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A New Antifungal Macrolide, Eushearilide, Isolated from *Eupenicillium shearii*

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Abstract In screening for antifungal substances, a new macrolide, eushearilide (**1**), was isolated from *Eupenicillium shearii* IFM54447. The structure of **1** was established to be 24-membered macrolide having a non-conjugated diene and a choline phosphate ester moetiy on the basis of detailed investigation of NMR, UV, IR and MS spectral data. Compound **1** showed antifungal activity against various fungi and yeasts, including human pathogens *Aspergillus fumigatus*, *Trichophyton* spp. and *Candida* spp.

Keywords *Eupenicillium shearii*, Eushearilide, Macrolide, Antifungal activity

Introduction

The incidence of life-threatening fungal infections has steadily increased in immunocompromised hosts such as HIV infected persons and cancer and transplant patients [1]. Invasive pulmonary aspergillosis and *Pneumocystis carinii* pneumonia are leading causes of death in bone marrow transplant recipients and in HIV-infected patients, respectively. Moreover, resistance to the azoles, which are the most widely used antifungals today, is attracting much attention. Therefore, there is a continuing need for new antifungal agents to overcome these fungal diseases. Screening for new antifungal substances from fungal sources was carried out using pathogenic filamentous fungi, *Aspergillus fumigatus* Fresenius IFM41362 and *Aspergillus niger* Van Tieghem IFM41398, and/or pathogenic yeasts, *Candida albicans* (Robin) Berkhout ATCC90028 and *Cryptococcus neoformans* (Sanfelice) Vuillemin ATCC90112. The chloroform - methanol (1 : 1) extract of freshly isolated *Eupenicillium shearii* IFM54447, cultivated on rice for 21 days at 25°C, showed antifungal activity against the above four test organisms. The purification of this extract led to the isolation of a new macrolide designated eushearilide (**1**) as the antifungal substance.

Results and Discussion

Eushearilide (**1**) was obtained as a white amorphous solid. High resolution time of flight mass spectrometry (HR-TOF-MS) for **1** gave a quasimolecular ion $[M+H]^+$ at *m/z* 544.3757 (calcd 544.3762) corresponding to the molecular formula $C_{29}H_{54}NO_6P$, which was consistent with ^1H, ^{13}C and ^{31}P NMR spectra. Infrared (IR) absorption at 2920 and 2850 cm^{-1} suggested the presence of aliphatic moiety and that at 1730 cm^{-1} (strong) suggested the presence of an ester carbonyl. The ^1H NMR spectrum of **1** exhibited 54

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non-exchangeable protons, including three equivalent tertiary (δ 3.21) and a secondary (δ 1.19) methyl groups and four olefinic protons (δ 5.36, 5.37, 5.39 and 5.50). The above olefins apparently possessed a *Z*-configuration at C-16, C-17 and an *E*-configuration at C-20, C-21 from the value of their coupling constants ($J_{16,17}$=7.8 Hz and $J_{20,21}$=15.1 Hz). The ^{13}C NMR spectrum of **1** showed four methyls (δ 19.7 and 54.7) including three equivalent methyls, 18 methylenes, two methines (δ 72.3 and 74.1) bearing oxygen functions, one carbonyl carbon (δ 171.8), and four tertiary olefinic carbon atoms (δ 126.5, 131.2, 131.8 and 134.7). A peak at δ −0.048 ppm in the ^{31}P NMR spectrum of **1** showed the presence of a phosphoryl or phosphoric acid moiety.

From the analysis of the ^{1}H-^{1}H COSY and HMBC spectra (Fig. 2) of **1**, the planar structure of eushearilide (**1**) was determined as a twenty-four membered macrolide with a non-conjugated diene and a choline phosphate ester moiety. The stereochemistry of **1** remains to be determined.

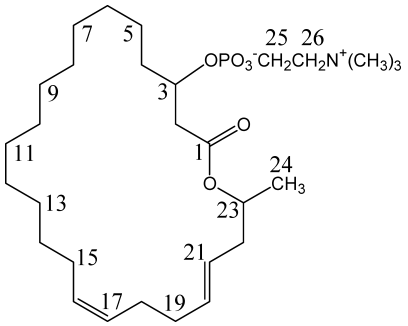

Fig. 1 Structure of eushearilide (**1**).

