## NOTE

# γ-Ionylidene-type sesquiterpenoids possessing antimicrobial activity against *Porphyromonas gingivalis* from *Phellinus linteus* and their absolute structure determination

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## INTRODUCTION

The oral bacteria *Porphyromonas gingivalis* (*P. gingivalis*) is well known as a periodontal pathogen. It not only causes periodontal disease but also plays a role in systemic disease including diabetes, respiratory illness, pregnancy complications and heart disease.[1] *P. gingivalis* has been identified as a key pathogen contributing to chronic periodontitis; its establishment in subgingival sites induces an inflammatory response that leads to gingival tissue destruction and the progressive loss of alveolar bone around the teeth.[2] Hence, discovering new growth inhibitors for these oral periodontal pathogens is of potential importance. Among the different potential sources of new bioactive compounds, mushrooms are of great interest because they contain a number of secondary metabolites with unique structures and interesting biological activities. Thus, mushrooms have been used as a valuable resource for the discovery of lead compounds in medicinal research.[3]

The medicinal mushroom *Phellinus linteus* (*P. linteus*, 'Meshimakobu' in Japanese), a member of the Hymenochaetaceae family, is a well-known fungus that is widely used in China, Korea and other Asian countries. Many studies have reported that this mushroom exhibits various pharmacological effects on several ailments including tumors, diabetes, inflammation and obesity.[4] Experimental demonstrations of antitumor,[5] immunomodulatory,[6] anti-inflammatory[7] and antioxidant[8] activities of *P. linteus* have also been reported. Moreover, a large number of bioactive compounds have been isolated and extracted from this medicinal mushroom. Some other classes of mushrooms also contain compounds (including polysaccharides, flavones, triterpenes and phenols) that have been reported to be responsible for various bioactivities exhibited by *P. linteus*.[9–12] Components in fruiting bodies and mycelia can be differentiated because the contents in *P. linteus* parasitizing on the trunk of *Morus alba* L. are noticeably higher than those on other trees. Therefore, we supposed that an isolation study based on *P. linteus* would be suitable for medical research.

In the course of our search for natural products with antimicrobial activity against *P. gingivalis*, the MeOH extract of the fruiting bodies of *P. linteus* was found to exhibit antimicrobial activity against *P. gingivalis*. A fractionation study on the MeOH extract of *P. linteus* resulted in the isolation of two known γ-ionylidene sesquiterpenoids, (−)-*trans*-γ-monocyclofarnesol (1) and (+)-γ-ionylideneacetic acid (2), as well as a new compound, Phellidene E (3). In this paper, we report the structures of 1, 2 and 3 together with their antimicrobial activities against *P. gingivalis*. (Figure 1)

## RESULTS AND DISCUSSION

The cultured mycelia of *P. linteus* were extracted sequentially with methanol at room temperature. The concentrated methanolic extract was diluted with distilled water and partitioned with chloroform and ethyl acetate. Each fraction was dried *in vacuo*. The AcOEt extract was chromatographed on silica gel and separated into fractions 1–10. The chloroform extract was chromatographed on silica gel and separated into fractions 1–10. Further purification by silica gel column chromatography and reversed-phase HPLC afforded known compounds 1, 2 and new compound 3.

Compound 1, $[\alpha]_D$ −6.8°, was afforded as a colorless oil. The molecular formula $C_{15}H_{26}O$ was determined from HR-FAB-MS data. The IR spectrum displayed absorptions at $3421\ cm^{-1}$ (−OH group) and $1643\ cm^{-1}$ (double bond). The $^1H$ and $^{13}C$ NMR spectra were identical to those reported in literature for (+)-*trans*-γ-monocyclofarnesol.[13,14] However, the value of $\alpha_D$ $[\alpha]_D$ (+18.2°) reported in the same paper was the opposite of that recorded for

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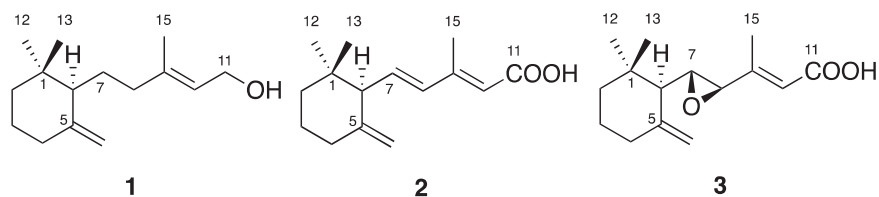E-mail: kobayashiy@pharm.kitasato-u.ac.jp

**Figure 1** Structures of compounds (−)-*trans*-γ-monocyclofarnesol (**1**), (+)-γ-ionylideneacetic acid (**2**) and Phellidene E (**3**).

