On Glumamycin, a New Antibiotic. VI.*1 An Approach to the Amino Acid Sequence

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(Received October 21, 1963)

In previous papers,[1-3] it was reported that glumamycin ($C_{59}H_{91}O_{20}N_{13}$, M. W. 1300[4]) was an acidic peptide consisting of 3-ITA[2] (1 mol.), L-Asp (3 mol.), L-MeAsp (1 mol.), Gly (2 mol.), L-Pro (1 mol.), L-Val (1 mol.), D-Pip (1 mol.) and α, β-Dab (2 mol.), and that, of the 2 mol. of α, β-Dab contained in glumamycin, the α- and β-amino groups of one molecule take part in the peptide combination, while the β-amino group of the other is free.

As the glumamycin was resistant to such enzymes as pepsin, trypsin, chymotrypsin and pronase, the antibiotic was subjected to partial hydrolysis with hydrochloric acid. The present paper deals with experiments designed to determine the amino acid sequence of glumamycin.

*1 Part XL of a series entitled "Studies on Antibiotics," Ed. by Sueo Tatsuoka.

*2 The abbreviations used are: 3-ITA, 3-isotridecenoic acid; MeAsp, β-methylaspartic acid; Pip, pipecolic acid; α, β-Dab, α, β-diaminobutyric acid; DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl.

1) M. Inoue, This Bulletin, 35, 1249 (1962).
2) M. Inoue, ibid., 35, 1255 (1962).
3) M. Inoue, ibid., 35, 1557 (1962).
4) M. Fujino, M. Inoue, J. Ueyanagi and A. Miyake, ibid., 38, (1965).

Experimental

The Partial Hydrolysis of Glumamycin with N Hydrochloric Acid. — One gram of glumamycin purified by the method reported in a previous paper[5] was heated with 10 ml. of N hydrochloric acid in an oil bath at 100°C for 4 hr. The mixture became turbid in about 2 hr., and an oily substance separated in 4 hr. The reaction mixture was then diluted with 40 ml. of water and extracted with three 30 ml.-portions of ethyl acetate and a 30 ml.-portion and two 20 ml.-portions of *n*-butanol in order to remove the oily substance. The aqueous layer (W-fraction) was lyophilized, and 100 mg. of this substance was dissolved in 2 ml. of 0.5 N acetic acid and poured on a column (2×40 cm.) Dowex-1×4 (200—400 mesh). The column was washed with 0.5 N acetic acid, and the effluent was collected in 5 ml.-portions with a fraction collector (Fig. 1).

The ethyl acetate layer was extracted three times with 20 ml. of a 2% sodium hydrogen carbonate solution, and the aqueous layer, after being adjusted to pH 2 with 2 N hydrochloric acid, was extracted again with ethyl acetate. The ethyl acetate

5) M. Shibata, T. Kanzaki, K. Nakazawa, M. Inoue, H. Hitomi, K. Mizuno, M. Fujino and A. Miyake, *J. Antibiotics* (*A*), 15, 1 (1962).

extract was evaporated to dryness, and the residue was washed with petroleum ether to give the E substance. The *n*-butanol extract was treated in the same manner to give the B substance.

The Partial Hydrolysis of Glumamycin with 6 N Hydrochloric Acid.—Four hundred milligrams of glumamycin was heated with 8 ml. of 6 N hydrochloric acid in a sealed tube at 100°C for 80 min.; the contents became turbid in about 40 min., and an oily substance separated in 60 min. The reaction mixture was diluted with 5 vol. of water and extracted with ethyl acetate to remove the fatty acid; after it had been diluted further with two times its volume of water, it was passed through a column (2×25 cm.) of Amberlite IR-120 (H-form) in order to adsorp the amino acids and peptides. The colum was washed with water to remove the hydrochloric acid and then eluted with 0.5 N aqueous ammonia, and the eluate was evaporated to dryness under reduced pressure at 35°C to give a sample for investigation by paper ionophoresis.