Table 1 Antifungal and antibacterial activities of eushearilide (**1**)

Microorganisms		Inhibition zone (mm)	Microorganisms		Inhibition zone (mm)
—Filamentous fungi—					
⟨Imperfect fungi and ascomycetes⟩			⟨Zygomycetes⟩		
Alternaria alternata	IFM 41348	21	*Absidia corymbifera*	IFM 41345	15
Arthroderma benhamiae	IFM 41160	20	*Cunninghamella elegans*	IFM 47050	20
Aspergillus flavus	IFM 41935	24	*Mucor ramosissimus*	IFM 46006	24
Aspergillus fumigatus	IFM 41362	14	*Rhizopus oryzae*	IFM 40515	12
Aspergillus fumigatus	IFM 47078	15			
Aspergillus fumigatus	IFM 49896	21	—Yeasts—		
Aspergillus fumigatus	IFM 51126	20	*Candida albicans*	IFM 47945	8
Aspergillus fumigatus	IFM 51357	21	*Candida albicans*	ATCC 90028	7
Aspergillus niger	IFM 41398	18	*Candida albicans*	ATCC 90029	7
Aureobasidium pullulans	IFM 4802	20	*Candida dubliniensis*	IFM 51756	11
Emericella nidulans	IFM 46997	12	*Candida glabrata*	IFM 46888	7
Exophiala dermatitidis	IFM 41479	17	*Candida guilliermondii*	IFM 46823	(14)
Fonsecaea pedrosoi	IFM 4887	11	*Candida kefyr*	IFM 46921	11
Fusarium oxysporum f. sp. *lactucae*	IFM 53787	17	*Candida krusei*	IFM 46834	7
Microsporum audouinii	IFM 41144	22	*Candida parapsilosis*	IFM 46863	9
Microsporum canis	IFM 45108	23	*Candida tropicalis*	IFM 46816	7.5
Penicillium citrinum	IFM 53298	19	*Cryptococcus neoformans*	ATCC 90112	10.5
Penicillium islandicum	IFM 41098	11	*Cryptococcus neoformans*	ATCC 90113	10
Penicilium marneffei	IFM 52703	20	*Saccharomyces cerevisiae*	IFM 40210	7
Phialophora verrucosa	IFM 4928	11	*Trichosporon asahii* var. *asahii*	IFM 48429	(16)
Pichia anomala	IFM 53788	9.5			
Pseudallescheria boydii	IFM 41901	26	—Bacteria—		
Trichophyton mentagrophytes	IFM 40951	18	*Staphylococcus aureus*	JCM 2151	(12)
Trichophyton rubrum	IFM 45802	18	*Escherichia coli*	JCM 1649	—
Trichophyton tonsurans	IFM 5275	20	*Pseudomonas aeruginosa*	JCM 5962	—
Trichophyton verrucosum	IFM 46798	14			

The parentheses mean hazy inhibition zone.

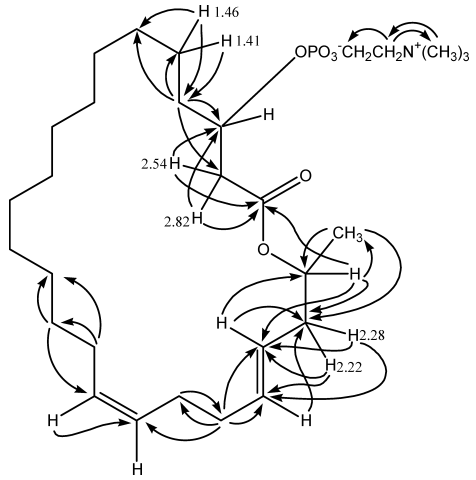

Fig. 2 HMBC correlations in eushearilide (**1**).

Antimicrobial Property

Since eushearilide (**1**) was insoluble in water, the antimicrobial activity was determined by the paper disc method, as described in the previous paper [2, 3]. The results are summarized in Table 1. Eushearilide (**1**) showed a broadrange of antifungal activity against various fungi and yeasts including human pathogens *Aspergillus fumigatus*, *Trichophyton* spp. and *Candida* spp. *etc.*, whereas only a trace of the antibacterial activity was observed.

Although many macrolide antibiotics have several conjugated double bonds and amino sugar moieties in the molecular structure (*cf.* amphotericin B [4]), eushearilide (**1**) is a macrolide antibiotic having a twenty-four membered ring, that has non-conjugated double bonds, no an amino sugar moiety and no hydroxyl groups on the ring structure. It is the first example to our knowledge of a twenty-four membered macrolide antibiotic having a choline phosphate ester moiety.

