VOL. 54 NO. 1, JAN. 2001 THE JOURNAL OF ANTIBIOTICS pp. 74 — 83

Novel Fungal Metabolites as Cell Wall Active Antifungals: Fermentation, Isolation, Physico-chemical Properties, Structure and Biological Activity

VINOD R. HEGDE,* JACK SILVER, MAHESH PATEL, VINCENT P. GULLO, RAYMOND YARBOROUGH, ERIC HUANG, PRADIP R. DAS, MOHINDAR S. PUAR, BETH J. DIDOMENICO and DAVID LOEBENBERG

Schering Plough Research Institute
2015 Galloping Hill Road, Kenilworth, NJ 07033 USA

(Received for publication August 16, 2000)

Two novel antifungal compounds, **1** (SCH 466457), and **2** (SCH 466456), active in a "cell wall" assay, were isolated from the fermentation broth of an unidentified fungus. The active compounds were separated from the broth filtrate by adsorption on a macroreticular resin and were purified on reverse phase HPLC. Detailed mass spectrometric and NMR experiments and degradative studies helped in elucidating the structures of these compounds. The compounds were identified to be peptides containing amino acids such as alanine, aminoisobutyric acid, proline, leucine, valine, glycine and a previously identified β-keto acid, 2-methyl 3-oxo-tetradecanoic acid.[5] Both compounds were active against *Candida*, dermatophytes and *Aspergillus* (Geometric Mean MIC's, 8.9, 20 and 16 μg/ml, and 64, 128 and 23 μg/ml, respectively for **1** and **2**).

During the past decade the search for novel molecules with activity against human pathogenic fungi has relied primarily on mechanistic based assays. Natural product screening provides the widest variety of novel structures within this paradigm. Biochemical assays, while exquisitely sensitive, may uncover whole-cell impermeant compounds. Whole cell, mechanistic assays are selective for permeable compounds and can target more than a single enzyme in a pathway, although 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 compounds from an unidentified fungus using a 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

These antifungals were produced by a taxonomically unidentified fungus.

Description of the WLD Assay

The WLD (Cell WaLl Defect) assay is designed to detect compounds that cause damage to the fungal cell wall. The host strain for the assay uses the model yeast, *S. cerevisiae*, strain SEY6210, transformed with plasmid pCYI-11.[2] The host strain has its native gene for invertase (*SUC2*) deleted, and a plasmid-borne copy of the *SUC2* gene that has its signal sequence deleted and hence cannot be secreted from the cell. Since yeast cells cannot metabolize sucrose directly, they must first secrete invertase, an enzyme that breaks down sucrose into glucose and fructose in the extracellular environment. Both six-carbon sugars are then easily absorbed and utilized by the cell. Thus, the assay strain as designed cannot grow on

* Corresponding: vinod.hegde@spcoyp.com

plates containing sucrose as a sole carbon source due to the fact that it cannot secrete invertase. In the assay, agar plates are prepared containing sucrose and are seeded with an inoculum ($\sim 5\times10^4$ cells/ml final concentration) of the assay strain. Wells are cut into the agar plates and test samples are applied to the wells. Cell growth is only possible if the cell wall is weakened enough to allow the passage of sucrose into the cell (or invertase out of the cell). Therefore, active compounds are scored as a ring of growth around a well.

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) proteus 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 fermentors and fermentation was carried out at 24°C with 450 rpm agitation and 4.5 liters/minute of aeration for 120 hours. The fermentation medium contained (g/liter) neopeptone 10, cerelose 40, $CaCO_3$ 4, and tap H_2O to 1 liter.

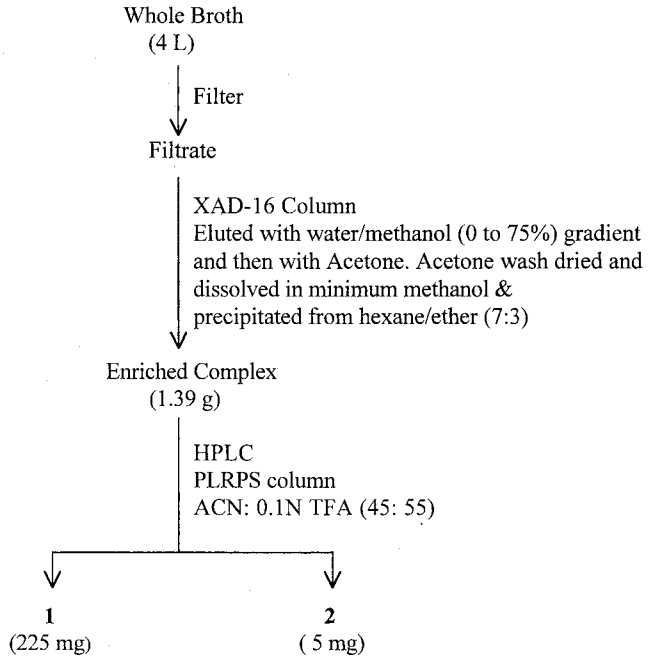
Fermentation Analysis

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. Four liters of fermentation broth were filtered and the filtrate was loaded on an XAD-16 column (2×5″). The column was washed with water and then eluted with a water/methanol gradient (methanol 0 to 75%) and finally stripped with acetone. The fractions were monitored by an agar diffusion assay using

