CHARACTERIZATION AND ACTIVE FRAGMENT OF PHENOMYCIN, AN ANTITUMOR POLYPEPTIDE ANTIBIOTIC

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The amino acid composition and molecular weight of phenomycin and degradation studies are reported in this paper. By STEIN-MOORE's and ultraviolet absorption methods, the molar ratio of amino acids in phenomycin were determined. The N-terminal amino acid is aspartic acid or asparagine. The C-terminal amino acid, determined with carboxypeptidase A and by hydrazinolysis, is tryptophan. The molecular weight is estimated to be about 10,000 by ARCHIBALD's method and from the amino acid analysis. The presence of D-amino acids in phenomycin is not certain. Proteolytic enzymes, pepsin, trypsin, chymotrypsin, pronase, nagarse and papain, digest and inactivate phenomycin. One of the fragments obtained by cyanogen bromide cleavage inhibits protein synthesis in a cell-free system of EHRLICH carcinoma cells almost as effectively as phenomycin.

Phenomycin is a basic polypetide antibiotic which is produced by *Streptomyces fervens* var. *phenomyceticus*[1]. The antibiotic exhibits significant inhibitory effect on the growth of EHRLICH carcinoma, sarcoma 180 (both ascitic and subcutaneous solid forms), and adenocarcinoma 755 in mice[2], but shows no activity against Gram-positive and negative bacteria at a concentration of 100 mcg/ml[1]. Phenomycin inhibits protein synthesis in tumor cells[3].

The production, purification and preliminary characterization of phenomycin have been reported in a previous paper[1]. Further characterization and degradation studies are now presented.

The molar ratio of amino acids in phenomycin, estimated from amino acid analysis and tryptophan analysis, is as follows:

Aspartic acid	10 (or 11)	Alanine	19	Phenylalanine	1
Threonine	7	Valine	3	Tryptophan	1
Serine	7 (or 8)	Methionine	2	Lysine	14
Glutamic acid	3 (or 4)	Isoleucine	4	Histidine	3 (or 4)
Proline	4	Leucine	4	Arginine	5
Glycine	4 (or 5)	Tyrosine	3		

This was calculated assuming one phenylalanine per molecule. On the basis of this amino acid composition, phenomycin has a molecular weight of 10,000~10,500. This value conforms with that obtained by ultracentrifugation analysis.

Phenomycin was dinitrophenylated, and after hydrolysis dinitrophenylaspartic acid was identified. Therefore, the N-terminal residue of phenomycin must be aspartic acid or asparagine. When ether-extracted aqueous part of the hydrolysate of

Fig. 1. Oxidation by D-glutamic-aspartic oxidase.

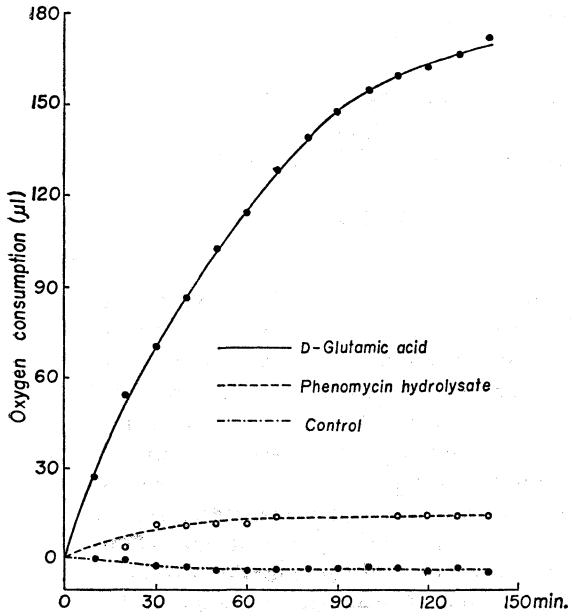


Fig. 2. Oxidation by non-specific D-amino acid oxidase.

