DEOXYMULUNDOCANDIN—A NEW ECHINOCANDIN TYPE ANTIFUNGAL ANTIBIOTIC

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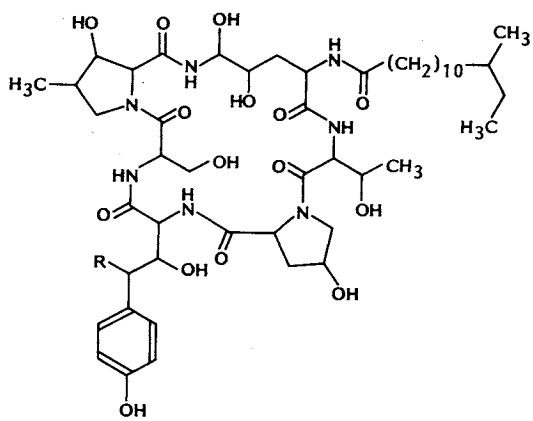
A new echinocandin type antifungal antibiotic, deoxymulundocandin, $C_{48}H_{77}N_7O_{15}$, was isolated from the culture filtrate and mycelia of a fungal culture, *Aspergillus sydowii* (Bainier and Sartory) Thom and Church var. nov. mulundensis Roy (Culture No. Y-30462). The structure was established by comparative GC-MS analyses of the derivatized acid hydrolysates of deoxymulundocandin and mulundocandin as well as by the high field NMR experiments (COSY, NOESY and DEPT).

In the course of our screening for new antibiotics from fungi, we have isolated an antifungal antibiotic, deoxymulundocandin (**1**), from a fungal culture *Aspergillus sydowii* (Bainier and Sartory) Thom and Church var. nov. mulundensis Roy (Culture No. Y-30462). Earlier we had reported the isolation and structure elucidation of a structurally related antifungal antibiotic, mulundocandin (**2**), from the same strain[1,2].

Isolation and Purification of Deoxymulundocandin

The microorganism was fermented in two stainless steel fermenters of 150 liters and 390 liters capacities with 95 liters and 270 liters of the production media as previously described[1]. After 66 hours the fermentation was terminated and the culture broth was centrifuged to separate the culture filtrate and the mycelial mass. Deoxymulundocandin, along with mulundocandin, was present in the culture filtrate as well as in the cell mass.

The mycelial mass (33.5 kg) was extracted with acetone (2 × 100 liters). The extract was concentrated under reduced pressure to 60 liters, diluted with water and then reextracted with ethyl acetate (4 × 60 liters). The culture filtrate (337 liters) was extracted with ethyl acetate (245 liters), the extracts were combined with the mycelial extract and then concentrated under reduced pressure to obtain an oil (253 g). Trituration of the oil with acetonitrile

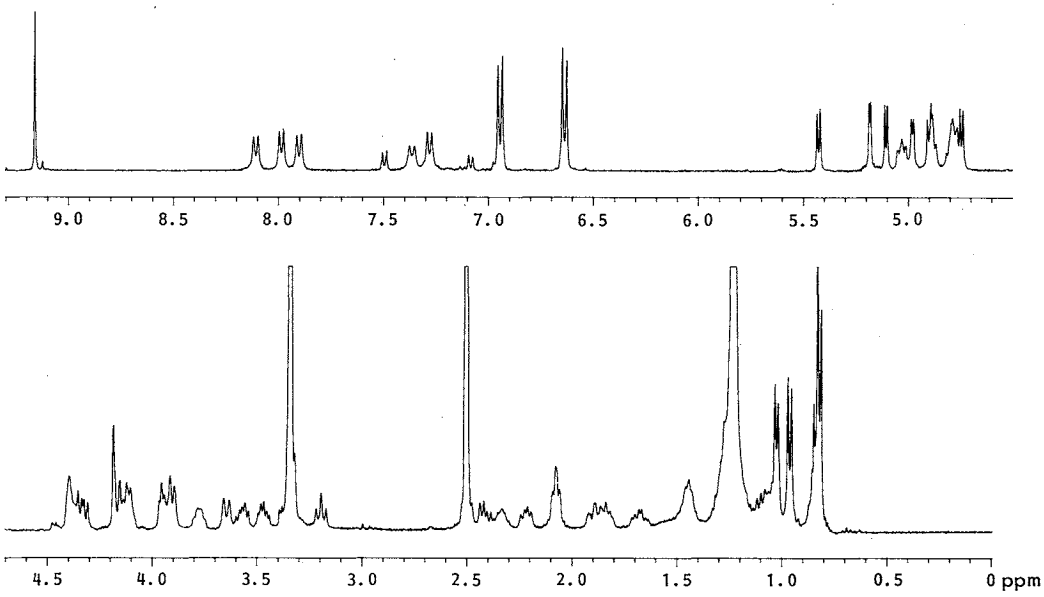


Deoxymulundocandin (**1**) R=H
Mulundocandin (**2**) R=OH

Table 1. TLC and HPLC comparison of deoxymulundocandin and mulundocandin.