**Table 1** NMR spectroscopic data (400 MHz, CDCl₃) of compounds (−)-*trans*-γ-monocyclofarnesol (1), (+)-γ-ionylideneacetic acid (2) and Phellidene E (3)

| | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| Atom | ¹H | ¹³C | ¹H | ¹³C | ¹H | ¹³C |
| 1 | | 35.6 | | 35.6 | | 35.9 |
| 2 | 1.35 ddd (14, 8.5, 5.5) | 39.0 | 1.35 ddd (14.0, 8.5, 5.5) | 39.0 | 1.36 ddd (13.0, 7.0, 6.0) | 39.1 |
| | 1.50 ddd (14, 5.5, 5.5) | | 1.50 ddd (14.0, 5.5, 5.5) | | 1.50 m | |
| 3 | 1.59 m | 23.2 | 1.59 m | 23.2 | 1.57 m | 23.4 |
| 4 | 2.05 ddd (13.5, 8.5, 5.5) | 34.4 | 2.05 ddd (13.5, 8.5, 5.5) | 34.4 | 2.03 ddd (13.5, 7.0, 6.0) | 35.1 |
| | 2.28 ddd (13.5, 5.5, 5.5) | | 2.28 ddd (13.5, 5.5, 5.5) | | 2.14 ddd (13.5, 6.0, 5.5) | |
| 5 | | 149.6 | | 149.6 | | 148.0 |
| 6 | 2.55 d (9.5) | 58.0 | 2.55 d (9.5) | 58.0 | 1.53 bd (8.5) | 57.2 |
| 7 | 6.31 dd (15.5, 9.5) | 137.3 | 6.31 dd (15.5, 9.5) | 137.3 | 2.92 dd (8.5, 2.0) | 60.5 |
| 8 | 6.14 d (15.5) | 135.3 | 6.14 d (15.5) | 135.3 | 3.04 dd (2.0, 1.0) | 59.3 |
| 9 | | 154.9 | | 154.9 | | 157.6 |
| 10 | 5.75 bs | 117.5 | 5.75 bs | 117.5 | 5.94 dq (1.0, 1.0) | 115.4 |
| 11 | | 172.9 | | 172.9 | | 171.9 |
| 12 | 0.90 s | 29.4 | 0.90 s | 29.4 | 1.04 s | 29.1 |
| 13 | 0.84 s | 23.6 | 0.84 s | 23.6 | 0.99 s | 23.8 |
| 14 | 4.54 d (1.5) | 109.0 | 4.54 d (1.5) | 109.0 | 4.52 d (1.0) | 109.2 |
| | 4.76 d (1.5) | | 4.76 d (1.5) | | 4.80 dd (1.0, 1.0) | |
| 15 | 2.33 s | 14.2 | 2.33 s | 14.2 | 2.11 d (1.0) | 15.0 |

Abbreviations: bd, broad doublet; bs, broad singlet; bq, broad quartet; d, doublet; dd, double doublet; ddd, double double doublet; m, multiplet; s, singlet.

isolated compound **1**. Thus, we assigned **1** as (−)-*trans*-γ-monocyclofarnesol (Table 1).

Compound **2**, $[\alpha]_D$ +11.7°, was afforded as a colorless oil. The molecular formula $C_{15}H_{22}O_2$ was determined from HR-FAB-MS data. The IR spectrum displayed absorptions at 3420 (−OH group), 1680 (α,β-unsaturated carbonyl moiety), 1645 and 1600 cm⁻¹ (*trans*-diene), and 1625 cm⁻¹ (double bond). During a comparison with the ¹H NMR spectrum of **1**, one olefinic proton peak was increased to assist the determination of structure **2** [(2*E*, 4*E*)-γ-ionylideneacetic acid]. The ¹H and ¹³C NMR spectra of compound **2** were identical to those previously reported for (2*E*, 4*E*)-γ-ionylideneacetic acid.[15] Thus, the stereochemistry of **2** was expected to be similar to that of **1**. We therefore assigned **2** as (+)-(2*E*, 4*E*)-γ-ionylideneacetic acid.

