Argadin, a New Chitinase Inhibitor, Produced by *Clonostachys* sp. FO-7314

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A new chitinase inhibitor, designated as argadin (1), was isolated from the cultured broth of a fungal strain FO-7314. The strain was identified as *Clonostachys* sp. from the morphological characteristics. Argadin was purified from the cultured mycelium by a combination of cation exchange, adsorption and gel filtration chromatographic methods. The structure of argadin was elucidated as cyclo(*N*^ω-acetyl-L-arginyl-D-prolyl-homoseryl-histidyl-L-2-aminoadipyl) in which homoseryl γ -methylene bonded to histidyl α -amino residue. The IC₅₀ value of argadin against *Lucilia cuprina* (blowfly) chitinase was 150 nM at 37 °C and 3.4 nM at 20 °C. Argadin arrested the moult of cockroach larvae upon injection into the ventral abdominal part.

Key words chitinase inhibitor; cyclic peptide; *Clonostachys*; argadin; microbial metabolite

Chitin occurs in fungi, some algae and many invertebrates including insects, but is not found in vertebrates. Thus chitin synthesis and degradation processes might be expected to be specific targets for fungicides and insecticides.^{1,2)} Though chitin synthesis inhibitors, such as fungicidal polyoxins and insecticidal benzoylphenylureas, are commercially available, chitin degradation inhibitors have not yet been used. Chitinase (EC 3.2.1.14) is a chitin degradation enzyme and hydrolyzes chitin into oligomer of *N*-acetylglucosamine. Only few inhibitors have been reported, e.g. allosamidin,³⁾ styloguanidine⁴⁾ and cyclo(L-arginyl-D-prolyl).⁵⁾ An inhibitor of chitinase would be expected to inhibit apolysis during the insect moulting and prevent maturation to the adult reproductive stage.

In the course of screening for new insecticides of microbial origin, we previously reported a new cyclic pentapeptide chitinase inhibitor, argifin (2), which was the first inhibitor produced by fungi and showed inhibition in a submicromolar range.⁶⁻⁸⁾ Our continuing screening efforts to find chitinase inhibitors were rewarded by finding of a new compound, argadin (1, Fig. 1), from the cultured broth of *Clonostachys* sp. FO-7314.

In this paper, we report on the isolation, structure elucidation and biological properties of 1 as well as the taxonomy of the producing organism.

Results and Discussion

Taxonomy of Strain FO-7314 The fungal strain was isolated from a soil sample collected on Oshima Island, Tokyo, Japan. Morphological properties were examined after incubation at 25 °C for 14 d on potato dextrose agar (Difco), cornmeal agar (Difco), Czapek agar (3.0% sucrose, 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O and 2.0% agar, pH 6.0), Miura's medium (0.1% glycerol, 0.08% KH₂PO₄, 0.02% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.02% KCl, 0.2% NaNO₃, 0.02% yeast extract and 1.5% agar, pH 6.0), and malt extract agar (2.0% malt extract, 0.1% peptone, 2.0% glucose and 2.0% agar, pH 6.0). The strain grew moderately to form white

colonies with diameters of 40—50 mm. Reverse of the colonies was white to pale yellow. The colonies were floccose to powdery on each medium. Microscopic observation showed that conidiophores (45—100×2.5—3.5 μ m) were branched two or more times, and their upper portions bore penicillate branches. Phialides that grew on the penicillate branches were enteroblastic, phialidic, obclavate and 10—14×1.5—3.0 μ m in size. Phialoconidia were ellipsoidal to cylindrical, 5.5—7.5×1.5—2.0 μ m in size, viscous and producing long chains more than 100 μ m length in a spicate form (Fig. 2).

From the above characteristics, strain FO-7314 was believed to belong to the genus *Clonostachys* and named

Fig. 1. Structure of Argadin (1) and Argifin (2)

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Dedicated to the memory of Dr. Kyosuke Tsuda.

Fig. 2. Electromicrograph of Phialoconidia of Strain FO-7314
Bar represents 10 μm .

Clonostachys sp. FO-7314. The culture was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology Japan, as FERM P-17200.

Fermentation and Isolation A stock culture of strain FO-7314 grown on potato dextrose agar was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium and incubated on a rotary shaker (210 rpm) at 27 °C for 4 d. One milliliter of the seed culture was transferred into each of eighty 500-ml Erlenmeyer flasks containing 100 ml of a production medium. The fermentation was carried out on a rotary shaker at 27 °C for 6 d. Compound **1** was produced mainly in mycelia.