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



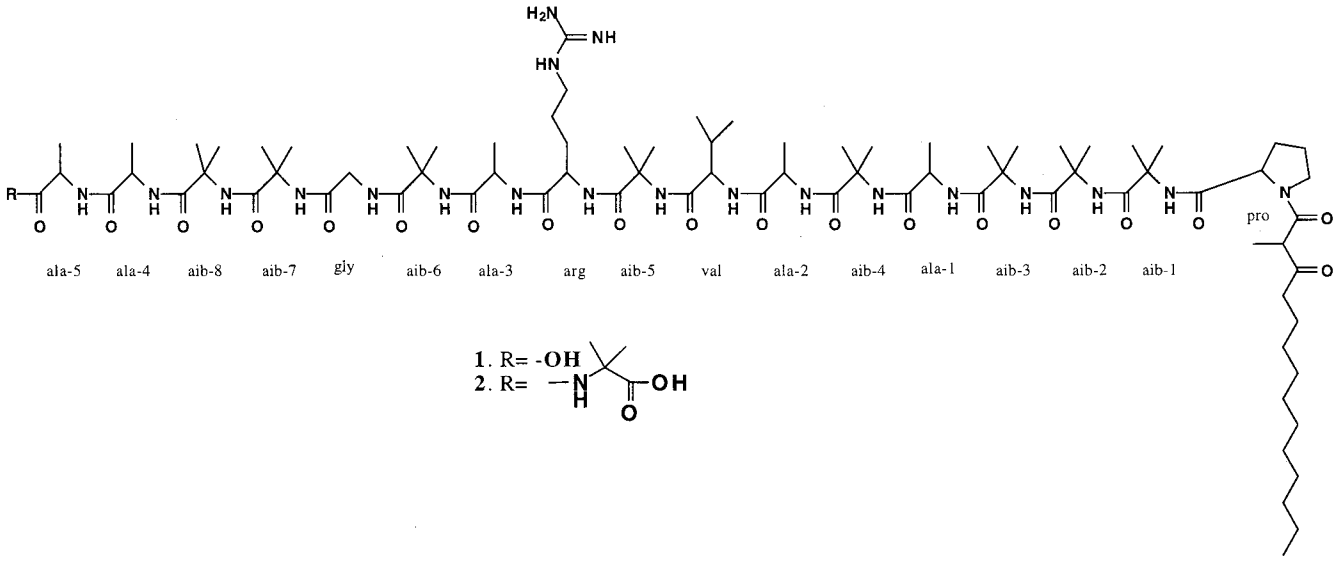
C. albicans. The acetone wash containing active material was dried under vacuum, dissolved in minimum methanol and solids were precipitated in hexane-ether mixture (7 : 3) to yield 1.39 g of solid. The active compounds were separated on a preparative PLRP-S polymeric column eluting with a mixture of acetonitrile and 0.1% aqueous TFA solution (45 : 55 v/v). The acetonitrile was removed from the individual peak eluates under vacuum and the remaining aqueous solutions yielded on freezing drying 225 and 5 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. The identity of the amino acids was determined by acid hydrolysis, stirring overnight at room temperature with 6 N HCl and subsequent TLC analysis of the hydrolysis products.

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 (six strains of *C. albicans* and



1. R= -OH
2. R=

two strains each of *C. parapsilopsis*, *C. krusei*, *C. stellatoidea*, *C. tropicalis*)] or 72 hours [*Aspergillus*, n=3, (*A. fumigatus*, *A. flavus*, *A. niger*) and dermatophytes, n=6 (*Trichophyton mentagrophytes*, *T. rubrum*, *T. tonsurans*)]. All cultures were incubated at 37°C.

