Antifungal Activity of a Polyacetylene against the Fungal Pathogen of Japanese Oak from the Liquid Culture of the Edible Mushroom, *Hypsizygus marmoreus*

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Three polyacetylene compounds, **1**, **2** and **3**, have been isolated from the organic extract of a liquid culture of *Hypsizygus marmoreus* (Peck.) Bigelow (Japanese name: Bunashimeji). The structures of **1**, **2** and **3** were determined by using spectroscopic methods. Compound **3** is new, and was identified as 8(*E*)-decene-4,6-diyn-1,2,10-triol. Compound **1** exhibits antifungal activity against the pathogenic fungus *Raffaelea quercivora* responsible for Japanese oak wilt.

Key words: Japanese Oak Wilt, *Hypsizygus marmoreus*, *Raffaelea quercivora*, Polyacetylene

Introduction

Japanese oak wilt (JOW) is a tree disease caused by a fungus called *Raffaelea quercivora*, which is vectored by the ambrosia beetle, *Platypus quercivorus* [1 – 3]. The first epidemic of JOW was documented in 1950 in Miyazaki Prefecture, Japan, and by 2005, with the exception of the northern district, Hokkaido Prefecture, JOW had spread throughout the country. In Honshu, Japan, predominantly in Yamagata, Fukui, Kyoto, and Tottori Prefectures, which all front on the Sea of Japan, major epidemics have spread during the last decade, and the disease incidence was reported to be as high as 33 000 m^3 in 2010, resulting in severe damage and economic loss [2]. The necrotic action of Japanese oak caused by *R. quercivora* is associated with the induction of discoloration of the xylem followed by the dysfunction of vessels [1, 3]. To date, the control of JOW has been based on fungicide treatments, which are aimed at reducing the inoculum potential in summer. In the fall and winter seasons, the fumigation of JOW logs with the *N*-methyl-*N*-(*m*-tolyl)dithiocarbamic acid sodium salt, burning, and chipping were the usual procedures for the control of the ambrosia beetle in Japan, as the larvae inhabit such logs during the winter. However, recently, logs infected with *R. quercivora* without fumigation have been used for log cultivations of mushrooms in Japan. There is no difference in the productivity of mushrooms on logs infected with *R. quercivora* and decay-free logs, although mushrooms (shiitake) cannot grow on logs exhibiting *Trichoderma*, which results in wood decay.

In our screening program on antifungally active components from the cultured mycelia of mushrooms, the EtOAc extract of *Hypsizygus marmoreus* (Peck.) Bigelow (Japanese: Bunashimeji) cultured on a liquid culture showed an appreciable suppressive effect against *R. quercivora*. Solvent partitions followed by repeated chromatographic fractionations of the extract, with monitoring of the antifungal activity, afforded the active compound 2(*E*)-decene-4,6,8-triyn-1-ol (**1**), together with the less active known compound, decene-4,6,8-triyn-1-ol (**2**), and a new compound **3** (Fig. 1). This paper describes the isolation and structure elucidation of **3** from a mycelia culture of *H. marmoreus*.

Results and Discussion

A culture broth (6.0 L) of *H. marmoreus* (Peck.) Bigelow was filtered, and the cultured filtrate was extracted with ethyl acetate (EtOAc). The EtOAc ex-

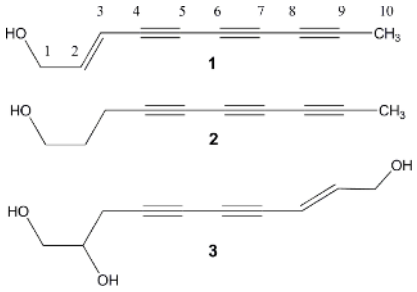

Fig. 1. Structures of **1**, **2** and **3**.

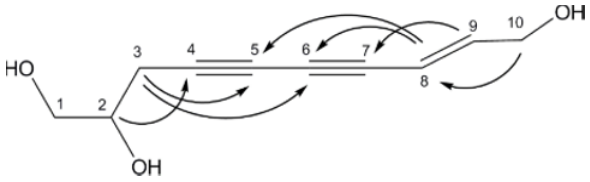

Fig. 2. HMBC correlations observed for **3**.

tract was chromatographed on silica gel and separated into 13 fractions. Purification of the fungal metabolites was guided by an antifungal activity test, UV absorption, and the characteristic coloration by thin-layer chromatography (TLC). The EtOAc layer was chromatographed on a silica gel column using a stepwise gradient of *n*-hexane-EtOAc. Further chromatographic studies using silica and ODS (octadecyl silica) gel yielded three compounds, **1**–**3**.

