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A New Nonadride Derivative, Dihydroepiheveadride, as Characteristic Antifungal Agent against Filamentous Fungi, Isolated from Unidentified Fungus IFM 52672

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In the screening of searching for new antifungal agents, a new nonaride compound, dihydroepiheveadride (**1**), was isolated from unidentified fungus IFM 52672 as the most potent antifungal principle from this organism.

The structure of **1** was established on the basis of spectroscopic and chemical investigation, as well as detailed comparison of the spectroscopic and physico-chemical data of the oxidized derivative (**3**) from **1** with those of heveadride (**2**). Compound **1** showed strong antifungal activity against various filamentous fungi including human pathogens *Aspergillus fumigatus*, *Penicillium marneffei* and *Trichophyton* spp. It also showed the growth inhibition activity against certain human pathogenic yeasts such as *Trichosporon* species, while it had weak or no antifungal activity against *Candida* spp. and *Cryptococcus neoformans*, and no antibacterial activity against *Bacillus subtilis* nor against *Escherichia coli*. The antifungal potencies of compounds **2** and **3** were found to be weaker than that of **1**.

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 a leading cause of deaths 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. In the screening for new antifungal substances from fungal sources against pathogenic filamentous fungi, *Aspergillus fumigatus* Fresenius IFM 41362 and *Aspergillus niger* van Tieghem H7160B, and/or pathogenic yeasts, *Candida albicans* (Robin) Berkhout IFM 40009 and *Cryptococcus neoformans* (Sanfelice) Vuillemin ATCC 90112, the chloroform - methanol (1 : 1) extracts of a freshly isolated unidentified fungus, IFM 52672, cultivated on rice for 21 days at 25°C, showed antifungal activity against *A. fumigatus* and *A. niger*.

Results and Discussion

The molecular formula of compound (**1**) was determined as $C_{18}H_{22}O_6$ by high resolution electron-impact ionization mass spectrometry (HREI-MS). The physico-chemical properties and the ^{1}H- and ^{13}C-NMR data of **1** are shown in Tables 1 and 2, respectively. The ^{1}H- and ^{13}C-NMR spectra of **1** was similar to those of heveadride (**2**)[2,3)], which was recently isolated from *Bipolaris heveae* CBS 241.93 (originally isolated from *Helminthosporium heveae* Petch[2)]), except for the extremely upfield shift of the carbon at C-12 (δ 98.7) in **1** instead of one of four carbonyl carbons in **2** and the new appearance of the ^{1}H-NMR signal at δ 6.04 (1H, s), corresponding to a proton attached to a

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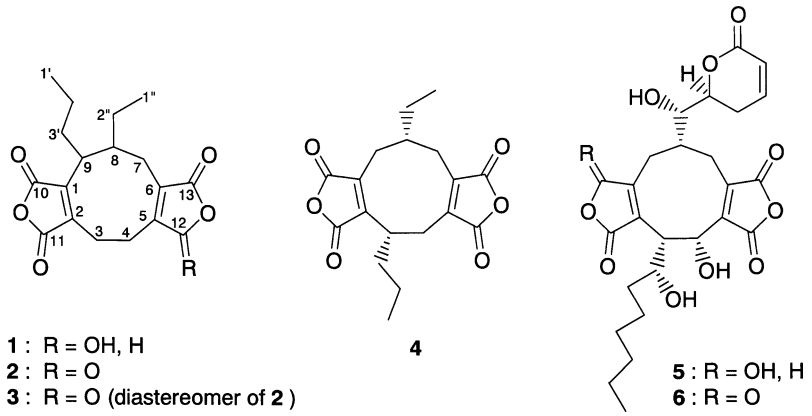
Table 1. Physico-chemical properties of compounds **1**~**3**.

	Dihydroepiheveadride (1)	Heveadride (2)	Epiheveadride (3)
Appearance	colorless crystalline powder (from diethyl ether)	colorless crystalline powder (from diethyl ether)	colorless crystalline powder (from diethyl ether)
Melting point (°C)	157-158	159.5-162	146-149
Molecular formula	$C_{18}H_{22}O_6$	$C_{18}H_{20}O_6$	$C_{18}H_{20}O_6$
HREI-MS(M^+)	334.1428 (Calcd. 334.1416)	332.1273 (Calcd. 332.1260)	332.1270 (Calcd. 332.1260)
CI-MS (isobutane)	335 (M+1)	333 (M+1)	333 (M+1)
IRν^{KBr} cm^{-1}	3450 (OH) 1840, 1770 (anhydride)	1834, 1761 (anhydride)	1830, 1763 (anhydride)
UV^{MeOH}nm(log ε)	251 (3.56), 204 (4.18)	246 (3.30), 204 (3.67)	246 (3.94), 204 (4.29)
CD $\Delta\varepsilon$(nm)	-5.76 (247)	+1.05 (220)	-13.16 (250)
$[\alpha]_D$	-91.3° (c=1.04, $CHCl_3$)	+59.3 ° (c=1.12, $CHCl_3$)	-170.9 ° (c=1.11, $CHCl_3$)
Solubility (Soluble) (Insoluble)	CH_2Cl_2, EtOAc, Me_2CO, EtOH, MeOH n-Hexane, H_2O	CH_2Cl_2, EtOAc, Me_2CO, EtOH, MeOH n-Hexane, H_2O	CH_2Cl_2, EtOAc, Me_2CO, EtOH, MeOH n-Hexane, H_2O

