THE CHEMISTRY OF ACTINOCARCIN. I

FURTHER CHARACTERIZATION AND C-TERMINAL STRUCTURE OF ACTINOCARCIN

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The amino-acid composition and molecular weight of actinocarcin, and C-terminal structure of actinocarcin are reported in this paper. By STEIN-MOORE's and ultraviolet-absorption methods, the molar ratio of amino acids in actinocarcin was determined. The N-terminal amino acids are alanine and valine. The C-terminal amino acid, determined by hydrazinolysis, is tryptophan. The molecular weight is about 11,000 as estimated by ARCHIBALD's method and amino acid analysis. Three fragments, named BrCN-I, BrCN-II and BrCN-III, were obtained by cyanogen bromide cleavage. The structure of BrCN-III, namely the C-terminal structure of actinocarcin was determined by EDMAN degradation

Actinocarcin is a basic polypeptide antibiotic which is produced by *Streptomyces* sp. 3654-JT_1 resembling *Streptomyces cinnamomeus*.[1] The antibiotic inhibits the growth of EHRLICH ascites carcinoma, but is not active against bacteria, fungi and yeasts.[1]

The production, purification and preliminary characterization of actinocarcin have been reported in a previous paper.[1] In this paper, further characterization and degradation studies as well as C-terminal structure determination of actinocarcin are described.

Amino-acid Composition and Molecular-weight Determination

The molar ratio of amino acids in actinocarcin, estimated from amino-acid analysis after acid hydrolysis and tryptophan analysis by the ultraviolet-absorption method,[2] was as follows;

Aspartic acid	13	Alanine	17	Phenylalanine	1
Threonine	4 (or 5)	Valine	3	Tryptophan	1
Serine	7	Methionine	2	Lysine	7 (or 8)
Glutamic acid	4	Isoleucine	5	Histidine	7
Proline	7	Leucine	4	Arginine	5
Glycine	5 (or 6)	Tyrosine	3		

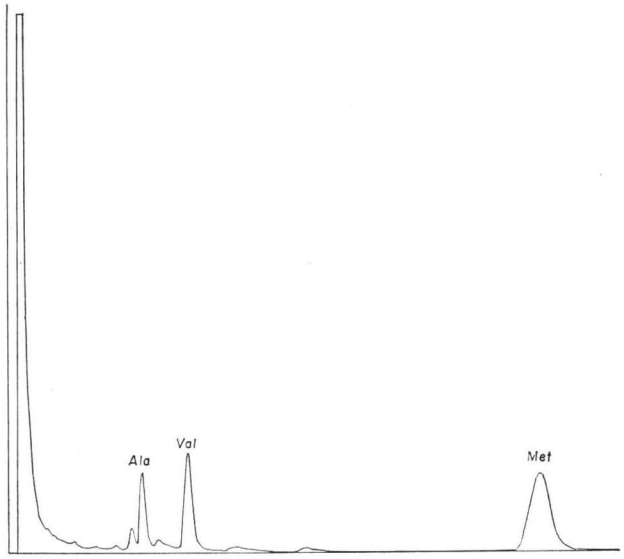
This result was calculated assuming that actinocarcin contains one phenylalanine per one molecule. On the basis of this amino-acid composition, it was established that actinocarcin is composed of about 100 amino acids.

The molecular weight of actinocarcin was estimated at 11,000 by ultracentrifugation analysis and it is in agreement with the value calculated from amino-acid composition of actinocarcin.

N- and C- Terminal Amino-acid Determination

Actinocarcin was reacted with dansyl chloride[4] (1-dimethylaminonaphthalene-5-sulfonyl chloride. abbreviated as DNS-Cl) and after acid hydrolysis of the reaction products, DNS-alanine

Fig. 1. GLC spectrum of PTH-amino acid removed at the first step of EDMAN degradation. Methionine (0.02 μM) was added as internal standard.



and DNS-valine were identified by silica gel thin-layer chromatography. To confirm this result, EDMAN degradation[5] of actinocarcin was performed with the automatic sequence analyzer JEOL-JAS-47K. The PTH-amino acid obtained from the first degradation step, was subjected to gas chromatography and the result is shown in Fig. 1.

PTH-alanine and PTH-valine were detected in about equal molar concentration. Thus, actinocarcin has two N-terminal amino acids, namely, valine and alanine.

Hydrazinolysis[6] of actinocarcin liberated one amino acid which was identified as tryptophan by amino-acid analysis using STEIN-MOORE's method.[7] Therefore, the C-terminal amino acid of actinocarcin is tryptophan.

Cyanogen Bromide Cleavage[8]

Actinocarcin contains two moles of methionine and was selectively cleaved at the methionine sites by cyanogen bromide into three fragments. The fragments were separated by Sephadex G-25 column chromatography and were named BrCN-I, BrCN-II and BrCN-III, respectively, according to the sequence of elution from the column. Amino-acid analysis after acid hydrolysis gave the following results.

