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NOTE

The cytotoxic and antifungal activities of two new sesquiterpenes, malfilanol A and B, derived from *Malbranchea filamentosa*

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Fungi of the genus *Malbranchea* belong to the family *Onygenaceae* and are taxonomically close to human and animal pathogenic fungi.[1] These facts prompted us to investigate the chemical constituents of *Malbranchea* fungi. We recently reported the isolation and structural characterization of 4-benzyl-3-phenyl-5*H*-furan-2-one as a vasodilator, malfilamentosides A and B as furanone glycosides and malbrancheosides A–D as triterpene glycosides, from the fungus *Malbranchea filamentosa* IFM41300.[2–4] Further purification of extracts of rice cultivated by the above fungus allowed us to isolate two new sesquiterpenes, designated malfilanol A (**1**) and B (**2**). Characterization of their structures, cytotoxic activities and antifungal activities are described in this paper.

The molecular formula of malfilanol A (**1**) (Figure 1) was determined as $C_{15}H_{24}O_3$ (four degrees of unsaturation) by high-resolution chemical-ionization mass spectrometry (HRCI-MS). Detailed analyses of the ^{1}H- and ^{13}C-NMR spectra and HMQC correlations of **1** revealed the presence of one exchangeable proton, three methyl units, five methylene units, two methine units, two quaternary carbons (one of which is oxygenated) and one olefine unit with an attached carboxylic acid (δ 172.6). These data accounted for the ^{1}H- and ^{13}C-NMR resonances and required the compound to be bicyclic. Interpretation of the ^{1}H–^{1}H COSY data led to the identification of two isolated proton spin systems corresponding to C-2–C-3 and C-5–C-7, including the branched C-9–C-11 chain subunits of structure **1**. The heteronuclear multiple bond coherence (HMBC) correlations (Figure 2) from H_3-12 to C-1, C-2, C-11; from H_3-13 to C-4, C-5, C-14; from H_3-14 to C-3, C-13; from H_2-2 to C-4, C-11; from H_2-3 to C-5; from H_2-5 to C-13, C-7; from H-7 to C-6, C-9, C-11, C-15; and from H_2-9 to C-7, C-8, C-10, C-11 enabled the determination of structure **1**.

Analysis of NOESY data (Figure 3) and ^{1}H NMR *J*-values enabled assignment of the relative configuration of malfilanol A (**1**). The coupling constant observed between H-6 and H-11 (2.8 Hz) indicated these two protons to be in a *cis* configuration. NOESY correlations of H-6 both with H_3-14 and with one proton of H_2-10 (δ_H 1.41) revealed that all these protons have a *syn* orientation. Further, the NOESY correlation between another proton of H_2-10 (δ_H 1.77) and H_3-12 was used to place these protons on opposite faces of the molecule against H-6, thereby establishing the relative configuration of malfilanol A (**1**).

The elemental composition of malfilanol B (**2**) was determined as $C_{16}H_{26}O_3$ (four degrees of unsaturation) on the basis of HRCI-MS and NMR data, which weighs 14 mass units higher than **1**. Analysis of ^{1}H- and ^{13}C-NMR data revealed the presence of a methoxy group (δ_C 51.4, δ_H 3.69) in **2**, instead of a hydroxyl group attached to C-15. On the basis of this consideration, the structure of **2** was presumed to be the methyl ester of **2**. Further, methylation of **1** with CH_2N_2 provided **2**, which was identical to the naturally occurring one, including the optical rotation (CD spectra). Therefore, malfilanol B (**2**) was identified as the methyl ester of **1**, and was established to have the same stereochemistry at C-1, C-6 and C-11 as **1**.

