THE FERMENTATION, ISOLATION AND CHARACTERIZATION OF MACROMOLECULAR PEPTIDE ANTIBIOTICS: AN-7A, -7B AND -7D

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A group of new macromolecular peptide antibiotics, named AN-7, was isolated from the culture broth of *Streptomyces griseoincarnatus* AJ9424.

AN-7 was fractionated into three different components, A, B and D. From 18 liters of culture broth (78 units/ml), 10 mg of AN-7A with a specific activity of 2,053 units/mg, 9 mg of AN-7B (1,167 units/mg) and 11 mg of AN-7D (6,225 units/mg) were obtained. All of these samples gave single bands on polyacrylamide gel electrophoresis. They are acidic polypeptides with molecular weights ranging from 12,400 to 13,000. Their UV absorption spectra showed maxima peaks at 280 nm and shoulders at 290 nm. Each AN-7 component has a nonprotein chromophoric component.

AN-7A, -7B and -7D have no antibacterial activity against the Gram-negative bacteria tested but strongly inhibit the growth of Gram-positive bacteria and *Escherichia coli* MP2, a macromolecule permeable mutant strain. The AN-7 components are mutagenic. These antibiotics inhibit the *in vitro* growth of L1210 cells (ED_{50} 0.13~0.18 μg/ml). AN-7A, -7B and -7D also inhibit the growth of L1210 cells in mice.

Macromolecular antibiotics were specifically screened by using macromolecule permeable bacteria, as reported previously[1].

Many macromolecular antibiotics can be grouped into three different types[2]. AN-1, AN-3 and AN-7 are representative antibiotics of each type. Based on studies of their modes of action, AN-1 and AN-7 were classified as antibiotics of the DNA-binding type and DNA-degrading type, respectively. AN-3 showed no apparent DNA-interacting property[2]. In the previous paper, we described fermentation, and physicochemical and biological properties of AN-3[3].

This paper deals with the isolation and characterization of the AN-7 complex. It became clear that AN-7 is a complex consisting of at least three different macromolecular antibiotics. This paper deals with the isolation and characterization of three of these components: AN-7A, AN-7B and AN-7D.

Materials and Methods

Microorganisms

Streptomyces griseoincarnatus AJ9424 was employed in this study for the production of AN-7. This strain was recently isolated and identified in our laboratory[2].

Fermentation Medium and Analyses

The fermentation medium was composed of 3% glucose, 0.5% Polypeptone, 0.5% dried yeast, 0.5% meat extract, 0.5% $(NH_4)_2SO_4$, 0.5% NaCl, 20 μg/ml $MnSO_4 \cdot 4H_2O$, 0.2% $MgSO_4 \cdot 7H_2O$ and 0.2% $CaCl_2 \cdot 2H_2O$; the pH was adjusted to 7.0 with aqueous NaOH.

Quantitative determination of antibacterial activity was done by the disk assay using UR3[1]. 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[2].

Fermentation, assaying of antitumor activity, and other analyses were carried out by methods previously described[3].

Results

Fermentation

Typical fermentation kinetics of AN-7 production are shown in Fig. 1. When necessary, NaOH solution was added jar fermentor to maintain the pH above 6.0 to ensure a good yield of AN-7.

Purification

The culture broth (18 liters) had an antibacterial activity of 78 units/ml at harvest. The mycelia was removed by centrifugation and pH of the supernatant was adjusted to 6.0 with HCl. Solid ammonium sulfate was added to the supernatant to 80 of % saturation to precipitate the antibiotic. After storing the solution at 4°C for 18 hours, the precipitate (1.2 kg wet weight) was harvested by centrifugation, dissolved in 1 liter of water, and dialyzed against running deionized water at room temperature for 48 hours. The crude AN-7 solution was chromatographed over DEAE-cellulose (Fig. 2) to yield three

Fig. 1. Time course of production of AN-7 by *S. griseoincarnatus* AJ9424.