Compound **3**, $[\alpha]_D$ +4.6°, was afforded as a colorless powder. The molecular formula $C_{15}H_{22}O_3$ was determined from HR-FAB-MS data. The IR spectrum displayed absorptions at 3400 (−OH group), 1650 and 1640 cm⁻¹ (double bond), and 1696 cm⁻¹ (α,β-unsaturated carbonyl moiety). The ¹H NMR spectra (Table 1) displayed typical resonances for two tertiary methyl groups at δ = 0.99 (3H, s), 1.04 (3H, s), a vinyl methyl group at δ = 2.11 (3H, d, *J* = 1.0 Hz), a terminal methylene group at δ = 4.52 (1H, d, *J* = 1.0 Hz), 4.80 (1H, dd, *J* = 1.0, 1.0 Hz) and an oxymethylene group at δ = 4.12 (2H, d, *J* = 7.0 Hz). In the ¹³C NMR spectra, 15 signals similar to those observed for **1** and **2** were recorded. This suggests that the carbon skeleton of **3** is γ-ionylidene sesquiterpenoid. Comparison of the ¹H and ¹³C NMR

data of **3** with those of **2**, revealed the disappearance of resonances for the double bond at C7–C8 in **2**, instead of the observation of a resonance at δ 2.92 (1H, dd, *J* = 8.5, 2.0 Hz), δ 3.04 (1H, dd, *J* = 2.0, 1.0 Hz), δ 60.5 (CH), δ 59.3 (CH), in **3**, suggesting that an epoxide structure at C7–C8 in **3**. Furthermore, detailed analyses of the HMBC spectra (Figure 2) confirmed the planar structure of **3**. The coupling constant of H-7 − H-8 (8.5 Hz) and the NOESY correlations between H-6/H₃-13, H-7/H₃-14 and H-6/H-8 suggest that H-7 and H-8 exhibit an anti-periplanar conformation. The E-geometry of the olefin at the C9–C10 position was determined by NOESY correlations between H-10/H-15. Furthermore, detailed analyses of the NOESY correlations of H-7/H-15 and H-8/H-10 were applied to determine the conformation of **3** (Figure 2). Thus, the relative configuration of **3** was elucidated as 6*R**,7*R**,8*R**.

The absolute stereochemistry of **3** was deduced from a comparison between the electronic CD (ECD)[16,17] and calculated spectra[18] (Figure 3). The conformational analyses of **3** started from MM2 force field structures was further optimized by semi-empirical PM3 calculations, and was finally refined by density functional theory (DFT, B3LYP/6-31G level) calculations to yield additional relevant conformers. The ECD spectra of **3** were simulated using time-dependent TD-density functional theory (B3LYP/DGDZVP level of theory) calculations on the relevant conformers.

A minimum value at a 260–280 nm area and a maximum value at a 220–240 nm area were observed as a result of the comparison between the experimental and calculated ECD spectra. In addition, the
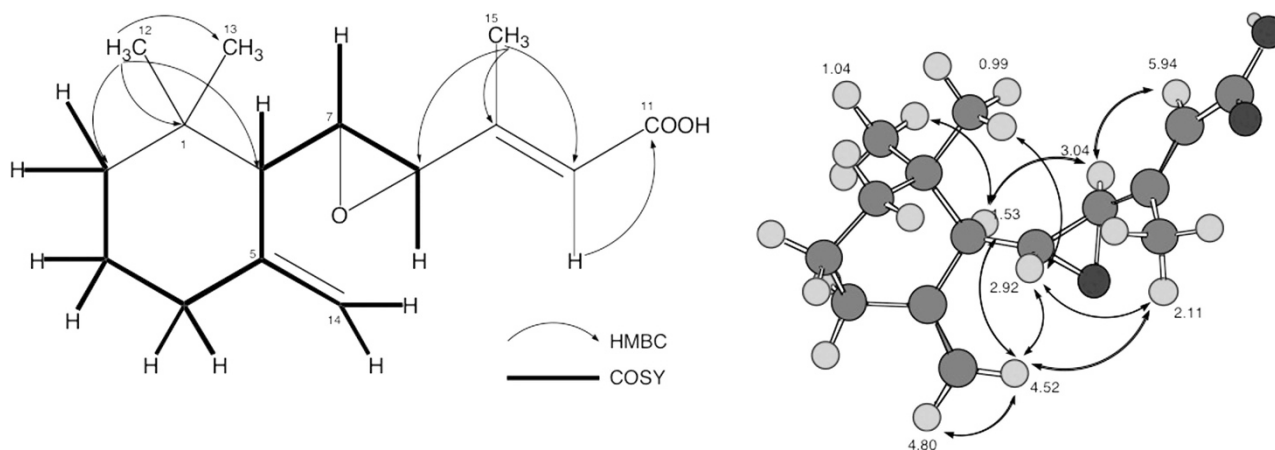
**Figure 2** Key $^1$H–$^1$H COSY and HMBC; and NOESY correlations of Phellidene E (**3**). A full color version of this figure is available at *The Journal of Antibiotics* journal online.
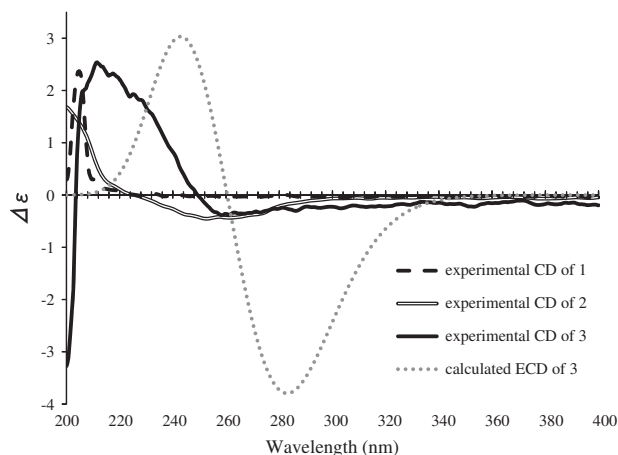