Results

Following the initial identification of an active extract, 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 466457 (**1**) a major component, mp 204°C; IR 3467, 3287, 2983, 1654, 1541, 1460, 1385, 1201 cm^{-1}. In Cs^+ ion liquid secondary ionization mass spectrum (SIMS), it displayed an intense ion at *m/z* 1701 $(M+H)^+$ revealing the molecular weight to be 1700. Peak matching measurements using high-resolution mass measurements showed the elemental composition to be $C_{80}H_{140}N_{20}O_{20}$ suggesting twenty-one degrees of unsaturation. The presence of multiple nitrogen atoms suggested that the compound might be a peptide. The compound was acid hydrolyzed with 6 N HCl at 110°C under nitrogen for 24 hours. Reaction mixture was extracted with ethyl acetate and aqueous solution was freeze dried. The freeze dried aqueous extract was analyzed for amino acids by 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 80 carbon atoms (Table 1). ^{13}C NMR APT experiment showed twenty sp^2 quaternary carbons (O=C<), eight sp^3 quaternary carbons (>C<), ten sp^3 methines (>CH–), seventeen methylenes and twenty five methyls in the molecule. Of those one carbonyl at δ 211.9, eighteen amide/carboxylic acid carbons at δ 170~178, one guanidino carbon at δ 157.8, eight aminoisobutyric acids with quaternary carbons (at δ 56.2 56.5, 56.6, 56.7, 56.8, 56.9, 57.0, 57.1), two methylenes at δ 47.6 (–N–CH_2–, proline) and δ 39.7 (–N–CH_2– gly), fifteen additional methylenes, nine (H–C–N) like methines and one (>*C*H–C) like methine, sixteen quartenary methyls, eight secondary methyls, and one primary methyl carbons. 2D (^1H-^1H) COSY, HMBC, HMQC, HMQC-TOCSY (15 msec) experiments allowed the assignment of most of the protons and carbon resonances. HMBC provided all the connectivities except for two amino acids, the terminal Ala- and Ala in between Aib and valine. The ^1H and ^{13}C-NMR data are presented in

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

Amino Acids	Carbon #	Chemical Shifts		Amino Acids	Carbon #	Chemical Shifts	
		^{1}H	^{13}C			^{1}H	^{13}C
MOTDA	1''		170.5 (S)	Glycine	1'		174.2 (s)
	2''	3.82 (q)	52.0 (d)		-NH-	8.39	
	2"-CH_3	1.48(d)	13.0 (q)		2'	3.05	39.7(t)
	3''		211.9 (s)				
	4''	2.66(t)	40.8 (t)	Valine	1'		172.2 (s)
	5''	1.62(m)	29.3 (t)		-NH-	8.22(d)	
	6''	1.25 (m)	23.6 (d)		2'	3.48	65.5(d)
	7''	1.25 (m)	29.2 (t)		3'	2.20	27.3(d)
	8''	1.25 (m)	29.4.(t)		4'	1.10	19.1 (q)
	9''	1.25 (m)	29.5 (t)		4'	1.01	20.4 (q)
	10''	1.25 (m)	29.5 (t)				
	11''	1.25 (m)	29.6 (t)	AIB			
	12''	1.25 (m)	31.9 (t)	aib-1			175.5(s)
	13''	1.31(m)	24.3 (t)		-NH-	7.55(s)	
	14''	0.89(t)	14.1 (q)				56.8(s)
							27.1(s)
Proline	1'		172.6 (s)				27.1(s)
	2'	4.29(dd)	57.1 (d)	aib-2			175.8(s)
	3'	1.98, 2.37	29.4 (t)		-NH-	7.5(s)	
	4'	2.05(m)	25.1 (t)				56.7(s)
	5'	3.42, 3.62	47.6 (t)				26.9(s)
							26.9(s)
Arginine	1'		174.2 (s)	aib-3			177.0(s)
	-NH-	8.27			-NH-	7.53(s)	
	2'	4.38	56.2 (t)				56.2(s)
	3'	1.89, 1.79	36.5 (t)				27.0(s)
	4'	1.70	25.0 (t)				27.0(s)
	5'	3.32	38.3 (t)	aib-4			175.5(s)
	6'		157.8 (s)		-NH-	7.40(s)	
	-NH-	7.17, 6.62					56.9(s)
							28.0(s)

Table 1. Continued.

Amino Acids	Carbon #	Chemical Shifts ^{1}H	Chemical Shifts ^{13}C	Amino Acids	Carbon #	Chemical Shifts ^{1}H	Chemical Shifts ^{13}C
Alanine							28.0(s)
ala-1			174.0(s)	aib-5			175.7(s)
	-NH-	7.86(d)			-NH-	8.22(s)	
		3.94	53.1(d)				57.1(s)
		1.52	22.4				28.1(s)
ala-2			175.3(s)				28.1(s)
	-NH-	8.48 (d)		aib-6			175.7(s)
		3.86	52.0(d)		-NH-	8.48(s)	
		1.49(d)	17.0				57.0(s)
ala-3			173.9(s)				28.1(s)
	-NH-	8.54(d)					28.1(s)
		3.92	54.1(d)	aib-7			176.4(s)
		1.52(d)	17.1		-NH-	8.48(s)	
ala-4			174.5(s)				56.6(s)
	-NH-	8.06(d)					28.0(s)
		4.2	51.2(d)				28.0(s)
		1.5(d)	16.7	aib-8			176.4(s)
ala-5			175.5(s)		-NH-	7.60(s)	
	-NH-	7.9					56.5(s)
		3.82	51.0				27.3(s)
		1.49(d)	16.5				27.3(s)

the Table 1.

