VOL. 53 NO. 6, JUNE 2000 THE JOURNAL OF ANTIBIOTICS pp. 609 — 614

Argifin, a New Chitinase Inhibitor, Produced by *Gliocladium* sp. FTD-0668

II. Isolation, Physico-chemical Properties, and Structure Elucidation

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(Received for publication January 27, 2000)

A new chitinase inhibitor, named argifin, was isolated from the cultured broth of a fungal strain *Gliocladium* sp. FTD-0668. Argifin was purified from the cultured mycelium by the combination of cation exchange, anion exchange, adsorption, and gel filtration chromatographic methods. The structure of argifin was elucidated as cyclo(N^{ω}-(*N*-methylcarbamoyl)-L-arginyl-*N*-methyl-L-phenylalanyl-β-L-aspartyl-β-L-aspartyl-D-alanyl) by NMR experiments and other spectroscopic analyses.

Chitinase is an essential enzyme for the insect ecdysis.[1] Inhibitors of chitinase would be expected to interrupt insect moulting and thus prevent maturation to adult reproductive stage. In the course of screening for chitinase inhibitors, we have found a new compound named argifin (**1**, Fig. 1), from the cultured broth of *Gliocladium* sp. FTD-0668.[2,3] In this paper, we report the isolation, physico-chemical properties, and structure elucidation of **1**.

Results

Isolation

The isolation procedure for **1** is outlined in Fig. 2. The cultured broth of FTD-0668 (8 liters) was filtered, and the mycelia was extracted with methanol (8 liters) and concentrated *in vacuo*. The liquid was diluted to 8 liters with water, neutralized, and charged on a Diaion SK1B cation exchange column (H^+ form, 800 ml, Mitsubishi Chemical Corp.). After washing with water, the active substance was eluted with 1 N NH_4OH. The eluate was neutralized and applied to a Diaion HP20 column (800 ml, Mitsubishi Chemical Corp.), and the column was washed with water and eluted with 20% methanol (800 ml). The eluate was then concentrated *in vacuo*, diluted with 8 liters of water, neutralized, and charged on a Dowex 1 anion exchange column (Cl^- form, 800 ml, Dow Chemical Co.). The column was washed firstly with 0.5 N HCl followed by 1 N HCl (800 ml). The latter eluate was neutralized and applied on an ODS column (Pegasil Prep ODS-7515-12A, 200 ml, Senshu Scientific Co.). After washing with water, the active substance was eluted with 100 ml of 20% methanol. The eluate was concentrated *in vacuo* to yield a white powder, that was then dissolved in a small amount of water and applied on a Sephadex G-10 column (600 ml, Amersham Pharmacia Biotech). The active fractions eluted with water were collected and concentrated *in vacuo* to yield a white powder of pure **1** (20.0 mg). It showed a single spot on a silica gel TLC (Merck 1.05715, developed with 80% *n*-propanol) detected by ninhydrin reagent after exposure to iodine vapor. It should be noted that **1** appeared as gray color using this reaction, and thus not strongly ninhydrin positive.

Physico-chemical Properties

Physico-chemical properties of **1** are summarized in Table 1. Compound **1** was a white powder, and gave a positive reaction Rydon-Smith reagent. It showed an end absorption and weak absorption at 225 nm in UV spectrum. The molecular formula of **1** was established as $C_{29}H_{41}N_9O_{10}$ on the basis of HR-FAB-MS (found $(M-H)^-$, 674.2930; calcd for $C_{29}H_{40}N_9O_{10}$, 674.2898). The IR spectrum of **1** indicated the presence of amide, carboxyl, and guanidine groups (1720, 1645, 1600, 1550, 1500 cm^{-1}). Chemical shifts in the ^1H and ^{13}C NMR of **1** are shown in Table 2.

Fig. 1. Structures of argifin (**1**).

