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Structure and biosynthesis of norneolambertellin produced by *Lambertella* sp. 1346

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ABSTRACT

Norneolambertellin (**1**) was isolated from a mycoparasite *Lambertella* sp. 1346. Combined analysis of the NMR spectra and chemical shift prediction based on molecular orbital calculation successfully revealed a novel pyrano[3,2-*c*]chromene-2,5-dione structure, which was further confirmed by X-ray crystallographic analysis. Isotopomer distribution analysis of the sample, prepared under labeling conditions, deduced its biosynthetic pathway.

The filamentous fungus, *Lambertella* sp. 1346 was found as a mycoparasite on another phytopathogenic fungus, *Monilinia fructigena*, on apple fruit.[1] Recently, we isolated lambertellols A, B,[2] C,[3] neolambertellin,[4] and sucroneolambertellin,[5] as well as the known antibiotic lambertellin (**2**),[6] from the culture broth of this mycoparasite. Our investigation also disclosed that lambertellols significantly inhibited the hyphal growth of the host *Monilinia fructigena*.[7] In the course of our studies investigating the mechanism of mycoparasitism, we isolated norneolambertellin (**1**), a novel pyrano[3,2-*c*]chromene-2,5-dione derivative, from *Lambertella* sp. 1346. The NMR spectroscopic analyses suggested two possible structures but did not support a final decision between them. The theoretical chemical shift calculation for the ^{13}C NMR spectra effectively indicated the more plausible structure, which was then verified by the X-ray crystallographic analysis. In this letter, we also report the biosynthetic route of **1** on the basis of the isotopomer distribution (ITD) in the mass spectra of the samples prepared under labeling conditions.

Norneolambertellin (**1**, 2.0 mg) was obtained from the ethyl acetate extracts of the culture broth of *Lambertella* sp. 1346 (5.0 L, cultured for 14 days). The EIMS gave the molecular ion signal at $m/z = 244.0372$, suggesting its molecular formula to be $C_{13}H_8O_5$. The ^1H and ^{13}CNMR spectra in $CDCl_3$ demonstrated 8 and 13 resonances to confirm the molecular formula. The ^1H NMR signal at 9.5 ppm (broad) disappeared upon the addition of D_2O, indicating a phenol group. This was supported by a strong adsorption at 3400 cm^{-1} in the IR spectrum. The ^1H NMR provided four aromatic proton resonances. The COSY spectrum disclosed that three of them were continuously arranged on the aromatic ring. The last aromatic proton was observed as a doublet ($J = 1.1$ Hz), which was coupled with methyl group (2.19 ppm), indicating a substructure [CH=C(CH_3)]. Detailed NMR analysis including the HMBC spectrum led to the tentative assignment of the eight quaternary carbons to provide two plausible structures **X** and **Y** (Fig. 1); however, neither could be discarded at that stage.

While the carbon resonance at 159.06 ppm was assigned to the carbonyl atom in the middle pyranone ring in structure **X** (indicated with the red arrow in Figure 1), this was assumed to be the ketene acetal carbon between the left and center rings (indicated by the blue arrow) when we considered structure **Y**. However, empirical chemical shift data for those individual carbons were not available.

The answer to this structural question was obtained by applying the ^{13}C chemical shift prediction based on *ab initio* molecular orbital calculations, which has become a powerful method in structural studies.[8] We used Spartan'06 (Wavefunction, Inc.) for the calculations.[9] After the structures of models **X** and **Y** were optimized with HF-631G**,[10] they were subjected to DFT B3LYP/6-31G**[11] to obtain their the theoretical ^1H and ^{13}C NMR chemical shifts (Fig. 1).

The experimentally observed ^{13}C chemical shifts were plotted versus those provided by theoretical calculations for isomers **X** and **Y**, providing plots **X** and **Y**, respectively (Fig. 2). Theoretical ^{13}C chemical shifts for isomer **X** showed higher conformity with