	Stationary phase	Mobile phase	Deoxymulundocandin	Mulundocandin
TLC	Silica gel (detection; I_2)	EtOAc-PrOH-H_2O (5:3:1)	Rf 0.76	Rf 0.69
		BuOH-AcOH-H_2O (4:1:1)	Rf 0.71	Rf 0.66
		$CHCl_3$-MeOH-17% NH_3 (2:2:1) (lower layer)	Rf 0.58	Rf 0.48
HPLC	10 μm ODS-Hypersil [(3+25) cm × 0.4 cm] (detection; 220 nm)	MeOH-0.2% aqueous $NaH_2PO_4 \cdot 2H_2O$-H_3PO_4 (75:25:0.1) Flow rate = 2 ml/minute	Rt 9.2 minutes	Rt 8.3 minutes

Fig. 1. 400 MHz ^{1}H NMR spectrum of deoxymulundocandin in DMSO-d_6 at 303 K.

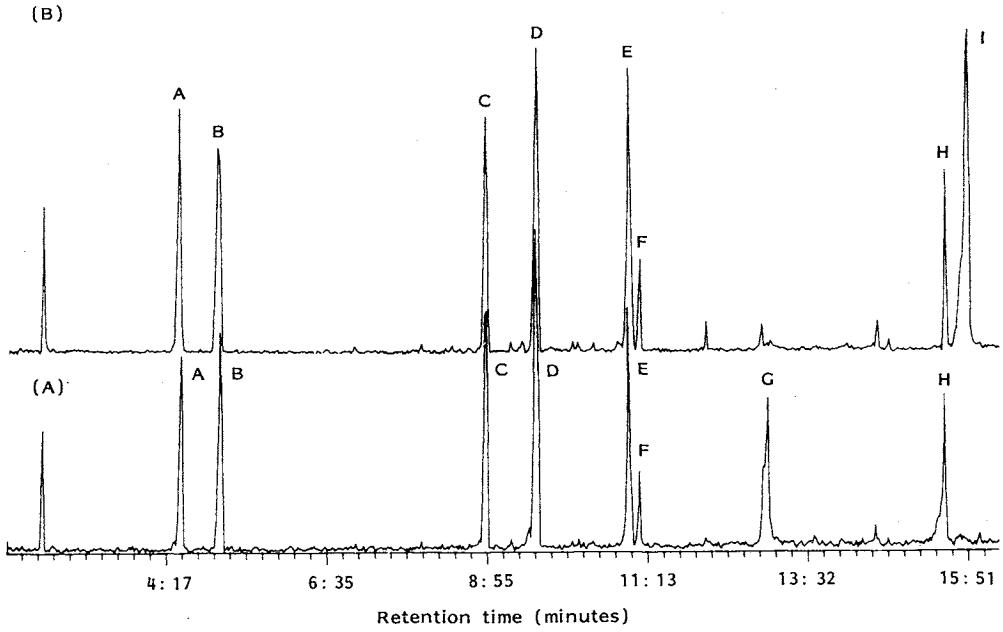


(3 × 5 liters) gave an insoluble solid. This solid was treated with methanol (2 × 1 liter) and the methanol soluble portion was concentrated to obtain the crude antibiotic (6 g). The concentrate, adsorbed on TLC grade silica gel, was loaded on to a silica gel column (TLC grade, 350 g, 3 cm × 85 cm) packed in ethyl acetate. The elution was carried out under pressure using ethyl acetate-propanol (5:2) followed by ethyl acetate-propanol (5:3). Bioactive fractions were analyzed by TLC (see Table 1) and fractions containing pure deoxymulundocandin were combined. Removal of solvent under reduced pressure gave the pure compound (85 mg).

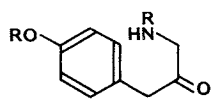
Physico-chemical Properties

Deoxymulundocandin (**1**), is a white amorphous powder having mp 167~168°C, $[\alpha]_D^{25}$ −28.23° (*c* 0.25, methanol), a molecular weight of 991 and a molecular formula of $C_{48}H_{77}N_7O_{15}$ (HRFAB-MS in the presence of lithium chloride gave $(M+Li)^+$ 998.552). The compound is soluble in methanol, dimethyl sulfoxide and *N*,*N*-dimethylformamide, but insoluble in other common organic solvents like acetone, acetonitrile, chloroform, ethyl acetate, petroleum ether *etc.* and also in water.