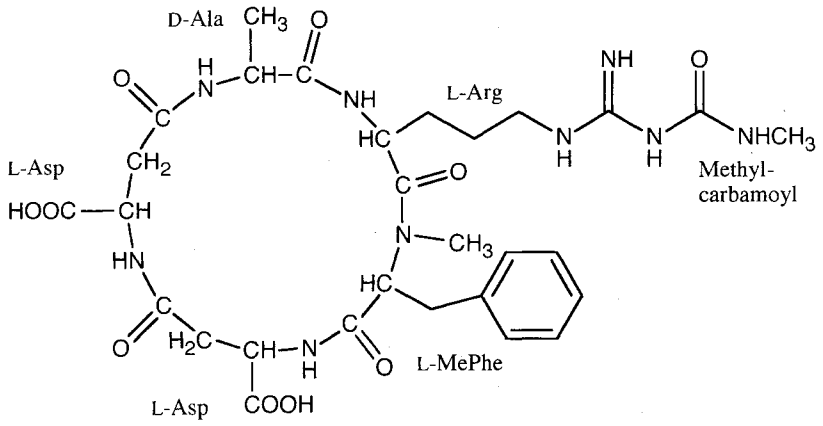
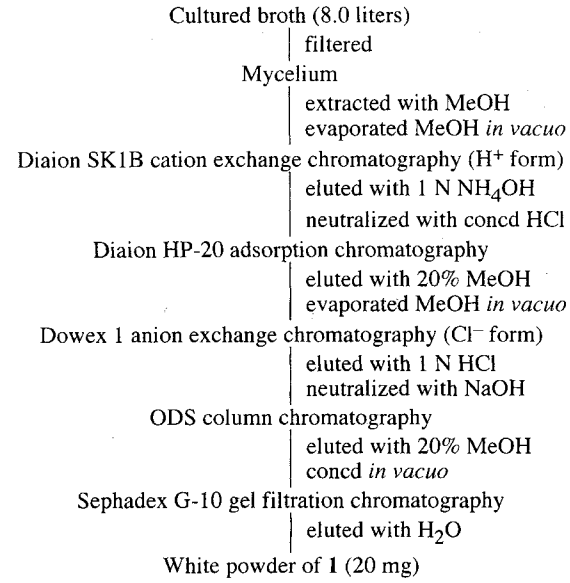


Table 1. Physico-chemical properties of **1**.

Appearance	White powder
Melting point	290°C (decomp.)
$[\alpha]_D^{25}$	–82.8° (*c* 0.1, H_2O)
Molecular weight	675.7
Molecular formula	$C_{29}H_{41}N_9O_{10}$
HR-FAB-MS (m/z)	found 674.2930 $(M-H)^-$ calcd 674.2898 (for $C_{29}H_{40}N_9O_{10}$)
UV $\lambda_{max}^{H_2O}$ nm (ε)	195 (55,700), 225 (sh., 5,100)
IR ν_{max} (KBr) cm^{-1}	3370, 3280, 3080, 2940, 1720, 1645, 1600,1550, 1500, 1450, 1400, 1260
Solubility	
soluble	H_2O
insoluble	Acetone, MeOH, $CHCl_3$
Color reaction	Rydon-Smith reagent + Nynhydrin reagent –

Fig. 2. Isolation procedure of **1**.



The HMQC experiment revealed the connectivity of each proton and carbon.

Structure Elucidation

In the DEPT spectra, **1** showed 3 methyl, 6 methylene, 10 methine, and 10 quaternary carbon signals. The ^1H NMR spectrum in D_2O showed 31 proton signals, and the spectrum in H_2O-D_2O (4 : 1) revealed an additional set of five active hydrogens. However, the remaining five hydrogens were not observed in either spectra.

Six partial structures **a**~**f** were elucidated by the ^1H-^1H COSY, ^1H-^{13}C HMBC, and ^1H-^{15}N-HMBC experiments in D_2O and H_2O-D_2O (4 : 1) (Fig. 3). The partial structures **a**~**f** were deduced to be arginine (**a**), *N*-methylphenylalanine (**b**), aspartic (or asparagine) (**c**), aspartic (or asparagine) (**d**), alanine (**e**), and *N*-methylcarbamoyl (**f**) residues, but the α-carbonyl of the arginine residue was not identified. At this stage, comparison with the molecular formula would indicate the six partial structures are deficient in one carbon (δ 174.2, suggested as carbonyl), two hydrogens, and three oxygens. Thus, two aspartic (or

Table 2. The ^{1}H, ^{13}C, and ^{15}N NMR data of **1**.

