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Atroviridins A~C and Neoatroviridins A~D, Novel Peptaibol Antibiotics Produced by *Trichoderma atroviride* F80317

I. Taxonomy, Fermentation, Isolation and Biological Activities

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Seven new peptaibols, atroviridins A~C composed of 20 residue amino acids and neoatroviridins A~D with 18 residues, were isolated from the culture broth of fungal strain F80317. The strain F80317 was identified as *Trichoderma atroviride* from its morphological and cultural characteristics. These compounds showed antimicrobial activity against Gram-positive bacteria and phytopathogenic fungi, and exhibited significant cytotoxicity to human cancer cell lines *in vitro*. Atroviridins showed significant membrane-perturbing activity responsible to their antibiotic action.

Peptaibols, a class of linear peptides, are characterized by high portion of α,α-dialkylated amino acids such as α-aminoisobutyric acid and isovaline, and contain an acyl substituted *N*-terminus and *C*-terminal amino alcohol. They result from a nonribosomal biosynthesis pathway involving peptide synthetases as multienzymic templates[1] and thus are produced as microheterogeneous mixtures of structurally related peptide analogues. Peptaibols are classified into three subclasses on the basis of their chain lengths. The long-sequence peptaibols such as alamethicins[2], trichosporins[3], trichorzins[4], chrysopermins[5] and longibrachins[6] contain 18~20 amino acid residues, including a single central proline[7,8]. The short-sequence peptaibols have 11~16 residues and several Aib-Pro motifs[9], such as emerimicins[10], zervamicins[11], antiamoebins[12], harzianins[13], tylopeptins[14] and bergofungins[15]. The lipopeptaibols are characterized by 7 or 11 residues with a high amount of glycine, *C*-terminal amino alcohol and an *N*-terminal acylated by an 8~10 carbon linear fatty acid instead of the acetyl group, such as trichogin[16] and trichodecenins[17].

This class of antibiotics is known as membrane-modifying and pore-forming peptide. They are organized in amphipathic helices, which interact with phospholipid bilayers and increase their permeability, either in the presence[18,19] or in the absence of voltage[20]. Their antibiotic activities are mainly directed against Gram-positive bacteria, and the long-sequence peptaibols exemplified by the prototypic alamethicin are the most potent. Also, it has been reported that peptaibols have various biological activities such as hemolysis[21], the uncoupling of oxidative phosphorylation in mitochondria[22], inhibition of ameba cell growth[12] and antimycoplasmic activities[23].

In search for novel antibiotic peptides, we have isolated two clusters of peptaibols, atroviridins A~C and neoatroviridins A~D (Fig. 1), from the culture broth of fungal strain F80317 and identified them as novel sequences. Atroviridins A~C are consisted of 20 amino acids and neoatroviridins A~D are composed of 18 amino acids. In this paper, we report the taxonomy of the

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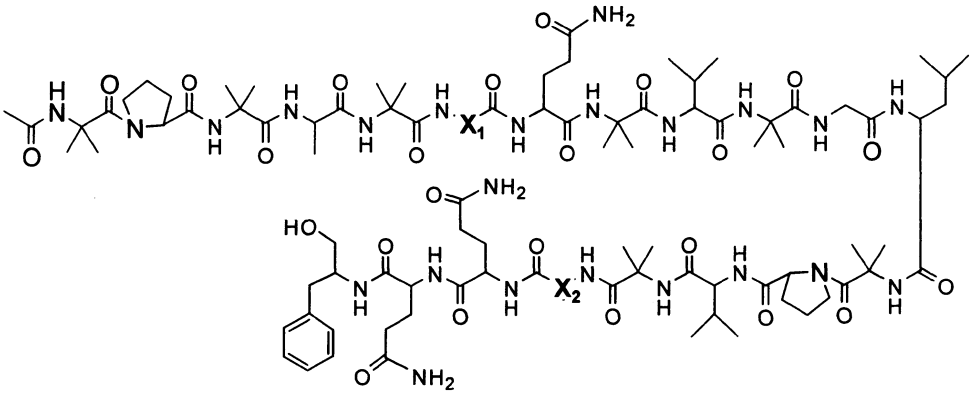
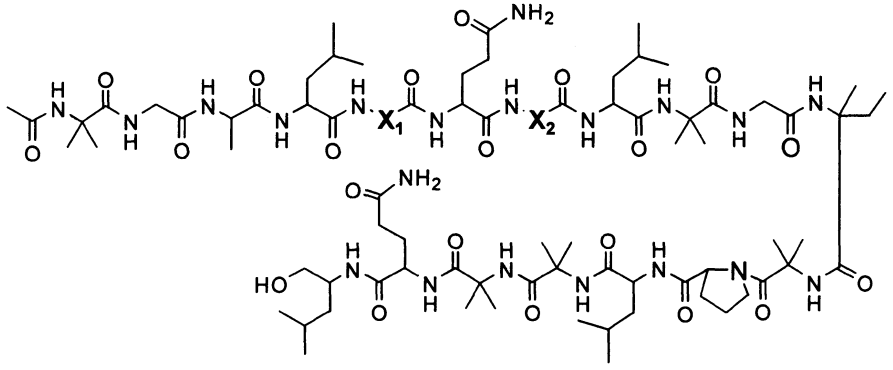
Fig. 1. Structures of atroviridins A~C and neoatroviridins A~D.



