of **6** might occur, which would result in the formation of lambertellin (**4**) after oxidative aromatization.

The retro-Michael ring opening of **1**, providing **6**, is likely to occur since this process involves an aromatization of the 2,3-dihydro-1,4-naphthoquinone moiety of **1**. In this example, the C2-methyne proton of **1** would be acidic enough to induce the retro-Michael reaction under neutral conditions. This mechanism would also explain the degradation of isolated **1** to **4** in the absence of TFA. When **1** was extracted with $CHCl_3$ from the HPLC fraction in the presence of 0.1% TFA, it is likely that there was contamination by trace TFA in the organic layer. The acid present in the $CHCl_3$ solution prevented the retro-Michael ring opening of **1** from occurring. The $CDCl_3$ solution used for NMR measurement would also contain this trace amount of TFA.

It also seemed possible that an isomerization between **5** and planar naphthol **7** would occur (Scheme 1). This equilibration would also bring about the racemization of both **2** and **3**. Although we have already established that both **2** and **3** were isolated in optically active forms,[5] the optical purity of each diastereomers has not been determined. Both **2** and **3** gradually decompose to **4**, even under neutral conditions. However, triol **8**, which was prepared from **2** by treatment with PtO_2/H_2,[5] could be converted into the corresponding MTPA esters. Separate treatment of **8** with (+)- and (−)-MTPACl (10 equiv) in the presence of DMAP gave bis-MTPA esters **9a** and **b**,[11] respectively. The hindered C4-OH did not react under these conditions. The ^1H NMR spectra of **9a** and **9b** as shown in Figure 5 were obtained after filtration of each reaction mixture through a short silica gel filter. These spectra indicated

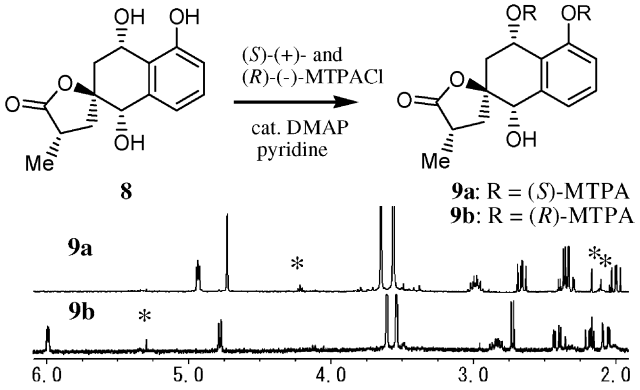


Figure 5. Formations of bis-MTPA esters **9a** and **9b**, and these ^1H NMR spectra in $CDCl_3$. *: Impurity.

that each reaction produced a single isomer, but these products were not identical. Since it can be concluded that both **2** and **3** are optically pure, it is not possible that there is any equilibration between **5** and **7**. The $\Delta\delta$ values in the extended Mosher protocol[12] are consistent with our previous stereochemical assignment based on the CD analysis.[5]

This conclusion was contradictory to our simple expectation that the aromatic nature of **7** would make it more stable than cyclohexenone **5**. Thus this issue was further investigated using theoretical calculations. Calculations were performed by ab initio models using the 6-31G* basis set by taking the accuracy into account. The initial geometries for these optimizations were obtained by a conformational search based on semi-empirical AM1 models.[13] The relative potential energies of **2**, **3**, **5**, and **7** that were obtained from those calculations are summarized in Table 1. Since these compounds have the same chemical composition ($C_{14}H_{12}O_5$), the energies provided by these calculations can be simply discussed as their potential energies. Lambertellols **2** and **3** were

Table 1. Relative potential energy of **2**, **3**, **5**, and **7** obtained by theoretical calculations (kcal/mol)

	Compound			
	2	**3**	**5**	**7**
Relative energy	−16.3	−14.7	0	+4.1

suggested to be much more stable than **5** by these calculations, which is consistent with the fact that we observed the lambertellols, but not compound **5**. Therefore, the equilibrium between lambertellols A, B (**2**, **3**) and **5** lies to left. Additionally, nonaromatic **5** is suggested to be 4.1 kcal/mol more stable than hydroquinone **7**. Accordingly, the contribution of **7** in these equilibria might not be considerable. We have not so far detected **5** or **7**.

As described in this report, we have isolated lambertellol C (**1**) as a labile biosynthetically related analogue of lambertellols from the culture broth of *Lambertella* sp. 1346. We have revealed that low concentrations of both lambertellols A and B inhibit hyphal germination of the host fungus *Monilinia fructicola*[6] Since we observed the slow production of **1** from **3** in methanol in vitro, lambertellol C is also a possible mediator of the mycoparasitism between *Lambertella* sp. and *Monilinia* sp. However, the biological properties of **1** have not been studied due to its instability even under mild conditions. Synthetic stable analogues of **1** might provide additional information about the identification of the species that directly affect the mycoparasitism. In this study, we have utilized the characteristic isotope profile of these ^{13}C-enriched natural products to search for novel compounds that are biosynthetically related to lambertellol. We also have demonstrated the efficiency of this LC–ESIMS method for the analysis of labile natural products. As seen in this study, LC–ESIMS may provide several cluster ions making this type of analysis complicated. However, detailed interpretation of the cluster ions provided additional support for the molecular weight of the compound.