**Figure 3** Experimental electronic CD (ECD) spectra of γ-monocyclofarnesol (**1**), γ-ionylideneacetic acid (**2**) and Phellidene E (**3**) overlaid with the calculated ECD spectra.

**Table 2 Antibacterial activity of sesquiterpenes against *Porphyromonas gingivalis* (*P. gingivalis*)**

| Compound | MIC ($\mu g\,ml^{-1}$) |
|---|---|
| *γ-Ionylidenes* | |
| γ-monocyclofarnesol (**1**) | 5.9 |
| γ-ionylideneacetic acid (**2**) | 34.1 |
| Phellidene E (**3**) | 155 |
| Hinokitiol | 25.0 |
| Triclosan | 3.13 |

measurement of the ECD spectrum of **1** and **2** resulted in the observation of a maximum value at a 200–240 nm area based on the stereochemistry at C6. Thus, the absolute stereochemistry of **3** was elucidated as 6*R*, 7*R*, 8*R*.

Compounds **1**–**3** were evaluated for their antibacterial activities against the periodontal bacteria *P. gingivalis*. The MIC was measured with hinokitiol and triclosan as positive controls (MIC: 25.0, 3.1 μg ml$^{-1}$, respectively, Table 2). Although compounds **2** and **3** exhibited weak effects on the growth of *P. gingivalis*, compound **1** markedly inhibited its growth (MIC: 5.9 μg ml$^{-1}$). These results support our hypothesis that the hydrophobic alkyl chain is an important moiety for the activity of γ-ionylidene sesquiterpenoid against *P. gingivalis*.

In conclusion, a new γ-ionylidene sesquiterpenoid (**3**) and two previously known γ-ionylidene sesquiterpenoid (**1** and **2**) were isolated from the fruiting bodies of *P. linteus*. Among these compounds, compound **1** exhibited potent antimicrobial activity against the oral pathogen *P. gingivalis*. Further studies are warranted to confirm the ability of products containing compound **3**, such as mouthwash, toothpaste and chewing gum, to alter the composition of oral microflora in humans, thereby improving their oral health.

## EXPERIMENTAL SECTION
### General information

Optical rotations ($[\alpha]_D$) were measured on a JASCO DIP-1000 digital polarimeter (Tokyo, Japan). IR and UV spectra were recorded on a JASCO density functional theory/IR 460 infrared (Tokyo, Japan) and a Hitachi U-2800 spectrophotometer (Tokyo, Japan), respectively. ECD data were recorded on a JASCO J720 (Tokyo, Japan). Positive and negative ion HR-FAB-MS measurements were recorded on JEOL JMS-AX505 HA and JMS-700 MS stations (Tokyo, Japan). EI-MS data were measured on a JEOL JMS-AX505 (Tokyo, Japan). $^1$H and $^{13}$C NMR experiments were performed on a Varian XL-400 (Palo Alto, CA, USA). The chemical shifts are expressed in p.p.m. downfield from the internal solvent peaks for CHCl$_3$ (7.26 p.p.m., $^1$H NMR), CH$_3$OH (3.31, 4.84 p.p.m., $^1$H NMR), CDCl$_3$ (77.0 p.p.m., $^{13}$C NMR) or CD$_3$OD (49.0 p.p.m., $^{13}$C NMR) and *J* values were measured in Hertz. The coupling patterns are denoted as s (singlet), d (doublet), dd (double doublet), ddd (double double doublet), t (triplet), dt (double triplet), q (quartet), m (multiplet) or br (broad). HPLC analysis was carried out using a Shimadzu SPD-10AV UV–vis detector (Kyoto, Japan) and a Waters HPLC-pump (Waters 515, Milford, MA, USA, UV; 254 nm) with a μ-BONDASPHERE 5μ C18–100 Å column (5 μm, 19 φ × 150 mm). Pre-coated silica gel plates with a fluorescent indicator (Merck 60 F254, Darmstadt, Germany) were used for analytical and preparative thin layer chromatography. Flash column chromatography was carried out with silica gel (Kanto Chemical, Tokyo, Japan, silica gel 60N, spherical neutral, 0.040–0.050 mm, Cat. No. 37 563–84).