BrCN-I	Aspartic acid	9	Alanine	14.5	Phenylalanine	1
	Threonine	3.3	Valine	3.3	Lysine	8
	Serine	6.5	Methionine	0	Histidine	5
	Glutamic acid	5	Isoleucine	2.3	Arginine	3.5
	Proline	6	Leucine	4.2	Homoserine	1
	Glycine	4.3	Tyrosine	2		
BrCN-II	Aspartic acid	3	Alanine	4	Arginine	1.5
	Threonine	1.3	Isoleucine	2.5	Homoserine	1
	Serine	1	Tyrosine	1		
	Proline	2	Histidine	2		
BrCN-III	Aspartic acid	1	Leucine	1		
	Glycine	1.4	Arginine	1		

N-Terminal amino-acid analysis by the dansyl method gave the following results.

BrCN-I: alanine and valine, BrCN-II: serine, BrCN-III: glycine

As BrCN-I has the same N-terminal amino acid as that of native actinocarcin, BrCN-I is the N-terminal residue of actinocarcin.

The UV spectrum of BrCN-III was identical with that of tryptophan, indicating that BrCN-III contains tryptophan as C-teminal amino acid because actinocarcin has one C-terminal tryptophan. Accordingly, amino-acid composition and the terminal amino acid of BrCN-III were determined as follows:

Amino-acid composition:				N-terminal	Glycine
Aspartic acid	1	Tryptophan	1	C-terminal	Tryptophan
Glycine	1	Arginine	1		
Leucine	1				

As mentioned above, BrCN-I is the N-terminal residue and BrCN-III is C-terminal in actinocarcin. Therefore, the sequence of three fragments in actinocarcin is determined as follows: BrCN-I—BrCN-II—BrCN-III

The Primary Structure Determination of BrCN-III

EDMAN degradation of BrCN-III was performed on automatic sequence analyzer, JEOL-JAS-47K and the thiazolinone derivative formed at each successive step of the EDMAN degradation was converted to phenylthiohydantoin by acid.[9] Each phenylthiohydantoin was identified by glc and tlc. The results are shown Fig. 2 and Fig. 3. The first step removed glycine, the second leucine and the third aspartic acd. As the C-terminus of BrCN-III is tryptophan, the residual arginine is the fourth amino acid. Accordingly, the primary structure (amino acid sequence) of BrCN-III is Gly—Leu—Asp—Arg—Trp.

Fig. 2. GLC spectrum of PTH-amino acids removed at each EDMAN degradation step.
Methionine (0.02 μM) was added as internal standard.

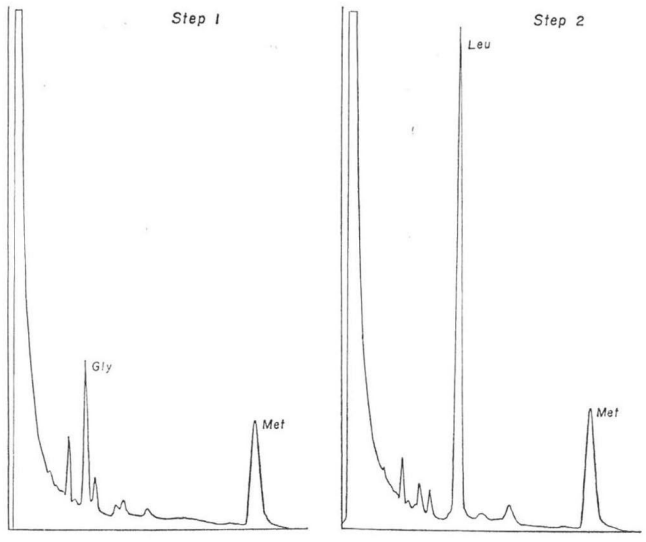
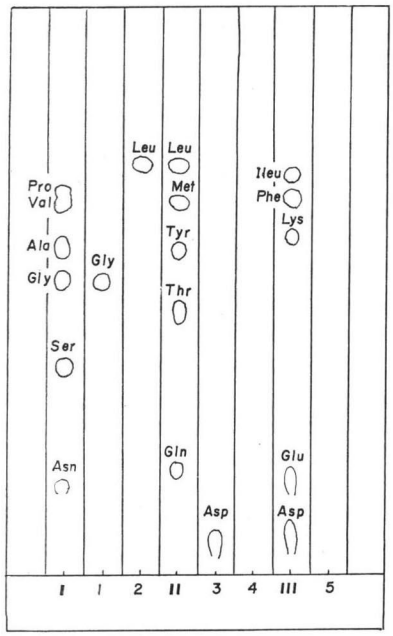


Fig. 3. TLC of PTH-amino acids removed at each EDMAN degradation step.



I, II, III = Standard PTH - amino acids.
I, 2, 3, 4, 5 = Number of steps

Experimental

Materials: Actinocarcin used in these experiments was prepared according to the method described in a previous paper.[1] Its purity was examined by celluose acetate film and disc electrophoresis.

