Activity of Armillarisin B *in vitro* against Plant Pathogenic Fungi

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The methanolic extract of the fruiting bodies of the mushroom *Armillariella tabescens* was found to show antifungal activity against *Gibberella zeae*. The active compound was isolated from the fruiting bodies of *A. tabescens* by bioassay-guided fractionation of the extract and identified as armillarisin B. Armillarisin B eventually corresponds to 2-hydroxy-2-phenylpropanediamide and its structure was confirmed on the basis of spectroscopic studies including 2D NMR experiments.

Key words: Armillariella tabescens, Armillarisin B, Antifungal Activity

Introduction

Research during the last decade has convincingly shown that natural products isolated from mushrooms play an important role, not only in pharmacology, but also in agriculture, as a rich source of bioactive components that can be used in crop protection (Wink, 1993; Luo *et al*., 2005). Strobilurin A and oudemansin A are fungicidal natural products found in the basidiomycete fungi *Strobilurus tenacellus* (Pers. Ex Fr.) Singer (Anke *et al*., 1977) and *Oudemansiella mucida* Hoehn (Musilek *et al*., 1969), respectively. The mushrooms, one of the main biological resources, are still an unexplored source for new agricultural chemicals.

Armillariella tabescens is a mushroom belonging to the family Tricholomataceae which is also called "Luminous Fungus" in China. *A. tabescens* has been used for the treatment of cholecystitis in traditional Chinese medicine, and armillarisin A (3-acetyl-5-hydroxymethyl-7-hydroxycoumarin) is the active component for curing cholecystitis (Wang *et al*., 2007). In continuation of our studies on basidiomycete-derived bioactive secondary metabolites employed as antifungal agents. The methanolic extract of the fruiting bodies of the mushroom *A. tabescens* was found to show antifungal activity against *Gibberella zeae*, *Colletotrichum ophiopogonis* and *Gloesporum fructigenum.* The active compound against *Gibberella zeae* was isolated from the fruiting bodies of *A. tabescens* by bioassay-guided fractionation of the extract and identified as armillarisin B. In this report, we describe the isolation, structural elucidation, and antifungal activity of armillarisin B.

Results and Discussion

The MeOH extract of *A. tabescens* showed fungitoxic activity against *Gibberella zeae*, *Colletotrichum ophiopogonis* and *Gloesporum fructigenum*. *Gibberella zeae* was the most sensitive to this fungal extract, and the active principle was armillarisin B. Armillarisin B showed a strong antifungal effect on mycelial growth of *Gibberella zeae*. However, it just exhibited weak antifungal activity against the other two plant pathogenic fungi.

Armillarisin B was first isolated in China by "Luminous Fungus" Cooperative Research Group of Kiangsu (1977) and proposed to be 2-hydroxy-2-phenylpropanediamide; however, its accurate chemical structure has not yet been elucidated owing to the deficiency of spectroscopic data. In our experiment, armillarisin B was obtained as white needles. Its HRESI-mass spectrum gave an ion $[M+Na]^+$ peak at *m/z* 217.0605 (calcd. for $C_9H_{10}N_2O_3Na$ 217.0589) and corresponded to a molecular formula of $C_9H_{10}N_2O_3$, requiring six degrees of unsaturation. The IR spectrum of armillarisin B showed peaks of $-NH_2$ (3436 cm^{-1}), -OH (3352 cm^{-1}), $-CONH_2$ (1680 cm^{-1}), and monosubstituted benzene (1580, 1452, 763, 692 cm^{-1}).

Table I. ^{1}H and ^{13}C NMR (in C_5D_5N) data of armillarisin B.

Position	δ_C	δ_H
1, 3	174.2	
2	80.2	
1'	142.1	
2', 4'	128.6	8.22 (1H, dd, 1.4, 7.2)
3', 5'	126.8	7.35 (m)
$-NH_2$		9.03 (br.s)

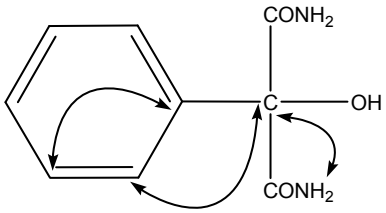

Fig. 1. Key HMBC correlations of armillarisin B.