The Partial Hydrolysis of DNP-glumamycin.—On hundred milligrams of DNP-glumamycin obtained by Sanger's method (described in Part IV[3]) was dissolved in 2 ml. of concentrated hydrochloric acid and they hydrolyzed by being heated in a seald tube at 80°C for 2 hr. The hydrolysate was diluted with 11 volumes of water, and an oily substance was removed by extraction with ethyl acetate. The aqueous layer was poured on a talc-celite (2 : 1) column (2×20 cm.),[6] whereby all the yellow substance were adsorbed. The column was washed with N hydrochloric acid, and the washings were concentrated to yield non-DNP-peptides. The yellow zone of the column was eluted with N hydrochloric acid-ethanol (1 : 4), and the eluate was evaporated to dryness to give DNP-peptides.

The Separation of Peptide Fragments by Paper Chromatography and Ionophoresis.—The mixture of peptide fragements (30—40 mg.) described above was applied, in the form of a narrow band, to Toyo Roshi No. 526 (10×40 cm.) and was submitted to ionophoresis in a pyridine-acetate buffer of pH 6.5[7] (300—400 V., 2—4.5 hr.). In this manner the various peptide fragments were separated and were located by dipping narrow guide strips cut from both long edges of the paper in a 0.25% ninhydrin solution in acetone. The bands were then eluted with 5% acetic acid (0.5 ml.), and the solutions were taken to dryness in vacuo over potassium hydroxide pellets and stored at 0°C. A portion of each peptide fraction was tested for homogeneity by paper chromatograph in *n*-butanol - acetic acid - water (12 : 3 : 5, solvent A) and *n*-butanol - acetic acid - pyridine - water (15 : 10 : 3 : 12, solvent B). If more than one component was revealed in a peptide fraction by either of these solvents, it was refractionated and the additional peptide components were isolated as has been described above.

The Characterization of Purified Peptides; Amino Acid Composition. — The amino acid components of each peptide were determined, after hydrolysis with 6 N hydrochloric acid in a sealed tube at 105°C for 20 hr., by paper chromatography using solvent A and *n*-butanol - pyridine - water (1 : 1 : 1, solvent C), and by paper ionophoresis in 2 N acetic acid at 300—400 V. for 3 hr. Quantitative amino acid analyses of peptides were performed according to Levy's method[8] as described in a previous paper.[2] The paper chromatography of fatty acid and its related compound was carried out in *n*-butanol saturated with 2N aqueous ammonia (solvent D) and located by a pH indicator (bromophenol blue).

***N*-Terminal Analyses.**—0.3—1.0 mg. of a peptide and 2 mg. of sodium hydrogen carbonate were dissolved in 0.2 ml. of water in a small test tube, and then 0.4 ml. of a 5% ethanolic solution of DNFB was added. After 2 hr., the reaction mixture was diluted with 0.6 ml. of water, extracted with ether to remove the unreacted DNFB, and, after being adjusted to pH 2 with 4 N hydrochloric acid, extracted three times with 1 ml. of ethyl acetate. (All the DNP-peptides treated in the present work were extractable with ethyl acetate.) The ethyl acetate extract was then washed twice with 0.5 ml. of water and evaporated to dryness in a small test tube. The residue was sealed in a tube with 6 N hydrochloric acid and heated at 100°C for 6—8 hr. The hydrolysate was dried over potassium hydroxide in a desiccator to remove hydrochloric acid, dissolved in 1 ml. of water, and shaken three times with ether to extract DNP-amino acid for paper chromatographic identification. The paper chromatography of DNP-amino acids was conducted by the two-dimensional ascending method using solvent C and a 1.5 M phosphate buffer (pH 6.0).

***C*-Terminal Analyses.**—1—3 mg. of a dried peptide was heated with 0.1—0.2 ml. of anhydrous hydrazine in a sealed tube at 100°C for 4—6 hr. The reaction mixture was dried over sulfuric acid in a desiccator to remove the excess hydrazine, and the free amino acid therein was determined by paper chromatography and paper ionophoresis after the amino acid hydrazide has been removed by enanthaldehyde.