Compound **1** was extracted with methanol from the mycelia from the cultured broth of FO-7314 (8 l). It was purified successively by a cation exchange column Diaion SK1B (NH_4^+ form, Mitsubishi Chemical Corp.), an adsorption column Diaion HP20 (Mitsubishi Chemical Corp.), a cation exchange column SP-Sephadex C-25 (NH_4^+ form, Amersham Pharmacia Biotech), an octadecyl silica (ODS) column (Pegasil ODS-7515-12A, Senshu Scientific Co.), a Sephadex LH-20 column (Amersham Pharmacia Biotech), and a Bio-Gel P-2 Gel column (fine, Bio-Rad). Twenty-five milligrams of a white powder of **1** was ultimately obtained. It showed a single spot on a silica gel TLC (Merck 1.05715, developed with 80% *n*-propanol) detected by ninhydrin reagent.

Structure Elucidation The molecular formula of **1** was established as $\text{C}_{29}\text{H}_{42}\text{N}_{10}\text{O}_9$ based on high resolution (HR)-FAB-MS. Compound **1** was soluble in acidic water and acidic dimethyl sulfoxide (DMSO), slightly soluble in water and DMSO, and insoluble in methanol, acetone and chloroform. Compound **1** gave positive reactions by both Rydon-Smith and ninhydrin reagents. A strong band at 1650 cm^{-1} in the IR spectrum indicated the presence of amide linkages.

Chemical shifts in the ^1H -, ^{13}C - and ^{15}N -NMR of **1** are shown in Table 1. The HMQC (heteronuclear multiple quantum coherence) experiment revealed the connectivity of each

Table 1. The ^1H -, ^{13}C - and ^{15}N -NMR Data of **1**^{a)}

Position	^{13}C ^{b)}	^1H ^{b,c)}	^{15}N ^{d)}
Acetyl			
C=O	172.7 s		
Me	24.1 q	2.10 s	
Arginine			
NH		8.58 d (6.8)	125.0
C=O	170.1 s		
α	51.6 d	4.10 m	
β	27.4 t	1.66 m, 1.73 m	
γ	24.3 t	1.50 m	
δ	40.7 t	3.25 m	
ϵ		9.16 t (4.8)	97.4
ζ	153.1 s		
η_1		11.78 s	133.3
η_2		8.68 br s	84.7
Proline			
N			137.4
C=O	171.1 s		
α	59.8 d	4.59 m	
β	27.9 t	1.75 m, 2.11 m	
γ	23.6 t	1.75 m, 3.28 m	
δ	46.0 t	3.31 m,	
		3.41 ddd (11.4, 11.4, 11.4)	
Homoserine			
NH		7.16 d (9.1)	123.1
C=O	172.9 s		
α	49.0 d	4.33 ddd (4.3, 9.1, 11.6)	
β	35.9 t	1.66 m,	
		2.63 ddd (6.2, 11.6, 19.4)	
γ	78.7 d	5.31 d (6.2)	
Histidine			
N			150.2
C=O	169.9 s		
α	57.2 d	4.58 m	
β	22.5 t	3.26 dd (9.6, 19.9),	
		3.36 dd (12.6, 19.9)	
γ	129.4 s		
π			179.1
δ	117.0 d	7.43 s	
ϵ	134.5 d	8.97 s	
τ		14.19 br s	172.1
2-Aminoadipic acid			
NH		8.27 d (7.9)	122.1
C=O	171.4 s		
α	53.2 d	4.08 m	
β	31.8 t	1.57 m, 1.66 m	
γ	20.7 t	1.43 m	
δ	33.1 t	2.17 ddd (9.1, 16.9, 21.3),	
		2.21 ddd (9.1, 16.9, 21.3)	
ϵ	174.3 s		

a) The spectra were obtained with Varian Inova 600 and Unity 400 spectrometers.

b) Solvent: DMSO- d_6 with 1.7% TFA. c) The coupling constants (Hz) are in parentheses. d) Solvent: H_2O - D_2O (4:1) with 2% TFA.

proton and carbon. In the DEPT (distortionless enhancement by polarization transfer) spectra, **1** showed 1 methyl, 11 methylene, 8 methine and 9 quaternary carbon signals. The ^1H -NMR spectrum showed 40 proton signals, whereas the remaining 2 hydrogens were not observed in either spectrum. The ^{15}N signals obtained by f_1 projection of the ^1H - ^{15}N HMBC (heteronuclear multiple bond coherence) indicated 10 nitrogens.