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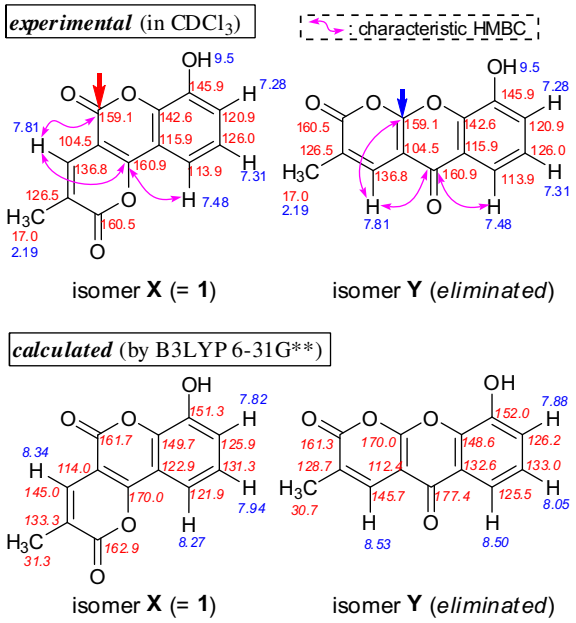


Figure 1. Structures of isomer **X** (=**1**) and isomer **Y** (eliminated); their experimental chemical shifts (top) and those obtained by calculation (bottom).

the experimental chemical shifts [r (correlation coefficient) = 0.9972] than those of isomer **Y** (r = 0.9913). After the calculated chemical shifts were corrected by slope and intercept, they were subtracted from those of the observed values to give $\Delta\delta$. The maximum $|\Delta\delta|$ for isomer **X** was 6.45 ppm, while that for **Y** was 8.77 ppm. The root mean square (RMS) of $\Delta\delta$ for **X** was 2.72 ppm, which was also lower than that of **Y** (RMS = 4.74 ppm). These studies clearly indicated that **X** should be the structure of **1**. Fortunately, **1** gave single crystals in the NMR sample tube (needle, mp = 237–238 °C), and the X-ray crystallographic analysis successfully verified structure **1** as shown in Figure 3.[12]

Since the structure of **1** resembles that of neolambertellin (**2**), isolated as a biosynthetic congener of lambertellol/lambertellin family,[3–5] **1** was assumed to be derived from **2** involving an elimination of the C6 carbon (*neolambertelin numbering*) (Scheme 1, path A). However, an elimination of the C5 carbon of **2** might also generate **1** (path B), and this pathway could not be disregarded at that stage of the investigation. We next investigated this issue. However, preparation of the ^{13}C-labeled samples for the NMR studies was not practical, taking the low yield of **1** (0.4 mg from 1.0 L of culture broth) into account. Therefore, we took advantage of the high sensitivity of MS spectrometry for these analyses.[13]

The ^{13}C-labeled **1** was prepared by culturing *Lambertella* sp. 1346 in a PS medium [prepared with potato broth (200 mL) and sucrose (4.0 g)] in the presence of ^{13}C-labeled sodium acetate (50 mg) for 48 h. The precultured *Lambertella* sp. 1346 was washed with sterile water prior to inoculation in order to minimize the contamination with non-labeled compounds present in the original preculture media.[14]

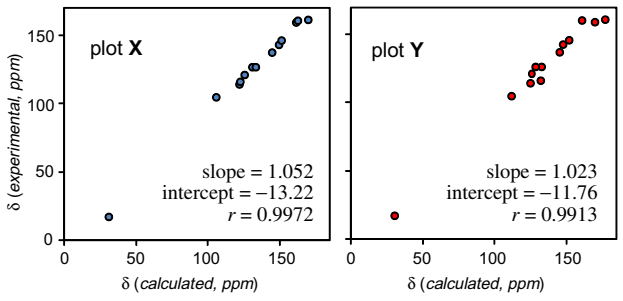


Figure 2. Plots of experimental versus calculated ^{13}C NMR chemical shifts for isomer **X** (plot **X**) and isomer **Y** (plot **Y**).