The ^{1}H NMR (Table I) spectrum of armillarisin B showed signals of five aromatic H atoms at δ_H 8.22 (2H, dd, J = 1.4, 7.2 Hz, H-2', 6'), δ_H 7.35 (2H, m, H-3', 5'), and δ_H 7.25 (1H, m, H-4'), and an $-NH_2$ group at δ_H 9.03 (br.s). The ^{13}C NMR (Table I) spectrum of armillarisin B showed six signals in all including three doublets at δ_C 174.2 (s), δ_C 128.6 (d) and δ_C 126.8 (d). The construction of the molecular framework was deduced from ^{1}H,^{1}H COSY, HMQC, and HMBC experiments. The HMBC correlations C (2) (δ_C 80.2)/H-C (2', 6') (δ_H 8.22), C (2) (δ_C 80.2)/H_2-N (δ_H 9.03), and C (1') (δ_C 142.1)/H-C (3', 5') (δ_H 7.35) were consistent with the structure shown in Fig. 1. Therefore, armillarisin B eventually corresponds to 2-hydroxy-2-phenylpropanediamide.

Experimental

General

UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. IR spectra were measured on a Bio-Rad FTS-135 spectrometer using KBr pellets. NMR spectra were recorded on Bruker AM-400 and DRX-500 spectrometers in C_5D_5N, with TMS as the internal standard. Mass spectra were recorded on VG Auto-spec-3000 and API QSTAR Pulsar spectrometers (Manchester, UK).

Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., China) and reversed-phase silica gel (Fuji Silica Chemical Ltd., Kasugai, Japan) were used for column chromatography. Fractions were monitored by TLC, and spots were visualized by heating the silica gel plates sprayed with 10% H_2SO_4 in ethanol.

Mushroom material

The fresh fruiting bodies of *A. tabescens* were collected at Funiu Mountain of Henan Province, People's Republic of China, in July 2008. The fungal identification was made by Prof. Jin-Wen Shen. A dried specimen was deposited in the Herbarium of Henan Agricultural University, Zhengzhou, P. R. China.

Antifungal activity of A. tabescens and armillarisin B in vitro

400 g air-dried fruiting bodies of *A. tabescens* were immersed in MeOH and left at room temperature for 48 h. Then the MeOH extract was decanted and evaporated. The MeOH extract dissolved in DMSO was tested for antifungal activity *in vitro* by the poison food technique. Potato dextrose agar (PDA) medium was used as the medium for all test fungi. The test pathogenic fungi were *Gibberella zeae*, *Colletotrichum ophiopogonis*, *Gloesporum fructigenum*, *Fusarium graminearum* Schw., *Alternaria alternata* f. sp. Mali, *Colletotrichum gloeosporioides* (Penz.) Sacc. and *Sclerotinia sclerotiorum*.

The MeOH extract at a concentration of 20 mg/ml (DMSO content 1%) was inoculated at the centre of agar discs containing the test fungi (4 mm diameter). Three replicate plates for each fungus were included. After incubation for 2 ~ 6 d, until the fungal growth in the control dishes was almost complete, the mycelial growth of fungi (mm) in both treated (T) and control (C) Petri dishes was measured diametrically in three different direction. The percentage of growth inhibition (I) was calculated using the following formula (Sztejnberg *et al.*, 1983):

$$I\ (\%) = [(C - T)/C] \cdot 100.$$

The MeOH extract was found to show fungitoxic activity against *Gibberella zeae*, *Colletotrichum ophiopogonis* and *Gloesporum fructigenum*. The percentages of growth inhibition were 81.9%, 65.3% and 66.5%, respectively, at 20 mg/ml.

In order to isolate the active principle against *Gibberella zeae*, the MeOH extract (20 g) was fractionated by column chromatography (silica gel) and eluted with petroleum ether, petroleum ether/acetone (9:1, 8:2, v/v), $CHCl_3$/MeOH (9:1, 8:2, v/v) and MeOH to give six fractions. The fraction (1.8 g) eluted with $CHCl_3$/MeOH (9:1, v/v) was confirmed by the mycelial growth inhibition test *in vitro* to be the active fraction, with a growth inhibition 76.3% at 1 mg/ml. Then the active fraction was submitted for further purification to reversed-phase column chromatography (RP-8, MeOH/H_2O 85:15) to give a pure compound (90 mg), namely armillarisin B.

Armillarisin B showed a significant antifungal effect on mycelial growth of *Gibberella zeae*, and the percentage of growth inhibition was 78.7% at 100 μg/ml, *in vitro*.

Physicochemical properties

White needles, m.p. 162–164 ºC. – UV (EtOH): λ = 250.5, 256.5, 263.0 nm. – IR (KBr): ν = 3436, 3352, 1680, 1580, 1452, 1127, 763, 692 cm^{-1}. – ^1H and ^{13}C NMR: see Table I. – HRESI-MS: *m/z* = 217.0605 $[M+Na]^+$ (calcd. for $C_9H_{10}N_2O_3Na$ *m/z* = 217.0589).

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