Antifungal activity was determined using the paper disk method, as described in a previous study.[5] Malfilanol A (**1**) and malfilanol B (**2**) showed specific antifungal activities against *Cryptococcus neoformans* (the diameters of the inhibition circles for **1** and **2** were 15 and 13 mm, respectively), but there was no antifungal activity against *Aspergillus fumigatus*, *A. niger* or *Candida albicans*, at 50 μg per disk. Malfilanol A (**1**) and B (**2**) were tested for cytotoxic activities against human umbilical vein endothelial cells (HUVEC) and A549 human lung cancer cells. Malfilanol A (**1**) and B (**2**) inhibited the cell proliferation of HUVEC with IC_{50} values of 14.6 and 19.8 μM respectively, while

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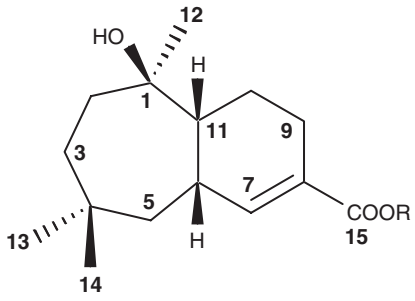

Malfilanol A (**1**) R = H

Malfilanol B (**2**) R = CH_3

Figure 1 Structures of malfilanol A (**1**) and B (**2**).

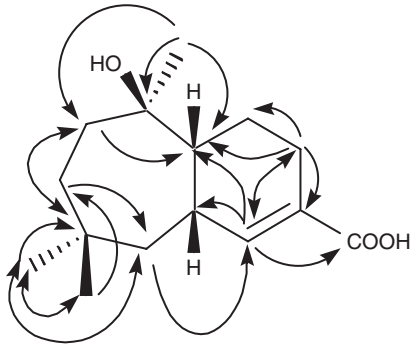

Figure 2 Important HMBC correlations of malfilanol A (**1**).

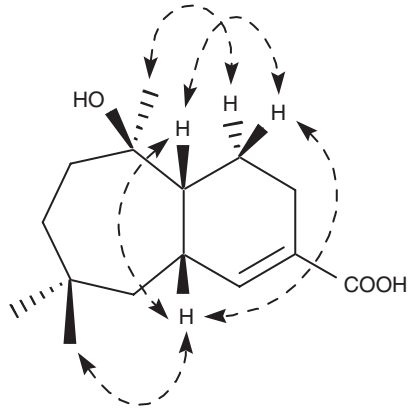

Figure 3 Important NOESY correlations of malfilanol A (**1**).

malfilanol B (**2**) inhibited proliferation of A549 cells with an IC_{50} value of 7.8 μM. Malfilanol A (**1**) showed no inhibition of A549 cell proliferation at 79.4 μM.

EXPERIMENTAL SECTION

General

Melting points were determined on a micro-melting point apparatus (Yanagimoto Ltd, Kyoto, Japan) and are uncorrected. CI-MS data were measured using a JMS-MS600W spectrometer (JEOL Co. Ltd, Tokyo, Japan). UV and IR spectra were recorded on an Ultrospec 2100 pro UV–visible spectrophotometer (Amersham Biosciences Ltd, Tokyo, Japan) and a JASCO FT/IR-4100 instrument (JASCO Co. Ltd, Tokyo, Japan), respectively. ^{1}H- and ^{13}C-NMR spectra were recorded using a JEOL Lambda-500 (500.00 MHz for ^{1}H, 125.25 MHz for ^{13}C, JEOL Co. Ltd) and/or a Bruker AV-400 spectrometer (400.13 MHz for ^{1}H, 100.61 MHz for ^{13}C, Bruker Biospin K. K., Kanagawa, Japan). Chemical shifts (δ) were measured in ppm using tetramethylsilane as an internal standard. CD curves were determined using a J-820 spectropolarimeter (JASCO Co. Ltd). Column chromatography was performed using Kieselgel 60 (Art. 7734, Merck Ltd, Tokyo, Japan) and Wakogel C-200 (Art. 237-00071, Wako Pure Chemical Industries, Ltd, Osaka, Japan). LPLC was performed using a Chemco Low-Prep 81-M-2 pump (Chemco Scientific Co. Ltd, Osaka, Japan) and a glass column (200×10 mm) packed with Silica gel CQ-3 (30–50 μm, Wako Pure Chemical Industries, Ltd). HPLC was performed using a Senshu SSC-3160 pump (flow rate, 7 ml min^{-1}, Senshu Scientific Co. Ltd, Tokyo, Japan) and a YMC-Pack PEGASIL Silica 60-5 column (300×10 mm, YMC Co. Ltd, Kyoto, Japan), equipped with a Shimamura YRD-883 RI detector (Shimamuratech Ltd, Tokyo, Japan). TLC was detected by UV light at 254 nm and/or by spraying with phosphomolybdic acid (5%)-ceric acid (trace) in 5% H_2SO_4 and then heating.

