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Novel Fungal Metabolites as Cell Wall Active Antifungals:

Fermentation, Isolation, Physico-chemical Properties, Structure and Biological Activity

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Two novel antifungals SCH 643432 (**1**), and **2**, were isolated from the fermentation broth of a fungus taxonomically classified as a *Paecilomyces varioti*. These compounds were separated from the fermentation broth filtrate by adsorption on a macroreticular resin XAD-16 (Amberlite). Purification and separation of the individual compounds were achieved by trituration of the extract with dichloromethane followed by preparative HPLC using reverse phase columns. Extensive FAB (Fast Atom Bombardment) and ESI (Electro Spray) mass spectrometric studies using fragmentation of various daughter ions, NMR experiments and degradative studies helped in elucidating the structure of compound **1**. Compound **2** is an isomer of SCH 643432 (**1**). They were identified as straight chains peptides containing several amino acids such as alanine, aminoisobutyric acid, proline, leucine, glycine and arginine. The *N*-terminal is terminated in a previously identified β-keto acid, 2-methyl 3-oxo tetradecanoic acid (MOTDA).[5] Both compounds were active against *Candida albicans*, other Candidas, dermatophytes and *Aspergillus* (Geometric Mean MIC's 4.00, 2.59, 3.56, 11.31 and 4.49, 4.00, 5.66, 16.0 μg/ml, respectively for **1** and **2**).

Since early 1900's the cure for fungal diseases were through discovering novel antifungal compounds that showed zone of inhibition on agar plates containing fungi like *C. albicans* and *Saccharomyces cerevisiae*. In last twenty years the search for novel molecules with activity against human pathogenic fungi has shifted to mechanistic based assays. Natural product screening provides one of the best source of novel structures within this paradigm. Whole cell, mechanistic assays are selective for permeable compounds and can target more than a single enzyme in a pathway. However sometimes active compounds present at extremely low concentrations or those with poor binding properties can be missed.[1]

Here we report the isolation of two novel antifungal compounds from the fermentation broth of a fungus using whole cell assay designed to identify compounds that cause fungal cell wall damage. We describe the fermentation conditions for production of the active metabolites, as well as their isolation, physico-chemical properties, structure elucidation and biological activity.

Materials and Methods

Microorganisms

The producing microorganism was isolated from a soil sample. The fungus producing this compound was identified as *Paecilomyces varioti*. based on taxonomic evaluation. The strain has been deposited in Schering culture collection with accession number SCF 1559.

Description of the WLD Assay

A complete description of WLD assay used has been described in our previous publication.[2]

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Fermentation Conditions

Fermentation studies were carried out in shake flasks. Stock cultures were maintained as frozen whole broths at −20°C in a final concentration of 10% glycerol. The inoculum medium for antifungal production contained (g/liter) proteous peptone 5, NaCl 5, KH_2PO_4 5, Difco yeast extract 3, Cerelose 20, soybean grits 5, Dow Corning emulsion B antifoam 0.5 ml, tap H_2O to 1 liter. The pH was adjusted to 7.0 prior to autoclaving. A 250 ml Erlenmeyer flask containing 70 ml of this medium was inoculated with 2.0 ml of the stock culture. The flasks were incubated at 24°C on a rotary shaker (New Brunswick Model # G-53, 3 tier, 2″ stroke) at 300 rpm for 96 hours. This seed culture was used to inoculate another 2-liter Erlenmeyer flask containing 500 ml of the same seed medium and the flask was incubated, as above for 96 hours.

Five percent of the second germination was used to inoculate 10-liter of fermentation medium containing (g/liter) Neopeptone 10, Cerelose 40, $CaCO_3$ 4, and tap H_2O to 1 liter. Fermentation was carried out at 24°C with 450 rpm agitation and 4.5 liters/minute of aeration for 120 hours.

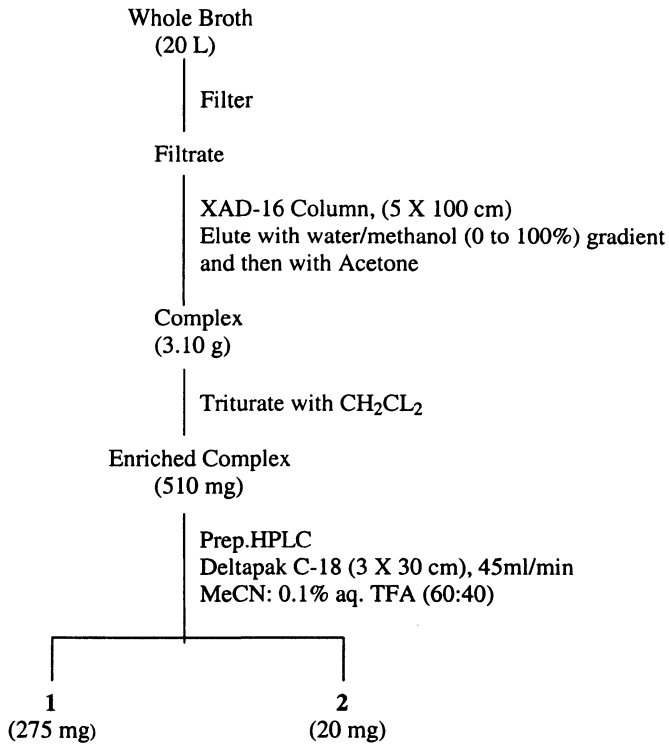
Fermentation Analysis

Mycelial growth was measured as packed cell volume (PCV) by centrifuging the fermentation broth at 5000 rpm for 35 minutes. The antibiotic production along with pH and packed cell volume were monitored at regular intervals. The production of antibiotic was measured by an agar diffusion well (12 mm) assay against *Candida albicans* 406.