The structure of **1** was confirmed using mass spectral data.[3] Electrospray mass spectrum showed the molecular ion at 1701 (Figure 2). Electrospray ionization CID-MS/MS experiments helped to elucidate the structure. Collision induced dissociation (CID) of doubly charged molecular ion (*m/z* 851) in electrospray mass spectrum showed distinct two fragment pairs of the molecular ion for example 421, 1281; 472, 1230; 506, 1197; 591, 1111; 662, 1040; 699, 1003; 748, 955; 784, 918. The fragments at *m/z* 1003 and 699 were predominant and were used for MS/MS studies. CID-MS/MS data of these two fragments are shown in Figure 3a and 3b respectively. CID-MS/MS of peak *m/z* 1003 in Figure 3a showed amino acid sequence as shown (Figure 3a) and a characteristic fragment at *m/z* 336. This fragment was characterized as prolyl-2-methyl -3-oxotetradecanoic acid (MOTDA) based on characteristic fragmentation of CID-MS/MS on 336 (Figure 4) fragment.[4] CID induced MS/MS on fragment 699 showed the fragments as shown in Figure 3b, suggested the sequence of second part of this molecule. The molecular formulas of the key fragments were confirmed by high resolution mass spectrum as in Table 2.

MOTDA–pro–aib–aib–aib–ala–aib–ala–val–aib–

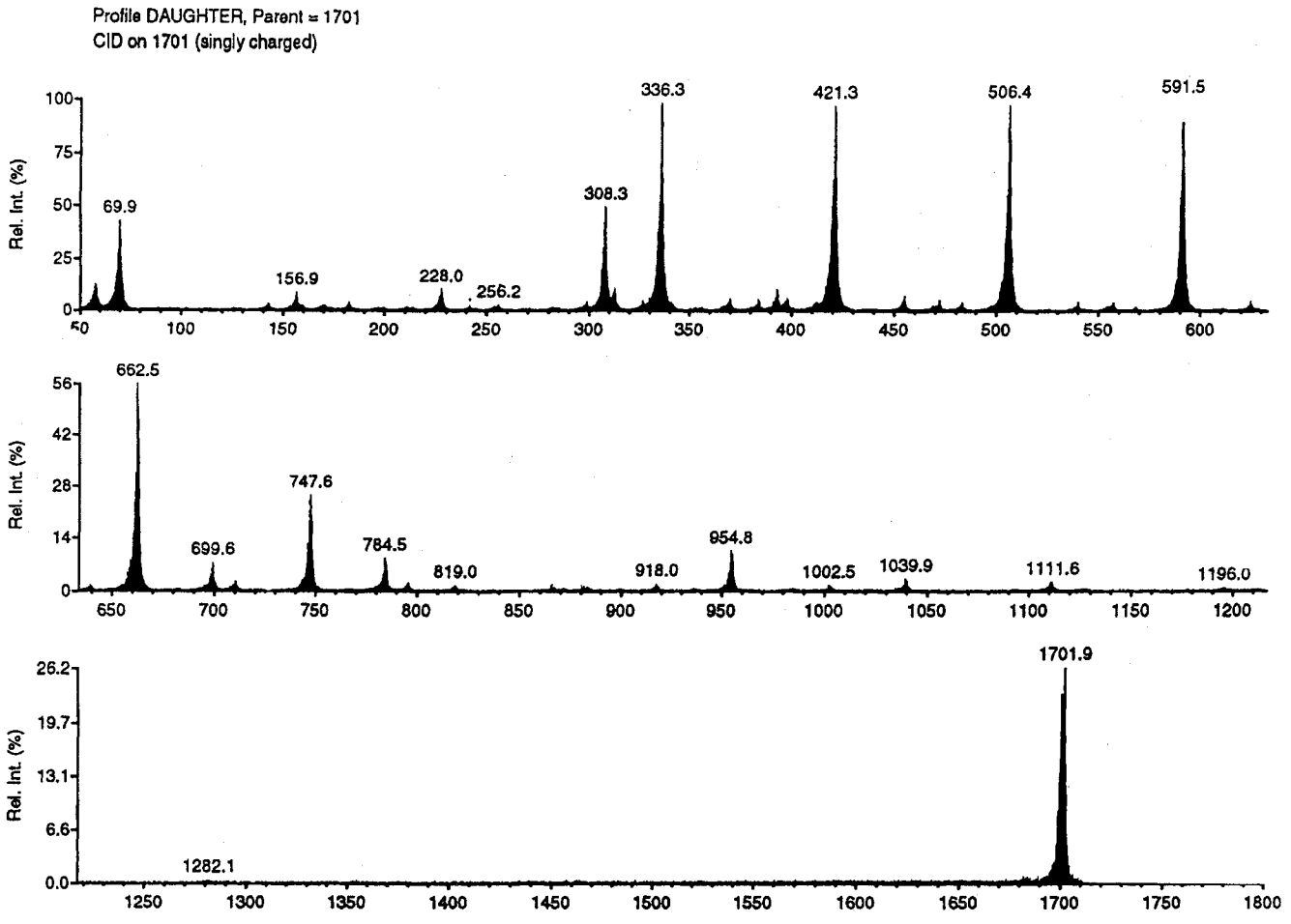
Fragment A

–arg–ala–aib–gly–aib–aib–ala–ala-OH

Fragment B

The attachment of two fragments at *N*-terminus of aib

Fig. 2. ESMS of compound **1**.



from fragment A and *N*-terminus of arginine of the fragment B was suggested by the fragment *m/z* 1230 which contains arginine and alanine of fragment A as shown in Figure 5.

Compound **2** (SCH 466456) showed molecular ion *m/z* at 1787 indicating molecular ion 1786. Presence of two fragments at 1003 and 784 and further analysis of ESMS (Electro-spray MS) spectral data established **2** with an additional amino acid aib (amino isobutyric acid)

Biological Activity

Compound **1** was significantly more active *in vitro* than compound **2** against all the microorganisms tested, *Candida*, *Aspergillus* and dermatophytes (Table 3). The compounds showed similar activity against both phases of *Candida* **1** (compare MIC values determined in SDB media, for yeast phase growth to that determined in EMEM media, for mycelial phase growth). Neither compound is particularly cidal as the MFC values are significantly higher than the MIC values.

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 polymeric column (styrene divinyl benzene based column, PLRP-S 2.5×30 cm) from Polymer laboratories, Amherst, MA.

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

Fig. 3. CID-MS/MS data of fragment *m/z* 1003 (a) and *m/z* 699 (b).