## Culture conditions for *P. linteus* mycelium

After sterilizing the cultivated fruiting body of *P. linteus*, a small piece of the stipe was inoculated onto a potato dextrose agar medium (39 g, distilled water = 1l). The developing colonies of the new hyphae (from the stipe) were transferred to malt agar in Petri dishes. The pure mycelia were subcultured for 2 weeks and grown in a 500 ml Erlenmeyer flask in 125 ml of medium containing 10 g sucrose, 30 g malt extract and 5 g yeast extract in 1 l of distilled water. Each flask was seeded with 5 of the 10 mm plugs cut from the potato dextrose agar culture.

## Extraction procedure and separation of the CHCl₃ and AcOEt extract of the mycelia

After 5 weeks of culture (100 flasks) (25 °C, dark, 63 r.p.m.), the mycelia (990.55 g, for wt) were harvested with nylon cloth, homogenized with MeOH in a Waring blender and allowed to stand for 1 week at room temperature. The homogenate was filtered and the residue was reextracted with the same solvent. The filtrates were combined and the organic solvent was removed under reduced pressure. The concentrated methanolic extract was then diluted with water and partitioned with $CHCl_3$ and AcOEt as follows: the residue was first extracted with $CHCl_3$ (X2 in a total of 2 l), dried *in vacuo* and evaporated to dryness to afford the $CHCl_3$ extract (My CHCl₃, 20.99 g). The resultant residue was then extracted with AcOEt (X4 in a total of 4.8 l), dried *in vacuo* and evaporated to dryness to afford the AcOEt extract (My EtOAc, 0.9557 g). The $CHCl_3$ extract (20.99 g) was first subjected to silica gel chromatography eluted with a toluene–AcOEt (19:1) mixture to afford fractions 1–10. Further purification of fraction 2 (17.7 g) was achieved by silica gel column chromatography to afford **2** (3.59 g). Fraction 4 (450.3 mg) was recrystallized from MeOH to afford a colorless powder and mother liquid that were concentrated to afford fraction 4-1. Both (−)-*trans*-γ-monocyclofarnesol (**1**) (39.4 mg) and Phellidene E (**3**) (80.5 mg) were isolated from Fracton44-1 by HPLC (solvent: 90% MeOH, flow rate: 6.0 ml min⁻¹).

**1**: Colorless oil; $[\alpha]_D^{22}$ −6.76° (*c* = 0.10, MeOH); IR (KBr) $\nu_{max}$ cm⁻¹: 3421, 2927, 2855, 1643; UV (MeOH) $\lambda_{max}$ nm (log ε): 204 (4.06); FAB-MS (positive) *m/z*: 222[M]⁺, 245[M+Na]⁺; 1H and 13C NMR data (Table 1); HR-FAB-MS (positive) *m/z*: Calcd for $C_{15}H_{26}O$ [M]⁺ = 222.1984; found = 222.1979.

**2**: Colorless solid; $[\alpha]_D^{28}$ +11.7° (*c* = 0.21, CHCl₃); IR (KBr) $\nu_{max}$ cm⁻¹: 3420, 2920, 1680, 1645, 1625, 1600; UV (MeOH) $\lambda_{max}$ nm (log ε): 250 (4.38); EI-MS *m/z*: 234[M]⁺; 1H and 13C NMR (Table 1); HR-EI-MS *m/z*: Calcd for $C_{15}H_{22}O_2$ [M]⁺ = 234.1620; found = 234.1624.

**3**: Colorless solid; $[\alpha]_D^{28}$ +4.55° (*c* = 0.22, MeOH); IR (KBr) $\nu_{max}$ cm⁻¹: 3400, 2925, 1696, 1650, 1640; UV (MeOH) $\lambda_{max}$ nm (log ε): 223 (4.23), 205 (4.21); FAB-MS (positive) *m/z*: 251 [M+H]⁺, 273 [M+Na]⁺: ₁H and 13C NMR data (Table 1); HR-FAB-MS (positive) *m/z*: Calcd for $C_{15}H_{23}O_3$ [M+H]⁺ = 251.1647; found = 251.1637.

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