Presence of partial structures **a**–**f** (Fig. 3) were elucidated by ^1H - ^1H COSY (correlation spectroscopy) and ^1H - ^{13}C HMBC experiments in DMSO- d_6 containing 1.7% trifluoroacetic acid (TFA). The partial structures **a**–**f** were deduced to be arginine (**a**), proline (**b**), homoserine (Hse, **c**), histidine

Fig. 3. Partial Structures of **1** Fig. 4. Structure of **1** Elucidated by ^1H - ^{13}C HMBC and ^1H - ^{15}N HMBC

(d), 2-aminoadipic (Aad, e) and acetyl (f) residues.

The connectivity of the partial structures **a**–**f** was determined from long-range couplings of ^1H - ^{13}C HMBC and ^1H - ^{15}N HMBC (Fig. 4). A long-range ^1H - ^{13}C coupling was observed from Pro α -CH (δ_{H} 4.59) to Arg carbonyl (δ_{C} 170.1), which suggested the linkage of Arg (**a**)–Pro (**b**). A long-range coupling between Hse NH (δ_{H} 7.16) and Pro carbonyl (δ_{C} 171.1) suggested the linkage of Pro (**b**)–Hse (**c**). Long-range couplings were also observed from Aad NH (δ_{H} 8.27) and Aad α -CH (δ_{H} 4.08) to His carbonyl (δ_{C} 169.9), which suggested the linkage of His (**d**)–Aad (**e**), and a long-range coupling between Arg α -NH (δ_{H} 8.58) and Aad carbonyl (δ_{C} 171.4) suggested the linkage of Aad (**e**)–Arg (**a**).

The homoseryl γ -carbon was shown to be a methine. The amide linkage between Hse (**c**)–His (**d**) and a bond between Hse γ -CH and His α -N were suggested by long-range couplings from Hse γ -CH (δ_{H} 5.31) to Hse carbonyl (δ_{C} 172.9) and His α -CH (δ_{C} 57.2) and from His α -CH (δ_{H} 4.58) to Hse γ -CH (δ_{C} 78.7), forming a γ -lactam. This partial structure was supported by long-range ^1H - ^{15}N couplings from Hse γ -CH and His α -CH protons to His α -N (δ_{N} 150.2). The γ -lactam structure was confirmed by the comparison of ^{13}C chemical shifts measured in D_2O with 2% TFA and

Table 2. Comparison of the ^{13}C Chemical Shifts of **1** Measured in D_2O with 2% TFA and H_2O - D_2O (4 : 1) with 2% TFA

Position	D_2O +TFA	H_2O - D_2O +TFA	Δ (ppm)
Aad ϵ	177.82	177.96	0.14
Ac C=O	174.64	174.82	0.18
Hse C=O	174.46	174.48	0.02
Pro C=O	173.29	173.36	0.07
Aad C=O	172.83	172.94	0.11
Arg C=O	172.48	172.51	0.03
His C=O	172.20	172.28	0.08
Arg ζ	152.86	153.12	0.26
His ϵ	133.96	134.19	0.23
His γ	127.82	127.93	0.11
His δ	116.98	117.11	0.13
Hse γ	79.88	79.99	0.11
Pro α	60.83	60.87	0.04
His α	57.68	57.75	0.07
Aad α	53.18	53.28	0.10
Arg α	52.57	52.69	0.11
Hse α	50.27	50.40	0.14

The spectra were obtained with a Varian Unity 400 spectrometer.

H_2O - D_2O (4 : 1) with 2% TFA (Table 2). Among seven carbonyl carbons, Hse carbonyl (δ_{C} 174.46 in D_2O -TFA) and Arg carbonyl (δ_{C} 172.48 in D_2O -TFA) signals showed small shifts (Δ 0.02–0.03 ppm), and the other carbonyls showed large shifts ($\geq \Delta$ 0.07 ppm). These deuterium isotope effects suggested that the former carbonyl carbons bonded tertiary nitrogens. Hse γ -CH (δ_{C} 79.88 in D_2O -TFA) also showed a large shift (Δ 0.11 ppm), which suggested that a hydroxyl bonded the methine.

Attachment of acetyl residue (**f**) to ω -terminal of Arg (**a**) was suggested from the ^1H - ^{13}C and ^1H - ^{15}N long-range couplings between Arg η_1 -NH (δ_{H} 11.78) and acetyl carbonyl (δ_{C} 172.7) and between acetyl CH_3 (δ_{H} 2.10) and Arg η_1 -NH (δ_{N} 133.3), respectively. The skeletal structure of **1** was thus elucidated. The two hydrogens missing in the ^1H -NMR were deduced to be hydrogens of Hse γ -OH and Aad ϵ -COOH from the above result.

