On Glumamycin, a New Antibiotic. IV. The Amino Acid Moiety*

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As we have reported previously[1-4], glumamycin[5], a new acidic peptide-type antibiotic, is composed of seven kinds of amino acids and one mono-unsaturated fatty acid. They are aspartic acid, α(L), β-methylaspartic acid, proline, valine, glycine, pipecolic acid, α, β-diaminobutyric acid, and 3-isotridecenoic acid.

In the present work, the nature of the amino acids was determined and an investigation was made of the free amino group of glumamycin.

Experimental

Preparation of the Sample for Determination of the Amino Acids.—Ten milligrams of glumamycin purified by counter current distribution[5] were hydrolyzed with 3 ml. of 6 N hydrochloric acid in a sealed tube at 110°C for 24 hr., and the hydrolysate was then treated in the usual way to remove the excess hydrochloric acid.

Determination of the Amino Acids by the Moore-Stein Method[6]. — First the determination was carried out by Moore-Stein's method. The results are shown in Table I.

Determination of the Amino Acids by the Paper-DNP Method.—Next, determination of the amino acids was conducted by the paper-DNP method.

A definite volume of the above hydrolysate was dinitrophenylated by the method of Levy[7]. The mixture of DNP-amino acids thus obtained was subjected to two-dimensional paper chromatography (Solvent system: first, *n*-butanol saturated with 2 N aqueous ammonia, and then a 1.5 M phosphate buffer[8]). The yellow spots on the paper chromatogram (Fig. 1) were cut out and extracted with 5 ml. of 1% sodium hydrogen carbonate solution[7]. Colorimetry of the extract was effected at 360 mμ for the usual amino acids, at 385 mμ for proline, and at 395 mμ for pipecolic acid. This determination was conducted on three sheets of paper chromatogram, and the amino acid contents were calculated from the mean values of the experimental millimole extinction coefficients obtained in the next experiment. The results are shown in Table III.

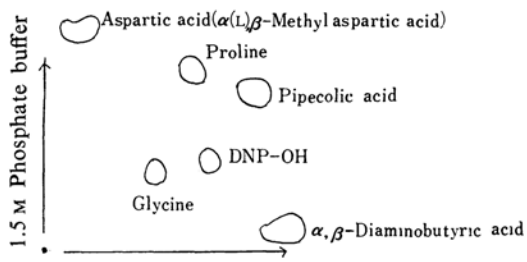

Fig. 1. Two dimensional paper chromatogram of DNP-derivatives of amino acids in 24 hr.-hydrolysate of glumamycin.

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1) M. Inoue et al., This Bulletin, 33, 1014 (1960).
2) M. Inoue, ibid., 34, 885 (1961).
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6) S. Moore and W. H. Stein, *J. Biol. Chem.*, 176, 367 (1948); ibid., 192, 663 (1951).
7) A. L. Levy, ibid., 213, 187 (1955); ibid., 213, 487 (1955); *Nature*, 174, 126 (1954).
8) K. Satake et al., *Kagaku no Ryoiki, Supplementum*, 34, 77 (1959).

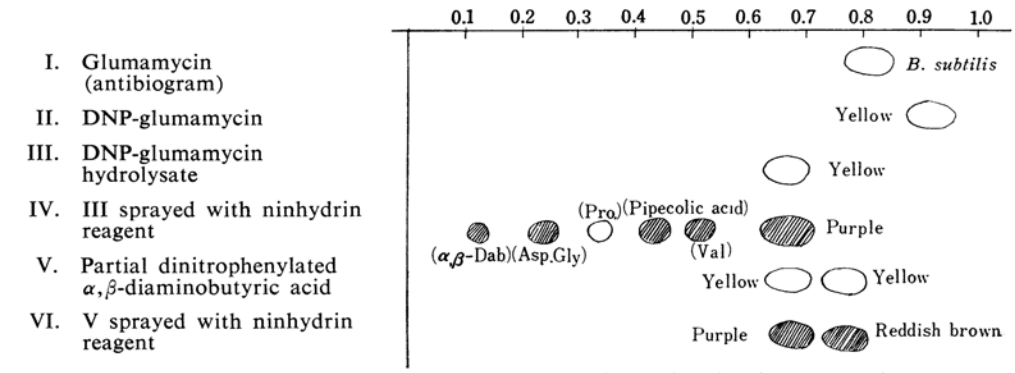

Fig. 2. Paper chromatograms of glumamycin and related compounds (Solvent system ; *n*-butanol, acetic acid, water (4 : 1 : 5)).

Measurement of the Experimental Millimole Extinction Coefficients of Various Amino Acids.—Aspartic acid, glycine, proline, valine, pipecolic acid, and α, β-diaminobutyric acid were dissolved in three consecutive concentrations. The solutions, after being dinitrophenylated by Levy's method[7], were subjected to two-dimensional paper chromatography (Solvent system: the same as has been mentioned above). The yellow sports were cut out and extracted in the same way as above. The experimental millimole extinction coefficients of the amino acids were calculated from the absorptions at their respective wavelengths, and finally the mean values of the solutions in three concentrations were obtained (Table II).

