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New Insecticidal Antibiotics, Hydroxyfungerins A and B, Produced by *Metarhizium* sp. FKI-1079

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Abstract New insecticidal antibiotics designated hydroxyfungerins A and B were isolated from the culture broth of a fungal strain *Metarhizium* sp. FKI-1079 together with a known compound, fungerin. The structures of hydroxyfungerins A and B were elucidated by spectroscopic studies including various NMR experiments. Hydroxyfungerins A and B showed growth inhibitory activity against brine shrimps, *Artemia salina*.

Keywords fungerin, insecticidal, brine shrimp, fungi, *Metarhizium* sp.

Introduction

Our research group has focused on discovery of biological active compounds from microbial metabolites [1～8]. In the course of our screening program for insecticidal antibiotics, we found that a fungal strain FKI-1079, which was isolated from soil collected at Yakushima, Kagoshima Prefecture, Japan, produced two new insecticidal antibiotics, named hydroxyfungerins A (**2**) and B (**3**), along with the known compound, fungerin (**1**) [9] (Fig. 1). The strain was identified as *Metarhizium* sp. from morphogical characteristics. Compounds **2** and **3** were structurally related to **1**, previously reported antifungal antibiotic active against *Penicillium chrysogenum*, *Colletorichum langenarium*, *Altenaria mali* and *Pyricularia oryzae* [9]. In this paper, the taxonomy of the producing fungus and the fermentation, isolation, structure elucidation and biological properties of hydroxyfungerins are described. Furthermore, biological activities of **1** previously reported to have antifungal activity are also described.

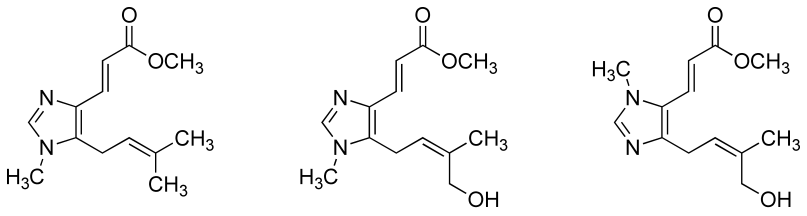

Fig. 1 Structures of fungerin (**1**) and hydroxyfungerin A (**2**) and B (**3**).

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Materials and Methods

General

NMR spectra were recorded on a Varian Inova 600 spectrometer ($^{2-3}J_{CH}$=8 Hz in HMBC). Chemical shifts are shown in δ values (ppm) relative to chloroform-d_1 at 7.26 ppm for ^{1}H NMR and at 77.0 ppm for ^{13}C NMR. FAB mass spectrometry was conducted on a JEOL JMS-AX505H spectrometer. UV and IR spectra were measured with a Beckman DU640 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively.

Taxonomic Studies of the Producing Organism

Strain FKI-1079 was isolated from a soil sample collected at Yakushima Island, Kagoshima Prefecture, Japan. For the taxonomic studies of the fungus, potato dextrose agar (PDA, Difco), malt extract agar (MEA), cornmeal agar (CMA, Difco) and Miura's medium (LcA) were used. Morphological observations were made under a light microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (JEOL JSM-5600). Color names and hue numbers were determined according to the Color Harmony Manual, 4th Ed [10].

Fermentation

A stock culture of strain FKI-1079 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (glucose 2.0%, yeast extract (Oriental Yeast Co.) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Daigo Nutritive Chemicals) 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0) and incubated on a rotary shaker at 27°C for 4 days. The main culture was initiated by transferring 2 ml of the seed culture into each of thirty 1-liter Roux flask containing 200 ml of production medium (rice (Nichiou syouji Co.) 150 g, $FeSO_4 \cdot 7H_2O$ 90 mg, $MnCl_2 \cdot 4H_2O$ 90 mg, $ZnSO_4 \cdot 7H_2O$ 90 mg, $CuSO_4 \cdot 7H_2O$ 90 mg, $CoCl_2 \cdot 6H_2O$ 90 mg and tap water 90 ml) and the fermentation was carried out at 27°C for 15 days on stationary culture.