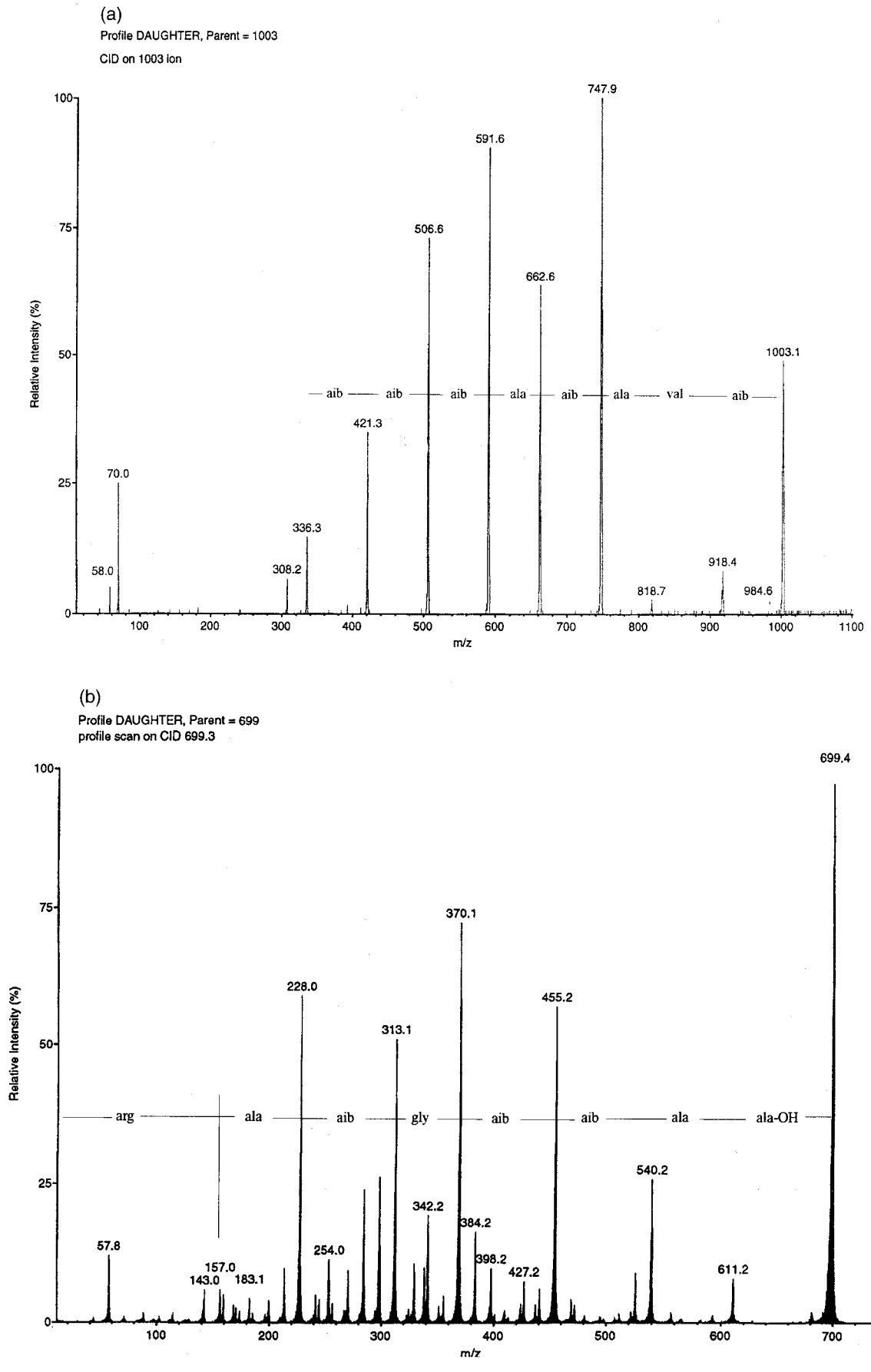


Fig. 4. CID-MS/MS data of fragment *m/z* 336.

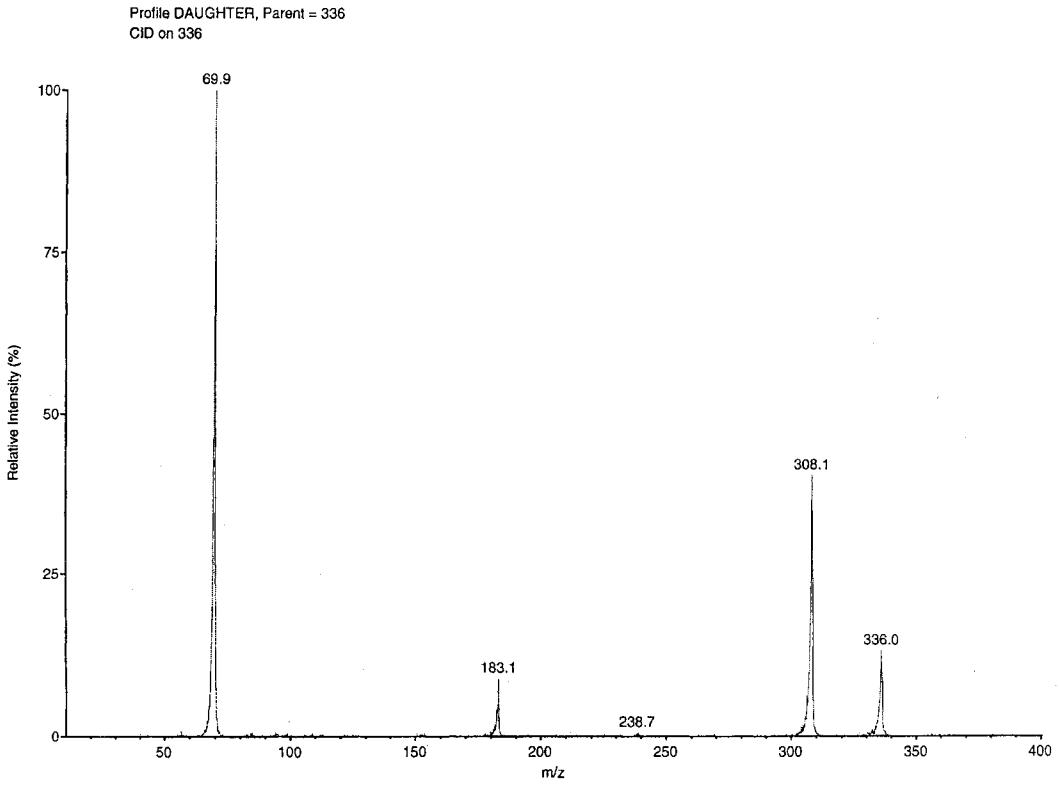


Table 2. Molecular formula of mass fragments confirmed by high resolution mass measurements.