Isolation

The steps leading to isolation and purification of these antifungals are shown in Figure 1. A typical twenty liter fermentation broth was filtered and the filtrate was loaded on an XAD-16 column (5×100 cm). The column was washed with water and then eluted with a water/methanol gradient (methanol 0 to 100%). The fractions were monitored by an agar diffusion assay using *C. albicans*. The 75% methanol and methanol eluate containing active material were combined, and solvent removed to yield 3.10 g of solid. This solid was triturated with dichloromethane (2 liters) overnight, filtered and solvent removed from the filtrate to yield 510 g of enriched active complex. The active compounds were separated on a Waters preparative Deltapak C-18 (3×30 cm) column eluting with a mixture of acetonitrile and 0.1% aqueous TFA solution (60 : 40 v/v). The acetonitrile was removed from the individual peak eluates under vacuum and the remaining aqueous solutions yielded on freezing drying

Fig. 1. Isolation scheme for compounds **1** and **2**.



275 mg and 20 mg of **1** and **2**, respectively.

Physico-chemical Properties

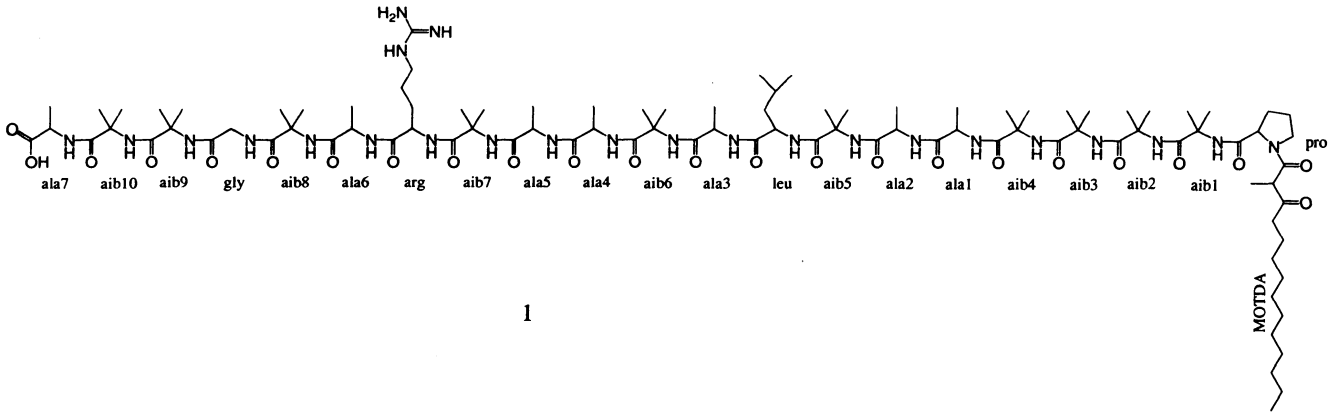
The physico-chemical properties of these antifungals are summarized in the experimental section. Compounds **1** and **2** were isolated as amorphous white powders soluble in methanol, chloroform, ethyl acetate, insoluble in hexane and water. They showed only end absorption in UV spectrum.

MIC/MFC Determinations: Minimal inhibitory and fungicidal concentrations were determined in Saboraud Dextrose Broth (SDB pH 5.0) and Eagles Minimum Essential Medium (EMEM pH 7.0) after 48 hours [*Candida*, n=14] or 72 hours [*Aspergillus*, n=3, and dermatophytes, n=6]. All cultures were incubated at 37°C.

Experimental

General Procedures

Solvents employed for chromatography were obtained from Fisher Scientific, Fair Lawn, NJ, 07410. The preparative reverse phase HPLC was carried out on a C-18 silica column (Deltapak, 1.9×30 cm) obtained from Waters



1

Corporation, Milford, MA 01757.

IR spectra were determined on a Nicolet FTIR model 10-MX instrument. Ultraviolet spectra were obtained by using a Hewlett Packard '8450 A' UV-vis spectrophotometer equipped with HP-9872B plotter. All Cs^+ ion liquid secondary ion mass spectra (SIMS) and high resolution mass measurements were obtained on a VG-ZAB-SE mass spectrometer using a glycerol-thioglycerol or *m*-nitrobenzyl alcohol matrix with the sample dissolved in dimethyl sulfoxide. Electrospray ionization (ESI) mass spectrometric data were obtained on a SCIEX API III mass spectrometer. NMR spectra were measured on Varian instruments, XL-300 operating at 300 and 75 MHz, XL-400 operating at 400 and 100.5 MHz for ^1H and ^{13}C NMR respectively. ^1H and ^{13}C NMR, spectra were recorded relative to TMS as an internal standard. COSY spectra were measured on a Varian XL-400 instrument.