Fig. 1. Structures of nonadrides and its derivatives.



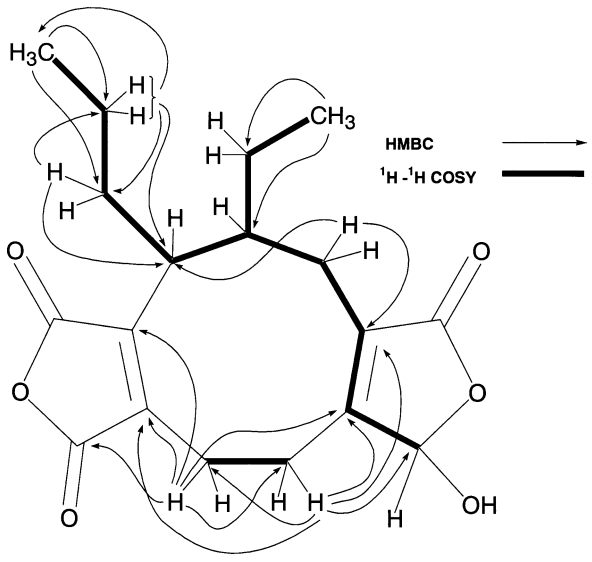
hemiacetal or acetal carbon (Table 2). The infrared (IR) absorption at 1840 cm^{-1} (weak) and 1770 cm^{-1} (strong) suggested the presence of a five-membered ring anhydride and a five-membered ester. These facts indicated that **1** could be the dihydro compound of **2**, *i.e.*, one of four carbonyls of two anhydride residues in **2** was reduced to a hemiacetal. From the detailed comparison of the ^{1}H- and ^{13}C-NMR signals of **1** with those of **2** and, from the analysis of the ^{1}H-^{1}H COSY and HMBC spectra of **1** (Fig. 2), the planar structure of compound (**1**) was elucidated.

In order to determine the stereochemical relationship between **1** and **2**, **1** was oxidized with pyridium chlorochromate (PCC). The ^{1}H- and ^{13}C-NMR spectra of compound (**3**) obtained was very similar to those of **2**, expect for the chemical shifts of the carbon signals at C-8 and C-9 (Table 2). The melting points, the optical rotation, and the CD value of **3** were different from those of **2** (Table 1). Therefore we concluded that **3** was a diastereomer of **2**,

Table 2. ^{1}H- and ^{13}C-NMR spectral data of dihydroepiheveadride (**1**), heveadride (**2**) and epiheveadride (**3**) in $CDCl_3$.

Carbon No.	Dihydroepiheveadride (1)		Heveadride (2)		Epiheveadride (3)	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	146.5		148.3		146.0	
2	146.5		145.1		146.2	
3	21.8	2.49 brt (12.5)	21.2	2.34 brt (12.8)	21.5	2.20 m
		3.08 ddd (12.5, 10.0, 1.2)		3.08 brt (12.8)		3.16 m
4	24.3	2.36 m	22.5	2.20 m	22.1	2.20 m
		2.73 m		3.23 dd (13.2, 8.6)		3.16 m
5	158.8		144.0		144.1	
6	130.6		144.5		145.0	
7	26.7	1.51m	26.1	2.12 brd (12.5)	28.0	1.76 brt (12.8, 12.5)
		2.64 brd (13.7)		2.88 brd (12.5)		2.90 dd (12.8, 3.0)
8	46.5	2.04 m	40.7	2.22 m	47.9	2.10 m
9	40.1	2.64 m	46.2	2.24 m	40.8	2.44 m
10	164.5		163.5		164.1	
11	165.8		164.8		165.2	
12	98.7	6.04 m	165.7		165.2	
13	171.8		165.7		164.8	
1'	13.9	0.86 t (7.1)	14.0	0.86 t (7.0)	13.9	0.87 t (7.3)
2'	21.7	1.14 m	21.8	1.04 m	21.8	1.15 m
		1.14 m		1.15 m		1.15 m
3'	31.5	1.51 m	30.8	1.62 m	31.6	1.56 m
		2.14 m		1.94 m		2.15 m
1"	12.9	1.06 t (7.1)	12.8	1.18 brs	12.9	1.08 t (7.3)
2"	23.2	2.04 m	22.5	1.20 m	23.5	0.98 m
		2.04 m		1.82 m		2.10 m

Fig. 2. HMBC and ^{1}H-^{1}H COSY correlations in dihydroepiheveadride (**1**).



designated as epiheveadride. The stereochemistry of **1** and **3** has not yet been determined.