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

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

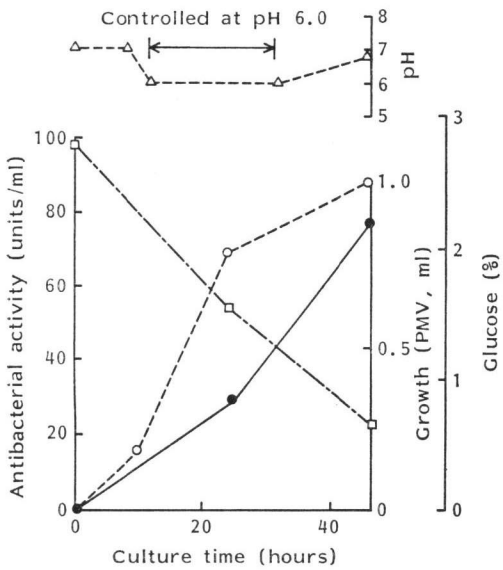
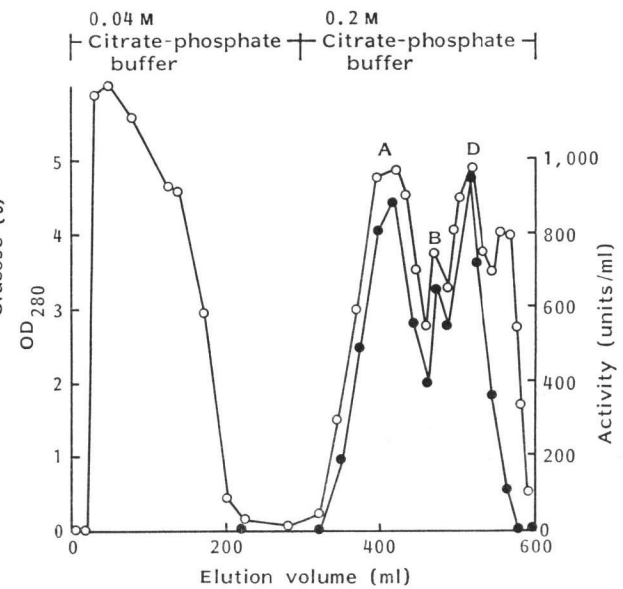


Fig. 2. Chromatography of AN-7 on a column of DEAE-cellulose.

The active fraction was applied to a Whatman DE-52 column (1.6×40 cm) which was equilibrated with 0.04 M citrate - phosphate buffer, pH 6.0. The column was washed with the same buffer and eluted with 0.2 M citrate - phosphate buffer (pH 6.0).

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



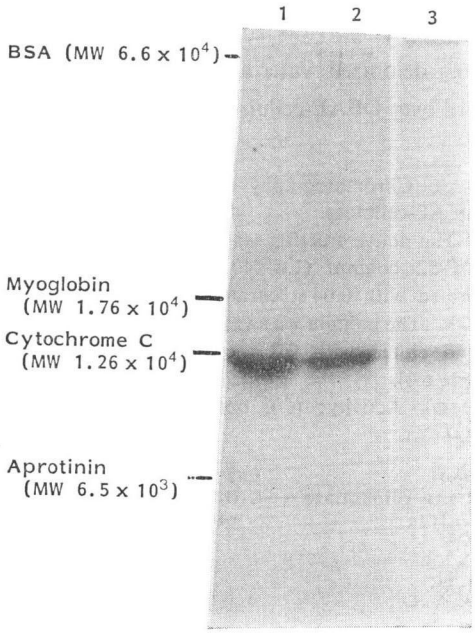
active fractions: AN-7A, -7B and -7D. Each fraction was concentrated to 5 ml, precipitated with ammonium sulfate, and the residue subjected to gel filtration of Bio-Gel P-30 (1.6×40 cm). The active fractions were dialyzed against distilled water at 5°C for 24 hours and lyophilized. Thus, three purified AN-7 components were obtained: 10 mg of AN-7A (2,053 units/mg), 9 mg of AN-7B (1,167 units/mg) and 11 mg of AN-7D (6,225 units/mg).

AN-7A, -7B and -7D each showed single bands on polyacrylamide gel electrophoresis (Fig. 3).

Fig. 3. SDS-polyacrylamide gel electrophoresis of AN-7.

Proteins were separated by electrophoresis on 20% acrylamide gel containing 1% SDS[5].

Lane 1, purified AN-7A; lane 2, purified AN-7B; lane 3, purified AN-7D.