The Hydrazinolysis of Glumamycin.—Ten milligrams of glumamycin was dried with 65 mg. of hydrazine sulfate over phosphorus(V) oxide in a desiccator for 2 days, sealed together with 0.5 ml. of anhydrous hydrazine in a tube, and heated at 60°C for 16 hr. Another hydrazinolysis was conducted in a similar manner except that hydrazine sulfate was not added and the reaction was effected at 100°C for 10 hr. In both cases, the reaction mixture was dried over sulfuric acid in a desiccator, the residue was dinitrophenylated by Sanger's method, and the reaction mixture was extracted fractionally and subjected to paper chromatography. In all cases, no DNP-amino acid deriving from the *C*-terminus was detected. As a control, glumamycin was hydrazinolyzed in the presence of several moles of glycine per mole of glumamycin, but in this case only DNP-Gly was clearly detected.

6) F. Sanger, *Biochem. J.*, **45**, 503 (1949).
7) F. Sanger and H. Tuppy, ibid., **49**, 463 (1951).
8) A. L. Levy, *Nature*, **174**, 126 (1954).

Results and Discussion

The Partial Hydrolysis of Glumamycin with N Hydrochloric Acid.—The partial hydrolysate of glumamycin with N hydrochloric acid was extracted successively with ethyl acetate (E-fraction) and *n*-butanol (B-fraction). The E-fraction was negative to the biuret and ninhydrin reactions and showed a fatty acid-like property, and on its paper chromatogram using solvent system D, only a spot of an acidic substance with an R_f value of 0.32 was detected. Next, the E-substance was completely hydrolyzed with hydrochloric acid and extracted with ether; it was found with the ether extract that the R_f 0.32 spot disappeared, while instead a spot (R_f 0.61) corresponding to 3-ITA was detected. When the aqueous layer of the ether extraction was subjected to paper chromatography using solvent systems A and B, a spot corresponding to Asp was detected. The E-substance gave no DNP-derivative by Sanger's method;[9] its hydrazinolysis by Akabori's method[10] released Asp (0.4 mol./mol., uncorrected) and in this case no di-DNP-Asp monohydrazide was detected by paper chromatography. From the facts mentioned above, the structure of E-substance was found to be 3-ITA→Asp.

The B-fraction was positive to the biuret reaction and weakly positive to the ninhydrin reaction, and its acid hydrolysis gave all the components of glumamycin. Therefore, sequence studies of the B-fraction were not carried out.

Paper ionophoresis of the aqueous fraction of the *n*-butanol extraction mentioned above (W-fraction) detected many ninhydrine positive substance assumed to be amino acids and peptides. Therefore, the fraction was developed on a column of Dowex-1×4 with 5 N acetic acid in order to separate it into a basic (W1), a neutral (W2) and three acidic fractions (W3—W5), as is shown in Fig. 1. Further, the basic fraction, W1, was fractionated by a combination of paper ionophoresis in a pyridine-acetate buffer (pH 6.5) (Fig. 2) and paper chromatography in solvent systems A and B. The chromatographically-pure peptides obtained in this way analyzed for constituent amino acids and *N*- and *C*-terminal groups. The results obtained are summarized in Table I. From the results concerning the W1 fraction, a partial structure of glumamycin could be deduced as follows:

H·Gly→α, β-Dab→Val→Pro→α, β-Dab(→Pip)

9) F. Sanger, *Biochem. J.*, **39**, 50 (1945).
10) S. Akabori, K. Ohno, T. Ikenaka, Y. Okada, H. Hanafusa, I. Haruna, A. Tsugita, K. Sugae and T. Matsushima, This Bulletin, **29**, 507 (1956).

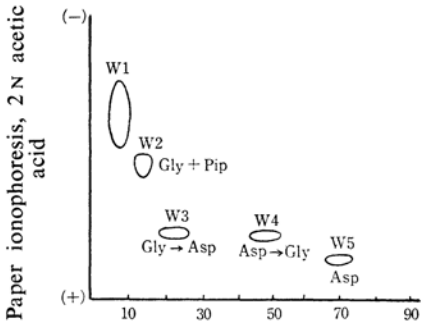

Fig. 1. Separation of partial acid (N HCl) hydrolysate by Dowex-1×4 column (2×40 cm) chromatography.
Each fraction of the effluent was subjected to paper ionophoresis in 2 N acetic acid (400 V., 2 hr.). W2—W5 were identified by making use of their authentic samples by paper chromatography and infrared spectrum.

