THE FERMENTATION, ISOLATION AND CHARACTERIZATION OF A MACROMOLECULAR PEPTIDE ANTIBIOTIC: AN-1

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A new macromolecular peptide antibiotic, named AN-1, was isolated from the culture broth of *Streptomyces albus* AJ9003.

From 18 liters of culture broth (110 units/ml activity) a 300 mg sample of AN-1 was obtained with a specific activity of 1,160 units/mg was obtained. AN-1 is a basic polypeptide with a molecular weight of 12,000, isoelectric point of pH 8.3, and gives a single band on SDS polyacrylamide gel electrophoresis. It is soluble in water but insoluble in ethanol, butanol and acetone. It was stable at pH 6~9 but very unstable at pH 2. The UV absorption spectrum shows a maximum at 280 nm.

AN-1 had no antibacterial activity against the Gram-positive and Gram-negative bacteria tested, but shows strong inhibitory activity toward *Escherichia coli* MP2, a macromolecule permeable mutant. In addition to being highly mutagenic, AN-1 inhibits the *in vitro* cell growth of L1210 (ED_{50} 0.41 μg/ml). However, AN-1 had no antitumor activity against mouse leukemia L1210 or Lewis lung carcinoma in mouse.

In the preceding papers, we reported the isolation and characterization of new protein antibiotics, AN-3 and AN-7[1,2].

This paper describes characteristics of another peptide antibiotic, AN-1. A comparison between AN-1 and other macromolecular peptide antibiotics is also made.

Materials and Methods

Microorganism

Streptomyces albus AJ9003 was employed in this study for the production of AN-1.

Fermentation and Analyses

The fermentation medium was composed of 2% glucose, 0.5% Polypepton, 0.5% dried yeast, 0.5% meat extract, 0.5% NH_4NO_3, 0.5% NaCl, 0.2 μg/ml $ZnSO_4 \cdot 7H_2O$ and 0.2% $CaCl_2 \cdot 2H_2O$; the pH was adjusted to 7.0 with NaOH.

Quantitative determination of antibacterial activity was done by the disk assay using UR3[3]. The diameter of inhibition zone was proportional to the logarithm of antibacterial activity. One unit of antibacterial activity was defined as the antibiotic concentration to give 10 mm (diameter) of inhibition zone[4].

Fermentation and assaying of antitumor activity were carried out by the methods described previously[1,2].

Results

Fermentation

Typical fermentation kinetics of AN-1 production are shown in Fig. 1. The pH of the culture broth was maintained above 6.0 by adding NaOH solution to the jar fermentor culture.

Purification

The culture broth (18 liters; activity of 110 units/ml) was centrifuged to remove mycelia. The pH of the supernatant was adjusted to 6.0 with HCl. Solid ammonium sulfate was added to the supernatant to 80% saturation to precipitate the peptide antibiotic. After holding the solution at 4°C for 18 hours, the precipitate (1.5 kg, wet weight) was harvested by centrifugation and dissolved in 2 liters of 0.02 M phosphate buffer (pH 7.2) containing 0.1 M NaCl. The antibiotic solution was dialyzed against 10 liters of the same buffer at 5°C for 48 hours. After dialysis, six liters of acetone was added to the dialysate to give 1.2 g of a precipitate which was dissolved in 200 ml of 0.02 M phosphate buffer (pH 7.2). This solution was applied to a column (1.6×40 cm) of DEAE-cellulose (Whatman DE-52). The column was then eluted with 0.02 M phosphate buffer (pH 7.2). The active fraction (about 200 ml) was dialyzed against 10 liters of 0.002 M acetate buffer (pH 5.0) at 5°C for 24 hours. The product was applied to a column of CM-cellulose (Whatman CM-32) and eluted with an increasing NaCl concentration (Fig. 2). Acetone was added to 74 ml of the active fraction to a final concentration of 75%. The precipitate was dissolved in 10 ml of water and subjected to gel filtration on a column (1.6×40 cm) of Bio-Gel P-30 using water as the eluent. The active fraction was dialyzed against distilled water at 5°C for 24 hours and then

Fig. 1. Time course of production of AN-1 by *S. albus* AJ9003.

The fermentation was carried out in a 30-liter jar fermentor. The pH was controlled with NaOH.

(●), Antibacterial activity; (○), growth (PMV stands for packed mycelial volume); (□), glucose; (△), pH.