The optical activity of each amino acid was elucidated by chiral HPLC of acid hydrolysate of **1**, which revealed the presence of 1 mol of L-arginine, D-proline and L-2-aminoadipic acid. Histidine and homoserine were not detected since they formed a γ -lactam complex that was not hydrolyzed to respective amino acid residues.

Combining the above results, the structure of **1** was elucidated as cyclo(N^{ω} -acetyl-L-arginyl-D-prolyl-homoseryl-histidyl-L-2-aminoadipyl) in which homoseryl γ -methylene bonded to histidyl α -amino residue.

Chitinase Inhibitory Activity Compound **1** inhibited chitinase from *Lucilia cuprina* (blowfly) dose dependently (Fig. 5). Inhibitory activity of **1** against *L. cuprina* chitinase was studied compared with that of argifin (**2**) and allosamidin³⁾ (Table 3). Compound **1** inhibited the chitinase with IC_{50} values of 150 nM at 37 °C and 3.4 nM at 20 °C, and the inhibitory activities were about thirty times more potent than those of **2**. Allosamidin showed inhibition with IC_{50} values of 2.3 nM at 37 °C and 0.40 nM at 20 °C. Therefore, **1** was only nine times weaker than allosamidin at 20 °C.

Cockroach Larvae Mortality by Injection Assay Compound **1** (20 or 2 $\mu\text{g}/1 \mu\text{l}$ of 0.1 N AcOH) was injected into twenty american cockroach (*Periplaneta americana*) larvae

Fig. 5. Inhibition of *Lucilia cuprina* Chitinase by **1**

The enzyme assay was carried out at 37 °C.

Table 3. *Lucilia cuprina* Chitinase Inhibition by Argadin, Argifin and Allosamidin

Compound	IC ₅₀ (nM)	
	37 °C	20 °C
Argadin (1)	150	3.4
Argifin (2)	3700	100
Allosamidin	2.3	0.40

Fig. 6. Efficacy of **1** against *Periplaneta americana* Cockroach Larvae

and compared with mock injected controls (1 μl of 0.1 N AcOH). Mortality was assessed at 1, 5 and 23 d after injection. There was unexpectedly high control mortality (35%) at day 1. Therefore, the numbers of dead larvae at day 5 and day 23 were summed and are shown in Fig. 6. The efficacy of **1** was shown against the larval stage of cockroaches dose dependently. No mortality was observed for control cases after day 1, while 60 and 38% mortality was observed for cockroaches injected with 20 μg and 2 μg of **1**, respectively.

Mortality at day 1 may be due to the use of an acidic solvent to dissolve **1** as well as to mechanical disruption of the cockroaches by the injection. We have reported a cockroach assay of argifin (**2**) using water as a solvent⁷; at that time, the control mortality at day 1 was only 12%. Therefore, the acidic solvent may have enhanced the control death.

Other Biological Activities Argadin did not inhibit the growth of the following microorganisms at 10 μg/disc: *Bacillus subtilis* PCI 219, *Micrococcus luteus* PCI 1001, *My-*

cobacterium smegmatis ATCC 607, *Staphylococcus aureus* FDA 209P, *Acholeplasma laidlawii* PG 8, *Bacteroides fragilis* ATCC 23745, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* P 3, *Xanthomonas campestris* pv. *oryzae* KB 88, *Candida albicans* KF 1, *Saccharomyces cerevisiae* KF 26, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581 or *Pyricularia oryzae* KF 180. Argadin also did not inhibit the growth of P388, KB or HL-60 cells at 25 μg/ml.

We isolated a new chitinase inhibitor, argadin (**1**), from the cultured broth of *Clonostachys* sp. FO-7314, after the isolation of **2** from the broth of *Gliocladium* sp. FTD-0668. Both **1** and **2** are cyclic pentapeptides containing *N*^ω-substituted-L-arginine. The guanidino group may be important for chitinase inhibition because known inhibitors, styloguanidines,⁴⁾ cyclo(L-arginyl-D-prolyl)⁵⁾ and compound A,⁹⁾ also have one or more guanidino groups. The producing strain of **2** was *Gliocladium* sp. which is close to the genus *Clonostachys*. Though there have so far been only two examples, the production of cyclic peptide chitinase inhibitors may be a genus specific event.

Chitinase is widely distributed in the group of bacteria, fungi, plants and arthropods, but it is not essential for mammals. Chitinase inhibitors would be expected to interfere with the life cycle of the former group, but not that of the latter. Therefore, cyclic pentapeptide containing *N*^ω-substituted-L-arginine could be an interesting lead for the development of novel insecticides.