	X_1	X_2
Atroviridin A	Alanine	α-Aminoisobutyric acid
B	Alanine	Isovaline
C	α-Aminoisobutyric acid	Isovaline



	X_1	X_2
Neoatroviridin A	α-Aminoisobutyric acid	α-Aminoisobutyric acid
B	Isovaline	α-Aminoisobutyric acid
C	α-Aminoisobutyric acid	Isovaline
D	Isovaline	Isovaline

producing strain, fermentation, isolation and biological activities of these compounds.

Materials and Methods

Taxonomic Studies

The producing strain F80317 was identified according to the BISSETT's method[24]. For taxonomical studies, strain F80317 was cultivated for 5 days at 24°C, and the growth and the colors of aerial mycelium, substrate mycelium and soluble pigment on various agar media including corn meal agar, Czapek's agar, malt extract agar, oat meal agar, potato dextrose agar, yeast-malt extract agar, potato sucrose agar and potato carrot agar were observed. All media except yeast-malt extract agar were commercially supplied by Difco Co. The morphology of spore chains was examined by light microscope (Nikon MicrophotFXA, Japan) using Amma's lactophenol (phenol crystals 20 g, lactic acid 20 g, glycerol 40 g, water 20 g) as a mounting fluid. Color names were guided from color standard of Nihon Shikisai Co.

Fermentation

The strain F80317 grown on potato dextrose agar was inoculated into 500-ml Erlenmeyer flasks containing 100

ml of the seed medium (glucose 2%, yeast extract 0.2%, peptone 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.01%, pH 5.7). The flasks were cultivated at 24°C for 4 days on a rotary shaker (120 rpm). Approximately two milliliters of the seed culture were inoculated into 500-ml Erlenmeyer flasks containing 100 ml of the same medium as above and cultivated at 24°C for 8 days on a rotary shaker.

Analytical Procedure

The content of atroviridins and neoatroviridins in the fermentation broth and the purity in purification steps were monitored by analytical HPLC. HPLC was carried out using a Maxsil C18 column (i.d. 10×250 mm) eluted with 90% aqueous MeOH. The flow rate was 0.9 ml/minute and the UV absorption was monitored using photodiode array detector. A mixture of atroviridins was detected at a retention time of 27.4 minutes and another mixture for neoatroviridins was evident at a retention time of 20.9 minutes.

Antimicrobial Activity

Antimicrobial activity was determined by the conventional paper disk (Advantec, 8 mm in diameter) method. The test microorganisms including Gram-positive bacteria, Gram-negative bacteria, yeast and phytopathogenic fungi were supplied from the Korean Collection for Type Cultures (KCTC) in the Korea Research Institute of Bioscience & Biotechnology (KRIBB), Korea. The paper disks containing each of 50 μg peptaibol antibiotics were placed on agar plate inoculated with the test organisms. Antibiotic activity was assessed by measuring the diameter of inhibition zone after 24 hours at 37°C for bacteria, whereas after 2~7 days at 27°C for fungi and yeast.