Experimental

ESI-TOF-MS was taken with a Bruker microTOF spectrometer. UV and IR spectra were recorded on a Hitachi U-3210 spectrometer and a JASCO IR-810 spectrometer, respectivity. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECA-800 (^{1}H, 800.14 MHz; ^{13}C, 201.20 MHz) spectrometer, using tetramethylsilane as an internal standard, and the ^{31}P NMR spectrum was recorded on a JEOL ECA-600 spectrometer. CD curves were determined on a JASCO J-600 spectropolarimeter. Column chromatography was performed using Wakogel C-200 (Art. 237-00071, Wako). High performance liquid chromatography (HPLC) was performed with a Senshu Scientific SSC-3160 pump (flow rate, 4 ml/minute), equipped with a Shimamura YRD-883 RI detector. HPLC analytical condition of Eushearilide was as follows [column: Inertsil ODS-3, 4.6×250 mm, GL sciences Inc.; mobile phase: MeOH - H_2O (9 : 1); flow rate: 1.0 ml/minute; column oven temperature: 40°C] TLC was conducted on pre-coated Kieselgel 60 F_{254} plates (Art. 5715; Merck) with solvent system $CHCl_3$ - MeOH - H_2O (6 : 4 : 1). Eushearilide was detected by spraying with 5%H_2SO_4 and then heating.

Isolation of Eushearilide (1) from *E. shearii* IFM54447

E. shearii IFM54447, kept by The Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, was cultivated for 21 days at 25°C on rice (450 g, using 3 Roux flasks). The cultivated rice was extracted with $CHCl_3$ - MeOH (1 : 1) and the evaporated extract suspended in water and partitioned with EtOAc. The EtOAc extract (15 g), which showed antifungal activity against *A. fumigatus*, was repeatedly chromatographed on silica gel (Wako, C-200) with $CHCl_3$ - MeOH, followed by preparative reverse-phase HPLC [column: Senshu Pack pegasil-ODS, 10×250 mm; mobile phase: MeOH - H_2O (9 : 1)] to give **1** (8 mg) along with a fraction including several lysophosphoglycerides.

Eushearilide (**1**): white amorphous solid; **1** was shown at Rt (15.2 minutes) and Rf value (0.42) in the above analytical condition. $[\alpha]_D^{25}$ +12.8 (*c* 0.75, MeOH); UV (MeOH): λ_{max} (log ε) 206 (3.23), 225 nm (2.82); CD (MeOH): $\Delta\varepsilon$ (nm) +0.36 (217); IR (film): ν_{max} 3400 (br), 2920 (s), 2850 (s), 1730 (s), 1230 (br), 1080 (s), 1060 (s), 850 (s) cm^{-1}; HR-TOF-MS (ESI positive) *m/z*: 544.3757 $[M+H]^+$; calcd. for $C_{29}H_{55}NO_6P$: 544.3762. ^{1}H-NMR (800.14 MHz, CD_3OD): δ 1.19 (3H, d, *J*=6.4 Hz, 24-H_3), 1.30 (16H, br s, 6, 7, 8, 9, 10, 11, 12, and 13-H_2), 1.36 (2H, m, 14-H_2), 1.41 (1H, m, 5-H), 1.46 (1H, m, 5-H), 1.64 (2H, m, 4-H_2), 2.00 (2H, br dd, *J*=6.0, 11.5 Hz, 15-H_2), 2.06 (2H, m, 18-H_2), 2.07 (2H, m, 19-H_2), 2.22 (1H, ddd, *J*=6.4, 7.3, 14.0 Hz, 22-H), 2.28 (1H, ddd, *J*=6.9, 7.3, 14.0 Hz, 22-H), 2.54 (1H, dd, *J*=8.2, 14.2 Hz, 2-H), 2.82 (1H, dd, *J*=4.2, 14.2 Hz, 2-H), 3.21 (9H, s, 27-CH_3), 3.62 (2H, m, 26-H_2), 4.26 (2H, m, 25-H_2), 4.54 (1H, m, 3-H), 4.87 (1H, br q, *J*=6.4 Hz, 23-H), 5.36 (1H, br d, *J*=7.8 Hz, 17-H), 5.37 (1H, br d, *J*=7.8 Hz, 16-H), 5.39 (1H, dt, *J*=7.3, 15.1 Hz, 21-H), 5.50 (1H, m, 20-H).; ^{13}C-NMR (201.20 MHz, CD_3OD): δ 19.7 (C-24), 25.4 (C-5), 28.5 (C-13), 29.2, 29.4, 29.6, 29.7×2, and 29.8 (C-7 to C-12), 29.5 (C-14), 30.1 (C-6), 32.8 (C-15), 33.6 (C-18), 33.9 (C-19), 36.1 (C-4), 40.0 (C-22), 41.9 (C-2), 54.7 (C-27), 60.3 (C-25), 67.5 (C-26), 72.3 (C-23), 74.1 (C-3), 126.5 (C-21), 131.2 (C-17), 131.8 (C-16), 134.7 (C-20), 171.8 (C-1).

Antibacterial and Antifungal Activities of 1

Antibacterial and antifungal activities were qualitatively determined using the agar diffusion method with paper discs (6 mm in diameter), loaded with 40 μg of **1** as described in the previous paper [2]. The test organisms used and the results are summarized in Table 1.

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