Amino-Acid Analysis: Actinocarcin (0.3 mg) was hydrolyzed with 0.5 ml of constant boiling HCl in a sealed and evacuated tube at 110°C for 20 hours. The hydrolysate was evaporated to dryness *in vacuo* and left overnight in a dessicator over NaOH. The residue was applied to a Hitachi amino-acid analyzer and the molar ratio of each amino acid was calculated according to the result of this amino-acid analysis. The molar ratio of tryptophan and tyrosine was determined by an ultraviolet absorption method.[2] The absorbancy of actinocarcin was measured at a concentration of 300 mcg/ml in 0.1 N NaOH at 280 nm and 294.4 nm. To correct the absorbancies at these wave lengths, the value was also measured at 340 and 370 nm. Contents of tryptophan and tyrosine were calculated by using the given molar extinction coefficients and these corrected absorbancies.

N-Terminal Amino-Acid Analysis: Actinocarcin (0.9 mg) was dissolved in 0.2 ml of 0.1 M triethylamine-H_2CO_3 buffer and 0.2 ml of 0.5% dansyl chloride[4] in acetone was added to the buffer solution. The reaction was allowed to proceed for 2 hours at 37°C. The reaction mixture was then dried *in vacuo* and the residue was dissolved in 0.5 ml of constant boiling HCl. Hydrolysis was carried out at 110°C for 18 hours in a sealed and evacuated tube. The hydrolysate was evaporated to dryness *in vacuo* and the residue was applied to the silica gel thin-layer plate using the solvent system, chloroform-benzyl alcohol-acetic acid (50:5:0.1, v/v). DNS-valine and DNS-alanine were identified by tlc.

C-Terminal Amino-Acid Analysis: Actinocarcin (1.3 mg) was dissolved in 0.5 ml of anhydrous hydrazine and left at 105°C in a sealed tube for 8 hours. Excess hydrazine was removed by lyophilization. The residue was dissolved in 2 ml of water and 0.5 ml of benzaldehyde was added. The mixture was immediately shaken vigorously for 1 hour at room temperature and the resulting hydrazides were removed by centrifugation. The supernatant was washed three times with ether to remove exess benzaldehyde and then lyophilized. The residue was dissolved in 1 ml of citrate buffer, pH 2.2, and the solution was subjected to amino-acid analysis.

Molecular Weight Determination by the ARCHIBALD's Method:[3] Actinocarcin (9 mg) was dissolved in 1.8 ml of 0.01 M NaCl and the solution was centrifuged at 29,400 rpm to observe the ARCHIBALD pattern. The same solution of actinocarcin was centrifuged at 21,410 rpm to observe the SCHLIEREN pattern. The molecular weight of actinocarcin was calculated by using the value obtained from the ultracentrifuge experiments described above.

Cyanogen Bromide Cleavage:[8] Actinocarcin (107 mg) was dissolved in 11 ml of 70% formic acid and 70 mg of crystalline cyanogen bromide was added. The reaction was allowed to proceed for 24 hours at room temperature in a tightly stoppered flask. After cyanogen bromide cleavage, 20 ml of water was added to the reaction mixture and the solution was evaporated *in vacuo* to remove excess cyanogen bromide and formic acid. The residue was dissolved

in 5 ml of water and the solution was lyophilized. Thus, 110 mg of cyanogen bromide cleavage products were obtained and these were separated by gel filtratioh on a Sephadex G-25 column (2.5 cm×80 cm) to yield three components, BrCN-I (78.1 mg), BrCN-II (12.4 mg) and BrCN-III (5.3 mg). Amino-acid analysis and N-terminal amino-acid determination of these were carried out by the similar procedure as described for actinocarcin.

EDMAN Degradation[5] of BrCN-III: BrCN-III (0.5 mg) was dissolved in 0.5 ml of distilled water and the solution was transferred to the reaction cup of an automatic sequence analyzer. Then EDMAN degradation of BrCN-III was carried out and repeated automatically by the sequence analyzer. The solution of the thiazolinone derivative formed at each degradation step was sent out from the instrument into the fraction tube. The solution was transferred to a centrifuge tube and was evaporated to dryness in a stream of N_2. One ml of 1 N HCl was added to the dry sample of the thiazolinone. After the tube was heated at 80°C for 10 minutes, the solution of the resulting phenylthiohydantoin (PTH) amino acid was extracted repeatedly with ethyl acetate (3×1 ml) and the extracts were combined. The organic phase was evaporated to dryness in a jet of N_2. Then the dry samples of PTH amino acids obtained by the procedure above described, were identified by silica gel thin-layer chromatography and gas chromatography. Xylene-isopropyl alcohol (1:1, v/v) was used as developing solvent for tlc. Gas Chrom Q was used as the solid support and SE-30 was used as the silicone liquid phase for glc.

EDMAN Degradation of Actinocarcin: Actinocarcin (4.5 mg) was used and the PTH amino acid obtained from the first degradation step was identified by gas chromatography by a procedure similar to that described for BrCN-III.

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