Cytotoxicity

In vitro cytotoxicity against a panel of human cancer cell lines was assessed according to a National Cancer Institute protocol. Human cancer cell lines PC-3 prostate, A549 lung, UACC62 melanoma, EL4 lymphomatic and K562 chronic myelogeneous leukemia were used for antitumor activity of atroviridins and neoatroviridins.

Each of the cell lines was suspended in the 10% calf serum-containing RPMI1640 medium and diluted to give the appropriate cell densities for inoculation onto 96-well microplates. The microplates containing the cells were preincubated for 24 hours at 37°C in 5% CO_2 to allow stabilization. Peptaibols were added into the microplates. Cells were incubated for 48 hours in the presence of peptaibols, and then cell growth was evaluated with an SRB (sulforhodamine B) assay. Adherent cell cultures were fixed by adding 100 μl of cold 50% trichloroacetic acid and incubating for 60 minutes at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and dried. A hundred microliter of 0.4% SRB solution dissolved in 0.1% acetic acid was added to each well and incubated for 30 minutes. Unbound SRB was removed by washing with 0.1% acetic acid, and then the plates were dried. Bound stain was solubilized with 10 mM Tris base (pH 10.5) and then read on a microplate reader at 540 nm. A dose-response curve was plotted and the concentration giving 50% inhibition (IC_{50}) was calculated.

Membrane Disrupting Activity against Artificial Vesicle

Carboxyfluorescein (CF)-encapsulated large unilamellar vesicles (LUV) composed of PC/PS (4:1, w/w) were prepared by the reverse-phase ether evaporation method[25] using 100 mM CF. To remove the free CF dye, the vesicles were passed through Bio-gel A 0.5 m (Bio-Rad, Richmond, USA) column (1.5×30 cm) using 50 mM potassium phosphate buffer (pH 7.4) as an eluting buffer. The separated LUV fraction, after appropriate dilution to a final concentration of 6.36 μM, was mixed with the peptaibol solution in a 2 ml cuvette at 25°C. The leakage of CF from the LUV was monitored by measuring fluoroscence intensity at 520 nm excited at 490 nm on a Shimadzu RF-5000 spectrofluorometer (Tokyo, Japan). The apparent percentage leakage value at fluoroscence intensity, F, was calculated by the following equation:

$$\% \text{ Leakage (apparent)} = 100 \times (F - F_0)/(F_t - F_0)$$

F_t denotes the fluoroscence intensity corresponding to 100% leakage after the addition of 20 μl of 10% Triton X-100. F_0 represents the fluoroscence of the intact vesicle.

Results and Discussions

Taxonomy

Taxonomical characteristics of the strain F80317 are summarized in Table 1. This fungus grew well on malt extract agar, potato dextrose agar and yeast-malt extract agar. Growing status of strain F80317 showed 7 cm of diameter in potato dextrose agar medium after 3 days of inoculation. Its colonies showed very rapid growth. Aerial mycelium was observed to be floccose and conidiation were tufted and forming compact pustules; white at first, eventually turning to dark green. Reverse color appeared to change slightly yellowish in potato dextrose agar and malt extract agar media after 5 days of inoculation.

Table 1. Cultural characteristics of the strain F80317.

Media	Growth	Mycelia	Reverse color	Soluble pigment
Corn meal agar	Poor	White, thin, submerged	Colorless	None
Czapek's solution agar	Poor	White, thin, submerged	Colorless	None
Malt extract agar	Good	White to green	Yellowish shaded	None
Oatmeal agar	Poor	White to yellow, submerged	Colorless	None
Potato dextrose agar	Good	Light yellow to green, raised at edge	Yellowish shaded	None
Yeast-malt extract agar	Moderate	White to yellow, at center, submerged	Colorless	None
Potato sucrose agar	Poor	White to light green	Colorless	None
Potato carrot agar	Poor	White to light green	Colorless	None

Table 2. Morphological characteristics of the strain F80317.