Physico-chemical Properties

All of them were obtained as almost colorless powders and which are soluble in water, but insoluble in organic solvents such as ethanol, butanol and acetone. They are positive in ninhydrin and biuret reaction, but negative in the anthrone and BLIX[4] reactions. These results indicate that they are peptides lacking sugar and aminosugar moieties.

The isoelectric points of these antibiotics were determined to be pH 3.2 by electrofocusing.

The molecular weights of AN-7A, -7B and -7D are 12,400, 13,000 and 12,400, respectively, as measured by gel filtration with Bio-Gel P-30. SDS-polyacrylamide gel electrophoresis gave a molecular weight of 12,500 for each.

Figs. 4, 5 and 6 show the IR, UV absorption and visible absorption spectra of these antibiotics. Their sets of spectra suggested a polypeptide nature.

AN-7 was shown to have a nonprotein chromophore which could be separated from its protein component (apoprotein) by the method employed for neocarzinostatin (NCS)[6~10]. The observed antibacterial activity appeared to be associated with the chromophore but not the apoprotein (data not shown). When these were detected by bioautography employing *Escherichia*

Fig. 4. IR spectra of AN-7 (KBr).

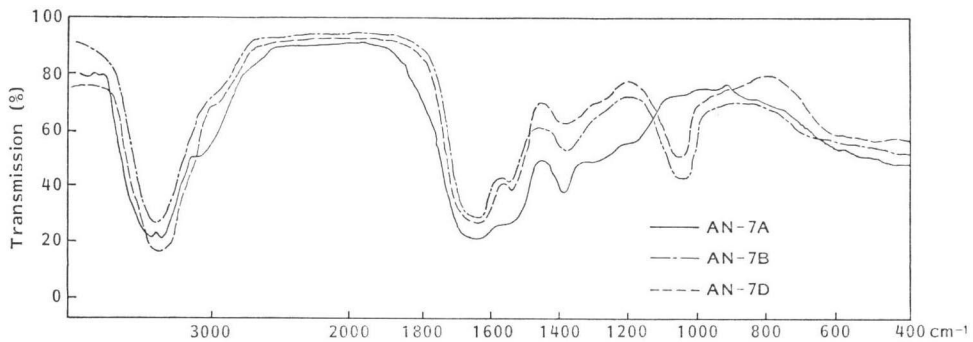


Fig. 5. UV absorption spectra of AN-7 in aqueous solution.

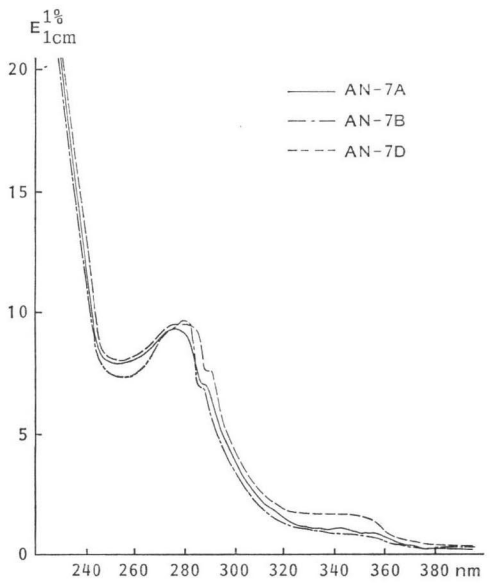


Fig. 6. UV-visible absorption spectra of AN-7D.

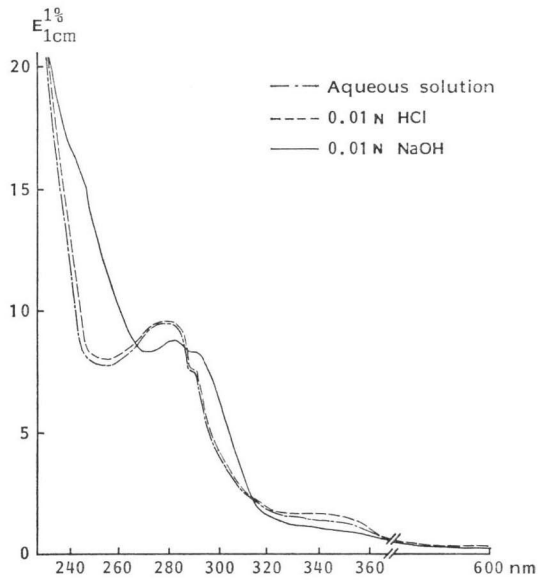


Table 1. Amino acid compositions of AN-7A, -7B and -7D.