Hydrolysis of **1**

SCH 466432 (**1**) (5 mg) was dissolved in 2 ml of 6 N HCl in a sealed tube under nitrogen and was heated at 110°C for 24 hours. The solution was diluted water and extracted with ethyl acetate. The ethyl acetate extract was dried, filtered and the filtrate was dried to yield the aliphatic acid, 2-methyl 3-oxo tetradecanoic acid (MOTDA).

The aqueous phase on freeze drying yielded the amino acids they were detected by TLC[3] and CIMS.

Results

Production of the antifungal in the fermentation was monitored using an agar diffusion assay against pathogenic yeast, *C. albicans* at different times during the fermentation process. The antifungal production in the fermentation peaked at 120 hours.

The complex obtained from XAD-16 extraction provided two related peptides. These compounds were similar in nature and were further purified and separated by using reverse phase chromatography on a polymeric column.

Structure Determination

SCH 643432 (**1**) a major component, mp >200°C; IR 3495, 3282, 2985, 1654, 1539, 1461, 1385, 1202 cm^{-1}. In Cs^+ ion liquid secondary ionization mass spectrum (SIMS), it displayed an intense ion at *m/z* 2028 $(M+H)^+$ revealing the molecular weight to be 2027. Peak matching measurements using high-resolution mass measurements showed the elemental composition to be $C_{95}H_{167}N_{24}O_{24}$ (obsd. 2028.5258 and calcd. for $C_{95}H_{167}N_{24}O_{24}$ 2028.5174) suggesting twenty-five degrees of unsaturation. Though the molecular weight is odd the compound has even number of nitrogen atoms because of the additional ^{13}C isotope mass. The presence of multiple nitrogen atoms suggested that the compound might be a peptide. It was hydrolyzed in a sealed tube with 6 N HCl at 110°C under nitrogen for 24 hours. Reaction mixture was extracted with ethyl acetate and aqueous solution was lyophilized. The freeze dried aqueous extract was analyzed for amino acids. TLC, CIMS and HPLC analysis indicated the presence of glycine, proline, arginine, alanine, leucine and an uncommon amino acid, which was identified as amino isobutyric acid.

The ^{13}C NMR spectrum measured in DMSO-d_6 at 100.5 MHz, revealed the presence of 95 carbon atoms (Table 1). ^{13}C NMR APT experiment showed twenty four sp^2 quaternary carbons (O=C$\lesssim$), ten sp^3 quaternary carbons ($\gtrsim$C$\lesssim$), twelve sp^3 methines ($\gtrsim$CH–), eighteen

Table 1. ^{13}C NMR spectrum of **1** and **2**.

Amino Acids	Carbon #	Chemical Shifts ^{1}H	Chemical Shifts ^{13}C	Amino Acids	Carbon #	Chemical Shifts ^{1}H	Chemical Shifts ^{13}C
MOTDA	1''		171.1 (s)	Glycine	1'		174.1 (s)
	2''	3.82 (q)	50.8 (d)		-NH-	7.52(d)	
	2''-CH_3	1.15(d)	12.5 (q)		2'	2.75	35.7 (t)
	3''		206.5 (s)	Leu	1'		171.7 (s)
	4''	2.66(t)	40.7 (t)		-NH-	8.08(d)	
	5''	1.62(m)	27.8 (t)		2'	3.48	50.8 (d)
	6''	1.2 (m)	22.0 (d)		3'	2.20	24.3 (t)
	7''	1.2 (m)	28.4 (t)		4'		24.1 (t)
	8''	1.2 (m)	28.6.(t)		5'	1.10	16.5 (q)
	9''	1.2 (m)	28.9 (t)		5'	1.01	16.4 (q)
	10''	1.2 (m)	28.9 (t)	AIB			
	11''	1.2 (m)	28.9 (t)	aib-1			172.3 (s)
	12''	1.2 (m)	31.2 (t)		-NH-	7.84(s)	
	13''	1.31(m)	24.9 (t)				55.9 (s)
	14''	0.84(t)	13.8 (q)				24.1 (s)
							24.1 (s)
Proline	1'		171.2 (s)	aib-2			172.0 (s)
	2'	4.29	60.2 (d)		-NH-	8.04(s)	
	3'	1.98, 2.37	35.0 (t)				55.9 (s)
	4'	2.05(m)	24.5 (t)				22.9 (s)
	5'	3.42, 3.62	47.5 (t)				22.9 (s)
				aib-3			175.6(s)
Arginine	1'		171.5 (s)		-NH-	7.95(s)	
	-NH-	7.48(d)					55.6 (s)
	2'	2.35	52.1 (d)				23.6 (s)
	3'		35.2 (t)				23.4 (s)
	4'		28.8 (t)	aib-4			171.6 (s)
	5'		35.6 (t)		-NH-	7.24(s)	
	6'		156.6 (s)				55.8 (s)
							25.6 (s)

methylenes and thirty one methyls in the molecule. The sp^2 quaternary carbons are represented by one carbonyl at δ 206.5, twenty two amide/carboxylic acid carbons at δ 170~178, one guanidino carbon at δ 156.6. All the sp^3 quaternary carbons are α-amino quaternary carbons of aminoisobutyric acids (δ 55.9, 55.9, 55.9, 55.9, 55.9, 55.8, 55.8, 55.8, 55.7, 55.6). Three, of the methylenes can be assigned as –N–CH_2– proline (δ 47.5), –CH_2 arginine (δ 35.7) and –N–CH_2– glycine (δ 35.6) and remaining fifteen methylenes are the part of the crowded up field aliphatic