Insecticidal Activity

Insecticidal activity was assayed by a microtiter-plate assay using brine shrimps, *A. salina* (Pfizer Consumer Inc) as reported previously [11]. Briefly, about 10 nauplii larvae hatched from eggs of brine shrimps were incubated with a test sample in a well of 96-well microplates at room temperature. After 48 hours, the motility of brine shrimps was assessed visually in comparison with controls (no samples).

Nematocidal Activity

Nematocidal activity was assayed by a microtiter-plate assay using the free-living nematode, *Caenorhabditis elegans*, as reported previously [11]. Briefly, *C. elegans* was cultivated on an agar plate covered with *Escherichia coli* for 4 days at 20°C and the grown nematodes were used for assay. These organisms were incubated with a sample in a well of a 96-well micoroplate at 20°C. After 48 hours, the motility of the nematoda was assessed visually under a microscope (×40, Olympus CK2) in comparison with controls (no samples).

Antimicrobial Activity

Antimicrobial activity against 14 species of microorganisms was measured by a paper disk method. The media for microorganisms are as follows: GAM agar (Nissui Seiyaku Co.) for *Bacteroides fragilis*; Waksman agar for *Mycobacterium smegmatis*; Bacto PPLO agar (Difco) supplemented with 15% horse serum, glucose 0.1%, phenol red (5 mg/ml) 0.2% and agar 1.5% for *Acholeplasma laidlawii*; nutrient agar for *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas areginosa* and *Xanthomonas oryzae*; a medium composed of glucose 1.0%, yeast extract 0.5%, and agar 0.8% for *Pyricularia oryzae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans* and *Saccharomyces cerevisiae*. A paper disk (i.d. 6 mm, Advantec) containing 10 μg of a sample was placed on the agar plate. Bacteria, except *X. oryzae*, were incubated at 37°C for 24 hours. Yeasts and *X. oryzae* were incubated at 27°C for 24 hours. Fungi were incubated at 27°C for 48 hours. Antimicrobial activity was expressed as the diameter (mm) of the inhibitory zone.

Results

Taxonomy of the Producing Organism

Colonies of strain FKI-1079 on the media were 20~30 mm in diameter after 7 days at 25°C, floccose to velutinous, white (a) or olive (1 pl) to dark olive (1 pn) in color. The reverse side was white (a) to light ivory (2 ca). On each medium, the conidiostroma, consisting of sporodochial conidiophores and conidia, were abundantly formed (Fig. 2). The conidiophores were composed of 3~4 branches of 40~50 mm in length. The cylindrical phialides were formed at the tip of branches and 10~15×2.0~2.5 μm in size. The phialospores were unicellular, cylindrical, pale green, produced in chains, adhering in tall, columnar aggregates and 6~7.5×2~3.0 μm in size. From the above characteristics, strain FKI-1079 was considered to belong

to the genus Metarhizium [12,13] and named *Metarhizium* sp. FKI-1079.

Isolation

The isolation procedure for the hydroxyfungerins is summarized in Fig. 3. The cultured broth (6 liters) was treated with EtOH (6 liters) for 1 hour. Then, the extracts were concentrated under reduced pressure to remove EtOH and extracted with EtOAc. After drying over Na_2SO_4, the organic layer was concentrated under reduced pressure to give a brown paste (3.9 g). This was applied to a silica gel column (120 g, Silica gel 60, 63~200 μm, Merck) prepared with $CHCl_3$, and eluted with $CHCl_3$ and $CHCl_3$ - MeOH. The fractions eluted with $CHCl_3$ - MeOH (50 : 1) were collected and concentrated under reduced pressure to give a yellow powder containing **1**. This powder (361 mg) was washed with hexane and treated with MeOH, and the MeOH soluble fraction was concentrated under reduced pressure to give a pale yellow powder. It was further purified by preparative HPLC (mobile phase: 40% CH_3CN; flow rate, 8 ml/minute; detection, UV 300 nm) using an ODS column under the following conditions: column (CAPCEL PAK C18 UG120: 20 i.d. ×250 mm, SISEIDO Co., Ltd.; temperature, 40°C). The active fraction eluted at 20 minutes was concentrated to dryness to give a white powder of pure **1** (25.5 mg). On the other hand, the fractions eluted with $CHCl_3$ - MeOH (20 : 1) were collected and concentrated under reduced pressure to give a yellow powder containing **2** and **3** (160 mg). This was applied to an ODS column (10 g, PEGASIL PREP ODS-7515-12-A, Senshu Co., Ltd) prepared with water, and eluted with CH_3CN - H_2O solvents. The fractions eluted with CH_3CN - H_2O (2 : 3) were collected and concentrated under reduced pressure to give a yellow powder containing **2** and **3** (18.3 mg). These compounds were finally separated and purified by preparative HPLC (mobile phase: 13% CH_3CN; flow rate, 8 ml/minute; detection, UV 300 nm) using an ODS column under the following conditions: column (CAPCEL PAK C18 UG120: 20 i.d. ×250 mm, SISEIDO Co., Ltd.; temperature, 40°C). The active fractions eluted at 47 and 49 minutes were concentrated to dryness to give white powders of pure **2** (1.2 mg) and **3** (0.8 mg), respectively.