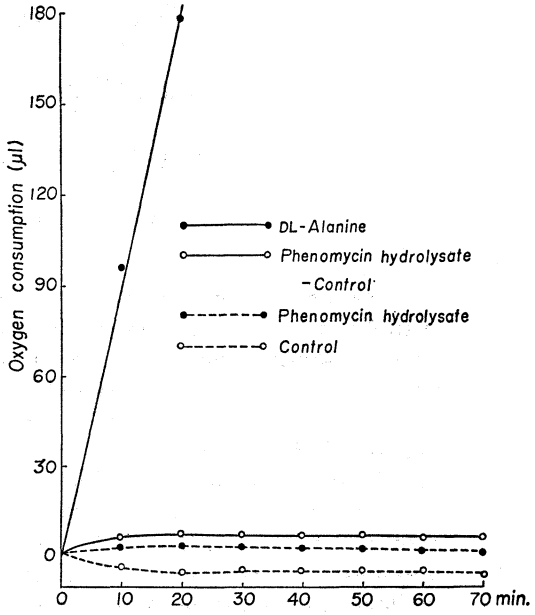
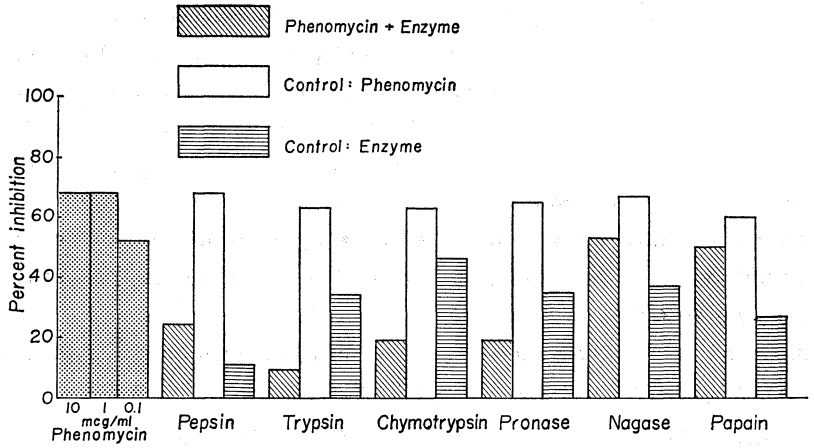


Fig. 3. Digestion by proteolytic enzymes.



dinitrophenylated phenomycin was subjected to amino acid analysis, amino acids were found in the following molar ratio (tryptophan was not determined):

Aspartic acid	11	Alanine	18	Phenylalanine	1
Threonine	7	Valine	3	Lysine	0.5
Serine	8	Methionine	1.5	Histidine	0
Glutamic acid	3 (or 4)	Isoleucine	4	Arginine	5
Proline	4	Leucine	4		
Glycine	4.5	Tyrosine	0		

Substantially no lysine remained in the hydrolysate of dinitrophenylated phenomycin and it indicates no branching from lysine in phenomycin. The small amount

(less than one mole) of lysine might be resulted from incomplete reactivity due to steric hindrance, or from the rapid decrease in solubility at the first stage of dinitrophenylation. The hydroxyl group of tyrosine and the imidazole imino group of histidine must be free in phenomycin because they were dinitrophenylated. The C-terminal determined by hydrazinolysis was tryptophan. The C-terminal tryptophan was liberated by carboxypeptidase A, treatment with carboxypeptidase A also liberated alanine, threonine, glycine, asparagine, and glutamic acid, as minor products.

The acid hydrolysate of phenomycin was subjected to oxidation by D-glutamic-aspartic oxidase and non-specific D-amino acid oxidase. As shown in Figs. 1 and 2, during the oxidation oxygen corresponding to one or two moles of D-amino acid was consumed. However, amino acid analysis of the hydrolysate of phenomycin after enzymatic oxidation showed no distinct decrease in any amino acid. Therefore, it is uncertain whether phenomycin contains D-amino acids.

Incubation of phenomycin with trypsin, chymotrypsin, pepsin or pronase reduced the activity when the reaction mixture was tested for its ability to inhibit protein synthesis in a cell-free system of EHRLICH carcinoma cells. Though the enzymes themselves showed inhibition of protein synthesis, as shown in Fig. 3, the experiment suggested that the activity of phenomycin was reduced by treatment with these enzymes. As seen in the same figure, nagarse and papain did not reduce the activity of phenomycin.