Fragment	High mass (m/z)		Mol. Formula
	Calc.	Obsd.	
699	699.4028	699.4007	$C_{29}H_{53}N_{11}O_9$
336	336.2539	336.2543	$C_{20}H_{34}NO_3$
228	228.1461	228.1468	$C_9H_{18}N_5O_2$

secondary ion mass spectra (SIMS) and high resolution mass measurements were obtained on a VG-ZAB-SE mass spectrometer using a glycerol - thioglycerol (1 : 1, v/v) or *m*-nitrobenzyl alcohol matrix with the sample dissolved in dimethyl sulfoxide. CIMS were obtained on an EXTREL ELQ400-1 quadrupole mass spectrometer using NH_3 gas. Electrospray Ionization (ESI) mass spectrometric data were obtained on a SCIEX API III mass spectrometer using argon gas for collision induced dissociation at cone voltage of 40 V. NMR spectra were measured on Varian XL-400 NMR spectrometer operating at 400 and 100.5 MHz for ^{1}H- and ^{13}C-NMR respectively. ^{1}H- and ^{13}C-NMR spectra were recorded relative to TMS as an internal standard. 2D COSY, HMBC, HMQC, HMQC-TOCSY (15 msec) were acquired using GE-400 wide bore NMR spectrometer.

Hydrolysis of **1**

SCH 466457 (**1**) (5 mg) was dissolved in 2 ml of 6 N HCl and the solution was heated at 110°C for 24 hours. The solution was diluted with water and extracted with ethyl acetate. The ethyl acetate extract was dried, filtered and the filtrate was dried.

The aqueous phase on freeze-drying yielded the amino acids and they were detected by TLC [Amino acids were analyzed by Thin Layer Chromatography on a Whatman's silica gel plate (20×20 cm) developed in a solvent mixture containing ethyl acetate, butanol, acetic acid and water (1 : 1 : 1 : 1)] and CIMS.

Physico-chemical Data for **1** and **2**

SCH 466457 (**1**): UV (MeOH) λ_{max}, nm: End absorption; IR (KBr) ν_{max} cm^{-1}: 3467, 3287, 2983, 1654, 1541, 1460,

Fig. 5. CID-MS/MS data of doubly charged ion *m/z* 851.5.