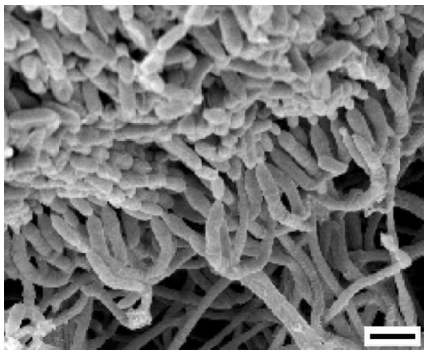

Fig. 2 SEM photomicrograph of sympodially produced conicedia of FKI-1079.

Bar represents 20 μm.

Physico-chemical Properties

The physico-chemical properties of **1**~**3** are summarized in Table 1. They are soluble in methanol, acetone, chloroform and DMSO. The molecular formulae of **1**~**3** were revealed to be $C_{13}H_{18}N_2O_2$, $C_{13}H_{18}N_2O_3$ and $C_{13}H_{18}N_2O_3$ by HR-FAB-MS, respectively. The UV spectra of **1**~**3** exhibited a characteristic absorption maximum at 305 nm in methanol. The IR spectra were also similar. These data indicated that

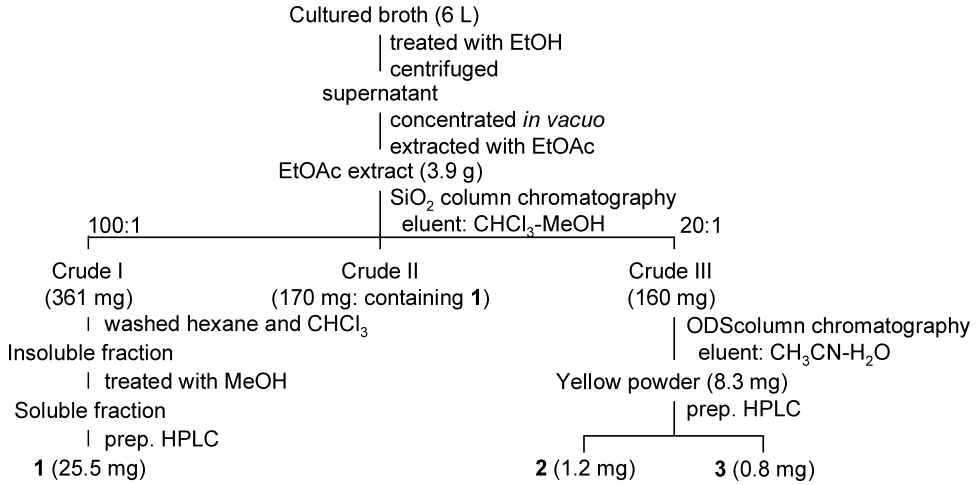

Fig. 3 Purification procedures of fungerin and hydroxyfungerins from the culture broth of *Metarhizium* sp. FKI-1079.

