# COMPLETE AMINO ACID SEQUENCE OF PHENOMYCIN, AN ANTITUMOR POLYPEPTIDE ANTIBIOTIC

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The primary structuture of phenomycin, an antitumor polypeptide antibiotic isolated from the culture broth of *Streptomyces fervens* var. *phenomyceticus*, was determined. Sequence analysis has revealed that it consists of 89 amino acid residues ($M_r$ 9,524) and no disulfide bridge is present. The sequence of phenomycin at residues 48~70 was found to be hydrophilic, being surrounded by hydrophobic regions at both sides. Furthermore, clustering of half the lysine residues in this hydrophilic region and marked basicity of phenomycin imply the external disposition of this region. Phenomycin does not show significant sequence homology to any known proteins including antitumor polypeptide antibiotics.

Phenomycin, a basic polypeptide antitumor antibiotic, was isolated from the culture broth of *Streptomyces fervens* var. *phenomyceticus* by NAKAMURA *et al.*[1]. It has a significant inhibitory effect on the growth of Ehlrich carcinoma, sarcoma 180 and adenocarcinoma 755 in mice[2]. It shows no antibacterial activity against Gram-positive and Gram-negative bacteria in the vicinity of 100 $\mu$g/ml[1]. The biological features of phenomycin are the inhibition of protein synthesis in turmor cells[1] and the inhibition of initiation of globin synthesis in rabbit cell-free system[3]. In the previous studies, the detailed physico-chemical characterization revealed that it is composed of a single polypeptide chain of $M_r$ 10,000 with either asparatic acid or asparagine as an amino terminus and tryptophan as a carboxyl terminus[4].

Here we will describe the complete amino acid sequence of phenomycin determined by enzymatic degradation and microsequencing of the resulting peptide fragments. The sequence studies, which depend on Edman degradation chemistry, are adapted to nanomolar amounts of samples.

## Materials and Methods

### Materials

Trypsin (Type III) and protease from *Staphylococcus aureus* strain V8 (Type XVII) were purchased from Sigma Chemical Co. Carboxypeptidase A was purchased from Worthington Biochemical Co.

### Production of Phenomycin

*S. fervens* var. *phenomyceticus* was cultured in a 500-ml Erlenmeyer flask containing 100 ml of medium consisting of starch 1.0%, peptone 1.0%, meat extract 1.0% and NaCl 0.5% (pH 7.4) at 27°C for 72 hours on a rotary shake at 200 rpm.

### Isolation of Phenomycin

The broth filtrate was treated with a cation exchange resin Amberlite IRC-50 (Na-type) according to

the previous method[1] with some modifications. Briefly, phenomycin was retained on the Amberlite IRC-50 column from the broth filtrate. After the column was completely washed with distilled water, phenomycin was eluted with 1.0 N HCl. The peak fractions with respect to antitumor activity *in vitro*, assayed using murine lymphoblastoma L5178Y cells, were pooled, dialyzed against distilled water and lyophilized. The partially purified powder, thus obtained, was further purified by reverse-phase (RP)-HPLC using a column (ODS-120T, 4×250 mm, Tosoh) and an aqueous solution containing 0.1% TFA and 30% acetonitrile ($CH_3CN$) as a mobile phase. The portion containing purified phenomycin was concentrated *in vacuo* to remove $CH_3CN$ and lyophilized.

Protease Digestion

The purified phenomycin (30 μg) was treated with trypsin in 0.1 M ammonium bicarbonate buffer (pH 8.2) at 37°C for 4 hours. The enzyme-substrate ratio was 1 : 50 (w/w). After the reaction was completed, the peptide fragments were separated by RP-HPLC with a gradient of $CH_3CN$ in distilled water containing 0.1% TFA. The digestion with V8 protease (enzyme-substrate ratio, 1 : 50 (w/w)) was carried out in 0.1 M ammonium bicarbonate buffer (pH 8.2) at 37°C for 48 hours.