The known compounds **1** and **2** were identified as 2(*E*)-decene-4,6,8-triyn-1-ol [4] and decene-4,6,8-triyn-1-ol [5] on the basis of their ^{1}H and ^{13}C NMR, ^{13}C-^{1}H COSY, and HMBC data (Fig. 1).

Compound **3** had the molecular formula $C_{10}H_{12}O_3$, as determined by HR-ESITOF-MS and NMR spectroscopy, thus requiring five degrees of unsaturation. The UV spectrum of **1** showed absorption maxima at 240, 253, 267, and 283 nm, suggesting the presence of an ene-diyne chromophore. The IR spectrum of **1** showed the presence of hydroxyl (3367 cm^{-1}) and acetylene (2237 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra showed 10 carbon signals including characteristic signals due to three sp^3 methylenes [δ_C = 25.1 (C-3), 62.7 (C-10), 65.9 (C-1)], two of which were linked to an oxygen atom, one oxymethine group [$\delta_C = 71.6$ (C-2)], two olefinic carbons [$\delta_C = 109.0$ (C-8), 147.2 (C-9)], and four quaternary carbons [$\delta_C = 81.4$ (C-4), 67.2 (C-5), 75.2 (C-6), 74.2 (C-7)]. The ^{1}H NMR spectrum, analyzed using ^{1}H-^{1}H COSY and HMQC data, indicated the presence of a -CH_2-CH-CH_2 linkage [$\delta_H = 2.47$ (dd, $J = 17.6$, 6.0 Hz, 1H, 3-H), 2.57 (dd, $J = 17.6$, 6.0 Hz, 1H, 3-H), 3.51 (d, $J = 5.5$ Hz, 1H, 1-H), 3.53 (d, $J = 5.5$ Hz, 1H, 1-H), 3.74 (m, 1H, 2-H)], and a -CH_2-CH=CH- linkage [$\delta_H = 4.12$ (dd, $J = 4.6$, 1.7 Hz, 2H, 10-H_2), 5.76 (d, $J = 15.9$ Hz, 1H, 8-H), 6.43 (dt, J= 15.9, 4.6 Hz, 1H, 9-H)]. In addition, the olefinic proton doublets with a coupling constant of 15.9 Hz could be attributed to the double bond in the *trans* configuration. For the connectivity of the partial structures, we conducted HMBC experiments (Fig. 2). The signals of H-9 correlated with C-7, H-2 with C-4, and H-3 with C-5 and 6, indicating a conjugated diyne system between C-3 and C-8. Thus, on the basis of these results, the structure of **3** was determined to be 8(*E*)-decene-4,6-diyn-1,2,10-triol. Compound **3** was closely related to 8(*E*)-decene-4,6-diyn-1,3,10-triol, which was isolated previously from the dried whole plant of *Bidens parviflora* Willd [6]. The biological activity of 8(*E*)-decene-4,6-diyn-1,3,10-triol has not been reported. Owing to the small amount of **3** in the sample, the absolute configuration of C-2 remains to be determined.

The antimicrobial activities of **1**, **2** and **3** were tested by using the agar dilution method. Compound **1** showed activity against *R. quercivora* JCM 11526 with a zone of inhibition of 12 mm in diameter at a concentration of 3 μg per disk. At a concentration of 50 μg per disk, **2** and **3** were inactive against *R. quercivora* JCM 11526. The behavior of **1** and **2** appeared to indicate that the activity is related to the change in the double-bond moiety at C-2 and C-3, suggesting that the presence of a conjugated system at the ene-triyne might be important for the antimicrobial activity of **1**. Further detailed biological studies on **1**–**3** are in progress.

Conclusion

Through bioactivity-guided fractionation and TLC analysis, we succeeded in the isolation and characterization of **1**, **2** and **3** from *H. marmoreus* (Peck.) Bigelow. Compound **3** had not been isolated previously from a natural source. Polyacetylenic compounds are widely distributed in the fungi of the group Basidiomycetes [7]. Some unsubstituted polyacetylenes are unstable at room temperature [7]. Although **1** was also unstable at ambient conditions and degraded to a yellow powder, we established that **1** exhibited inhibitory

activity against the pathogenic fungus *R. quercivora* JCM 11526 at this assay.