Table 1 Physico-chemical properties of **1** to **3**

	1	**2**	**3**
Appearance	Pale yellow powder	Pale yellow powder	Pale yellow powder
Molecular weight	234	250	250
Molecular formula	$C_{13}H_{18}N_2O_2$	$C_{13}H_{18}N_2O_3$	$C_{13}H_{18}N_2O_3$
HRFAB-MS			
Calcd	234.1368 $(M)^+$	250.1317 $(M)^+$	250.1317 $(M)^+$
Found	234.1360 $(M)^+$	250.1325 $(M)^+$	250.1317 $(M)^+$
UV λ_{max} nm (ε) in MeOH	300 (28,000)	305 (27,000)	305 (27,200)
IR υ_{max} cm^{-1} (KBr)	2775, 1866, 1704, 1630, 1432, 1384, 1303	3430, 2766, 1702, 1689, 1433, 1303	3421, 2782, 1700, 1674, 1435, 1323
Solubility			
Soluble	$CHCl_3$, MeOH, acetone, EtOAc, DMSO	$CHCl_3$, MeOH, acetone, EtOAc, DMSO	$CHCl_3$, MeOH, acetone, EtOAc, DMSO
Insoluble	Hexane, H_2O	Hexane, H_2O	Hexane, H_2O

Table 2 ^1H and ^{13}C NMR chemical shifts of **1** to **3**

Position	**1**		**2**		**3**	
	^{13}C chemical shifts (ppm)[a]	^1H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a,c]	^1H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a,c]	^1H chemical shifts (ppm)[b]
1	168.5	s —	168.4	s —	168.0	s —
2	114.2	d 6.65 d (*J*=15.4 Hz)	116.4	d 6.67 d (*J*=15.6 Hz)	115.0	d 6.79 d (*J*=15.6 Hz)
3	135.2	d 7.72 d (*J*=15.4 Hz)	133.4	d 7.57 d (*J*=15.6 Hz)	132.0	d 7.55 d (*J*=15.6 Hz)
4	134.0	s —	133.0	s —	133.5	s —
5	—	—	—	—	—	—
6	138.0	d 7.5 s	138.2	d 7.78 br.s	137.8	d 8.17 br s
7	—	—	—	—	—	—
8	134.2	s —	133.7	s —	133.4	s —
9	22.4	t 3.51 d (*J*=7.0 Hz)	22.6	t 3.55 d (*J*=6.6 Hz)	22.5	t 3.47 d (*J*=6.6 Hz)
10	119.3	d 5.13 t (*J*=7.0 Hz)	122.0	d 5.19 t (*J*=6.6 Hz)	118.6	d 5.36 t (*J*=6.6 Hz)
11	134.3	s —	137.6	s —	138.9	s —
12	25.5	q 1.87 s	61.6	t 4.29 s	67.8	t 4.00 s
13	18.0	q 1.83 s	21.6	q 1.82 s	14.0	q 1.78 s
OCH_3	51.4	q 3.87 s	51.5	q 3.77 s	51.8	q 3.75 s
NCH_3	31.6	q 3.66 s	32.3	q 3.64 s	32.8	q 3.67 s

[a] Chemical shifts are shown with reference to $CDCl_3$ as 77.0 ppm. [b] Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm. [c] ^{13}C chemical shifts and the multiplicit of the ^{13}C NMR signals were determined from HMQC and HMBC data.

these compounds are structurally related. Compound **1** was identified as the known compound fungerin previously reported as an antifungal antibiotic [9].

Structure Elucidation

The structures of **2** and **3** were elucidated by extensive NMR experiments in $CDCl_3$. The assignment of the ^1H and ^{13}C NMR signals was facilitated by ^1H-^1H COSY and ^{13}C-^1H HMQC experiments (Table 2). The data for **1** are also shown in Table 2 for comparative purposes.

Structure of **2**: The ^1H and ^{13}C NMR spectra (Table 2) resembled those of **1** except for the proton and carbon

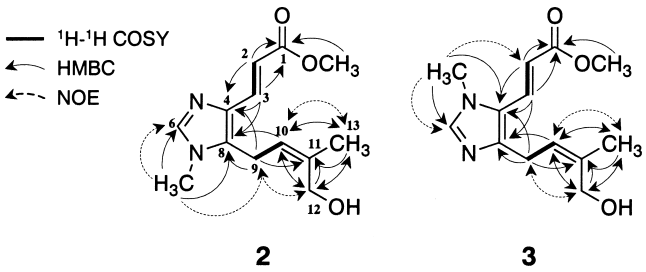

Fig. 4 ^{1}H-^{1}H COSY, HMBC and NOE experiments for **2** and **3**.