Amino Acid Sequence Determination

Phenomycin and the peptide fragments obtained by the enzymatic degradation were sequenced using an automated gas-phase sequencer (Applied Biosystems, model 470A). Anilinothiazolinones were converted to phenylthiohydantoins (PTH) and the PTH-amino acids recovered from each cycle of Edman degradation were identified by RP-HPLC over a Brownlee C-18 column (2.1×220 mm) at 55°C. The solvent and gradient systems were as follows. Solvents: (A) 5% THF, 0.6% acetic acid, 0.00625% trimethylamine, pH 4.0; (B) $CH_3CN$. Linear gradient of solvent A from 88 to 61% in 18 minutes at 0.2-ml/minute flow rate followed by 61% solvent A for an additional 7 minutes at the same flow rate was employed.

*C*-Terminal Analysis

A suspension of carboxypeptidase A (treated with phenylmethylsulfonyl fluoride) in distilled water was centrifuged at 5,000 rpm for 1 minute and the supernatant was removed to eliminate free amino acids. The precipitated carboxypeptidase A was dissolved in 0.2 M *N*-ethylmorpholine buffer, pH 8.4, containing 10 μg/ml phenomycin. The enzyme-substrate ratio was 1 : 10 (mol/mol) and the digestion was performed at 37°C for 16 hours. The released amino acids were determined by RP-HPLC using a column (Ultrasphere ODS-DABS, 4.6×250 mm, Beckman) after being derivatized dimethylaminoazobenzene-4′-sufonyl amino acids[5,6].

Amino Acid Analysis

Phenomycin (25 μg) was hydrolyzed with 6 N HCl containing 1% phenol in the Millipore Picotag system at 110°C for 18 hours. The hydrolysates were dried and analyzed on a Beckman model 7300 amino acid analyzer.

## Results

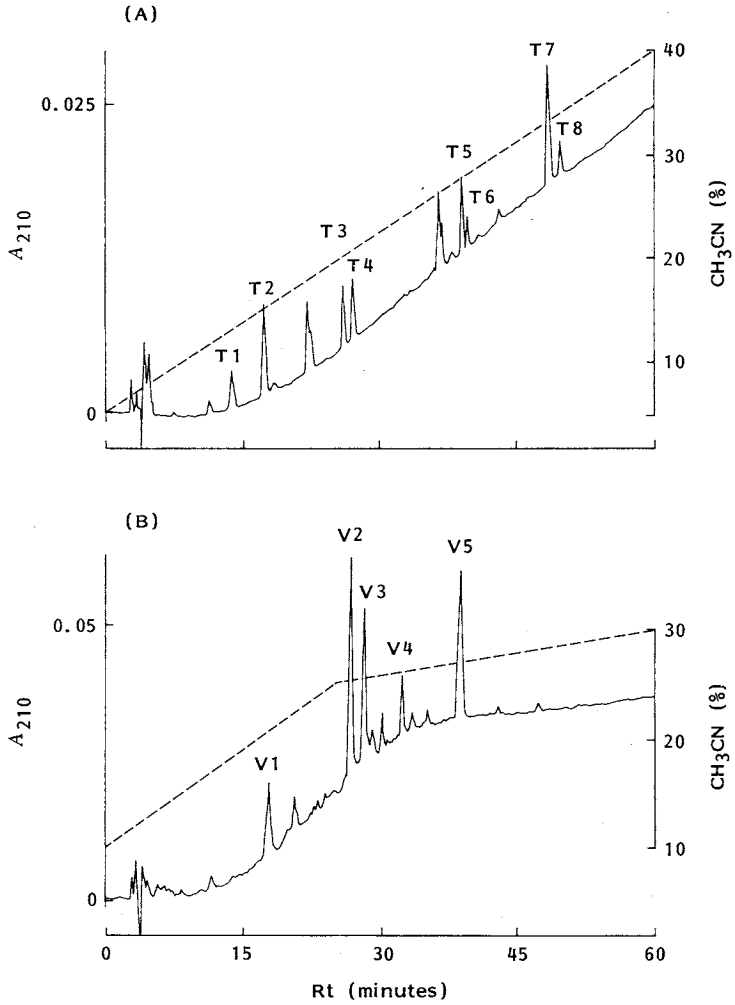
### Isolation and Sequencing of Phenomycin Peptide Fragments

The purified phenomycin was digested with trypsin or V8 protease and the peptide fragments produced were separated by RP-HPLC. As shown in Fig. 1, we obtained eight tryptic fragments and five V8 fragments designated T1 to T8 and V1 to V5, respectively. Table 1 illustrates the sequencing data of phenomycin and the peptide fragments. The automated Edman degradations of phenomycin revealed its *N*-terminal amino acid sequence up to 34 residues, which in turn allowed the alignment of peptide fragments T3, T2 and V2.