Fig. 2. GC comparison of the derivatized acid hydrolysates of deoxymulundocandin (run A) and mulundocandin (run B).



The compound, spotted on silica gel TLC plate shows no characteristic coloration with benzidine-periodic acid, Ehrlich reagent, PAULY's reagent and ninhydrin spray; the $KMnO_4$ and $FeCl_3$-$K_3Fe(CN)_6$ spray gives positive color reactions. Deoxymulundocandin gives UV absorption (λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 225 (74), 276 (16.5), 282 sh (13.5); $\lambda_{max}^{MeOH+NaOH}$ nm ($E_{1cm}^{1\%}$) 224 (72.5), 241 sh (37.5), 277 (15), 282 (14.5), 294 (8)) and an IR spectrum (ν_{max}^{KBr} cm^{-1} 3400, 1670, 1650, 1540, 1455, 1390, 1240 and 1080). The 400 MHz ^1H NMR spectrum is shown in Fig. 1. The above properties indicate that the compound is an echinocandin type antibiotic—similar to mulundocandin (**2**).



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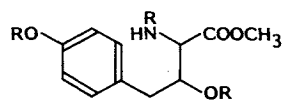

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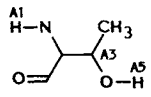
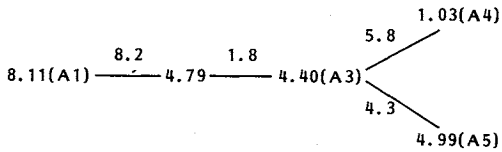
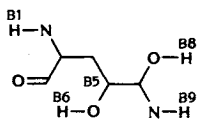
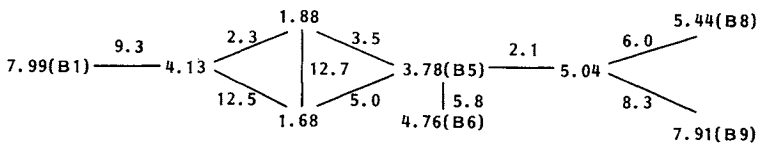
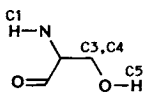
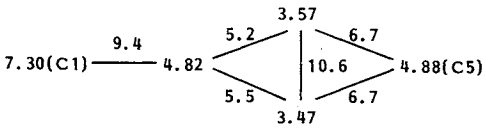
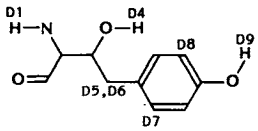
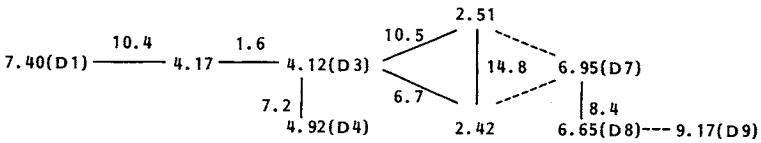
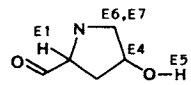
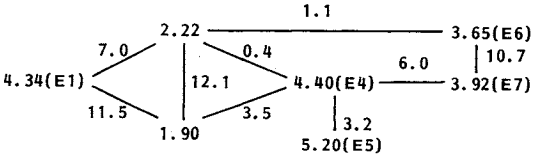
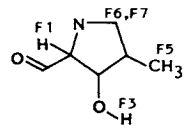
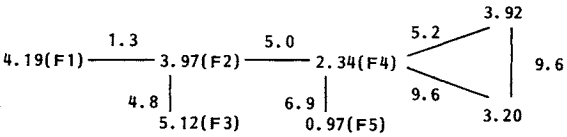
R = $COCF_3$