Table 1. Continued

Amino Acids	Carbon #	Chemical Shifts ^1H	Chemical Shifts ^{13}C		Amino Acids	Carbon #	Chemical Shifts ^1H	Chemical Shifts ^{13}C
Alanine								25.5 (s)
Ala-1			173.8(s)		aib-5			173.1 (s)
	-NH-	8.20(d)				-NH-	7.68(s)	
		3.94	50.0 (d)					55.9 (s)
		1.52	22.7 (q)					25.4 (q)
Ala-2			175.2 (s)					25.2 (q)
		8.16 (d)			aib-6			172.6 (s)
		3.86	49.2 (d)			-NH-	8.58(s)	
			16.5 (q)					55.9 (s)
Ala-3			171.1 (s)					26.6 (q)
	-NH-	7.62(d)						26.4 (q)
		3.92	50.1(d)		aib-7			173.3 (s)
			22.8 (q)			-NH-	8.40(s)	
Ala-4			174.2 (s)					55.8 (s)
	-NH-	7.63(d)						26.2 (q)
		4.2	50.0 (d)					25.8 (q)
		1.5(d)	22.6 (q)		aib-8			174.9 (s)
Ala-5			175.4 (s)			-NH-	7.94(s)	
	-NH-	7.14(d)						55.7 (s)
			49.2 (d)					25.1 (q)
			17.0 (q)					25.0 (q)
Ala-6			176.0 (s)		aib-9			174.1 (s)
	-NH-	7.20(d)				-NH-	8.06(s)	
			50.0 (d)					55.9 (s)
			21.6 (q)					24.9 (q)
Ala-7			169.3 (s)					24.8 (q)
	-NH-	7.73(s)			aib-10			175.1 (s)
			49.6 (d)			-NH-	7.80(s)	
			17.0 (q)					55.8 (s)
								24.6 (q)
								24.3 (q)

** The carbon with similar chemical shifts may be interchanged*

carbon region of the CMR spectrum. Ten of the twelve sp^3 methines are located in the region of α-amino methines (H–C–N) of amino acids and the remaining two are aliphatic $\geq$CH– located in the upfield region. The ^{13}C NMR spectrum revealed twenty quaternary (from aminoisobutyric acid), ten secondary (from alanine, leucine

and aliphatic chain) and one primary methyl carbons. 2D (^{1}H-^{1}H) COSY, HMBC, HMQC, HMQC-TOCSY (15 msec) experiments allowed the assignment of most of the protons and carbon resonances. HMBC provided most of the connectivities. The data are presented in the Table 1.

The structure of **1** was confirmed using mass spectral data[4~6] and our past experience in these kind of peptides.[2] Electrospray mass spectrum of **1** using 0.1% TFA showed the molecular ion at 2028 (M+H, Figure 2) and three other strong peaks at *m/z* 1353, 1016 and 676 (Figure 2) suggesting this mass spectral environment breaks the compounds in two main fragments at *m/z* 1353 and 676. The daughter ion peak at *m/z* 1016 appears to be due to a divalent parent ion. The analysis of peaks at *m/z* 1353 and 676 helped in establishing the structure of **1**. FAB MS/MS analysis of the fragment at *m/z* 676 in the FAB mass spec using solvent mixture (3NBA+DMSO+TFA), showed fragmentation as shown in Figure 3. The spectrum showed fragments at *m/z* 591, 507, 421, 336, 308. Analysis of fragment peaks suggested that fragmentation patterns is similar to that encountered in another cell wall active antifungal SCH 466457.[7] For SCH 466457 the CID induced fragment at *m/z* 1003 on MS/MS analysis showed fragments at *m/z* 747, 662, 591, 507, 421, 336 and 308. The fragment ion at *m/z* 336 has been well characterized.[2,4~6] The mass fragments *m/z* 336, 308 are characteristics of prolyl-2-methyl-2-oxotetradecanoic acid.[5] The presence of 2-methyl-2-oxotetradecanoic acid (MOTDA) was confirmed by hydrolysis followed by isolation. This fragmentation pattern compared with that of **1** revealed that **1** contains this part of SCH 466457. The fragments at *m/z* 336, 421, 507 and 591 suggested four amino acids with *m/z* 85 revealing successive four identical amino acids, α-amino isobutyric acid (aib).