Amino acids	Content (%)		
	A	B	D
Asp	12.8	12.2	12.0
Thr	10.4	10.5	10.1
Ser	6.6	7.3	7.2
Glu	7.8	6.6	7.1
Pro	5.0	4.1	4.6
Gly	7.4	8.0	8.1
Ala	9.8	10.8	10.3
Val	7.9	8.9	8.1
Met		Not detected	
Ile	2.3	1.8	2.2
Leu	7.3	6.5	6.6
Tyr	3.2	0.9	1.9
Phe	5.2	5.5	5.4
Lys	4.0	2.0	2.0
His	1.3	0.7	0.9
Arg	5.7	4.4	4.0
Cys	—*	—	2.4
Trp	—	—	3.5

* Not determined.

coli UR3, Rf values of chromophore of AN-7A, -7B and -7D on a silica gel thin-layer plate developed with chloroform - methanol (1 : 1) were 0.70, 0.62 and 0.26. Their UV absorption spectra showed maxima at 275, 283, 290, 300, 332, 366 and 390 nm. Therefore, their chemical structures appear to be different but resemble each other.

The amino acid composition of the AN-7 components is illustrated in Table 1. Most notable is the lack of methionine in these antibiotics.

Biological Activity

Antibacterial Activity

Antibacterial activities of AN-7 and NCS (reference compound) are summarized in Table 2. The AN-7 antibiotics showed no antibacterial activity against Gram-negative bacteria, but were inhibitory to Gram-positive bacteria, MP2 (a macromolecule permeable mutant of *E. coli* W3876) and UR3 (a *uvr*A and *rec*A double mutant derived from MP2). AN-7D has similar antibacterial activity as compared to NCS (Table 2).

Mutagenic Activity

The mutagenic activity of AN-7 was examined by an assay to quantitatively measure the induction of valine resistant mutants. AN-7A, -7B and -7D were clearly mutagenic as shown in Fig. 7. AN-7

Table 2. Antibacterial activity of AN-7A, -7B and -7D.

Microorganism	Minimum inhibitory concentration (μg/ml)			
	AN-7A	AN-7B	AN-7D	NCS
E. coli W3876	>100	>100	>100	>100
〃 MP2	8	10	4	2
〃 UR3	1.5	2	0.3	0.14
Bacillus subtilis ATCC 6633	2	3	3	15
Micrococcus luteus ATCC 9341	1	1	1	2
Staphylococcus aureus FDA 209P	100	>300	20	10
Pseudomonas aeruginosa ATCC 10145	>100	>100	>100	>100

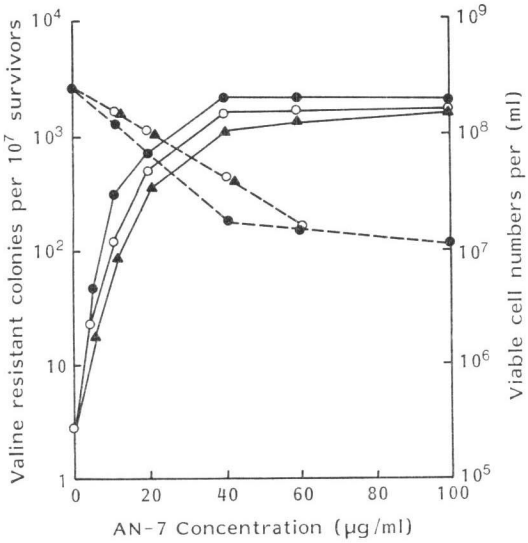
Table 3. Antitumor activity of AN-7A, -7B and -7D.

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-7A	0.15	25	125~234	25	na
AN-7B	0.18	25	125~177	25	na
AN-7D	0.13	2.5~50	170~300	25	na
NCS	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.

Fig. 7. Mutagenic activities of AN-7.

Mutagenic activity was measured by the appearance of valine resistant colonies to the concentration of AN-7 during incubation (abscissa)[8].
(○), AN-7A; (▲), AN-7B; (●), AN-7D; (——), valine resistant colonies; (------), viable cell number.



increased the mutation frequency a few hundred fold at a concentration of 40 μg/ml or higher.