Colony	- floccose in malt extract agar, potato dextrose agar - aerial mycelium scanty in Czapek's agar - growing rapidly, more than 7cm in 3 days, at 24℃, on potato dextrose agar and malt extract agar media - odor : not detected
Hyphae	- hyaline, moderate smooth-walled, 3-5mm
Conidiation	- appearing granular, forming compact pustules - white at first, rapidly turning dark green - reverse are yellowish shade in potato dextrose agar and malt extract agar media
Conidia	- 3.0 X 3.8(-4.3) X (2.3-)2.6 - 3.4(-3.5) μm - subglobose to ovate - green, smooth (observed in X 1,000)
Phialide	- (5.7-)5.9 - 10.1(-12.3) X 2.5 - 3.3(-3.5) μm - subulate to lageniform - not crowded - mostly in verticils of 2 or 3
Chlamydospore	- 5-8 μm diameter - globose to subglobose - frequently found - intercalary or terminal, solitary

Morphological characteristics of the strain F80317 are shown in Table 2. In the light microscopic morphological observation, chlamydospore existed frequently and conidiophores were relatively narrow and flexuous with primary branches arising at regular intervals, usually paired or three branched, short and not extensively rebranched. Phialides showed mostly in verticils of 2 or 4, more or less lageniform. Conidia was dark green, smooth-walled to distinctly verrucose, subglobose to ellipsoid.

From the above taxonomical characteristics, the strain F80317 was identified as *Trichoderma atroviride* and thus named *T. atroviride* F80317.

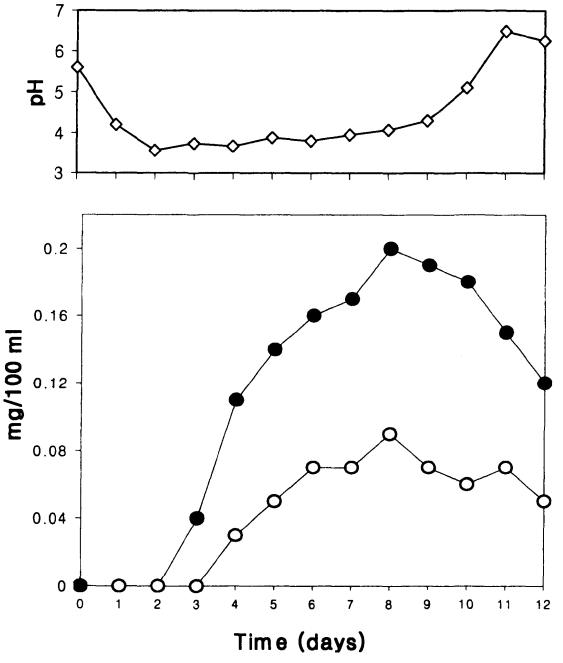
Fermentation

The fermentation of *T. atroviride* F80317 was carried out as described in Materials and Methods using 500-ml Erlenmeyer flasks because of lower yield of peptaibols in

jar fermentation. The fermentation was continued for 12 days at 24°C with agitation of 120 rpm. The cell growth of this strain showed rapid increase at 5 days and reached a maximum at 8 days.

A typical time course production of atroviridins and neoatroviridins in 500-ml Erlenmeyer flask are shown in Fig. 2. The production of atroviridins A~C started after 2 days of cultivation, showed rapid increase of production and reached a maximum at 8 days of cultivation. The yield of neoatroviridins gradually increased after 3 days and reached a maximum at 8 days of cultivation. After 9 days of cultivation, the pH of the culture broth showed a tendency of steep increase, and the production of peptaibols was decreased with rising pH.