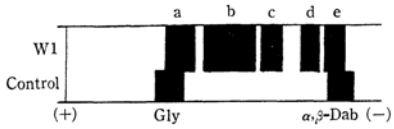

Fig. 2. Separation of W1 fraction by paper ionophoresis in pyridine-acetate buffer, pH 6.5, 300 V., 4.5 hr.

The Partial Hydrolysis of Glumamycin with 6 N Hydrochloric Acid.—The partial hydrolysate of glumamycin with 6 N hydrochloric acid was extracted with ethyl acetate in a manner similar to that used in the extraction of the partial hydrolysate with N hydrochloric acid. The aqueous fraction was passed through a column of Amberlite IR-120 (H-form), the absorbed substances were eluted with 0.5 N aqueous ammonia, and the eluted substances were fractionated by paper ionophoresis in a pyridine-acetate buffer (pH 6.5) (Fig. 3), followed by paper chromatography using solvent systems A and B. The components and the *N* terminal residues of the peptides thus separated were further investigated, as has been mentioned before; the results obtained are summarized in Table II.

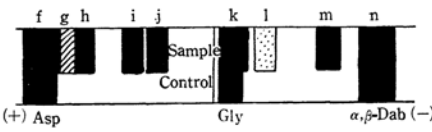

Fig. 3. Separation of partial acid (6 N HCl) hydrolysate by paper ionophresis in pyridine-acetate buffer, pH 6.5, 300 V., 5 hr.

TABLE I. AMINO ACID SEQUENCE OF PEPTIDES ISOLATED FROM PARTIAL ACID (N HCl) HYDROLYSATE OF GLUMAMYCIN

Fractions (W1 : a—e) obtained by ionophoresis at pH 6.5 (Fig. 2) were separately chromatographed in *n*-butanol - acetic acid - water (solvent A) or *n*-butanol - pyridine - acetic acid - water (solvent B). Fractions c and d were chromatographically pure, e was separated into e_1 and e_2; a and b were not identified.

Peptide		R_f in A	R_f in B	Amino acid composition* and deduced partial structure**
W1 :	a			all the amino acids constituting glumamycin were detected
	b			
	c	0.36	0.34	Gly·(α, β-Dab, Val)·Pro
	d	0.34	0.28	Gly·α, β-Dab·Val
	e : 1	0.21	0.21	Gly·(α, β-Dab, Val, Pro)·α, β-Dab
	e : 2	0.41	0.45	Gly·(α, β-Dab, Val, Pro, α, β-Dab, Pip)

* Analyzed by paper chromatography and ionophoresis.
** *N*-Terminal was analyzed by Sanger's DNP-method and *C*-terminal was analyzed by hydrazinolysis.

TABLE II. PEPTIDES AND AMINO ACIDS ISOLATED FROM PARTIAL ACID (6N HCl) HYDROLYSATE OF GLUMAMYCIN

Fractions obtained by ionophoresis at pH 6.5 (Fig. 3) were separately chromatographed in *n*-butanol - acetic acid - water (solvent A) or *n*-butanol - pyridine - acetic acid - water (solvent B) and gave the additional components as shown.

Peptide and amino acid		R_f in A	R_f in B	Amino acid composition*	Deduced partial structure**
f		0.20	0.17	Asp	—
g		0.22	0.25	MeAsp, Asp	MeAsp·Asp
h :	1	0.12	0.13	Gly (1), Asp (1)	Gly·Asp
	2	0.15	0.13	Asp (1), Gly (1)	Asp·Gly
i		0.12	0.16	MeAsp, Asp (2), Gly (1)	MeAsp·(Asp, Gly, Asp)
j		0.19	0.24	Asp (2), Gly (1)	Asp·(Gly, Asp)
k		—	—		
l		—	—		
m :	1	—	0.23	α, β-Dab, Val, Pro	α, β-Dab·(Val, Pro)
	2	—	0.27	α, β-Dab, Val, Pro	?
n :	1	—	0.16	Gly, α, β-Dab	Gly·α, β-Dab
	2	—	0.20	α, β-Dab	—

* Analyzed by paper chromatography and ionophoresis. Molar ratio of amino acids was determined by Levy's DNP-method.
** *N*-Terminal was analyzed by Sanger's DNP-method.