Collision induced dissociation (CID) of doubly charged molecular ion (*m/z* 1015) in electrospray mass spectrum showed several fragment pairs of the molecular ion, for example *m/z* 506, 1524; 591, 1439; 629, 1400; 676, 1353; 747, 1282; 856, 1172; 903, 1126; 942, 1087. Several predominant peaks were used for MS/MS studies. The mass spectrum of FAB-MS/MS of peak *m/z* 1172 is shown in the Figure 4. This spectrum includes *m/z* 676 and other small fragments as shown in Figure 3, the *N*-terminal side of this peptide. The spectrum also shows higher fragments at *m/z* 747, 818, 903, 1087 and 1172 suggesting the consecutive difference between the fragments *m/z* 71, 71, 85, 184, and 85. The cleaved fragments with *m/z* 71 represent alanine and *m/z* 85 amino isobutyric acid as shown in Figure 5a. The fragment *m/z* 184 must be due to two amino acids or an amino acid with an even number of nitrogen atoms. This can represent several combinations of different amino acids, but hydrolysis of **1** shows the presence of leucine, suggesting that this fragment must be a combination of leucine and alanine as shown in the Figure 5a. The structure

Fig. 2. CID-MS/MS data of doubly charged ion of **1**.

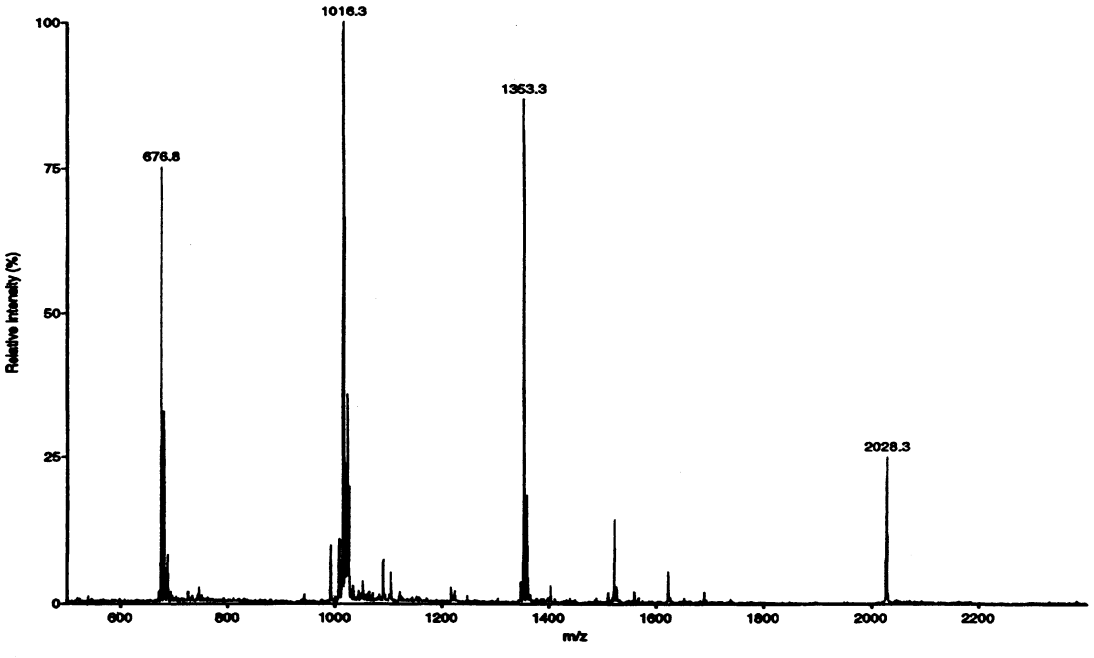


Fig. 3. FAB-MS/MS data of fragment *m/z* 676.