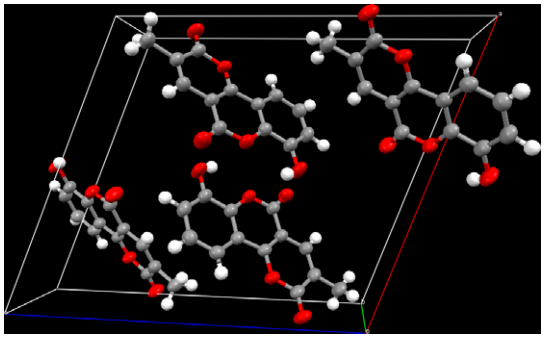
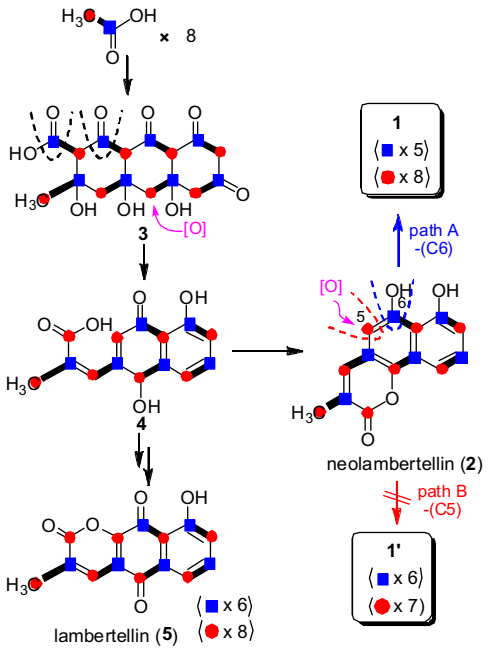


Figure 3. Structure of **1** by crystallographic analysis.



Scheme 1. Proposed biosynthesis of **1**.

After the culture broth was extracted with ethyl acetate, the extracts were directly subjected to GC–EIMS. First, the labeled extracts prepared with sodium [2-^{13}C]acetate were analyzed (The extracts, **1**, and **5** prepared under labeling conditions with sodium [2-^{13}C]acetate were abbreviated to [2-^{13}C]-labeled extracts, [2-^{13}C]-labeled **1** and [2-^{13}C]-labeled **5**, respectively, for convenience). Although the total ion chromatogram (TIC) of the [2-^{13}C]-labeled extracts gave many peaks, the ion chromatograms choosing m/z = 248 and 260 were effective to detect **1** and **5** selectively.[15] Both GC peaks for [2-^{13}C]-labeled **1** and [2-^{13}C]-labeled **5** provided their MS spectra with sufficient quality for further analysis (Fig. 4). The ions M+4 were chosen in the ion chromatograms because of the high incorporation level of the labeled acetate. Similar experiments were performed employing the sample prepared with sodium [1-^{13}C]acetate (Similarly, [1-^{13}C]-labeled **1** and [1-^{13}C]-labeled **5** are used for convenience). The observed relative peak intensities of labeled **1** and labeled **5** were plotted against the molecular ions (Fig. 5).

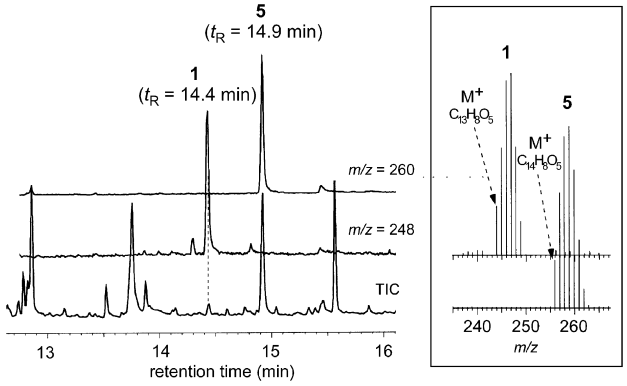


Figure 4. Total ion and mass chromatograms (m/z = 248 and 260) of the crude extracts prepared with sodium [2-^{13}C]acetate (left), and the EIMS spectra of the peak at 14.4 and 14.9 min (right) [column: RESTEK Rtx®-1 (0.25 mm ID × 30 m) at 100 °C for 5 min; then increasing at 9 °C/min].

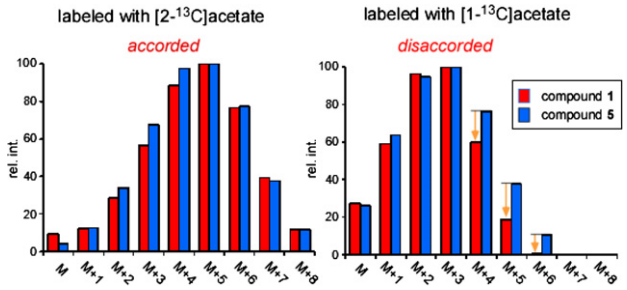


Figure 5. Comparison of experimental relative peak intensities between **1** and **5** in GC–MS.