TABLE III. PEPTIDES AND AMINO ACID OBTAINED FROM PARTIAL ACID HYDROLYSATE OF DNP-GLUMAMYCIN

DNP-peptide (Fig. 5)		Amino acid composition*	Deduced partial structure**
DNP-C :	a	β-DNP-α, β-Dab Val, Pro	β-DNP-α, β-Dab·(Val, Pro)
	b	β-DNP-α, β-Dab Val, Pro, α, β-Dab	β-DNP-α, β-Dab·(Val, Pro, α, β-Dab)
Non-DNP - Pertide (Fig. 6)			
n :	1	Val, Pro, α, β-Dab, Pip	Val·(Pro, α, β-Dab, Pip)
	2	α, β-Dab	—

* Analyzed by paper chromatography and ionophoresis.
** *N*-Terminal was analyzed by Sanger's DNP-method. Non-*N*-terminal α, β-Dab was detected as β-mono-DNP-α, β-Dab by paper chromatography in the analysis of peptides DNP-C·b and n·1 respectively.

From the results on peptides f—j summarized in Table II, and also from the results of the partial hydrolysis with N hydrochloric acid shown in Fig. 1, the presence of the H·MeAsp →Asp→Gly→Asp·OH sequence was presumed. Results on the other peptides, m and n in Table II, were in accord with the sequence deduced from the results of the partial hydrolysis with N hydrochloric acid.

The Partial Hydrolysis of DNP-glumamycin with Concentrated Hydrochloric Acid. — DNP-Glumamycin prepared by Sanger's method[8] was hydrolyzed partially with concentrated hydrochloric acid, and the resulting oily substance was removed with ethyl acetate. The DNP-peptides were separated from non-DNP-peptides on a column packed with a mixture of talc and celite (2 : 1) and subjected to paper chromatography to give four yellow spots, as Fig. 4 shows. All the amino acids

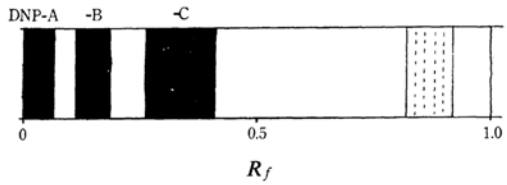

Fig. 4. Paper chromatogram of DNP-peptides obtained from partial acid hydrolysate of DNP-glumamycin.
Solvent system : *n*-butanol saturated with 2 N aqueous ammonia

constituting glumamycin were detected in the hydrolysate of DNP-A and -B and Val, Pro, α, β-Dab, β-DNP-α, β-Dab and a small amount of the other amino acids in that of DNP-C. The DNP-C was subjected to paper ionophoresis using a pyridine-acetate buffer (pH 6.5). It was separated into a neutral (DNP-C·a) and a basic (DNP-C·b) fraction, as is shown in Fig. 5. Analyses of these fractions are

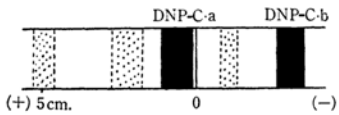

Fig. 5. Separation of DNP-C fraction by paper ionophoresis in pyridine-acetate buffer, pH 6.5, 300 V., 6 hr.

shown in Table III. From the results described above and from those of the partial hydrolysis of glumamycin, it was possible to formulate the partial sequence in the neighborhood of the free β-amino group in glumamycin as follows :

$$\rightarrow \alpha\text{-Dab} \rightarrow \text{Val} \rightarrow \text{Pro} \rightarrow \underset{\beta\text{-NH (free)}}{\alpha\text{-Dab}} \rightarrow \underset{\beta\text{-NH}}{\alpha\text{-Dab}} \leftarrow$$

Next, the non-DNP-peptide fraction from the above-mentioned talc-celite column was passed through a column of Amberlite IR-4B (OH form) in order to remove acidic amino acids and peptides. Peptides and amino acids in the effluent were adsorbed on a colum of Amberlite IR-120 (H from) and eluted with 0.5 N aqueous ammonia to give a sample for paper ionophoresis. The results are shown in Fig. 6. Analyses of the isolated substances are shown in Table III.