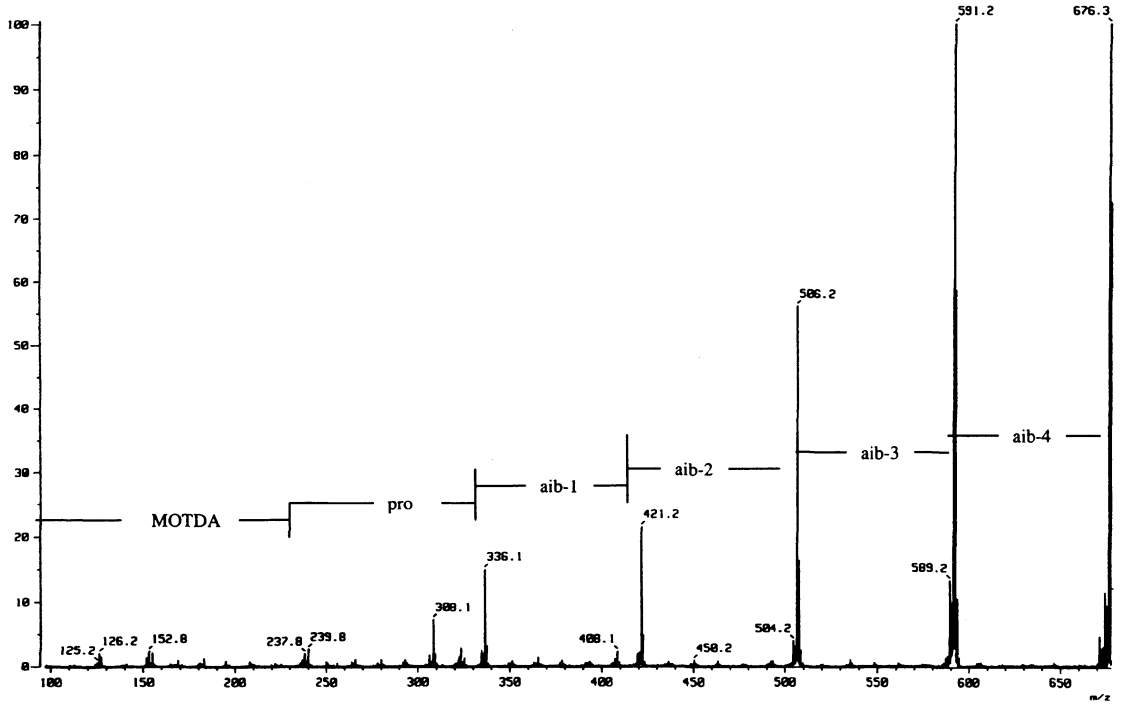
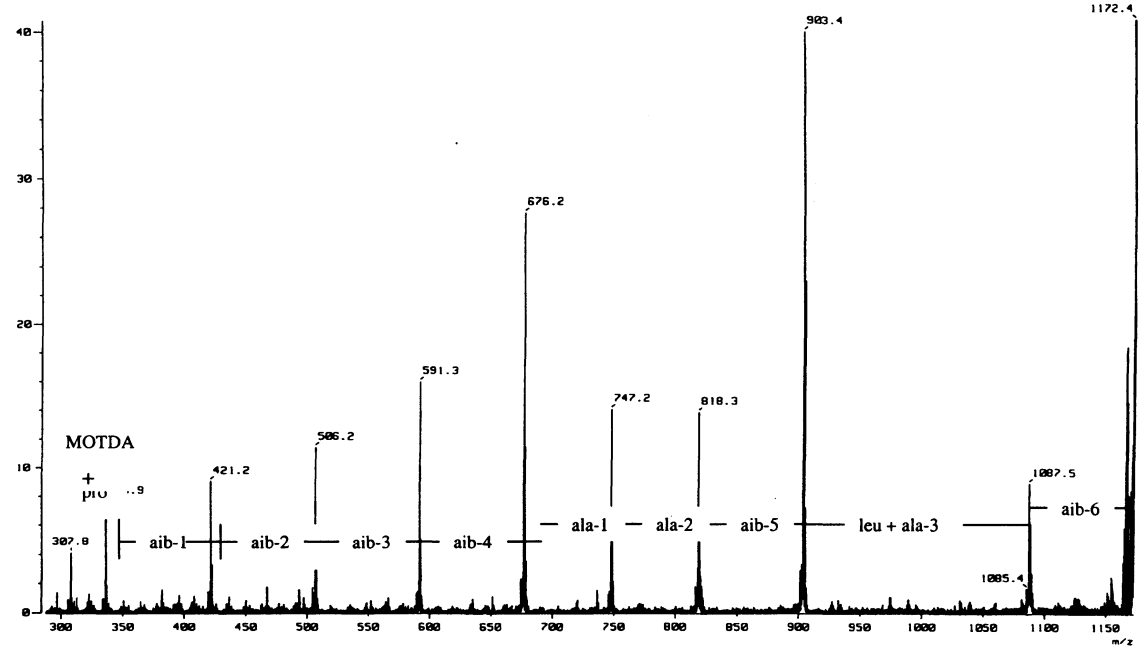


Fig. 4. FAB-MS/MS data of fragment *m/z* 1172.



of this fragment has been established further by several other mass spectral fragmentation techniques. Slow scan/high OR mass spectrum of the compound is shown in Figure 6. This spectrum shows peak at *m/z* 1015 (1015−903=112, leu) and no 974 (974−903=71, ala) peak confirming the position of leucine in the molecule. High resolution mass measurement of the *m/z* 1172 confirms the molecular formula of this fragment as shown in Table 2.

The mass spectrum of CID-MS/MS of peak *m/z* 856 showed peaks at 785, 714, and 629 among the other peaks, and the CID-MS/MS spectrum of fragment peak *m/z* 629 is shown in Figure 7. This spectrum shows fragments at *m/z* 157, 228, 299, 370, 455, 540 suggesting the amino acid sequence as shown in Figure 7 and the amino acid sequence for the fragment B with *m/z* 856 can be established as in Figure 5b.

The molecular formulas of the key fragments were confirmed by high resolution mass spectrum as in Table 2.

The attachment of two fragments at *C*-terminus of aib6 from fragment A and *N*-terminus of ala4 of the fragment B was suggested by the fragment *m/z* 942 which contains ala4 of the fragment B and aib6 of the fragment A as shown in Figure 6.

Compound **2** showed molecular ion *m/z* 2028 $(M+H)^+$, suggesting this compound is structural isomer of (**1**). Structure of this compound is still under investigation.

Fig. 5a. Amino acid sequence revealed by fragment *m/z* 1172.

—aib6—ala3—leu—aib5—ala2—ala1—aib4—aib3—aib2—aib1—pro—MOTDA

Fragment A

Fig. 5b. Amino acid sequence revealed by fragment *m/z* 856.

——aib7——arg——ala6——aib8——gly——aib9——aib10——ala7——OH

Fragment B

Fig. 6. Collision induced electrospray MS of **1**.