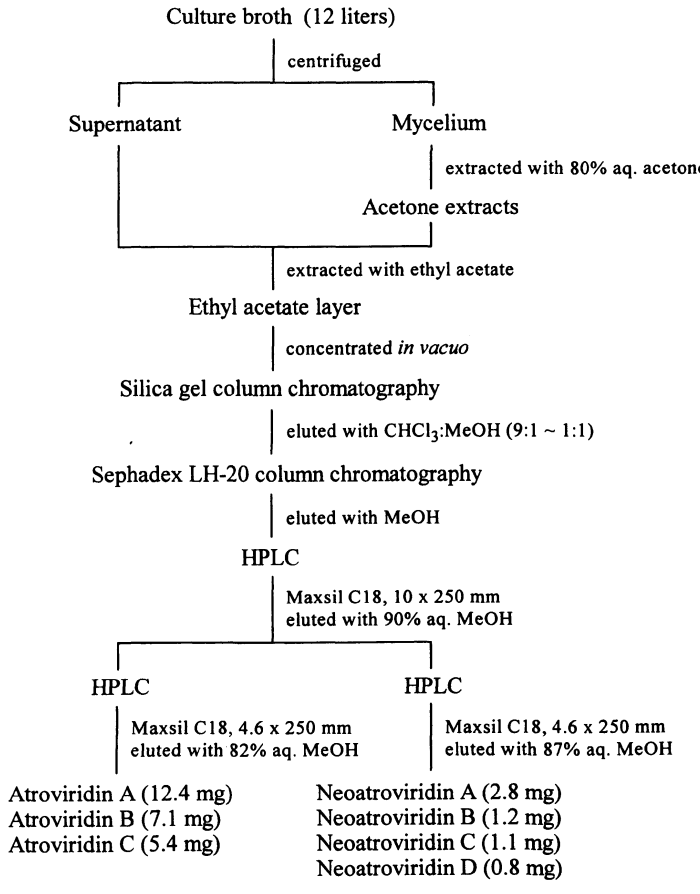
Fig. 2. Fermentation profiles of *Trichoderma atroviride* F80317 for the production of atroviridin (●) and neoatroviridin (○) groups.



Isolation and Purification

Atroviridins and neoatroviridins were isolated from the culture broth of strain F80317 as shown in Fig. 3. Twelve liters of culture broth were centrifuged at 6,000 *g* for 10 minutes and the precipitated mycelium was extracted with 5 liters of 80% aqueous acetone. The extract was filtered and

Fig. 3. Purification procedure of atroviridins A~C and neoatroviridins A~D.



concentrated *in vacuo* for elimination of acetone. The resulting aqueous solution was extracted three times with 3 liters of ethyl acetate. The supernatant was also extracted with ethyl acetate. Both ethyl acetate extracts were combined and concentrated *in vacuo* to dryness. The crude oily extract was subjected to a column of silica gel and eluted with $CHCl_3$: MeOH (100 : 1~MeOH only, stepwise). The $CHCl_3$: MeOH (7 : 3) eluate showed significant antimicrobial activity. This active fraction was concentrated and then applied to Sephadex LH-20 column chromatography eluting with MeOH. The active fraction was applied to HPLC with a reverse phase column (Maxsil C18, i.d. 10×250 mm) eluting isocratically with 90% aqueous MeOH to afford two active fractions. Each of the two active eluates was further purified by the slower HPLC using ODS column (Maxsil C18, i.d. 4.6×250 mm) eluting with 87% aqueous MeOH to give atroviridins A (12.4 mg), B (7.1 mg) and C (5.4 mg), and eluting with 82% aqueous MeOH to afford neoatroviridins A (2.8 mg), B (1.2 mg), C (1.1 mg) and D (0.8 mg). The structures of atroviridins A~C have been reported[26] and the structures of neoatroviridins A~D are in preparation.

Biological Activities

Antimicrobial Activity and Cytotoxicity

Antimicrobial activities of atroviridins and neoatroviridins were assessed by the agar diffusion method. These compounds exhibited strong antifungal activity against some plant pathogenic fungi such as *Curvavularia inaqualis*, *Collectotrichum dematium* and *Fusarium oxysporum*, and moderate activity against *Verticillium dahliae*, *Aspergillus niger* and *Cladisporum* sp. These compounds also showed significant antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, whereas they exhibited no activity against Gram-negative bacteria and yeast. Antimicrobial spectrum of atroviridins and neoatroviridins is described in Table 3.

The *in vitro* cytotoxicity of atroviridins and neoatroviridins against a panel of tumor cell lines was investigated according to NCI protocol. Although these compounds were less active than adriamycin which was used as a control, they showed significant cytotoxicity against human cancer cell lines such as prostate (PC-3), melanoma (UACC62) and leukemia (K562) with IC_{50} values in the range of 2~4 μg/ml (Table 4).