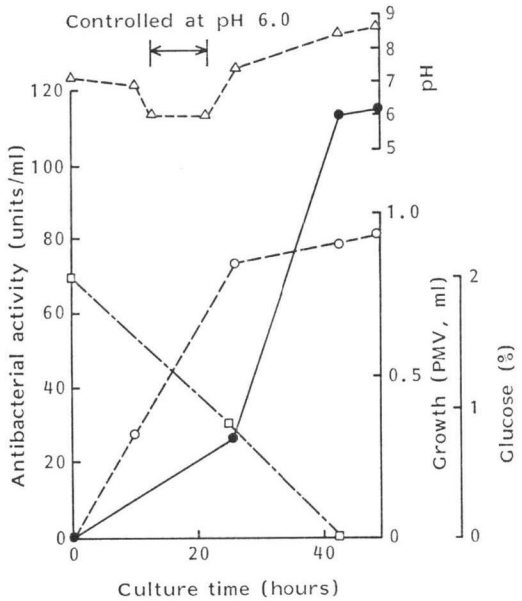


Fig. 2. Chromatography of AN-1 on a CM-cellulose column.

The active fraction obtained by chromatography on a DE-52 column was applied on a CM-32 column, 1.6×40 cm, equilibrated with 0.002 M acetate buffer, pH 5.0. Elution was effected with 0.02 M acetate buffer (pH 5.0), followed by an increasing linear NaCl concentration gradient in the same buffer.

(●), Activity; (○), optical density at 280 nm (OD_{280}).

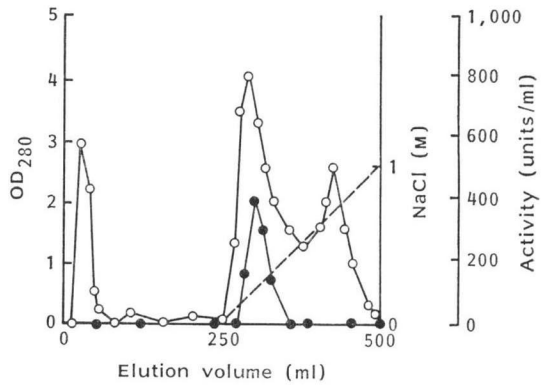
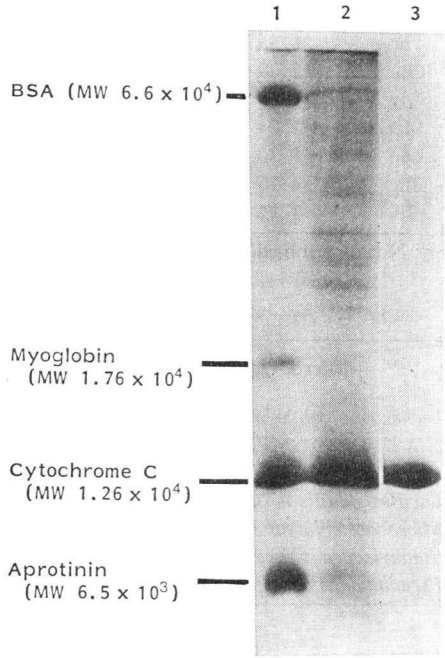


Fig. 3. SDS-polyacrylamide gel electrophoresis of AN-1.

Proteins were separated by electrophoresis on 20% acrylamide gel containing 1% SDS.

Lane 1, marker proteins; 2, precipitate with ammonium sulfate from culture supernatant; 3, purified AN-1.



lyophilized. The purified antibiotic (300 mg) had an activity of 1,160 units/mg.

The purified sample of AN-1 gave a single band on polyacrylamide gel electrophoresis, indicating its homogeneity (Fig. 3).

Physico-chemical Properties

AN-1 is a white solid that is soluble in water, but insoluble in organic solvents such as ethanol, butanol and acetone. It is positive in ninhydrin and biuret reactions, but negative in the anthrone and BLIX reactions. These results indicate the peptide nature, without sugar or aminosugar, of AN-1.

The isoelectric point of AN-1 is about pH 8.3 as determined by electrofocusing. Its molecular weight was determined to be 11,500 and 12,500 by gel filtration and SDS polyacrylamide gel electrophoresis, respectively.

Figs. 4 and 5 show the IR spectrum and UV absorption spectrum of AN-1, respectively. The UV absorption shows $E^{1\%}_{1cm}$ 10.6 at 280 nm in aqueous solution and 0.01 N HCl, and $E^{1\%}_{1cm}$ 10.8 at 280 nm in 0.01 N NaOH. Both spectra also suggested that AN-1 is a peptide.

The amino acid composition of AN-1 is shown in Table 1.