As we have reported, the expected intensity of each isotopomer of the labeled samples in the MS spectra can be expressed by a function including the number of carbons and oxygens in the molecule (N_C and N_O, respectively), number of the carbons to be labeled (N_L), and average incorporation rate (x) as the parameters.[14] The N_O for **1** and **5** are given by their molecular formulae and were identical (N_O = 5). When we utilized the GC–MS of crude extracts, the incorporation rates (x) were expected to be identical between **1** and **5** in any labeling experiments because of the common biosynthetic intermediate **4**. These parameters do not affect the ITDs in the present analyses. Naturally occurring ^{13}C increases the ion $[M+1]^+$ by $1.1 \times N_C$ (%), but this would not lead to a considerable change in ITDs, even though the N_C for **1** and **5** are not identical. The increment of [M+1] for **1** by natural ^{13}C is estimated to be 14.3% (1.1 × 13), while that for **5** should be 15.4% (1.1 × 14). The difference is only 1% when we compare them with the corresponding base signals. The difference of N_C also affects the larger ions $[M+n]^+$ ($n \geqslant 2$), but the influence should be much smaller. Thus, we expected that ITD is affected by only N_L and that the difference enabled us to distinguish between paths **A** and **B** (Table 1).

It was found that the ITD of [2-^{13}C]-labeled **1** was quite similar to that of [2-^{13}C]-labeled **5**. This indicated that the N_L for **1** and **5** are identical to support path **A**. On the other hand, the ITD for [1-^{13}C]-labeled **1** showed a lower correlation with that of [1-^{13}C]-labeled **5**, suggesting a different N_L in the experiment using [1-^{13}C]acetate. In [1-^{13}C]-labeled **1**, the intensities of isotopomers larger than M+4 were considerably smaller than those of [1-^{13}C]-labeled **5**. This observation was also consistent with path **A** but not with path **B**. Thus, we concluded that **1** was derived by losing the C6 carbon of **2**.[16]

Table 1
Comparison of expected ITDs

	Additive	N_L for **1**	N_L for **5**	Expected ITD of **1** and **5**
Path A	[1-^{13}C]acetate	5	6	*Disagree*
	[2-^{13}C]acetate	8	8	*Agree*
Path B	[1-^{13}C]acetate	6	6	*Agree*
	[2-^{13}C]acetate	8	7	*Disagree*

ITD, isotopomer distribution; N_L, number of carbons to be labeled.

Biological examination revealed that **1** did not exhibit distinct inhibition of the growth against the host *M. fructigena*, so the relationship between this compound and the mycoparasitism has remained unclear. However, **1** inhibited growth of phytopathogenal fungus *Cochlibolus miyabeanus* at a concentration of 50 μg/mL.

In these studies, we revealed a novel metabolite **1** from mycoparasite *Lambertella* sp. 1346. The combined analysis of NMR spectra with the theoretical chemical shift prediction effectively elucidated the structure of **1**. We also demonstrated the biosynthetic analysis by comparing ITDs of the labeled samples. Elucidating the detailed biosynthetic cascades of secondary metabolites are requisite for their medicinal and agrichemical applications. Since the GC–MS/LC–MS results are extremely sensitive, the present analysis will become a powerful methodology for biosynthetic investigations of the minor natural products.

Acknowledgments

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10. Only the most stable conformer was taken into consideration for each chemical shift calculations, because other conformers around their hydroxyl groups involved much higher energies in both structures **X** and **Y** [Δ*E*: 4.5 kcal/mol (isomer **X**), 2.9 kcal/mol (isomer **Y**)].
11. Leach, A. R. *Molecular Modeling Principles and Applications*, 2nd ed.; Pearson Education: Essex, 2001.
12. CCDC 688448 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
13. Although labeling patterns are usually analyzed with ^{13}C NMR spectra, this analysis was not economically practical in that case because preparation of the labeled **1** for the ^{13}C NMR with allowable quality required more quantity of the labeled acetate.
14. Murakami, T.; Sasaki, A.; Fukushi, E.; Kawabata, J.; Hashimoto, M.; Okuno, T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2591.
15. The GC–MS conditions did not provide distinct signals of lambertellols A, B, C, and neolambertellin due to decomposition.
16. It is hard to discard completely the alternate pathway providing **1** from **4** involving loss of the same carbon (the carbonyl carbon of the semiquinone ring). However, this pathway is unfeasible by taking its stability into account. Compound **4** is theoretical intermediate and we have not detected. That was quite readily transformed into lambertellols. See Ref. 2.