

# 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 moiety 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

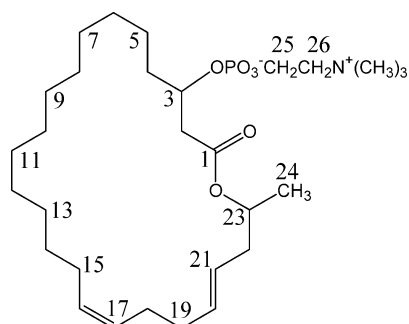
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non-exchangeable protons, including three equivalent tertiary ( $\delta$  3.21) and a secondary ( $\delta$  1.19) methyl groups and four olefinic protons ( $\delta$  5.36, 5.37, 5.39 and 5.50). The above olefins apparently possessed a *Z*-configuration at C-



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

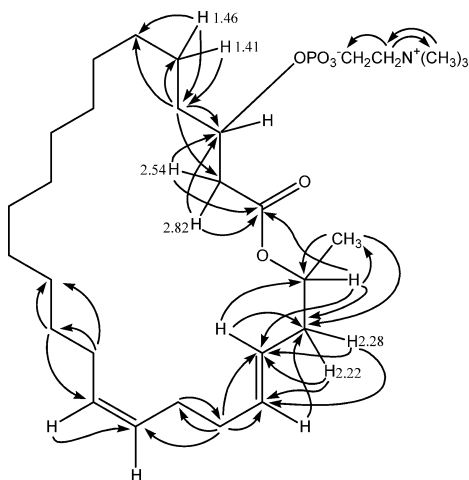
16, C-17 and an *E*-configuration at C-20, C-21 from the value of their coupling constants ( $J_{16,17}=7.8$  Hz and  $J_{20,21}=15.1$  Hz). The  $^{13}\text{C}$  NMR spectrum of **1** showed four methyls ( $\delta$  19.7 and 54.7) including three equivalent methyls, 18 methylenes, two methines ( $\delta$  72.3 and 74.1) bearing oxygen functions, one carbonyl carbon ( $\delta$  171.8), and four tertiary olefinic carbon atoms ( $\delta$  126.5, 131.2, 131.8 and 134.7). A peak at  $\delta$   $-0.048$  ppm in the  $^{31}\text{P}$  NMR spectrum of **1** showed the presence of a phosphoryl or phosphoric acid moiety.

From the analysis of the  $^1\text{H}$ - $^1\text{H}$  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.