Effect of AN-7 on Synthesis of DNA, RNA and Protein

The effect of AN-7 on the synthesis of cellular macromolecules was determined by their effect on the incorporation of radiolabelled precursors into macromolecules of MP2. As indicated in Fig. 8, incorporation of [^{3}H]thymidine into DNA was markedly inhibited at the concentration of 5 μg/ml; under similar conditions RNA and protein synthesis were not affected. Thus the primary action of AN-7D appears to be inhibition of DNA syntheses. Similar results were obtained for AN-7A and -7B.

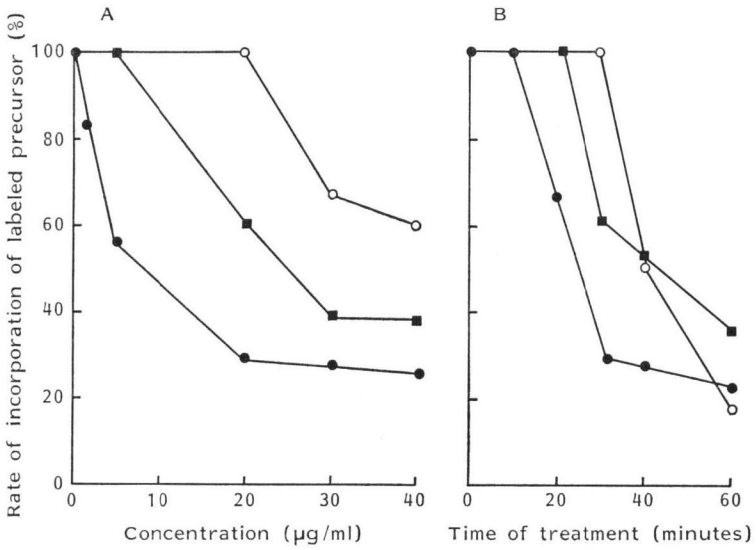
Antitumor Activity

Antitumor activities of AN-7 were examined *in vitro* and *in vivo*. Neocarzinostatin and bleomycin were also tested as reference antitumor agents.

Fig. 8. Inhibitory effect of AN-7D on the incorporation of radiolabeled precursors into macromolecules of *E. coli* MP2.

(A) Cells were incubated at the indicated concentrations of AN-7D for 30 minutes. (B) Cells were incubated for the indicated times with 20 μg/ml of AN-7D. After incubation and washing of cells, labeled precursor was added and cultivation continued. Aliquots of cultures were withdrawn and the incorporation of labeled precursors measured as previously described[8].

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



Each of the peptide antibiotics tested was inhibitory toward L1210 leukemia cells *in vitro*. AN-7A, -7B, -7D and neocarzinostatin were active *in vivo*, whereas bleomycin was not. The inverse pattern of activity was observed in the murine Lewis lung carcinoma (Table 3).

Discussion

AN-7A, -7B and -7D are acidic, colorless polypeptides and are different from many known macromolecular antibiotic[8]. Several properties of AN-7 are similar to those of NCS. Both the AN-7 components and NCS are composed of an acidic polypeptide with similar molecular weights and a non-protein chromophore.

However, AN-7D, the most well characterized antibiotic in the AN-7 group, differs from NCS in the following points; the isoelectric point (Ip of AN-7D and NCS were 3.2 and 3.3, respectively), lack of histidine in NCS, and the chromophoric color (chromophores of AN-7D and NCS were pink and yellow, respectively). Furthermore, both antibiotics strongly inhibited the growth of Gram-positive bacteria and L1210 cells, although AN-7D was much less toxic toward mammalian cells (Table 3).

AN-7A and -7B are similar to AN-7D, but differ in elution profile on DEAE-cellulose, IR absorption, and MIC against various bacteria (Figs. 2, 4, Table 2). These antibiotics differ from NCS in the same manner as AN-7D. Therefore, the AN-7 antibiotics are novel macromolecular antibiotics.

Determination of the amino acid sequence of the apoprotein and the structure of the AN-7 chromophore is in progress. Additional efforts are under way to clone the gene encoding AN-7.

Acknowledgments

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