### Amino Acid Sequence of Phenomycin

The alignment of the sequenced peptide fragments of phenomycin is shown in Fig. 2. Although the

Fig. 1. Isolation of the peptide fragments generated by proteinase digestion.




(A) Peptides generated by trypsin digestion were separated by RP-HPLC with a TSK-gel (ODS-120T, 4×250 mm, Tosoh) using a linear gradient of 5 to 40% $CH_3CN$ in 0.1% TFA in 60 minutes at a flow rate of 1 ml/minute. The peaks collected for the amino acid sequence analysis were designated T1 to T8 in an elution order. (B) Peptides generated by *Staphylococcus aureus* V8 digestion were separated with the same column as trypsin fragments using linear gradients of 10 to 25 and 25 to 30% $CH_3CN$ in 0.1% TFA in 25 and additional 35 minutes, respectively, at a flow rate of 1 ml/minute. The peaks subsequently subjected to the amino acid sequence analysis were designated V1 to V5.

residue at position 36 could not be identified by the results of fragment T8 sequence analysis, it was determined as asparatic acid from the results for fragment V2. Carboxypeptidase A released tryptophan (0.39 mol/mol protein) and threonine (0.06 mol/mol protein) after 16-hour digestion, indicating the *C*-terminal sequence to be -Thr-Trp. This result is compatible with the previous findings[4] that the *C*-terminal amino acid is tryptophan. No microheterogeneity was observed in the sequence of phenomycin.

The amino acid composition of phenomycin is shown in Table 2. The number of residues of each amino acid obtained from amino acid analysis was in good agreement with that calculated on basis of amino acid sequence.

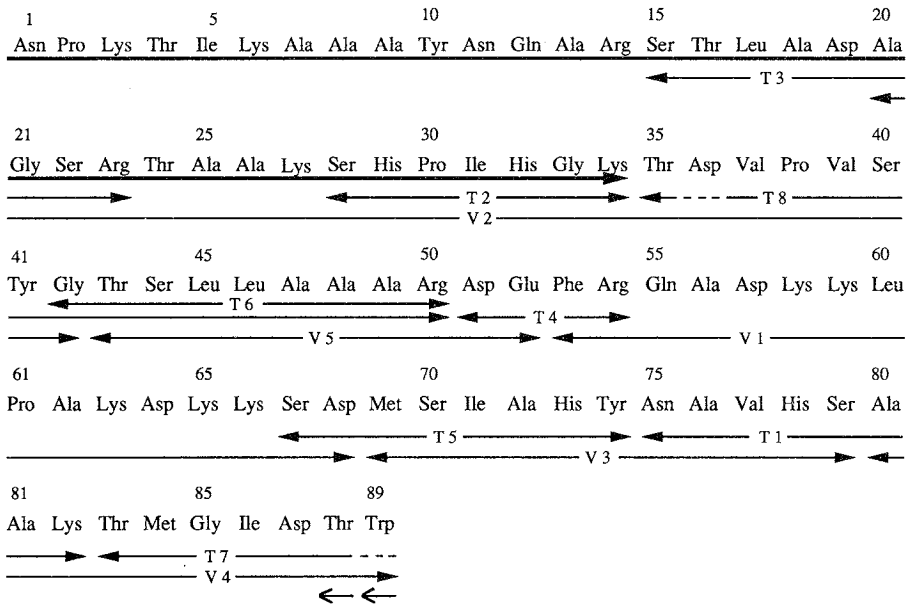
Table 1. Edman amino acid sequencing of phenomycin.