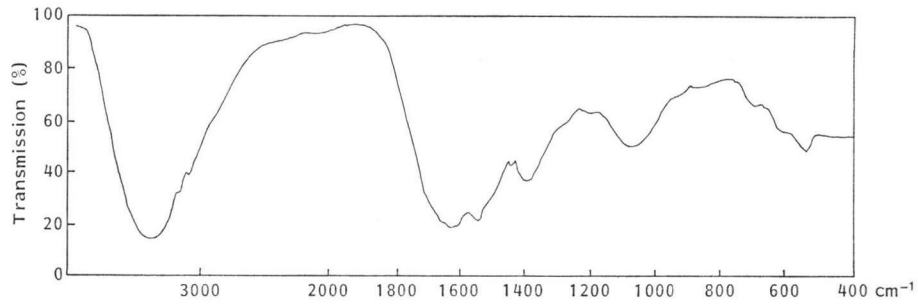
AN-1 is stable at pH 6~9, but markedly unstable at pH 2.

Biological Properties

Antibacterial Activity

The antibacterial activity of AN-1 is summarized in Table 2. AN-1 had no antibacterial activity against the Gram-positive or Gram-negative bacteria tested. On the other hand, AN-1 showed strong antibacterial activity toward MP2, a macromolecule permeable mutant, which was derived from *Escheri-*

Fig. 4. IR spectrum of AN-1 (KBr).



chia coli W3876 and toward UR3, a *uvr*A and *rec*A double mutant derived from MP2.

Mutagenic Activity

The mutagenic activity of AN-1 was quantitatively measured by the induction of valine resistant mutants[8]. AN-1 was found to be

Fig. 5. UV absorption spectrum of AN-1.

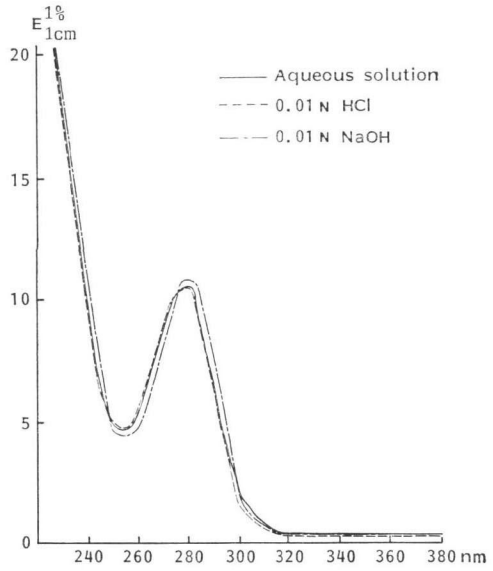


Table 1. Amino acid composition of AN-1.

Amino acid	Content (%)	Amino acid	Content (%)
Lys	5.66	Asp	10.58
Arg	5.64	Ser	5.12
Thr	6.86	Pro	4.62
Glu	12.20	Ala	8.96
Gly	6.10	Val	7.34
Met	1.04	Ile	4.18
Leu	8.30	Tyr	4.20
Phe	4.16	Cys	nd
His	1.96	Trp	nd

nd: Not determined.

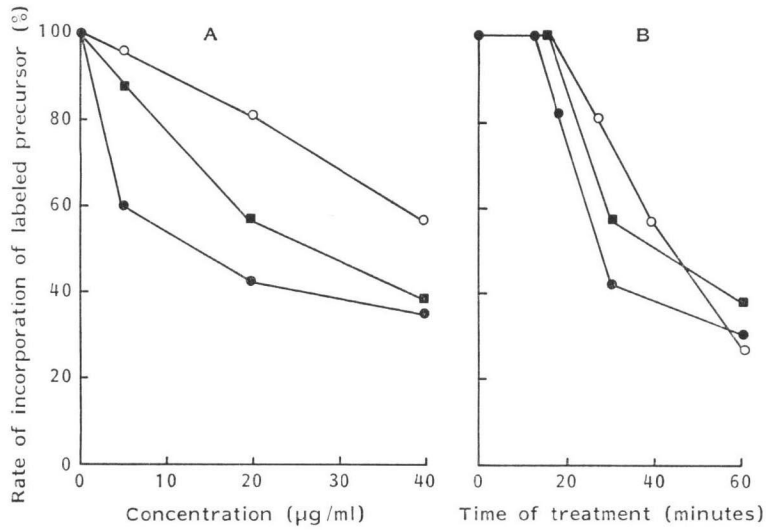
Table 2. Antibacterial spectrum of AN-1.

Microorganism	MIC (μg/ml)
Escherichia coli W3876	>100
〃 MP2	2
〃 UR3	0.3
Bacillus subtilis ATCC 6633	>100
Micrococcus luteus ATCC 9341	>100
Staphylococcus aureus FDA 209P	>100
Pseudomonas aeruginosa ATCC 10145	>100

Fig. 6. Inhibitory effect of AN-1 on incorporation of radioactive precursors into macromolecules in *E. coli* MP2.