Structure Elucidation

The acid hydrolysate of deoxymulundocandin was converted to the methyl ester which was then trifluoroacetylated. This sample in methylene chloride was compared with a similarly prepared sample from mulundocandin by GC-MS (Fig. 2). All the peaks in GC were characterized by their mass spectra in the CI as well as EI modes. Seven peaks (peaks A ~ F and peak H) were common in the two GC runs; these were identified as derivatized amino acids namely threonine (peak A), serine (peak B), 3-hydroxy-4-methylproline (peak C), 4-hydroxyproline (peak D), 4-oxoproline (peak E, an artifact derived from 4,5-dihydroxyornithine), epimeric 4-hydroxyproline (peak F, an artifact) and 12-methyltetradecanoic acid (peak H). The two runs were different in the formation of **3** (peak I), a derivatized dehydration-decarboxylation product of 3,4-dihydroxyhomotyrosine, in the case of mulundocandin and of **4** (peak G), a derivatized 3-hydroxyhomotyrosine, in the case of deoxymulundocandin. This suggested

Table 2. Spin correlation of the amino acid units of deoxymulundocandin—chemical shift (ppm) of the spins and coupling constants (Hz) between the connected spins for each structural unit are shown.

Amino acid unit	Spin correlations
	
	
	
	
	
	

the presence of 3-hydroxyhomotyrosine in deoxymulundocandin instead of 3,4-dihydroxyhomotyrosine which is present in mulundocandin—the rest of the structural units being identical.

The 400 MHz ^{1}H NMR spectrum (DMSO-d_6/303 K) (Fig. 1) and the COSY of deoxymulundocandin identified the spin systems (Table 2) and were in conformity with the proposed structural units of **1**. The ^{13}C NMR spectrum of both the compounds were identical[2] except for the signals from the homotyrosine residue part (Table 3).

Table 3. Comparison of the chemical shifts and multiplicities of the homotyrosine unit in the ^{13}C NMR spectra of deoxymulundocandin and mulundocandin.

Carbon atom	Mulundocandin	Deoxymulundocandin
2	53.58/d	55.17/d
3	73.18/d	72.32/d
4	74.99/d	39.19/t
2′,6′	132.45/d	130.18/d
3′,5′	114.61/d	114.81/d

The NOE (NOESY spectrum) of **1** and **2** were nearly identical[2] suggesting the same connectivity sequence of the fatty acid and the amino acids in the two compounds. Further, since the ^{1}H-^{1}H coupling constants observed were also simlar to **2**[2], the overall conformation of the cyclic peptide must also

Table 4. MIC values of deoxymulundocandin.

Test organisms	MIC (μg/ml)	Test organisms	MIC (μg/ml)
Staphylococcus aureus 209P	200	*Cladosporium* species	0.75
Escherichia coli 9632	>200	*Cercospora beticola* 71	6.25
Pseudomonas aeruginosa	>200	*Botrytis cinerea* 47	6.25
Candida albicans	1.5	*B. cinerea* 57	50
Saccharomyces cerevisiae	3.1	*B. cinerea* 211	25
Aspergillus niger	3.1	*B. cinerea* 212	25
Penicillium italicum	25	*Trichophyton mentagrophytes*	<0.18
P. digitatum 135	>200	*Microsporum gypseum*	0.18
Fusarium culmorum 100	>200	*M. canis*	<0.18
F. nivale	>200		

be similar in the two compounds. Deoxymulundocandin then can be represented by the structure **1** and is a new echinocandin type of antibiotic[3].

Biological Properties

Deoxymulundocandin (**1**), possesses antifungal properties and the MICs required to inhibit the different fungal strains are given in Table 4.

Experimental

Preparation of Derivatized Acid Hydrolysates

Deoxymulundocandin in 6 N aqueous hydrochloric acid was heated at 110°C for 6 hours and the hydrolysate was concentrated under nitrogen. The concentrate was esterified with 3 N methanolic hydrochloric acid by heating at 110°C for 1 hour. The solvent was removed under nitrogen. The crude was heated with perfluoroacetic anhydride at 110°C for 0.5 hour, evaporated partially under nitrogen and then diluted with methylene chloride for the GC-MS analysis.

Mulundocandin was hydrolyzed and derivatized in a similar manner.

GC-MS of the Derivatized Acid Hydrolysates

GC conditions: 30 m DB-17 fused silica capillary column with 1 bar helium pressure, split 1 : 50 and column temperature gradient of 80~290°C with 10°C/minute heating rate.

Peak A (*N,O*-Bistrifluoroacetate Derivative of Threonine Methyl Ester)

CI-MS: *m/z* 326 (100%, $(M+H)^+$, 294 $(M+H-CH_4O)^+$, 212 $(M+H-HOCOCF_3)^+$, 152 $(M+H-C_2H_4O_2-HOCOCF_3)^+$; EI-MS: *m/z* 266 $(M-COOMe)^+$, 185 $(M^{+\cdot}-CH_2{=}CHOCOCF_3)$, 152 (100%, $(M-COOMe-HOCOCF_3)^+$).

