Structures and Biological Activities of Novel Antibiotic Peptaibols Neoatroviridins A-D from *Trichoderma atroviride* F80317

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Abstract Four new antibiotic peptaibols, named neoatroviridins A (1), B (2), C (3), and D (4), have been isolated from the culture broth of *Trichoderma atroviride*. Their amino acid sequences were determined mainly by mass spectrometry in combination with NMR studies. The absolute stereochemistry of neoatroviridins was established as L by GC analysis of their acid hydrolysates with derivatization, except for isovaline which was in D. Neoatroviridins, composed of 18 amino acid residues, showed significant membrane-perturbing activity responsible for their antibiotic action, which was comparable to that of alamethicin, a well-known 20-residue peptaibol.

Key words: Peptaibol antibiotic, *Trichoderma atroviride*, neoatroviridins, structure determination, membrane-perturbing activity

Peptaibols are characterized by high proportion of α,α-dialkylated amino acids such as α-aminoisobutyric acid (Aib) and isovaline (Iva), and contain an acyl substituted N-terminus and C-terminal amino alcohol. They are synthesized from a nonribosomal biosynthetic pathway, involving peptide synthetases as multienzymic templates [9], and therefore, are produced as microheterogeneous mixtures of structurally related peptide analogues.

Peptaibols are classified into three subclasses on the basis of their chain lengths; 1) long-sequence peptaibols with 18–20 amino acid residues [1, 7, 12, 14], 2) short-sequence peptaibols having 11–16 residues and several Aib-Pro motifs [5, 13, 15], and 3) lipopeptaibols composed of 7 or 11 residues with a large amount of glycine, C-terminal amino alcohol, and an N-terminal acylated by an 8–10 carbon linear fatty acid [3].

This class of antibiotics has a strong tendency to form amphipathic helices and interact with phospholipid bilayers, resulting in transmembrane channels in the lipid bilayer membrane either in the presence or absence of voltage, and are responsible for antimicrobial activity [2, 16]. The antibiotic mechanism suggests that peptaibols may be potential antibiotics against multidrug resistance pathogenic bacteria.

In the search for novel antibiotic peptides, we have isolated a group of peptaibols, neoatroviridins A (1), B (2), C (3), and D (4) (Fig. 1) together with atroviridins, from the culture broth of *Trichoderma atroviride* F80317 and identified their novel sequences using tandem mass and NMR experiments. We previously reported the taxonomy, fermentation, isolation, and biological properties of these compounds [10, 11]. In this paper, we describe the sequence determination and membrane-perturbing activity of these neoatroviridins.

**MATERIALS AND METHODS**

General Procedures

NMR spectra were obtained using the UNITY 500 NMR spectrometer with samples dissolved in DMSO-d$_6$ with
TMS as an internal standard. The chemical shifts are given in ppm (d) values. FAB and tandem mass spectra were obtained on a JEOL JMS-HX 110A/HX110A mass spectrometer. HPLC was carried out using a Metasil 5u ODS column (φ4.6×250 mm), and was eluted with 87% aqueous MeOH at the flow rate of 2.0 ml/minute. UV absorption was monitored using a photodiode array detector.

**Extraction and Isolation**

Neoatroviridins were isolated from the culture broth of strain F80317. Twelve liters of culture broth were centrifuged at 6,000 ×g for 10 min, and the precipitated mycelium was extracted with 5 l of 80% aqueous acetone. The extract was filtered and concentrated in vacuo to eliminate acetone. The resulting aqueous solution was extracted three times with 3 l 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 silica gel column and eluted with CHCl₃:MeOH (100:1/MeOH only, stepwise). The CHCl₃:MeOH (7:3) eluate showed significant antimicrobial activity. This active fraction was concentrated and then applied to a Sephadex LH-20 column, eluting with MeOH. An active fraction was subjected to HPLC with a reverse phase column (Maxsil C18, φ10×250 mm), eluting isocratically with 82% aqueous MeOH to afford an antibiotic cluster. The active eluate was further purified by HPLC using an ODS column (Maxsil C18, φ4.6×250 mm), 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).