(A) MP2 cells were incubated with the indicated concentrations of AN-1 for 30 minutes. (B) Cells were incubated for the indicated times with 20 μg/ml of AN-1. After incubation, a labeled precursor was added to the culture and the cultivation was continued. Aliquots of the culture were withdrawn and tested for the incorporation of labeled precursors as previously described[1].

(○), [^{3}H]Leucine; (■), [^{3}H]uracil; (●), [^{3}H]thymidine.



highly mutagenic (data not shown); it increased the mutation frequency a few hundred fold at the concentration of 40 μg/ml or higher.

Effect of AN-1 on Synthesis of DNA, RNA and Protein

AN-1 was examined for its effects on the synthesis of DNA, RNA and protein in MP2, using [^{3}H]-thymidine, [^{3}H]uracil and [^{3}H]leucine as their precursors. As shown in Fig. 6, DNA synthesis is inhibited by AN-1 at a lower concentration and more rapidly than the inhibition of RNA and protein syntheses. Therefore, the primary action of AN-1 appears to be on DNA synthesis.

Antitumor Activity

AN-1 was examined for antitumor activity *in vitro* and *in vivo* (Table 3). Neocarzinostatin and bleomycin served as reference antitumor agents.

Although AN-1 strongly inhibits the *in vitro* growth of L1210 cells, it had no antitumor activity against L1210 *in vivo*. AN-1 and neocarzinostatin were not inhibitory toward the *in vivo* growth of Lewis lung carcinoma in mouse, whereas bleomycin was active.

Table 3. Antitumor activity of AN-1.

Antibiotics	*In vitro*	*In vivo*			
	L1210	L1210 leukemia		Lewis lung carcinoma	
	ED_{50} (μg/ml)	Dose (mg/kg)	ILS (%)*	Dose (mg/kg)	Reduction of tumor size (%)
AN-1	0.41	1~25	na**	25	na
Neocarzinostatin	0.10	0.25	155~174	0.25	na
Bleomycin	0.85	5	na	5~10	52~68

* ILS: Increase in life span, ILS of control is 100%.

** na: Not affected.

Table 4. Comparison of properties of macromolecular peptide antibiotics.

Property	AN-1	AN-3	AN-7D	NCS*1	AUR*2	MCR*3	Sporamycin[12~14]
MW	11,500~12,500	12,000~12,500	12,400~12,500	10,700	12,500	12,500	about 10,000
Isoelectric point	pH 8.3	pH 7.6	pH 3.2	pH 3.3	pH 5.4	pH 5.4	Alkaline
Amino acid composition (amino acid(s) lacking)			Met	Met and His	Met and Arg	Met and Arg	His, Arg, Met and Pro
Chromophore	Absent ?	Absent ?	Present	Present	Present	Absent ?	Present
λ_{max} in the UV absorption (nm)	280	280	280	274 and 350	273 and 357	280	252, 258, 265, 267 and 275
Antibacterial activity	MP2	MP2	MP2 and Gram(+)	Gram(+)	Gram(+) and Gram(−)	Gram(+)	Gram(+)
Antitumor activity							
In vitro	L1210		L1210				
In vivo	?	Lewis lung carcinoma	L1210	L1210 P388	L1210 Lewis lung carcinoma	L1210 P388 Lewis lung carcinoma	L1210 P388 Meth A

*1: Neocarzinostatin[5,6,8], *2: auromomycin[9], *3: macromomycin[7,10,11].

Discussion

AN-1 is a basic, colorless polypeptide. A nonprotein chromophore could not be detected in the purified AN-1 sample by the method used for NCS and AN-7 (data not shown). AN-1 thus appears to be a new protein antibiotic with DNA interacting activities for the reasons previously discussed[1,4]. AN-1 showed a strong inhibitory effect on L1210 cells *in vitro*, but no *in vivo* antitumor activity against mouse leukemia L1210 and Lewis lung carcinoma. Both AN-7 and AN-3 have *in vivo* activity against these tumor systems.

Using a new assay system, we have shown that a variety of macromolecular antibiotics, mostly of a proteinaceous nature, are produced by Actinomycetes[4]. A summary of the physico-chemical and biological properties of the well characterized protein antibiotics found by us and others is given in Table 4. Some of these large molecular weight antibiotics show strong antitumor activities *in vivo* against only certain types of cancer cells. Therefore, our initial supposition that macromolecular antibiotics have highly specific and powerful activities seems to be justified.

Recently, many detailed studies on the mechanism of action of NCS and macromomycin have demonstrated their abilities to affect the structure and function of DNA[5~7]. Based on the knowledge of the mode of action and variability of these protein antibiotics, we may hopefully improve the methods of utilizing these antibiotics as anticancer drugs and continue to seek more useful antibiotics among microbial products.

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