Position	in D_2O			in D_2O+TFA	
	^{13}C	^{1}H[a]	^{15}N	^{13}C	^{1}H
Methylcarbamoyl					
Me	26.0 q	2.76 s		25.9 q	2.76 s
NH		6.99 br. s[b]	86.3		
C=O	155.1 s			155.1 s	
Arg					
NH		8.34 d (*J*=7)[b]	125.7		
C=O	174.2 s			174.1 s	
α	48.6 d	4.34 dd (*J*=14, 2)		48.6 d	4.29 dd
β	26.3 t	–0.43 m, 1.08 m		26.6 t	–0.28 m, 1.13 m
γ	23.9 t	1.21 m, 1.43 m		23.9 t	1.14 m, 1.40 m
δ	40.5 t	3.01 m		40.6 t	3.02 m
ζ	153.5 s			153.5 s	
MePhe					
NMe	29.6 q	2.87 s	122.5	29.8 q	2.88 s
C=O	170.6 s			170.1 s	
α	62.4 d	5.10 dd (*J*=11, 3)		62.1 d	5.13 dd
β	33.3 t	3.06 m, 3.14 dd (*J*=14, 3)		33.3 t	3.06 m, 3.17 dd
γ	137.6 s			137.3 s	
δ_1, δ_2	129.6 d	7.23 d (*J*=7)		129.6 d	7.24 d
ε_1, ε_2	129.0 d	7.36 dd (*J*=7, 7)		129.0 d	7.37 dd
ζ	127.1 d	7.29 dd (*J*=7, 7)		127.1 d	7.29 dd
Asp 1					
NH		8.42 d (*J*=5)[b]	128.5		
C=O	178.3 s			174.9 s	
α	52.7 d	4.36 dd (*J*=12, 3)		50.3 d	4.57 dd
β	36.0 t	2.79 dd (*J*=17, 12) 2.95 dd (*J*=17, 3)		34.9 t	2.88 dd, 3.08 dd
γ	170.5 s			171.2 s	
Asp 2					
NH		7.75 d (*J*=9)[b]	127.8		
C=O	177.2 s			174.0 s	
α	52.1 d	4.49 dd (*J*=14, 3)		49.7 d	4.81 dd
β	38.7 t	2.41 dd (*J*=14, 14) 2.70 dd (*J*=14, 3)		37.6 t	2.53 dd, 2.80 dd
γ	172.2 s			171.2 s	
Ala					
NH		8.10 d (*J*=6)[b]	133.6		
C=O	175.2 s			175.1 s	
α	49.5 d	4.18 q (*J*=7)		49.5 d	4.19 q
β	16.6 q	1.32 d (*J*=7)		16.7 q	1.31 d

a: The coupling constants (Hz) are in parentheses.

b: Signals of amine protons were observed in H_2O-D_2O (4:1).

asparagine) residues were suggested as aspartic residues. The ^{13}C NMR spectrum in 0.4% trifluoroacetic acid D_2O solution (pH 4) was measured to elucidate whether these aspartic residues were linked to their adjacent amino acids at α- or β-carbonyl carbons. Among the carbonyl carbons of the aspartic residues, the two carbons of δ 178.3 (Asp 1 (**c**) C=O) and δ 177.2 (Asp 2 (**d**) C=O) shifted by more than 3 ppm highfield in acidic solution as shown in Table 2. However, the carbon signals δ 170.5 (Asp 1 C=O) and δ 172.2 (Asp 2 C=O) shifted only 0.7 ppm and 1.0 ppm respectively in acidic solution. We therefore deduced that the former carbons are represented as carboxylic acids, and Asp 1 (**c**) is linked to the adjacent amino acid by β-amide bond. The two carbonyls of Asp 2 (**d**) could not be identified as α or β in Fig. 3. However long-range ^{1}H-^{15}N coupling was observed between Asp 2 β-CH_2 and Ala NH

Fig. 3. Partial structures of **1**.