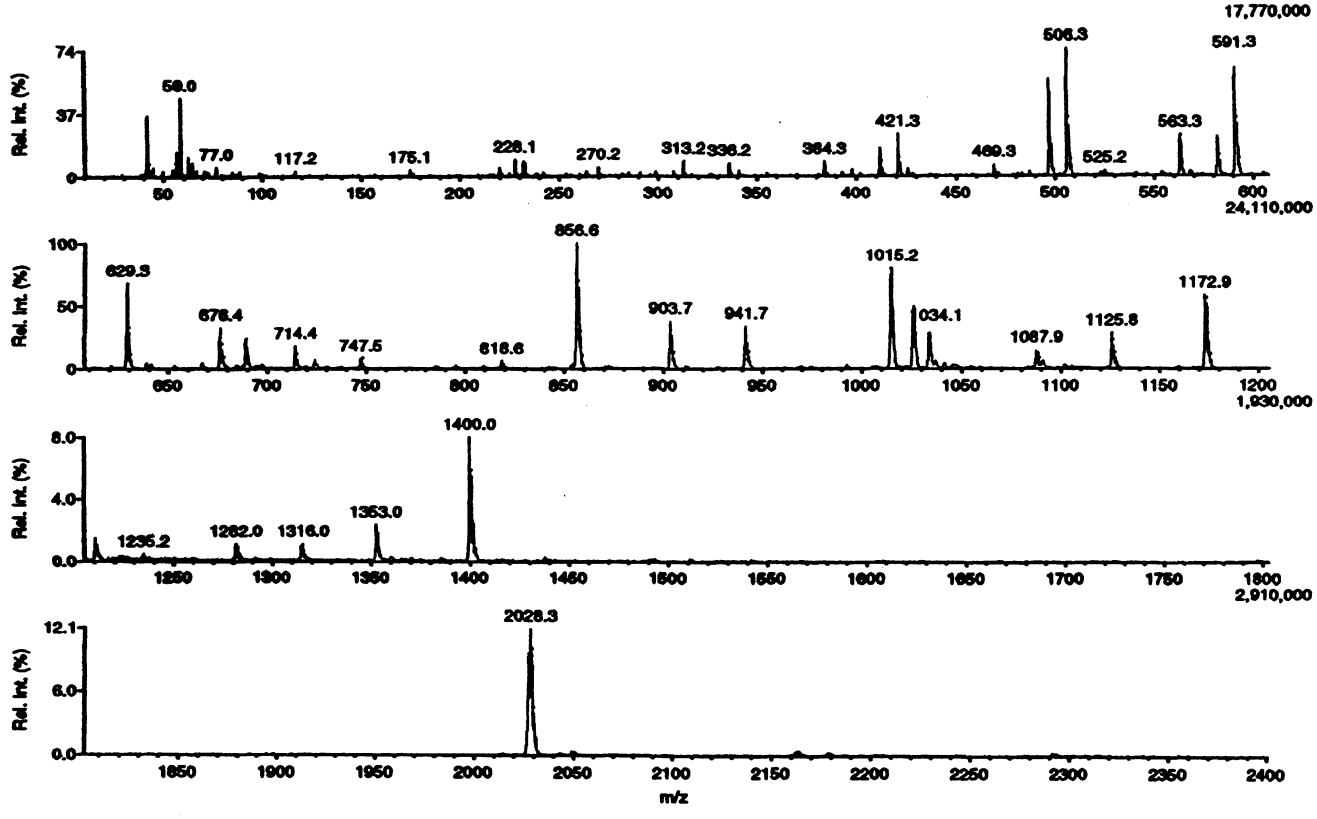


Fig. 7. CID-MS/MS data of fragment *m/z* 629.