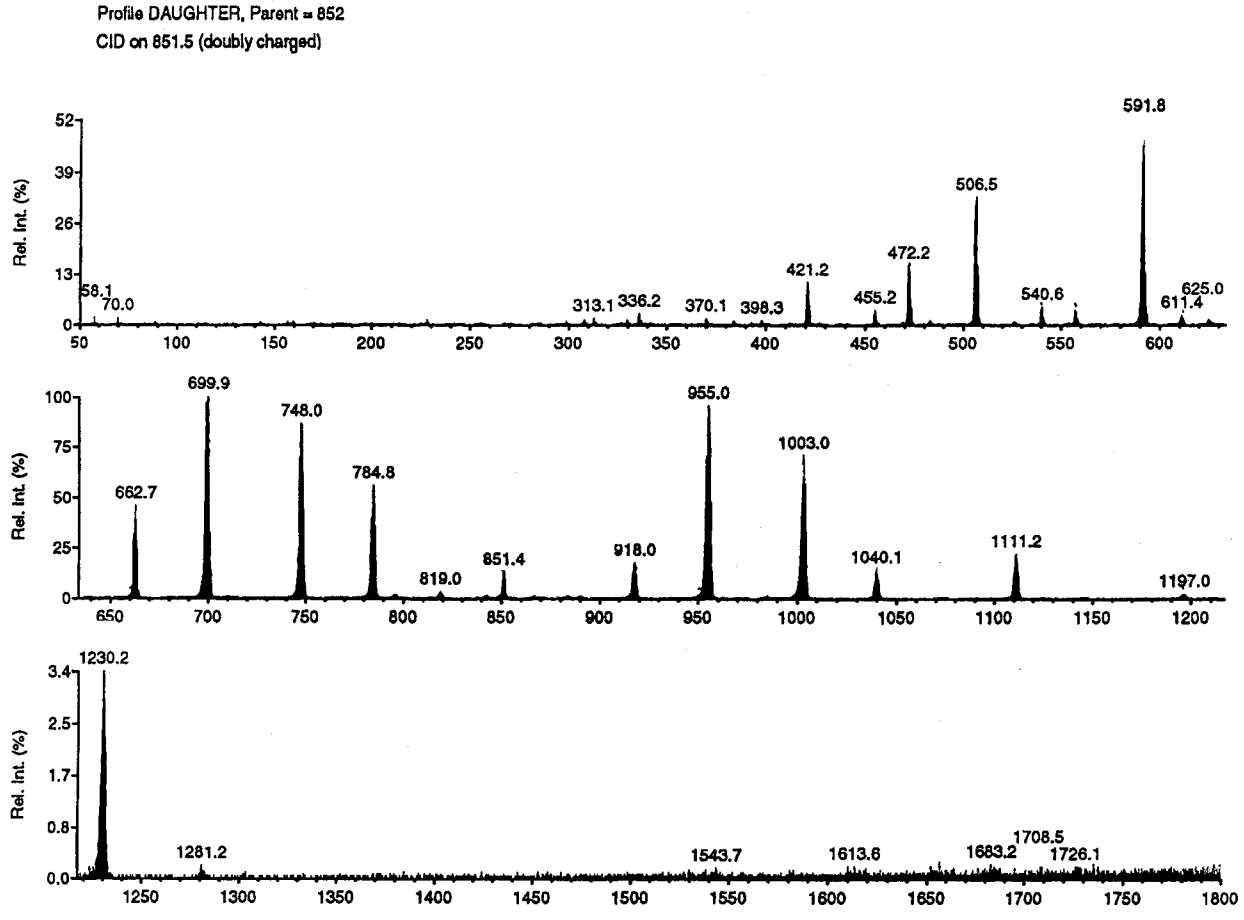


Table 3. Antifungal activity evaluation.

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 hour (*Candida*) or 72 hours (dermatophytes and *Aspergillus*) incubation.

Organism (no. isolates)	Geometric Mean MICs (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)	8.91	36	6.4	≥16	64	≥128	≥23	≥32
Other Candida[a] (8)	8.70	≥38			51	≥101		
Dermatophytes[b] (6)	20	40			≥101	≥128		
Aspergillus[c] (3)	16	≥51			≥81	≥128		

a - 2- *C. parapsilopsis*, *C. krusei*, *C. stellatoidea*, *C. tropicalis*
b - 2- *Trichophyton mentagrophytes*, *T. rubrum*, *T. tonsurans*
c - *A. fumigatus*, *A. flavus*, *A. niger*

MIC=minimum inhibitory concentration; MFC=minimum fungicidal concentration.

1385, 1201; FAB MS: *m/z* 1701 $(M+H)^+$.

SCH 466456 (**2**): UV (MeOH) λ_{max}, nm: End absorption; IR (KBr) ν_{max} cm^{-1}: 3275, 2923, 1653, 1541, 1462, 1201; FAB MS: *m/z* 1787 $(M+H)^+$.

Discussion

Recently, novel antifungal peptides isolated from various sources have been reviewed in detail.[5] Fungi produce antifungals like helioferins[6] and aureobasidins[7,8] 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 amino terminal attached to a β-keto acid and they contain the unnatural amino acid, amino *iso*-butyric acid. All these peptides contain at least one proline residue.

Acknowledgments

We wish to thank SCOTT EMR for his kind gift of strain SEY6210.

References

1) KIRSCH, D. & B. J. DIDOMENICO: Mechanism Based Screening for Discovery of Novel Antifungals. Discovery of Natural Products with Therapeutic Potential. Ed., GULLO, V., pp. 177~223, Butterworth, Inc., New York, 1993.