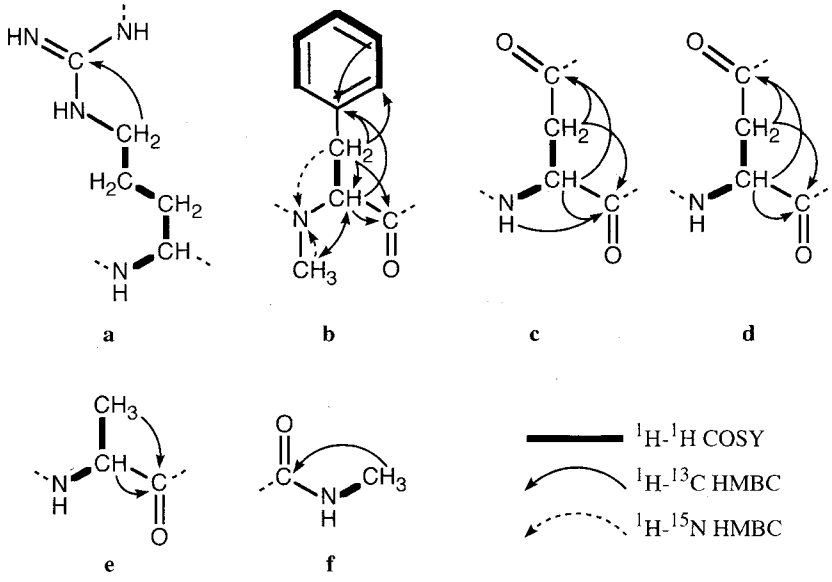
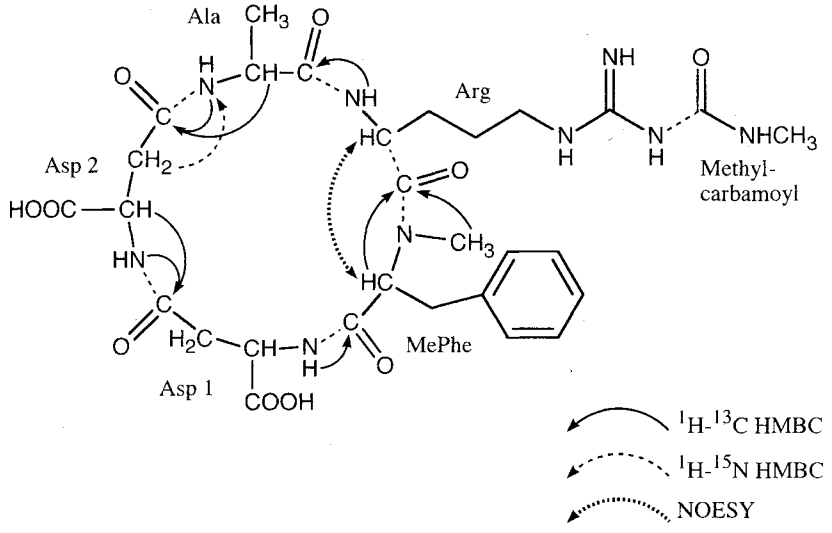


Fig. 4. Structure elucidation of **1**.



(Fig. 4), which suggested that Asp 2 was β-amide bonded with an adjacent Ala.

The linkages of the partial structures **a**~**f** were elucidated as shown below. A long-range ^{1}H-^{13}C coupling was observed between Asp 1 NH and MePhe carbonyl, which suggested the linkage of MePhe (**b**)-Asp 1 (**c**) as shown in Fig. 4. Long-range couplings between Asp 2 NH and Asp 1 carbonyl and between Asp 2 α-CH and Asp 1 carbonyl suggested the linkage of Asp 1 (**c**)-Asp 2 (**d**). Long-range couplings between Ala NH and Asp 2 carbonyl and between Ala α-CH and Asp 2 carbonyl confirmed the linkage of Asp 2 (**d**)-Ala (**e**) described above. A long-range coupling between Arg NH and Ala carbonyl suggested the linkage of Ala (**e**)-Arg (**a**). Long-range couplings were also