| Peptide denomination | Sequence results |
|---|---|
| T1 | Asn-Ala-Val-His-Ser-Ala-Ala-Lys<br>78 120 148 20 50 93 112 27 |
| T2 | Ser-His-Pro-Ile-His-Gly-Lys<br>219 29 212 231 20 304 124 |
| T3 | Ser-Thr-Leu-Ala-Asp-Ala-Gly-Ser-Arg<br>286 112 502 203 70 370 326 135 63 |
| T4 | Asp-Glu-Phe-Arg<br>133 182 574 26 |
| T5 | Ser-Asp-Met-Ser-Ile-Ala-His-Tyr<br>162 52 241 66 190 126 11 29 |
| T6 | Gly-Thr-Ser-Leu-Leu-Ala-Ala-Ala-Arg<br>132 29 61 113 127 98 92 104 12 |
| T7 | Thr-Met-Gly-Ile-Asp-Thr<br>46 182 109 253 21 37 |
| T8 | Thr-ND-Val-Pro-Val-Ser-Tyr-Gly-Thr-Ser-Leu-Leu-Ala-Ala-Ala-Arg<br>5 8 9 9 2 1 3 1 1 3 3 1 1 1 trace |
| V1 | Phe-Arg-Gln-Ala-Asp-Lys-Lys-Leu-Pro-Ala-Lys-Asp-Lys-Lys-Ser-Asp<br>796 32 214 532 63 243 454 550 139 370 178 44 169 172 136 10 |
| V2 | Ala-Gly-Ser-Arg-Thr-Ala-Ala-Lys-Ser-His-Pro-Ile-His-Gly-Lys-Thr-Asp-Val-<br>866 480 375 23 66 70 70 105 144 49 239 122 36 222 134 34 109 191<br>Pro-Val-Ser-Tyr-Gly<br>105 182 63 71 30 |
| V3 | Met-Ser-Ile-Ala-His-Tyr-Asn-Ala-Val-His-Ser<br>769 395 692 643 14 201 111 335 395 3 76 |
| V4 | Ala-Ala-Lys-Thr-Met-Gly-Ile-Asp-Thr-Trp<br>533 506 294 79 498 338 487 87 53 30 |
| V5 | Thr-Ser-Leu-Leu-Ala-Ala-Ala-Arg-Asp-Glu<br>225 528 836 945 856 981 960 575 424 153 |
| *N*-Term | Asn-Pro-Lys-Thr-Ile-Lys-Ala-Ala-Ala-Tyr-Asn-Gln-Ala-Arg-Ser-Thr-Leu-Ala-<br>87 112 80 27 201 132 205 262 256 56 55 73 195 13 101 17 131 137<br>Asp-Ala-Gly-Ser-Arg-Thr-Ala-Ala-Lys-Ser-His-Pro-Ile-His-Gly-Lys-<br>11 160 74 51 11 7 74 96 31 27 1 15 29 2 22 23 |

The yield of PTH-amino acid at each cycle is shown below the residue in picomoles.
*N*-Term: Sequencing of the *N*-terminal end of phenomycin.
ND: No detection of PTH-amino acid at the cycle.

Fig. 2. Summary of sequencing studies on phenomycin produced by *Streptomyces fervens* var. *phenomyceticus*.

⟶: *N*-Terminus of the protein, ⟷: cleaved peptides (T, tryptic peptides; V, staphylococcal protease-cleaved peptides), ←: carboxypeptidase digestion. Dashed lines indicate undetermined residues.



Taken these results together, the primary structure of phenomycin was unambiguously determined as illustrated in Fig. 2.

## Discussion

In the present study, we have isolated phenomycin from the culture broth of *S. fervens* var. *phenomyceticus* and determined its primary structure by enzymatic degradation and automated Edman degradation. Sequence analysis indicated that phenomycin consists of 89 amino acid residues, the MW being 9,524. Disulfide bridge is absent in the molecule, since no cystein residue is observed.