More than ten nonadrides have been isolated from fungal sources, for example, byssochlamic acid (**4**) originally from *Byssoclamys fulva* Olliver and Smith[4] and *Byssoclamys nivea*[5] and heveadride (**2**) from *Helminthosporium heveae* Petch[2]. However, there is only one example of a partially hydrogenated nonadride, which is rubratoxin A (**5**) originally isolated from *Penicillium rubrum* along with the typical nonadride, rubratoxin B (**6**)[6]. Dihydroepiheveadride (**1**) is the second example of a partially hydrogenated nonadride to our knowledge.

Antimicrobial Property

Since none of the three compounds **1**~**3**, dissolve in water, the antimicrobial activity was determined by the paper disc method, as described in the previous paper[7]. The results are summarized in Table 3. Compound **1** showed strong antifungal activity against various filamentous fungi including the human pathogens *A. fumigatus*, *P. marneffei* and dermatophytes *T. rubrum* and *T. mentagrophytes* at a

Table 3. Antimicrobial activities of compounds **1**~**3**.

Microorganisms	Diameter of Inhibition Zone (mm)					
	1		2		3	
	5 μg	100 μg	5 μg	100 μg	5 μg	100 μg
<Filamentous fungi>						
Arthroderma benhamiae IFM 41160	20		10		9	
Aspergillus flavus IFM 41935	26		−		−	
Aspergillus fumigatus IFM 41243	20		−		−	
Aspergillus fumigatus IFM 41362	20		−		−	
Aspergillus fumigatus IFM 47078	23		−		−	
Aspergillus niger IFM 41398	21		−		−	
Cladophialophora carrionii IFM 4808	−	26	−	−	−	−
Emericella nidulans IFM 46997	24		−		−	
Epidermophyton floccosum IFM 46637	13		9		8	
Fonsecaea pedrosoi IFM 4887		−		−		−
Fusarium oxysporum IFM 53787	10		−		−	
Fusarium solani IFM 52712	12		9		9	
Microsporum canis IFM 45108	25		10		9	
Penicillium marneffei IFM 52703	21		−		−	
Penicillium marneffei IFM 52697	11		−		−	
Phialophora verrucosa IFM 4928		−		−		−
Scedosporium apiospermum IFM 52028	17		11		11	
Trichophyton mentagrophytes IFM 40951	30		12		12	
Trichophyton raubitschekii IFM 45579	20		12		8	
Trichophyton rubrum IFM 45802	17		13		11	
Trichophyton tonsurans IFM 5275	28		12		9	
Trichophyton verrucosum IFM 46798	10		−		−	
Trichophyton violaceum IFM 46913	25		16		13	
< Yeasts >						
Candida albicans ATCC 90028	−	16	−	15	−	−
Candida albicans ATCC 90029	−	19	−	19	−	−
Candida glabrata IFM 40217		−		−		−
Candida guilliermondii IFM 46823	−	8	−	−	−	−
Candida kefyr IFM 46921	−	12	−	8	−	−
Candida krusei IFM 46834	−	15	−	−	−	−
Candida parapsilosis IFM 46863		−		−		−
Candida tropicalis IFM 46816		−		−		−
Cryptococcus neoformans ATCC 90112	−	14	−	11	−	9
Saccharomyces cerevisiae IFM 40210	−	20	−	−	−	−
Pichia anomala IFM 53788		−		−		−
Trichosporon asahii IFM 48429	15		10		—	
Trichosporon asteroides IFM 48608	10		—		—	
< Bacteria >						
Bacillus subtilis ATCC 6633		−		−		−
Escherichia coli B		−		−		−

Paper disc (i. d. 6 mm) was used.
The minus (−) means no inhibition.

concentration of 5 μg/disc. In the activity test of **1** for yeasts at 5 μg/disc, antifungal activity was observed on the two species, *Trichosporon asahii* and *Trichosporon asteroides*, but was not observed for such other human pathogens as *Candida albicans* and *Cryptococcus neoformans*. On the other hand, **1** inhibited the growth of several *Candida* species such as *C. albicans*, *C. krusei* and also *C. neoformans* at 100 μg/disc. However, **1** did not show growth inhibition activity against of *Candida* species, *C. glabrata* and *C. tropicalis* nor dematiaceous fungi as *Fonsecaea pedrosoi* and *Phialophora verrucosa*.