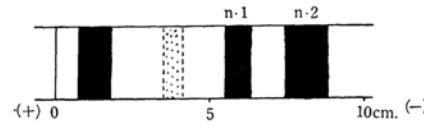

Fig. 6. Separation by paper ionophoresis of non-DNP-peptides obtained from partial acid hydrolysate of DNP-glumamycin.
Pyridine-acetate buffer, pH 6.5, 300 V., 3.5 hr.

From the results summarized in Table III, the structure of non-DNP-peptide n·1 may be as follows ;

$$\text{H-Val} \rightarrow \text{Pro} \rightarrow \underset{\beta\text{-NH}_2}{\alpha\text{-Dab}} \rightarrow \text{Pip-OH}$$

The Elucidation of the Sequence.—The partial structure of glumamycin, inferred from the sequence of amino acids in the peptide fragments obtained by the partial hydrolysis of glumamycin and DNP-glumamycin, may be represented by 3-ITA→Asp·(OH), (H)·Gly→ β-NH$_2$ β-NH$_2$
α-Dab→Val→Pro→α-Dab→Pip·(OH) and (H)·MeAsp→Asp→Gly→Asp·(OH). All the amino acid residues constituting glumamycin are contained in the above three peptides. Glumamycin was subjected to the hydrazinolysis-DNP method of Akabori in the presence and in the absence of hydrazine sulfate, but no DNP-amino acid derived from *C*-terminal was detected in either case. Therefore, the three peptides above are presumed to be arranged in a cyclic form in glumamycin.

From these facts, the structure of glumamycin may be supposed to be one of the following two ;

$$\text{(A)}\quad \text{3-ITA} \rightarrow \text{Asp} \rightarrow \text{Gly} \rightarrow \underset{\beta\text{-NH}_2}{\alpha\text{-Dab}} \rightarrow \text{Val} \rightarrow \text{Pro} \rightarrow \alpha,\beta\text{-Dab} \begin{cases} \nearrow \text{Pip} \rightarrow \text{MeAsp} \searrow \\ \nwarrow \text{Asp} \rightarrow \text{Gly} \swarrow \end{cases} \text{Asp}$$

$$\text{(B)}\quad \text{3-ITA} \rightarrow \text{Asp} \rightarrow \alpha,\beta\text{-Dab} \begin{cases} \nearrow \text{Pip} \rightarrow \text{MeAsp} \rightarrow \text{Asp} \rightarrow \text{Gly} \searrow \\ \nwarrow \text{Pro} \leftarrow \text{Val} \leftarrow \underset{\beta\text{-NH}_2}{\alpha\text{-Dab}} \leftarrow \text{Gly} \swarrow \end{cases} \text{Asp}$$

Fundamentally these structures of glumamycin are similar to those of the antibiotics of the polymixin series[11,12] but they are reverse in the relation of functional groups; that is, in the former, one amino group and four carboxyl groups are free, while in the latter only the amino groups are free. It is interesting to compare these points with their antibacterial spectra; glumamycin is active only to Gram-positive microorganisms, and the antibiotics of polymixin, only to Gram-negative microorganisms.

Summary

To elucidate the amino acid sequence of glumamycin, glumamycin and DNP-glumamycin have been partially hydrolyzed with hydrochloric acid, and various peptides have been isolated by column chromatography on ion-exchange resin or talc-celite, paper chromatography and paper ionophoresis. From the structures of the peptides, two possible structures (A and B) for glumamycin have been presumed.

The author wishes to express his thanks to Dr. Sueo Tatuoka, the Director of the Research Laboratories, Dr. Satoru Kuwada and Dr. Kuniyoshi Tanaka for their guidance and encouragement, and to Drs. Koichi Nakazawa, Akira Miyake, Jisaburo Ueyanagi, Michitaka Inoue and Motoo Shibata for their kind advice.

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12) K. Vogler, R. O. Studer, W. Lergier and P. Lanz, *Helv. Chim. Acta*, **43**, 1751 (1960).