A homology search of the proteins (Swiss-Prot Protein Seqence Database) revealed no significant homology to any known proteins including antitumor polypeptide antibiotics such as neocarzinostatin[7,8], auromomycin[9], *etc.* These antibiotics consist of chromophores and apoproteins: the chromophores alone manifest antitumor activity to a lesser extent than the intact antibiotics, which are significantly enhanced in the presence of the apoproteins[10]. In contrast, phenomycin does not contain any chromophore, as confirmed by the UV spectrum[1].

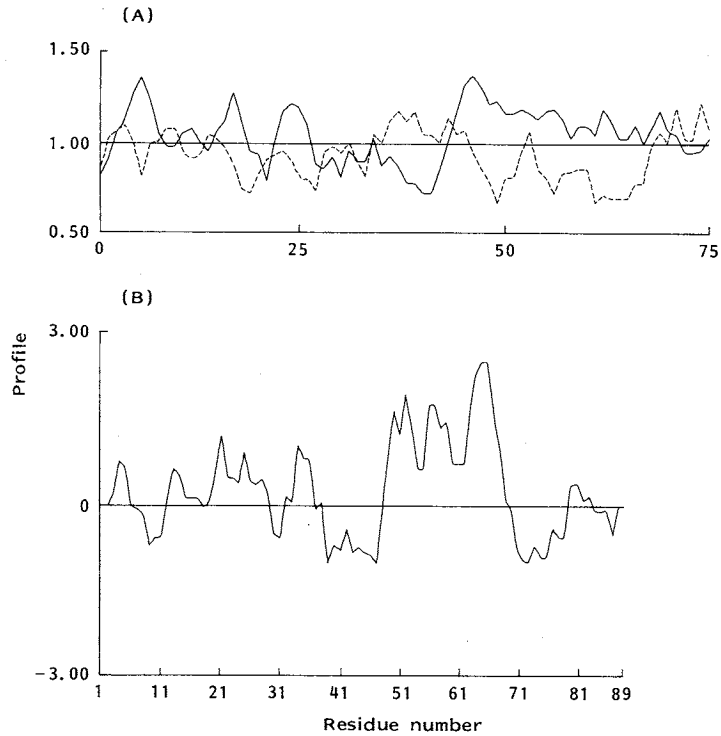
α-Helix structure is predicted for phenomycin at residues 44~71 according to the interpretation of CHOU and FASMAN[11] (refer Fig. 3(A)). Fig. 3(B)

Table 2. Amino acid compositions of phenomycin.

| Residue | Phenomycin | Residue | Phenomycin |
|---|---|---|---|
| Asx | 10.29 (10) | Val | 2.91 ( 3) |
| Glx | 3.36 ( 3) | Met | 2.01 ( 2) |
| Ser | 7.63 ( 8) | Ile | 3.77 ( 4) |
| Thr | 6.97 ( 7) | Leu | 4.34 ( 4) |
| Arg | 4.09 ( 4) | Phe | 1.00 ( 1) |
| Gly | 4.26 ( 4) | Trp | ND ( 1) |
| Ala | 17.35 (17) | Lys | 10.51 (10) |
| Cys | 0.00 ( 0) | His | 3.80 ( 4) |
| Pro | 4.11 ( 4) | Tyr | 2.71 ( 3) |

Numbers in parentheses are obtained on the basis of the amino acid sequence.
ND: Not detected.

Fig. 3. Predicted conformational and hydrophilicity profiles of phenomycin.
(A) Conformational profile. —— Helix, ------ sheet. (B) Hydrophilicity profile.



shows the hydrophilicity profile obtained by the method of HOOP and WOODS[12]. It is obvious that the sequence of phenomycin at residues 48~70 containing many lysine residues is hydrophilic. Moreover, this region represents amphiphilic α-helix on an Edmundson helical wheel[13] (data not shown). It is proposed that those regions with a rigid amphiphilic secondary structure, such as an α-helix and a β-sheet, will be surface-active at biomembranes and lipid-water interfaces[14]. These findings suggest that the Lys-rich region at residues 48~70, which is predicted to construct amphiphilic α-helix structure, may play a important role in the biological activity of the antibiotic. The identification of the active site of phenomycin is now being investigated using chemically synthesized phenomycin fragments.

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