The antifungal spectra of compounds **2** and **3** at 5 μg/disc against the filamentous fungi were similar to that of **1**, but the potencies were lower.

For the yeasts, none of the three compounds show antifungal activity at 5 μg/disc. Compounds **1** and **2** produced similar spectra at 100 μg/disc, but the potency in **2** was relatively lower. On the other hand, no antifungal activity was observed in **3**, except for *C. neoformans*. Antibacterial activity of **1**～**3**, against *Bacillus subtilis* or *Escherichia coli* was not found.

These results have suggested that reduction of the carbonyl group at C-12 in nonadrides is more related to the intensity of the antifungal activity than stereochemical difference of side-chains at C-8 and C-9. The antifungal activity of **1** against filamentous fungi may contribute to discovery of a new molecular mechanism of action for antifungal agents.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. EI- and CI-MS were taken with a JEOL JMS-MS600W 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 Lambda-500 (^{1}H, 500.00 MHz; ^{13}C, 125.25 MHz) spectrometer, using tetramethylsilane as an internal standard. CD curves were determined on a JASCO J-600 spectropolarimeter. Column chromatography was performed using Kieselgel 60 (Art. 7734, Merck) and Wakogel C-200 (Art. 237-00071, Wako). Low-pressure liquid chromatography (LPLC) was performed with a Chemco Low-Prep 81-M-2 pump and glass column (200×10 mm) packed with Silica gel CQ-3 (30～50 μm, Wako). TLC were vizualized by UV light at 254 nm and/or by spraying with 5%-H_2SO_4 and then heating.

Antifungal Screening Assay of the Fungal Extracts.

The antifungal assay was performed by the paper disc method against *C. albicans* IFM 40009, and *C. neoformans* ATCC 90112, *A. fumigatus* IFM 41362, *A. niger* H7160B, as test organisms. About 220 source strains were cultured at 25°C for 21 days in a test-tube containing 10 g of moist rice. The cultivated rice was extracted with CH_2Cl_2-MeOH (1 : 1) and the organic layer was evaporated *in vacuo*. The extracts were spotted on the paper discs (8 mm diameter) at 2.5 mg/disc and placed on the assay plates. The fungi were cultivated in Potato Dextrose (PD) agar at 25°C. After 24～72 hours incubation, zones of inhibition (mm in diameter) were recorded.

Isolation of Dihydroepiheveadride (**1**) from Unidentified Fungus IFM 52672

The fungus IFM 52672 was cultivated on rice (500 g, using 5 Roux flasks) for 28 days at 25°C. The cultivated rice was extracted with $CHCl_3$-MeOH (1 : 1), and the evaporated extract (18 g) was suspended with water, and extracted with hexane (350 ml), benzene (350 ml), $CHCl_3$ (350 ml), acetone (350 ml), and methanol (350 ml), sequentially. The hexane and benzene extracts showed antifungal activity against *A. fumigatus*. The combined residue of the hexane and benzene extracts was repeatedly chromatographed on silica gel (Wako, C-200) with benzene-chloroform, followed by recrystallization from diethyl ether to give compound **1** (315 mg), designated dihydroepiheveadride. Physico-chemical properties and the ^{1}H-, ^{13}C-NMR signal assignments of **1** are summarized in Tables 1 and 2, respectively.

Oxidation of Dihydroepiheveadride (**1**) by PCC (Pyridium Chlorochromate)

The CH_2Cl_2 solution (3 ml) of dihydroepiheveadride (**1**) (53 mg) was added to a CH_2Cl_2 suspension (12 ml) of PCC (350 mg) and the mixture was refluxed for 2 hours with stirring. The reaction mixture was filtered, and the filtrate was evaporated *in vacuo*. The residue obtained was chromatographed on silica gel (Kieselgel 60, Merck) with CH_2Cl_2, followed by recrystallization from diethyl ether to give epiheveadride (**3**) (41 mg). Physico-chemical properties of **3** are summarized in Table 1, and the ^{1}H-, ^{13}C-NMR signal assignments are summarized in Table 2.

Antibacterial and Antifungal Activities of Compounds **1**～**3**

Antibacterial and antifungal activities were *semi*-quantitatively determined using the agar diffusion method with paper disc (6 mm in diameter), loaded with 5 and/or

100 μg of compound as described in the previous paper[7]. The test organisms used and the results are summarized in Table 3.

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