2) BANTA, L. M.; J. S. ROBINSON, D. J. KLIONSKY & S. D. EMR: Organelle assembly in yeast: Characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. J. Cell Biology 107: 1369~1383, 1988
3) ROEPSTORFF, P.; P. HOJRUP & J. MOLLER: Evaluation of fast atom bombardment mass spectrometry for sequence determination of peptides. Biomed. Mass Spec. 12: 184~189, 1985
4) CHRISTNER, C.; M. ZERLIN, U. GRAFE, S. HEINZE, G. KULLERTZ, G. FISCHER: Lipohexin, a new inhibitor of prolyl endopeptidase from *Moeszia lindtneri* (HK-0054) and *Paecilomyces* sp. (HKI-0055; HKI-0096): II. Inhibitory activities and sphecificity. J. Antibiotics 50: 384~389, 1997
5) DE LUCCA, A. J. & T. J. WALSH: Antifungal peptides: Novel therapeutic compounds against emerging pathogens. Antimicrob. Agents and Chemother. 43: 1~11, 1999
6) GRÄFE, U; W. IHN, M. RITZAU, W. SCHADE, C. STENGEL, B. SCHLEGEL, W. F. FLECK, W. KÜNKEL, A. HÄRTL & W. GUTSCHE: Helioferins; novel antifungal lipopeptides from mycogone rosea: Screening, isolation, structures, and biological properties. J. Antibiotics 48: 126~133, 1997
7) TAKESAKO, K.; K. IKAI, F. HARUNA, M. ENDO, K. SHIMANAKA, E. SONO, T. NAKAMURA, I. KATO & H. YAMAGUCHI: Aureobasidins, new antifungal antibiotics. Taxonomy, fermentation, isolation, and properties. J. Antibiotics 44: 919~924, 1991
8) KATSUSHIGE, I.; K. TAKESAKO, K. SHIOMI, M. MORIGUCHI, Y. UMEDA, J. YAMAMOTO & I. KATO: Structure of aureobasidin A. J. Antibiotics 44: 925~933, 1991