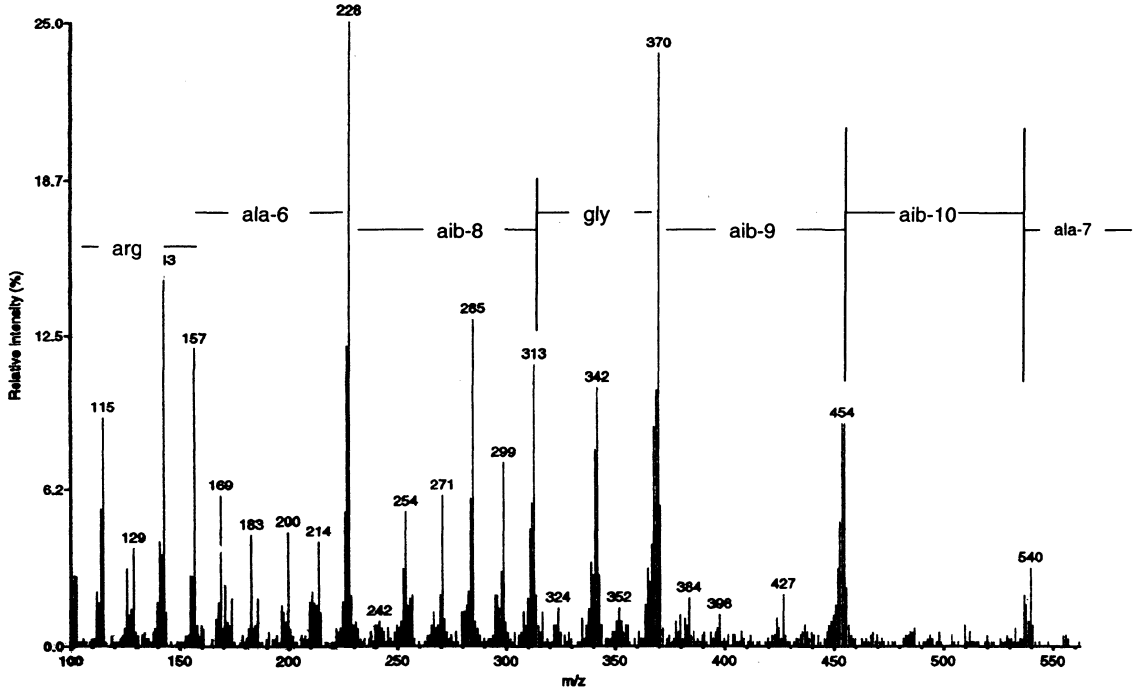


Table 2. Molecular formula confirmed by high resolutiom mass spectrum.

Fragment	High mass (m/z)		Mol. Formula
	Calc.	Obsd.	
1172	1172.7659	1172.7615	$C_{59}H_{102}N_{11}O_{13}$
1128	1128.6919	1128.6979	$C_{49}H_{92}N_{16}O_{14}$
858	858.5161	858.5176	$C_{36}H_{58}N_{13}O_{11}$
676	676.4649	676.4634	$C_{36}H_{62}N_5O_7$
336	336.2539	336.2543	$C_{20}H_{34}NO_3$
228	228.1461	228.1468	$C_9H_{18}N_5O_2$

Biological Activity

Both compounds **1** and **2** were active WLD (Cell WaLL Defect) assays with minimum active concentration of 2.5 and 2.5 μg/dose for **1** and **2** respectively. Compounds **1** and **2** showed good *in vitro* activity against all the microorganisms tested, *Candida*, *Aspergillus* and dermatophytes (Table 3). These compounds showed similar activity against both phases of *Candida* (compare MIC values determined in SDB media, for yeast phase growth to that determined in EMEM media, for mycelial phase growth). Both compounds appear to be cidal as the MFC values are comparable to the MIC values. In mice compound **1**, showed efficacy at 2, 150 and 1.8 mpk dose concentration in *ip*, *sq*, *iv* modes respectively.

Conclusion

Investigators have exploited artificial lipid bilayer membranes to characterize interactions of drugs with cellular membranes.[7~9] A series of natural products of microbial origin such as linear and cyclic peptides, polyethers and polyene macrolides have been shown to improve ion penetration through biological membranes *via* formation of pores or carriers thus enabling passive ion fluxes from inside to outside of the cell or *vice versa*.[9] Amongst the pore forming agents, the peptaibols[10,11] display particularly effective structures due to their helical

Table 3. *In vitro* activity of compound **1** and compound **2**.

Organism (no. isolates)	Geometric Mean MIC's (mcg/mL)							
	Compound 1				Compound 2			
	SDB (5.0)		EMEM (7.0)		SDB (5.0)		EMEM (7.0)	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans (6)	4.00	7.12	0.71	5.66	4.49	8.98	0.89	17.96
Other Candida[a] (8)	2.59	4.36			4.00	9.51		
Dermatophytes[b] (6)	3.56	4.49			5.66	8.98		
Aspergillus	11.3	13.45			16.0	26.91		

a - 2- *C. parapsilopsis, C. krusei, C. stellatoidea, C. tropicalis*
b - 2- *Trichophyton mentographytes, T. rubrum, T. tonsurans*
MIC=minimum inhibitory concentration; MFC=minimum fungicidal concentration.
In vitro activity of compound **1**, and compound **2** against 14 *Candida* isolates in two media (SDB for yeast phase growth and EMEM for mycelial phase growth), 6 dermatophytes and 3 *Aspergillus* isolates determined in liquid microtitre after 48 hours (*Candida*) or 72 hours (dermatophytes and *Aspergillus*) incubation.

peptide backbone comprised mainly from the hydrophobic amino acids like α-amino butyric acid, isoleucine, leucine and valine. Moreover even some hydrophilic constituents such as glutamic acid, glutamine, alanine, arginine and proline could be included.[12~18] Usually, the nitrogen terminus of the 12 to 20 amino acid peptide chain is acylated while the carbon terminal end is reduced to the pertinent amino acid alcohol.

Recently, novel peptide antifungals isolated from various sources have been reviewed in detail.[19] Fungi produce antifungals like helioferins[20] and SCH 217048.[21] The fungal metabolites SCH466457[2] and lipohexin[5] have structure similar to SCH 643432 (**1**). Bacteria produce antifungals like polymyxin, colistin and polypeptin containing cyclic peptides linked to a hydroxy fatty acid moeity. Echinocandins, produced by various *Aspergillus* species, are cyclohexapeptides linked to linolenic, myristic or palmatic acids. Compounds **1** and **2** are straight chain peptides with the amino terminal attached to a β-keto acid and they contain the unnatural amino acid, amino isobutyric acid. All these peptides contain at least one proline residue. The peptide structure of this novel compound **1** appears to be similar to that of other peptaibols.[4,10,11]

Acknowledgments

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