Experimental Section

General experimental procedures

Optical rotation was measured with a Horiba model SEPA-300 polarimeter, IR spectra were recorded with a JASCO J-20A spectrophotometer, and UV spectra were recorded with a Shimadzu UV mini-1240 instrument. Mass spectra were obtained with a Synapt G2 mass spectrometer, and ^{1}H and ^{13}C NMR spectra were obtained with a Jeol EX-400 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was conducted on ODS (octadecyl silica, chromatorex-ODS, Fuji Silysia Chemical, Ltd., Japan) and silica gel 60 (Kanto Chemical Co., Inc., Japan). TLC was performed on a precoated silica gel plate (Merck), and spots were detected by spraying 10 % vanillin in H_2SO_4 followed by heating.

Fungus and cultivation

The cultivated edible mushroom, *H. marmoreus* (Peck.) Bigelow, was purchased at a local market in Tsuruoka City, Yamagata, Japan. Pieces of fresh fruiting bodies were surface-sterilized with EtOH, and placed on potato dextrose agar plates. Plates were incubated at 25 °C for 7 days, and colonies appearing on the plates were isolated. The producing strain *H. marmoreus* (Peck.) Bigelow was grown on slant of potato dextrose agar. A loopful of the culture was transferred into 65 Sakaguchi flasks (500 mL) containing 100 mL of a medium consisting of 40 g of malt extract, 40 g of glucose, and 1.0 g peptone per 1 liter of water. The inoculated flask was incubated at 25 °C for 4 weeks on a rotary shaker.

Extraction and isolation of compounds

6.0 L of culture broth of *H. marmoreus* (Peck.) Bigelow was separated from the mycelia by filtration. The filtrate was extracted with EtOAc. The organic layer was concentrated *in vacuo* to give an oily residue (11.4 g). The residue was subjected to silica gel column chromatography with mixtures of *n*-hexane-EtOAc, and mixtures of EtOAc-MeOH to give fractions 1 to 13 (Fr. 1 – 13). Fr. 8 (70 % MeOH eluate, 710 mg) was further chromatographed on silica gel by eluting with $CHCl_3$ and an increasing volume of EtOAc to afford 40 – 50 % EtOAc eluates (99 mg). These fractions were combined and rechromatographed on ODS with $CHCl_3$-MeOH (90 : 10, v/v) to yield compounds **1** (2.6 mg) and **2** (4.6 mg). Fr. 10 (10 % ErOAc eluate, 1.0 g) was further chromatographed on silica gel with mixtures of $CHCl_3$-EtOAc. The 20 – 30 % EtOAc eluates (100 mg) were further purified by ODS column chromatography with mixtures of H_2O-MeOH (10 % stepwise gradient) to obtain 3.0 mg of compound **3**.

*8(E)-Decene-4,6-diyn-1,2,10-triol (**3**)*

Off-white amorphous powder. – $[\alpha]_D^{20} = +17^\circ$ ($c = 1.0$, MeOH). – UV (MeOH) λ_{max} ($\lg \varepsilon$) = 240 (3.6), 253 (3.7), 267 (3.7), 283 (3.7) nm. – IR (KBr): ν = 3367, 2873, 2237, 1455, 1417 and 1033 cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.47 (dd, J = 17.6, 6.0 Hz, 1H, 3-H), 2.57 (dd, J = 17.6, 6.0 Hz, 1H, 3-H), 3.51 (d, J = 5.5 Hz, 1H, 1-H), 3.53 (d, J = 5.5 Hz, 1H, 1-H), 3.74 (m, 1H, 2-H), 4.12 (dd, J = 4.6, 1.7 Hz, 2H, 10-H_2), 5.76 (d, J = 15.9 Hz, 1H, 8-H), 6.43 (dt, J = 15.9, 4.6 Hz, 1H, 9-H). – ^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$) : δ = 25.1 (C-3), 62.7 (C-10), 65.9 (C-1), 67.2 (C-5), 71.6 (C-2), 74.2 (C-7), 75.2 (C-6), 81.4 (C-4), 109.0 (C-8), 147.2 (C-9). – HRMS ((+)-ESI-TOF): m/z = 203.0725 (calcd. 203.0684 for $C_{10}H_{12}O_3Na$, $[M+Na]^+$).

Antimicrobial activity

The test microorganism was *Raffaelea quercivora* JCM 11526. Antimicrobial assays were carried out by the paper disk diffusion method using a published protocol [8]. Triforine (*N*,*N*′-[piperazine-1,4-diylbis(2,2,2-trichloroethane-1,1-diyl)]bisformamide) was used as a positive control (the zone of inhibition of 12 mm in diameter at a concentration of 0.02 μg per disk).

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