Further chemical and biological studies of the mycoparasitism discussed here are under investigation in our laboratories.

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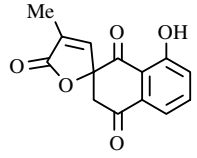
7. Twenty milligrams of sodium [1-^{13}C]acetate was added into the medium prepared by the standard condition with potate (40 g), sucrose (4.0 g), and water (200 mL).
8. The similar cluster ions were observed in the cases of known **2**, **3**, and **4** by performing the HPLC under the same conditions. (**2** and **3**: $m/z = 261$ $[MH]^+$, 279 $[MH+H_2O]^+$, 521 $[2M+H]^+$, and 539 $[2M+H_2O+H]^+$, **4** $m/z = 257$ $[MH]^+$, 275 $[MH+H_2O]^+$, 298 $[MH+CH_3CN]^+$, 531 $[2M+H_2O+H]^+$).
9. The spectral data of **1** are follows; ^1H NMR ($CDCl_3$) δ 1.98 (1H, d, $J = 1.5$ Hz, C3′-CH_3), 3.28, 3.49 (each 1H, δ, $J = 16.6$ Hz, C2H_2), 7.08 (1H, q, $J = 1.5$ Hz, C4′*H*), 7.35 (1H, dd, $J = 1.0$, 8.5 Hz, C5*H*), 7.60 (1H, dd, $J = 1.0$, 7.5 Hz, C7*H*), 7.72 (1H, dd, $J = 7.5$, 8.5 Hz, C6*H*), 11.86 (1H, s, O*H*), ^{13}C NMR ($CDCl_3$) δ 10.8 (*C*H_3), 46.6 (*C*2), 86.6 (*C*3), 117.3 (*C*8a), 119.7 (*C*7), 125.1 (*C*5), 132.7 (*C*3′), 133.4 (*C*4a), 137.6 (*C*6), 144.8 (*C*5′), 161.8 (*C*8), 171.5 (*C*2′), 188.4 (*C*1), 197.6 (*C*4) ppm. EIMS (rel. int., %) $m/z = 258$ (6.6), 69 (83), 45 (100).
10. Structure **I** was eliminated by the transformation of **1** into **4**.



I

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11. The ^1H NMR spectral data of **9a** and **b** are follows: Compound **9a**: δ 1.26 (3H, δ, $J = 7.3$ Hz, CH_3), 2.00 (1H, dd, $J = 10.3$, 13.7 Hz, C4′*H*H), 2.32 (1H, dd, $J = 2.9$, 14.6 Hz, C2*H*H), 2.38 (1H, dd, $J = 5.4$, 14.6 Hz, C2*H*H), 2.66 (1H, d, $J = 9.8$, 13.7 Hz, C4′*H*H), 2.99 (1H, m, C3′*H*), 3.56, 3.65 (each 3H, q, $J = 1.6$ Hz, CH_3O × 2), 4.73 (1H, s, C4*H*), 4.93 (1H, dd, $J = 2.9$, 5.4 Hz, C1H), 7.21 (1H, d, $J = 8.3$ Hz, C5*H* or C7*H*), 7.25–7.62 (13H, C5*H* or C7*H*, C6*H*, C4′H, and 10 aromatic protons for MTPA moiety). Compound **9b**; δ 0.69 (3H, d, $J = 7.3$ Hz, CH_3), 0.73 (1H, dd, $J = 10.9$, 13.4 Hz, C4′*H*H), 2.08 (1H, dd, $J = 2.2$, 15.1 Hz, C2*H*H), 2.18 (1H, dd, $J = 9.5$, 13.4 Hz, C4′*H*H), 2.41 (1H, dd, $J = 4.6$, 15.1 Hz, C2*H*H), 2.73 (1H, d, $J = 6.8$ Hz, C4O*H*), 2.84 (1H, m, C3′*H*), 3.54, 3.61 (each 3H, q, $J = 1.6$ Hz, CH_3O × 2), 4.78 (1H, d, $J = 6.8$ Hz, the coupling disappeared upon the addition of D_2O, C4*H*), 5.99 (1H, dd, $J = 2.2$, 4.6 Hz, C1*H*), 7.16 (1H, d, $J = 7.6$ Hz, C5*H* or C7*H*), 7.25–7.62 (13H, C5*H* or C7*H*, C6*H*, C4′H, and 10 aromatic protons for MTPA moiety).
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