Fermentation, extraction and isolation

Rice (Akitakomachi, 560 g) was moisturized with water for 30 min and then dispensed to four Roux flasks, and sterilized by steaming under pressure. *M. filamentosa* IFM 41300 was inoculated in these Roux flasks and cultivated at 25 °C for 21 days, after which the rice was extracted with acetone and the organic layer was evaporated *in vacuo*. The residue (42 g) was suspended in water and extracted with AcOEt. The evaporated residue was extracted sequentially with hexane (100 ml), benzene (100 ml), CH_2Cl_2 (100 ml) and MeOH (100 ml). The benzene extract (2 g) was chromatographed using a silica gel column (solvent system: CH_2Cl_2/acetone (30:1), (10:1), (5:1), (3:1), (1:1) and acetone) to yield 13 fractions. Fraction 11 (the acetone eluate) was re-chromatographed using HPLC with a silica gel (CH_2Cl_2/ethanol (8:1)) to give malfilanol A (**1**: 4.0 mg). The hexane extract (1 g) was chromatographed using a Sephadex LH-20 column (solvent system: hexane/$CHCl_3$ (1:4) 400 ml, $CHCl_3$/acetone (4:1) 200 ml, (3:2) 200 ml, and acetone 300 ml) to yield nine fractions. Fraction 3 ($CHCl_3$: acetone (4:1), 219 mg) was chromatographed using HPLC with a silica gel (hexane/acetone (4:1)) to give malfilanol B (**2**: 5.0 mg).

Malfilanol A (1)

Colorless amorphous solid. CI-MS *m/z* (%): 253.1833 ((M+H)$^+$, 253.1804 for $C_{15}H_{25}O_3$). UV λ_{max} (MeOH) nm (ε): 218 (4.00). IR ν_{max} (KBr) cm^{-1}: 3420, 2945, 1690 and 1650. CD (MeOH) $\Delta\varepsilon$ (nm): +0.76(249) and −7.72(215). $[\alpha]_D^{25}$: −96° (c 0.525, MeOH). ^{1}H-NMR ($CDCl_3$): δ 0.86 (3H, s, 13-H_3), 0.96 (3H, s, 14-H_3), 1.05 (1H, d, *J*=15.0 Hz, 5-H), 1.20 (1H, m, 2-H), 1.21 (3H, s, 12-H_3), 1.22 (1H, m, 3-H), 1.41 (1H, m, 10-H), 1.46 (1H, m, 5-H), 1.54 (1H, td, *J*=14.0, 9.0 Hz, 2-H), 1.69 (1H, btd, *J*=13.1, 2.8 Hz, 11-H), 1.73 (1H, m, 3-H), 1.77 (1H, dd, *J*=13.1, 6.7 Hz, 10-H), 2.16 (1H, m, 9-H), 2.40 (1H, dd, *J*=18.0, 5.8 Hz, 9-H), 2.69 (1H, m, 6-H) and 6.97 (1H, dd, *J*=5.8, 1.2 Hz, 7-H). ^{13}C-NMR ($CDCl_3$): δ 19.6 (C-10), 25.1 (C-9), 29.3 (C-14), 30.1 (C-12), 31.4 (C-6), 32.0 (C-13), 32.7 (C-4), 34.2 (C-3), 34.5 (C-2), 41.3 (C-5), 48.3 (C-11), 74.2 (C-1), 126.9 (C-8), 147.8 (C-7) and 172.6 (C-15).