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

Microorganisms	Inhibition zone (mm)	Microorganisms	Inhibition zone (mm)
—Filamentous fungi—			
(Imperfect fungi and ascomycetes)		(Zygomycetes)	
<i>Alternaria alternata</i>	IFM 41348 21	<i>Absidia corymbifera</i>	IFM 41345 15
<i>Arthroderma benhamiae</i>	IFM 41160 20	<i>Cunninghamella elegans</i>	IFM 47050 20
<i>Aspergillus flavus</i>	IFM 41935 24	<i>Mucor ramosissimus</i>	IFM 46006 24
<i>Aspergillus fumigatus</i>	IFM 41362 14	<i>Rhizopus oryzae</i>	IFM 40515 12
<i>Aspergillus fumigatus</i>	IFM 47078 15		
<i>Aspergillus fumigatus</i>	IFM 49896 21	—Yeasts—	
<i>Aspergillus fumigatus</i>	IFM 51126 20	<i>Candida albicans</i>	IFM 47945 8
<i>Aspergillus fumigatus</i>	IFM 51357 21	<i>Candida albicans</i>	ATCC 90028 7
<i>Aspergillus niger</i>	IFM 41398 18	<i>Candida albicans</i>	ATCC 90029 7
<i>Aureobasidium pullulans</i>	IFM 4802 20	<i>Candida dubliniensis</i>	IFM 51756 11
<i>Emericella nidulans</i>	IFM 46997 12	<i>Candida glabrata</i>	IFM 46888 7
<i>Exophiala dermatitidis</i>	IFM 41479 17	<i>Candida guilliermondii</i>	IFM 46823 (14)
<i>Fonsecaea pedrosoi</i>	IFM 4887 11	<i>Candida kefyr</i>	IFM 46921 11
<i>Fusarium oxysporum</i> f. sp. <i>lactucaea</i>	IFM 53787 17	<i>Candida krusei</i>	IFM 46834 7
<i>Microsporium audouinii</i>	IFM 41144 22	<i>Candida parapsilosis</i>	IFM 46863 9
<i>Microsporium canis</i>	IFM 45108 23	<i>Candida tropicalis</i>	IFM 46816 7.5
<i>Penicillium citrinum</i>	IFM 53298 19	<i>Cryptococcus neoformans</i>	ATCC 90112 10.5
<i>Penicillium islandicum</i>	IFM 41098 11	<i>Cryptococcus neoformans</i>	ATCC 90113 10
<i>Penicillium marneffeii</i>	IFM 52703 20	<i>Saccharomyces cerevisiae</i>	IFM 40210 7
<i>Phialophora verrucosa</i>	IFM 4928 11	<i>Trichosporon asahii</i> var. <i>asahii</i>	IFM 48429 (16)
<i>Pichia anomala</i>	IFM 53788 9.5		
<i>Pseudallescheria boydii</i>	IFM 41901 26	—Bacteria—	
<i>Trichophyton mentagrophytes</i>	IFM 40951 18	<i>Staphylococcus aureus</i>	JCM 2151 (12)
<i>Trichophyton rubrum</i>	IFM 45802 18	<i>Escherichia coli</i>	JCM 1649 —
<i>Trichophyton tonsurans</i>	IFM 5275 20	<i>Pseudomonas aeruginosa</i>	JCM 5962 —
<i>Trichophyton verrucosum</i>	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 broad range 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, respectively.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a JEOL ECA-800 ( $^1\text{H}$ , 800.14 MHz;  $^{13}\text{C}$ , 201.20 MHz) spectrometer, using tetramethylsilane as an internal standard, and the  $^{31}\text{P}$  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,  $4.6 \times 250$  mm, GL sciences Inc.; mobile phase: MeOH - H<sub>2</sub>O (9 : 1); flow rate: 1.0 ml/minute; column oven temperature: 40°C] TLC was conducted on pre-coated Kieselgel 60 F<sub>254</sub> plates (Art. 5715; Merck) with solvent system CHCl<sub>3</sub> - MeOH - H<sub>2</sub>O (6 : 4 : 1). Eushearilide was detected by spraying with 5% H<sub>2</sub>SO<sub>4</sub> 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<sub>3</sub> - 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<sub>3</sub> - MeOH, followed by preparative reverse-phase HPLC [column: Senshu Pack pegasil-ODS,  $10 \times 250$  mm; mobile phase: MeOH - H<sub>2</sub>O (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.  $[\alpha]_{\text{D}}^{25} + 12.8$  (*c* 0.75, MeOH); UV (MeOH):  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 206 (3.23), 225 nm (2.82); CD (MeOH):  $\Delta\epsilon$  (nm) +0.36 (217); IR (film):  $\nu_{\text{max}}$  3400 (br), 2920 (s), 2850 (s), 1730 (s), 1230 (br), 1080 (s), 1060 (s), 850 (s) cm<sup>-1</sup>; HR-TOF-MS (ESI positive) *m/z*: 544.3757 [M+H]<sup>+</sup>; calcd. for C<sub>29</sub>H<sub>55</sub>NO<sub>6</sub>P: 544.3762.  $^1\text{H}$ -NMR (800.14 MHz, CD<sub>3</sub>OD):  $\delta$  1.19 (3H, d, *J*=6.4 Hz, 24-H<sub>3</sub>), 1.30 (16H, br s, 6, 7, 8, 9, 10, 11, 12, and 13-H<sub>2</sub>), 1.36 (2H, m, 14-H<sub>2</sub>), 1.41 (1H, m, 5-H), 1.46 (1H, m, 5-H), 1.64 (2H, m, 4-H<sub>2</sub>), 2.00 (2H, br dd, *J*=6.0, 11.5 Hz, 15-H<sub>2</sub>), 2.06 (2H, m, 18-H<sub>2</sub>), 2.07 (2H, m, 19-H<sub>2</sub>), 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<sub>3</sub>), 3.62 (2H, m, 26-H<sub>2</sub>), 4.26 (2H, m, 25-H<sub>2</sub>), 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);  $^{13}\text{C}$ -NMR (201.20 MHz, CD<sub>3</sub>OD):  $\delta$  19.7 (C-24), 25.4 (C-5), 28.5 (C-13), 29.2, 29.4, 29.6, 29.7 $\times$ 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  $\mu\text{g}$  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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