**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 [17] using 100 mM CF. To remove free CF dye, the vesicles were passed through a Bio-gel A 0.5 m (Bio-Rad, Richmond, U.S.A.) 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

![Fig. 2. FAB-mass spectra of neoatroviridins A (a), B (b), C (c), and D (d).](image-url)
the LUV was monitored by measuring fluorescence intensity at 520 nm, excited at 490 nm, on a Shimadzu RF-5000 spectrofluorometer (Tokyo, Japan). The apparent percentage leakage value at fluorescence intensity, F, was calculated by the following equation:

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\% \text{ Leakage (apparent)} = 100 \times \frac{F - F_0}{F_0}
\]

F denotes the fluorescence 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 DISCUSSION**

**Structure Determination of Antibiotic Peptaibols Neoatroviridins**

The acid hydrolysate of the compound 1 reacted with ninhydrin, suggesting that this compound has a peptidic character. Amino acid analysis of the total acid hydrolysate of 1 provided the following amino acid composition; Ala (1), Gly (2), Aib (7), Iva (1), Leu (4), Glx (2), Pro (1). In addition to amino acid analysis, the lipophilicity and NMR spectra suggested that 1 belonged to the peptaibol class of antibiotics. The 1H NMR spectrum of 1 in DMSO-d_6 exhibited peaks due to 7 Aibs at δ 1.33–1.45, several α-protons at δ 3.5–4.3, and 22 exchangeable protons that were collapsed on shaking with D.O in the low-field region between δ 6.7 to 10.8. The COSY, TOCSY, and HMBC spectra also revealed the presence of the above amino acids, and Glx residues were assigned as Gln on the basis of the presence of four ε-protons at δ 7.21, 7.11, 6.73, and 6.71 for two carbamoyl groups. The molecular formula C_{18}H_{37}N_{7}O_{7} of 1 was deduced from FAB-mass data in combination with amino acid composition. The sequence of 18 amino acid residues for 1 was determined by mainly FAB and tandem mass analyses. The FAB-mass measurement in positive-ion mode gave an [M+Na]^+ ion peak at m/z 1754 and daughter ions derived from the N-terminal at m/z 128, 185, 256, 369, 454, 667, 780, 865, 922, 1021 and 1106 corresponding to AcAib-Gly-Ala-Leu-Aib-(Gln-Aib)-Leu-Aib-Gly-Iva-Aib, along with a fragment ion peak at m/z 624 corresponding to a counterpart of m/z 1106, as shown in Fig. 2a. The collision-induced dissociation (CID) spectrum of m/z 1106 gave an additional fragment peak at m/z 582, revealing -(Gln-Aib)- sequence, but not -(Aib-Gln)-. Also, the CID spectrum of m/z 624 showed the fragment ion peaks at m/z 70, 211, 296, 381, and 509 to assign the C-terminal sequence as Pro-Leu-Aib-Gln-Leuol (Fig. 3). Therefore, the structure of neoatroviridin A was established to be a new 18-residue peptaibol with sequence of AcAib-Gly-Ala-Leu-Aib-Gln-Aib-Leu-Gly-Iva-Aib-Pro-Leu-Aib-Aib-Gln-Leuol.

Compounds 2 and 3 differ from 1 in that they contained additional Iva instead of Aib, and 4 has 2 moles of Iva instead of 2 moles of Aib in 1. The structures of 2–4 were determined by comparison of their mass spectra with that of neoatroviridin A. The FAB-mass spectra of 2–4 showed a common fragment ion at m/z 624, which were generated from the Aib-Pro bond with a labile tertiary amide link, revealing that these compounds had the identical C-terminal sequence, Pro-Leu-Aib-Gln-Leuol. Also the N-terminal sequences of 2–4 were assigned by detailed FAB-mass analyses in combination with amino acid compositions, as shown in Fig. 2. Therefore, the structures of neoatroviridins were established as novel 18-residue peptaibols. These sequences were consistent with intensive 2D-NMR spectral data, including COSY, TOCSY, HMBC, and NOESY.

The absolute stereochemistry of component amino acids of 1–4 was determined to be L by GC analysis of their acid hydrolysates with derivatization, except for isovaline which was in D.

**Membrane-Perturbing Activity of Neoatroviridins**

Compounds 1–4 exhibited significant antimicrobial activity against Gram-positive bacteria and some plant pathogenic fungi [10]. Antimicrobial activity seems to be caused by their structural features, which consist of many hydrophobic amino acids such as Aib and Iva to form a helical conformation, resulting in membrane-perturbing action. Hence, we estimated the membrane-disrupting activity of 1–4 against artificial vesicle. Thus, carboxyfluorescein (CF)-encapsulated large unilamellar vesicles (LUV) composed of PC/PS (4:1, w/w) were prepared by the reverse-phase ether evaporation method using 100 mM CF, and the [lipid]/[peptide] R ratios for CF leakage at 20 min were assessed for convenient comparison of the leakage rate of 1–4 [4]. As a result, although 1–4 exhibited less leakage rate than alamethicin, a 20-residue peptaibol which was used as a control, these compounds showed significant CF leakage rate (Fig. 4).
Increase of hydrophobicity in 4 by replacement of Aib residues for Iva resulted in somewhat enhancement of membrane perturbation. Several peptides are known to exhibit significant anticancer activity [6, 8]. The anticancer activities and structure-activity relationship of 1–4 are under investigation.

REFERENCES