Table 3. Antimicrobial spectrum of atroviridins A (**1**), B (**2**) and C (**3**), and neoatroviridins A (**4**), B (**5**), C (**6**) and D (**7**).

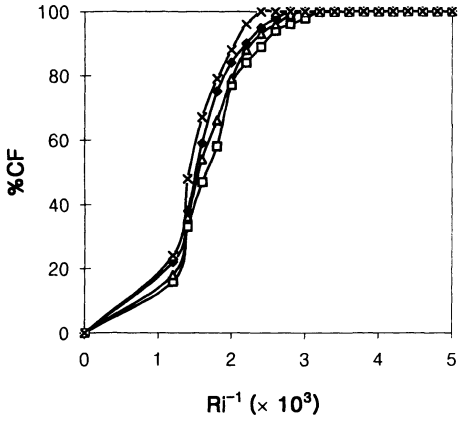
Test organisms[a]	Diameter of inhibition zone (mm)[b]						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Pathogenic fungi							
Curavularia inaqualis	18	14	14	16	15	15	18
Collectotrichum dematium	14	12	14	17	16	14	14
Verticillium dahliae	10	12	10	10	12	10	9
Phytophthora infestans	13	12	15	13	12	15	14
Aspergillus niger	12	12	12	12	12	12	10
Cladisporum sp.	10	12	12	15	14	12	14
Fusarium oxysporum	14	14	12	14	14	12	9
Gram positive bacteria							
Bacillus subtilis	15	17	13	18	18	15	16
Staphylococcus aureus (KCTC1916)	18	10	14	22	20	22	18
Staphylococcus aureus (KCTC1928)	12	14	17	18	16	19	16
Gram negative bacteria							
Escherichia coli	ND[c]	ND	ND	ND	ND	ND	ND
Salmonella typhimurium	ND	ND	ND	ND	ND	ND	ND
Yeast							
Candida albicans	ND	ND	ND	ND	ND	ND	ND

[a] Fungi, bacteria and yeast were cultured on PDA agar, LB agar and SAB agar media, respectively.
[b] Determined by the agar diffusion method with 50 μg compounds on a 8 mm disc.
[c] ND: none detected (no activity).

Table 4. Cytotoxicity of atroviridins A (**1**), B (**2**) and C (**3**), and neoatroviridins A (**4**), B (**5**), C (**6**) and D (**7**) against human cancer cell lines.

Cell lines	IC_{50} (μg/ml)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	Adriamycin
Prostate (PC-3)	4.18	3.89	3.95	4.82	4.11	3.35	6.13	0.12
Lung (A549)	9.12	8.79	8.55	9.21	9.49	9.55	9.22	0.04
Melanoma (UACC62)	4.01	4.41	3.97	5.75	6.22	6.42	5.88	0.04
Lymphoma (EL4)	14.56	13.12	13.97	12.42	11.98	11.14	12.47	0.09
Leukemia (K562)	3.84	4.21	3.92	2.12	2.33	2.19	2.57	0.05

Fig. 4. Atroviridins-induced CF leakage from PC/PS (4 : 1) vesicles at t=20 minutes different Ri^{-1}=[peptide]/lipid.



Atroviridins A (◆), B (□), C (△) and alamethicin (×).

In antimicrobial activity and cytotoxicity, there were no difference between 20-residue peptaibols for atroviridins A~C and 18-residue peptaibols for neoatroviridins A~D.

Membrane Perturbing Activity

It has been known that long chain peptaibol is more efficient than short chain one to penetrate cell membrane. For the leakage rate of atroviridins having more long chain sequence than neoatroviridins, we estimated the [lipid]/[peptide] R_i ratios for 50% leakage at 20 minutes[27]. In result, atroviridins A~C showed significant membrane perturbing activity, which was comparable to neutral peptaibol alamethicin, as shown in Fig. 4. Antimicrobial activity is most likely to be caused by structural features of atroviridins and neoatroviridins, which consist of many hydrophobic amino acids such as α-aminoisobutyric acids and isovaline to form helical conformation and to result in membrane-perturbing action, as reported for other peptaibols.

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