Thus, the enzymatic fragmentation of phenomycin to yield an active fragment was unsuccessful. However, phenomycin contains two moles of methionine and cyanogen bromide cleavage yielded an active fragment. Five fragments were obtained by cyanogen bromide cleavage. Two of them are thought to be the result of incomplete cleavage, since only three fragments should have been produced if cleavage were complete. These five fragments were separated by gel filtration. The main fragment showed the same activity as phenomycin in inhibiting protein synthesis in a cell-free system of EHRLICH carcinoma cells. The other fragments showed lower activity. Amino acid analysis after acid hydrolysis of this fragment gave the following results:

Aspartic acid	10	Alanine	18	Phenylalanine	1
Threonine	6	Valine	3	Lysine	11
Serine	8	Methionine	0	Histidine	4
Glutamic acid	4	Isoleucine	3	Arginine	4 (or 5)
Proline	2	Leucine	4		
Glycine	3 (or 4)	Tyrosine	3		

Phenomycin differs from antimicrobial peptide antibiotics obtained from Actinomycetes, in the following respects: (1) The N-terminal α-amino and C-terminal α-carboxyl groups of phenomycin are free; (2) phenomycin is digested by animal and plant proteolytic enzymes, losing its biological activity; (3) no novel amino acid can be detected by STEIN-MOORE's method in phenomycin. Most peptide antibiotics which have antimicrobial activity contain novel or D-amino acids, have a cyclic structure or no free terminal amino acid if linear, and are not susceptible to animal

or plant proteolytic enzymes (except polymixins which can be digested by bacterial proteinase such as nagarse[4]).

Experimental

Materials: Phenomycin used in these experiments was prepared according to the method described in a previous paper[1]. Its purity was examined by cellulose acetate film and disc electrophoresis, ultracentrifugation and N-terminal amino acid analysis[1].

Carboxypeptidase A, pepsin, trypsin and chymotrypsin were purchased from Sigma Chemical Company, U.S.A., pronase from Kaken Chemical, Tokyo, and nagarse from Nagase Company, Osaka. Two kinds of D-amino acid oxidase are gifts of Dr. MIZUSHIMA of the Institute of Applied Microbiology, the University of Tokyo. Bio-Gel was obtained from Calbiochem, U.S.A.

Amino Acid Analysis: Two mg of phenomycin was hydrolyzed with 2 ml of constant boiling HCl in a sealed and evacuated tube at 110°C for 24 hours. The hydrolysate was evaporated to dryness *in vacuo* and left overnight in a desiccator over NaOH. The residue was applied to a Hitachi amino acid analyzer type KLA-3B. Tryptophan and tyrosine were determined by ultraviolet absorption method[5]. The optical density of phenomycin was measured at a concentration of 200 mcg/ml in 0.1 N NaOH at 280 mμ and 294.4 mμ by Shimadzu Spectrophotometer QV-50. To correct the O. D. value at these wave lengths, the O. D. value was also measured at 370 and 340 mμ and contents of tryptophan and tyrosine were calculated by using the given molar extinction coefficients[5]. The value for methionine is supported by the results of elemental analysis[1]. No cystine was found by the STEIN-MOORE's method.

N-Terminal Amino Acid Analysis: Dinitrophenyl phenomycin was prepared by the usual method[6]. Phenomycin (10 mg) was dissolved in 1 ml of H_2O with 10.0 mg of $NaHCO_3$ and to this was added 2 ml of a 7.5 % ethanolic solution of 1-fluoro-2,4-dinitrobenzene (FDNB). The reaction mixture was stirred for 4 hours on a magnetic stirrer in the dark. Then 2 ml of H_2O was added and the mixture extracted five times with ether at room temperature to remove excess FDNB. The DNP-antibiotic was precipitated by addition of 3 drops of concentrated HCl and the suspension again extracted with ether to remove dinitrophenol. The residue was evaporated to dryness and 1.8 ml of constant boiling HCl added. The material was hydrolyzed in a sealed and evacuated tube at 105°C for 16 hours. Water (4 ml) was added to the hydrolysate and the DNP-amino acid extracted five times with ether and washed with H_2O. The extract was examined by paper chromatography using the *tert*-amyl alcohol-pathalate buffer (pH 5.0) system of BLACKBURN and LOWTHER[7]. The residual water layer was examined with a Hitachi amino acid analyzer type KLA-3B. The DNP-amino acid extracted with ether was identified as DNP-aspartic acid by paper chromatography.

C-Terminal Amino Acid Analysis by Carboxypeptidase A: Phenomycin (5 mg) was dissolved in 1 ml of H_2O and the pH adjusted to 7.5 with 0.1 N NaOH. To this was added 0.5 ml of a carboxypeptidase A solution which was prepared according to GLADNER and NEURATH[8]. The reaction mixture was adjusted to pH 7.5 with 0.1 N NaOH and incubated at 25°C for 12 hours with shaking. After incubation, 2 mg of $NaHCO_3$ and 0.6 ml of 5 % ethanolic solution of FDNB were added to the reaction mixture and stirred at 25°C for 2 hours in the dark. The DNP-amino acids were extracted by the usual method and identified as tryptophan by paper and thin-layer chromatographies. DNP-alanine, threonine, glycine, asparagine and glutamic acid were also detected as minor products.