observed between MePhe NCH_3 and the remaining carbonyl (δ 174.2) and between MePhe α-CH and the same carbonyl, which suggested that the carbonyl was linked to the nitrogen of MePhe. The partial structure **a** suggested an arginine lacking a carbonyl, and the chiral HPLC experiment indicated that **1** had arginine as shown later. Therefore, the carbonyl was deduced to be an α-carbonyl arginine. The index of hydrogen deficiency of **1** was 14, which indicates that **1** is a cyclic peptide. So the linkage of Arg (**a**)-MePhe (**b**) was also suggested. Moreover, the NOESY experiment revealed the NOE between Arg α-CH and MePhe α-CH. Thus, the linkage of Arg-MePhe was confirmed. Consequently, the remaining carbamoyl residue (**f**) was linked to guanidino nitrogen of Arg, and thus the planar structure of **1** was elucidated.

It is interesting that one proton signal of Arg β-CH_2 was observed at extremely highfield (δ–0.43). The proton may be positioned on the benzene ring of MePhe and shielded.

The optical activity of each amino acid was elucidated by chiral HPLC using acid hydrolysate of **1**. The experiment revealed the presence of 1 mol of L-arginine, *N*-methyl-L-phenylalanine, D-alanine, and 2 mol of L-aspartic acid. Therefore, the absolute structure of **1** was elucidated as cyclo(N^{ω}-(*N*-methylcarbamoyl)-L-arginyl-*N*-methyl-L-phenylalanyl-β-L-aspartyl-β-L-aspartyl-D-alanyl).

Only few arginine-containing pentapeptides (*e.g.* nodularin from a cyanobacteria[4] and plactins A~D from a fungi[5]) have been reported. However, their ω-amino residues are not substituted.

After allosamidin, a potent chitinase inhibitor from *Streptomyces* sp., was discovered,[6,7] several inhibitors have been reported. Styloguanidines from *Stylotella aurantium*[8] and compound A from *Dysidea* sp.[9] had guadinino residue, and both were produced by marine sponges. Cyclo(L-arginyl-D-prolyl) from *Pseudomonas* sp.[10] and butenolides from *Streptomyces antibioticus*[11] were also reported to inhibit chitinase, though their inhibitions were weak. To the best of our knowledge, argifin (**1**) is the first chitinase inhibitor isolated from fungi. Since styloguanidines, compound A, cyclo(L-arginyl-D-prolyl), and **1** have a guanidino residue, it may be important for chitinase inhibition.

Experimental

General

NMR spectra were obtained with a Varian Unity 400 spectrometer using D_2O, H_2O-D_2O (4:1), and 0.4% TFA in D_2O as solvents. Mass spectrometry was conducted on a JEOL JMS-AX505 HA spectrometer. UV and IR spectra were measured with a Shimadzu UV-240 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively. Optical rotation was recorded on a JASCO model DIP-181 polarimeter. Melting point was measured with a Yanaco micro melting point apparatus MP-S3.

Acid Hydrolysis of 1

Compound **1** (100 μg) was hydrolyzed with 6 N HCl - 1% phenol vapor at 120°C for 11 hours. The reaction mixture was concentrated to dryness, dissolved in a small amount of water, and charged on a chiral HPLC: column, Sumichiral OA-5000 (Sumika Chemical Analysis Service, i.d. 4.6× 150 mm); mobile phase, 1 mM $CuSO_4$ for Arg and Ala, 2 mM $CuSO_4$ - MeOH (85 : 15) for MePhe and Asp; flow rate 1.0 ml/minute; detection, UV 254 nm.

The amino acids peaks were identified by co-injection of each authentic optically pure sample. The molar ratios of amino acids were calculated from the peak area.

Acknowledgement

We are grateful to Ms. AKIKO HATANO. Ms. CHIKAKO SAKABE, and Ms. AKIKO NAKAGAWA, School of Pharmaceutical Sciences, Kitasato University, for measurements of NMR and mass spectra. This work was supported in part by a grant from Japan Keirin Association.

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