Malfilanol B (2)

Colorless crystalline powder (m.p. 109 °C). CI-MS *m/z* (%): 267.1950 ((M+H)$^+$, 267.1960 for $C_{16}H_{27}O_3$). UV λ_{max} (MeOH) nm (ε): 220 (3.92). IR ν_{max} (KBr) cm^{-1}: 3465, 2950, 1715 and 1650. CD (MeOH) $\Delta\varepsilon$ (nm): +0.80(247) and −4.84(218). $[\alpha]_D^{25}$: −68° (c 0.365, MeOH). ^{1}H-NMR ($CDCl_3$): δ 0.86 (3H, s, 13-H_3), 0.96 (3H, s, 14-H_3), 1.05 (1H, d, *J*=15.0 Hz, 5-H), 1.19 (1H, m, 2-H), 1.21 (3H, s, 12-H_3), 1.23 (1H, m, 3-H), 1.42 (1H, m, 10-H), 1.45 (1H, m, 5-H), 1.53 (1H, m, 2-H), 1.67 (1H, btd, *J*=12.3, 3.3 Hz, 11-H), 1.73 (1H, m, 3-H), 1.77 (1H, bdd, *J*=12.3, 6.9 Hz, 10-H), 2.16 (1H, m, 9-H), 2.41 (1H, dd, *J*=18.4, 6.0 Hz, 9-H), 2.68 (1H, bs, 6-H), 3.69 (3H, s, $COOCH_3$) and 6.85 (1H, bd, *J*=5.9 Hz, 7-H). ^{13}C-NMR ($CDCl_3$): δ 19.6 (C-10), 25.4 (C-9), 29.2 (C-14), 30.0 (C-12), 31.2 (C-6), 32.0 (C-13), 32.7 (C-4), 34.2 (C-3), 34.5 (C-2), 41.5 (C-5), 48.4 (C-11), 51.4 ($COOCH_3$), 74.0 (C-1), 127.5 (C-8), 145.5 (C-7) and 168.1 (C-15).

Methylation of malfilanol A (1) with CH_2N_2

An excess ethereal solution (2 ml) of diazomethane was added to an ether solution (0.5 ml) of malfilanol A (**1**) (1.0 mg), and the solution was stirred for 1 min followed by evaporation to give malfilanol B (**2**) (0.8 mg).

Cytotoxicity assay

Cells were seeded into 96-well microplates at 3000 cells per well, allowed to attach for 4–6 h and then incubated in Dulbecco's modified Eagle's medium (Invitrogen Co. Ltd, Carlsbad, CA, USA) for A549 human lung cancer cells, or in endothelial cell growth medium-2 Single Quots medium (Lonza Co., Ltd, Valais, Switzerland) supplemented with 10% fetal bovine serum, penicillin G (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and amphotericin B (0.25 μg ml^{-1}) for HUVEC until they were 80% confluent. The media were supplemented with the indicated concentrations of isolated compounds for 48–72 h. Cell proliferation was measured using the Cell Counting Kit8 (Dojindo, Kumamoto, Japan) to count living cells by combining WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) and 1-methoxy PMS (1-methoxy-phenazine methosulfate).[6] Briefly, after the medium was removed, 10 μl of Cell Counting Kit8 solution was added to each well, and the plates were incubated for 4 h, then cell numbers were obtained by scanning with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Antifungal assay using the paper disk method

The antifungal assay was performed using a method reported earlier.[5] The antifungal assay was performed using the paper disk method against *A. niger* IFM 41398, *A. fumigatus* IFM 41362, *C. albicans* IFM 40009 and *C. neoformans* ATCC 90112 as test organisms. Malfilanol A (**1**) and malfilanol B (**2**) were applied to the paper disk (diameter: 8 mm) at 50 μg per disk and the disks were placed on the assay plates. The test organisms were cultivated in potato dextrose agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) at 25 °C. After 48–72 h of incubation, zones of inhibition (the diameter measured in millimeters) were recorded.

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