C-Terminal Amino Acid Analysis by Hydrazinolysis[9]: Phenomycin (10 mg) was dissolved in 0.5 ml of hydrazine and left at 100°C for 8 hours in a sealed tube. After hydrazinolysis, excess hydrazine was removed *in vacuo* in a desiccator over H_2SO_4. The dried reaction mixture was dissolved in 3 ml of H_2O and 0.6 ml of benzaldehyde was added. The solution was stirred at room temperature for 2 hours. Excess benzaldehyde

and the resulting hydrazones were extracted five times with ether and the aqueous phase dried in a desiccator. The liberated amino acid was dissolved in 5 ml of H_2O and 150 mg of $NaHCO_3$, then 0.15 ml of FDNB and 10 ml of ethanol added. The reaction mixture was stirred at room temperature for 2 hours in the dark[9]. The DNP-amino acid was extracted by the usual method and identified as tryptophan as in the experiment with carboxypeptidase A. In this case, no other DNP-amino acid was detected.

Molecular Weight Determination by ARCHIBALD's Method[10]: Phenomycin (10 mg) was dissolved in 1 ml of 0.05 M tris buffer (pH 7.30; $\mu=0.1$) and centrifuged at 40,400 rpm. From the sedimentation pattern, the molecular weight of phenomycin was calculated as 9,900 using the following constants: partial specific volume=0.75 and density=1.00.

D-Amino Acid Assay: The acid hydrolysate from 3 mg of phenomycin was dissolved in 0.2 ml of a borate-phosphate buffer (pH 8.0) and the pH adjusted to 8.0 with 1 N NaOH. The solution was incubated with D-glutamic-aspartic oxidase at 30°C in WARBURG's apparatus and oxygen consumption measured manometrically[11]. For non-specific D-amino acid oxidase, 0.01 M pyrophosphate buffer (pH 8.30) was used and oxygen consumption measured by the same method. As shown in Figs. 1 and 2, both D-amino acid oxidases consumed oxygen corresponding to the amount which expected if 1~2 moles of D-amino acids were present in phenomycin. No distinct decrease of aspartic acid, glutamic acid and other amino acids were detected by amino acid analysis after the digestion.

Digestion by Proteolytic Enzymes: Phenomycin was incubated with trypsin or chymotrypsin at pH 7.5 (pH was adjusted with 5 % $NaHCO_3$), pronase at pH 7.5 in 0.1 M phosphate buffer, nagarse at pH 7.1 in SORENSEN's phosphate buffer or pepsin at pH 2.0 of 0.01 N HCl at 37°C for 24 hours. The ratio of phenomycin and these enzymes was 100 : 1 by weight. For digestion by papain, 1 % NaCN and 1/10 weight of papain was used. Fig. 3 shows that phenomycin was inactivated by treatment with proteolytic enzymes. Pepsin, trypsin, chymotrypsin or pronase decreased the activity to less than one hundredth. Nagarse and papain digestion caused a smaller loss of activity than the other enzymes. The enzymes themselves showed considerable inhibitory activity.

Cyanogen Bromide Cleavage[12]: Phenomycin (60 mg) was dissolved in 6 ml of a CNBr solution (9.27 mg/ml 0.1 N HCl) and incubated at 25°C for 24 hours. The reaction mixture was lyophilized and the residue separated and purified by gel filtration using Bio-Gel P-6 column of 1.5 cm × 43 cm. The reaction mixture gave five ninhydrin-positive spots on a paper chromatogram (*n*-BuOH - pyridine - AcOH - H_2O, 15 : 10 : 3 : 12) at Rf=0.023, 0.14, 0.23, 0.34 and 0.61. The main component (Rf=0.023) was recovered from fractions 34 and 35 (volume of each fraction was 2 ml) by lyophilization. This fraction was shown to be also a basic peptide by cellulose acetate film electrophoresis using pH 7.0 buffer (0.01 M phosphate and 0.1 M NaCl). It moved 1.7 cm to the cathode within one hour when phenomycin moved 2.2 cm (2 mA/cm). This fragment showed almost equal activity to phenomycin at the same concentration, whereas other fractions showed very low activity. Amino acid analysis of the main component showed that this component had lost about 10 amino acids.

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