signals of C-12. The ^{13}C-^{1}H long-range couplings of ^{2}J and ^{3}J in the HMBC experiments are shown in Fig. 4, giving the following results. The cross peaks from 12-H_2 (δ 4.29) to the sp^2 methine carbon C-10 (δ 122.0), the sp^2 quaternary carbon C-11 (δ 137.6) and the methyl carbon C-13 (δ 21.6), from 10-H (δ 5.19) and 13-H_3 (δ 1.82) to oxymethylene carbon C-12, (δ 61.6) indicated that **2** has a hydroxymethyl residue at C-12 instead of the methyl group of **1**. From NOE experiments as shown in Fig. 4, NOEs were observed between 9-H_2 and 12-H_3 and 10-H and 13-H_3, indicating that the double bond between C-10 and C-11 has the *Z*-isomer. Furthermore, an NOE was observed between the *N*-methyl proton and 9-H, consistent with attachment of the *N*-methyl group at position 7 of the imidazole ring. Taking these data together, the structure of **2** was elucidated as shown in Fig. 4.

Structure of **3**: The ^{1}H and ^{13}C NMR spectra (Table 2) resembled those of **2** except for the proton signal of 6-H (δ 8.17). The analysis of HMBC experiments revealed the structure of **3** as shown in Fig. 4. The ^{13}C-^{1}H long-range couplings were observed from the *N*-methyl proton (δ 3.67) to the sp^2 methine carbon C-6 (δ 137.8) and the sp^2 quaternary carbon C-8 (δ 133.4), indicating that the *N*-methyl group of **3** is attached at position 5 of the imidazole ring. Furthermore, an NOE was observed between *N*-methyl proton and 2-H_2, supporting the proposed structure.

Biological Activitiess

Insecticidal activity

The insecticidal activities of **1**~**3** were studied in a microplate assay using the brine shrimp *Altemia salina*. Minimum growth inhibitory concentrations (MIC) were summarized in Table 3. Compound **1** was the most potent with a MIC value of 0.39 μg/ml (1.7 μM). Compounds **2** and **3** exhibited moderate inhibition with MICs of 6.25 μg/ml (25 μM).

Table 3 Incecticidal and nenatocidal activities of **1** to **3**

Compound	MIC (μg/ml)	
	A. salina	*C. elegans*
1	0.39	6.25
2	6.25	>50
3	6.25	>50

Nematocidal Activity

The nematocidal activities of **1**~**3** were studied in a microplate assay using the free-living nematode, *Caenorhabditis elegans*. The MICs are summarized in Table 3. Compound **1** was the most potent with a MIC value of 6.25 μg/ml (27 μM), while **2** and **3** showed almost no activity at 50 μg/ml.

Antimicrobial Activity

Compounds **1**~**3** showed no antimicrobial activity against 14 microorganisms as described at 10 μg/6 mm disk.

Discussion

Fungerin (**1**) was originally isolated as an antifungal antibiotic from a *Fusarium* sp., and subsequently reported [9]. As shown in this paper, **1** was also produced by a different fungus a *Metarhisium* sp. FKI-1079. Visoltricin, a positional isomer at the *N*-methyl position of **1**, was reported to show anti-*A. salina* activity and cholinesterase inhibitory activities [14, 15]. However, a recent synthetic study resulted in a revised structure identical to **1** [16]. In this sense, we only confirmed the anti-*A. salina* activity of **1** as described above, and the potency (MIC 1.3 μM) seemed almost the same as that (0.85 μM) reported [15]. Interestingly, the *N*-methyl position of the imidazole ring in hyroxyfungerin B is the same as that of original visoltricin. Therefore, it may be plausible that visoltricin is produced by this fungus, although it was not isolated from the culture broth.

Regarding the biological activities of fungerins, a similar order of potency was observed in insecticidal and nematocidal activities (Table 3); **1** is the most potent, followed by **2** and **3**. These results suggested that the hydroxy residues in the terminal isoprene moiety is detrimental to these biological activities. As reported previously, **1** causes M phase arrest in Jurkat cells (a cell line from human T cell leukemia) by inhibiting microtubule polymerization [17]. Therefore, these anti-*A. salina* and

anti-*C. elegans* activities might be caused by the microtubule action by **1**.

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