Peak B (*N,O*-Bistrifluoroacetate Derivative of Serine Methyl Ester)

CI-MS: *m/z* 312 (100%, $(M+H)^+$, 280 $(M+H-CH_4O)^+$, 198 $(M+H-HOCOCF_3)^+$, 138 $(M+H-C_2H_4O_2-HOCOCF_3)^+$; EI-MS *m/z* 252 $(M-COOMe)^+$, 184 $(M-CH_2OCOCF_3)^+$, 138 (100%, $(M-COOMe-HOCOCF_3)^+$).

Peak C (*N,O*-Bistrifluoroacetate Derivative of 3-Hydroxy-4-methylproline Methyl Ester)

CI-MS: *m/z* 352 (100%, $(M+H)^+$), 320 $(M+H-CH_4O)^+$, 292 $(M+H-C_2H_4O_2)^+$, 178 $(M+H-C_2H_4O_2-HOCOCF_3)^+$; EI-MS: *m/z* 351 $(M^{+\cdot})$, 292 (100%, $(M-COOMe)^+$), 178 $(M-COOMe-HOCOCF_3)^+$.

Peak D (*N,O*-Bistrifluoroacetate Derivative of 4-Hydroxyproline Methyl Ester)

CI-MS: *m/z* 338 (100%, $(M+H)^+$), 306 $(M+H-CH_4O)^+$, 278 $(M+H-C_2H_4O_2)^+$, 164 (M+H-

$C_2H_4O_2-HOCOCF_3)^+$; EI-MS: *m/z* 278 $(M-COOMe)^+$, 164 (100%, $(M-COOMe-HOCOCF_3)^+$).

Peak E (*N*-Trifluoroacetate Derivative of 4-Oxoproline Methyl Ester)

CI-MS: *m/z* 240 (100%, $(M+H)^+$), 208 $(M+H-CH_4O)^+$, 180 $(M+H-C_2H_4O_2)^+$, 152 $(M+H-C_2H_4O_2-CO)^+$; EI-MS: *m/z* 239 $(M^{+\cdot})$, 207 $(M-CH_4O)^+$, 180 $(M-COOMe)^+$, 152 (100%, $(M-COOMe-CO)^+$).

Peak F (*N,O*-Bistrifluoroacetate Derivative of Epimeric 4-Hydroxyproline Methyl Ester)

CI-MS and EI-MS: Identical to those obtained for peak D.

Peak G (*N,O,O'*-Tristrifluoroacetate Derivative of 3-Hydroxyhomotyrosine Methyl Ester (**4**))

CI-MS: *m/z* 514 (100%, $(M+H)^+$), 400 $(M+H-HOCOCF_3)^+$, 367, 340, 227; EI-MS: *m/z* 399 (M' $(M'^{+\cdot}-HOCOCF_3)$), 367 $(M'^{+\cdot}-HOMe)$, 340 $(M'-COOMe)^+$, 227 (100%, $(M'-COOMe-H_2NCOCF_3)^+$), 203 $(CF_3COOC_6H_4CH_2)^+$.

Peak H (Methyl 12-Methyltetradecanoate)[2]

CI-MS: *m/z* 257 $(M+H)^+$; EI-MS: *m/z* 256 $(M^{+\cdot})$, 199 $(M-C_4H_9)^+$, 143 $((CH_2)_6COOMe)^+$, 87 $(CH_2=CHC(OH)OMe)^+$, 74 (100%, $(CH_2=C(OH)OMe)^+$).

Peak I (*N,O*-Bistrifluoroacetate of 2-Oxo-3(4-hydroxyphenyl)propyl Amine (**3**))

CI-MS: *m/z* 358 (100%, $(M+H)^+$), 245 $(M+H-NH_2COCF_3)^+$, 203 $(M+H-NH_2COCF_3-C_2H_2O)^+$; EI-MS: *m/z* 357 $(M^{+\cdot})$, 203 (100%, $(CF_3COOC_6H_4CH_2)^+$), 126 $(CH_2NHCOCF_3)^+$.

Discussion

Deoxymulundocandin is a cyclic peptide with a fatty acid side chain belonging to the echinocandin class of antifungal antibiotics. The compound is structurally related to mulundocandin; it contains 3-hydroxyhomotyrosine unit in place of the 3,4-dihydroxyhomotyrosine unit of mulundocandin. The molecular weight and the structural formula of this antibiotic is different from any of the known echinocandin type of antibiotics and hence deoxymulundocandin is described as a new compound of the echinocandin class.

Acknowledgment

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