Experimental

General NMR spectra were obtained with Varian Inova 600 and Unity 400 spectrometers at room temperature using DMSO-*d*₆ with 1.7% TFA, H₂O-D₂O (4:1) with 2% TFA and D₂O with 2% TFA as solvents.

¹H-¹⁵N HMBC was recorded on a Varian Unity 400 spectrometer equipped with pulse field gradients and an inverse detection 5 mm probe. Other conditions were as follows: sample 8.0 mg, solvent 0.6 ml of H₂O-D₂O (4:1) with 2% TFA, *f*₁ × *f*₂ = 13037 × 3507 Hz, *t*₁ × *t*₂ = 64 × 1024 points, transients = 2032, repetition time = 1.5 s, relaxation delay optimized for *J* = 6 Hz.

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 IR 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.

4-Methylumbelliferone and 4-methylumbelliferyl-β-D-N,N',N''-triacylchitotriose were obtained from Sigma Chemical Co.

Fermentation and Isolation The seed medium was composed of 2.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co.), 0.1% agar, 0.2% yeast extract (Oriental Yeast Co.), 0.05% MgSO₄ · 7H₂O and 0.1% KH₂PO₄ (pH 5.7 prior to sterilization). The production medium was composed of 5.0% maltose, 3.0% dry yeast (Gist-brocades), 1.0% KBr, 0.5% KH₂PO₄ and 0.05% MgSO₄ · 7H₂O (pH 6.0 prior to sterilization).

The cultured broth of FO-7314 (8 l) was filtered, and the mycelia were extracted with methanol (8 l) and concentrated *in vacuo*. The extract was diluted to 8 l with water, neutralized, and charged on a Diaion SK1B cation exchange column (NH₄⁺ form, 800 ml). After washing with water, the active substance was eluted with 0.5–2.0 N NH₄OH. The eluate was neutralized and applied on a Diaion HP20 column (800 ml). The column was washed with water and eluted with 10–80% methanol. The eluate was concentrated *in vacuo* and dissolved in a small amount of 15 mM ammonium acetate. The solution was charged on an SP-Sephadex C-25 cation exchange column (NH₄⁺ form, 800 ml). After washing with 50 mM ammonium acetate, the active substance was eluted with 100 mM ammonium acetate. The active fractions were collected and applied on an ODS column (Pegasil ODS-7515-12A, 200 ml). After washing with water, the active substance was eluted with 100 ml of 40% methanol. The eluate was concentrated *in vacuo* and dissolved in a small amount of 65% methanol. The solution was charged on a Sephadex LH-20 column (800 ml) and eluted with 65% methanol. The ac-

tive fractions were collected and concentrated *in vacuo*, then dissolved in a small amount of 0.05% acetic acid, charged on a Bio-Gel P-2 Gel column (fine, 800 ml), and eluted with 0.05% acetic acid. The active fractions were collected and concentrated *in vacuo* to yield a white powder of pure **1** (25 mg).

Argadin (**1**): White powder, $[\alpha]_D^{25} +52.1^\circ$ ($c=0.1$, H_2O). $C_{29}H_{43}N_{10}O_9$ (m/z 675.3214 ($M+H$)⁺; Calcd for $C_{29}H_{43}N_{10}O_9$, 675.3214) by HR-FAB-MS. mp 270 °C (dec.). UV λ_{max} (H_2O) nm (ϵ): 200 (31400). IR ν_{max} (KBr) cm^{-1} : 3310, 3220, 1710, 1650, 1575, 1530, 1435, 1390, 1245.

Acid Hydrolysis of 1 Compound **1** (100 μ g) was hydrolyzed with 6 N HCl–1% phenol vapor at 120 °C for 11 h. 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 $\times$ 150 mm); mobile phase, 1 mM $CuSO_4$; flow rate 1.0 ml/min; detection, UV 254 nm.

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

Assay Methods for Biological Activities Chitinase inhibitory activity was measured as described previously.⁷⁾ In brief, 20 μ l of 100 mM sodium phosphate buffer, pH 7.0, 30 μ l of crude chitinase solution (prepared from late pupae of the blowfly, *Lucilia cuprina*), and 50 μ l of 80 μ M 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotriose in the buffer were placed in each well of a microplate, and incubated with or without the sample at 37 °C for 10 min or 20 °C for 15 min. Fluorescence (excitation at 355 nm, emission at 460 nm) was measured by a fluorometer (Fluoroscanner II, Labsystems), and the rate of 4-methylumbelliferone production was corrected by calibrating the quenching ratio of each sample using the mixture of the sample and 4-methylumbelliferone.

Assay methods for antimicrobial activity and cockroach larvae injection were also described previously.⁷⁾

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