Hydrolysis of DNP-Glumamycin. — Ten milligrams of DNP-glumamycin obtained by Sanger's method[9] were hydrolyzed with 3 ml. of 6 N hydrochloric acid in a sealed tube at 110°C for 24 hr. The hydrolysate was diluted and treated with ether in the usual way. No ether-soluble DNP-amino acid was obtained. The yellow aqueous layer was evaporated in a dark place, to remove the excess hydrochloric acid. The residue thus obtained was submitted to paper chromatography (Fig. 2).

Paper Chromatography.—The paper chromatograms (I—VI) in Fig. 2 were obtained by developing them with the solvent system *n*-butanol, acetic acid and water (4 : 1 : 5). All the processes were conducted by the descending method.

Partial Dinitrophenylation of α, β-Diaminobutyric Acid.—α, β-Diaminobutyric acid[3] isolated from the hydrolysate of glumamycin was dinitrophenylated by Sanger's method[9], but the reaction time was limited to 30 min. The reaction mixture was immediately shaken with ether to remove the unreacted dinitrofluorobenzene and di-DNP-α, β-diaminobutyric acid. The yellow aqueous layer thus obtained was submitted to paper chromatography.

Results and Discussion

The amino acids contents of glumamycin was estimated by the Moore-Stein method ; as a result, 4 mol. of aspartic acid, 1 mol. of valine, and 2 mol. of glycine were detected, as is shown in Table I. The other amino acids were not detected, probably because α(L), β-methylaspartic acid was eluted in the same fraction as that of aspartic acid, because pipecolic acid was not colored favorably with ninhydrin, and because α, β-diaminobutyric acid was not eluted by this method. That ammonia was not detected means that asparagine does not exist therein despite the presence of 4 mol. of aspartic acid.

TABLE I. AMINO ACIDS COMPOSITION OF GLUMAMYCIN DETERMINED BY MOORE-STEIN'S METHOD

Amino acid	Calcd. ratio in 48 hr. hydrolysate	Assumed ratio of amino acid residue
Aspartic acid	4.23	4
Glycine	2.12	2
Valine	1.00	1
Proline	1.05	1
Amino N	0	0

Next, the determination was carried out by the paper-DNP method. The DNP-derivatives of the amino acids in glumamycin hydrolysate were obtained by Levy's method, and their two-dimensional paper chromatograms are shown in Fig. 2. The DNP-derivatives of the two acidic amino acids, i. e. α(L), β-methylaspartic acid and aspartic acid were not separated clearly, so their ratio could not be determined. However, the ratio of the two compounds was estimated to be about one to three or more, judging from the sizes of their spots colored with ninhydrin in the paper chromatography of the acidic amino acid fraction of glumamycin hydrolysate. The spots of the DNP-derivatives of valine and pipecolic acid also overlapped, but the ratio of their contents could be calculated since their absorption maxima were different. This method of determining amino acids was carried out

9) F. Sanger, *Biochem. J.*, **39**, 507 (1945); ibid., **45**, 563 (1949).

TABLE II. EXPERIMENTAL mM EXTINCTION COEFFICIENT

Amino acid	Experimental	Extinction coefficient $k\times10^{-3}$ at λ_{max}	Recovery %
Aspartic acid	15.74	18.1	87
Glycine	15.39	17.0	90
Valine	15.75	18.0	87
Proline	16.80	18.5	91
α,β-Diaminobutyric acid	20.85	29.2	71
Pipecolic acid	15.51	15.8	98

TABLE III. AMINO ACID COMPOSITION OF GLUMAMYCIN DETERMINED BY PAPER-DNP METHOD

Amino acid	Calcd. ratio	Assumed ratio of amino acid residue
Aspartic Acid	4.12	4
Glycine	1.94	2
Proline	1.00	1
Valine	0.89	1
Pipecolic acid	0.97	1
α,β-Diaminobutyric acid	1.93	2

after confirming the fact that the dinitrophenylation of each amino acid and the elution of the product were reproducible with an error of $\pm4\%$, and that, therefore, this method was usable. The results are shown in Table II. From the experimental data, the nitrogen content of the amino acids was calculated; from the recovery rate of nitrogen (108%), the molecular ratio shown in Table III seems reasonable and is in accord with the results obtained by the Moore-Stein method.

On the basis of the above results, glumamycin is considered to contain 4 mol. of aspartic acid (including α(L), β-methylaspartic acid), one mol. each of proline, valine and pipecolic acid, and 2 mol. each of glycine and α,β-diaminobutyric acid; judging from the analytical results[3], 1 mol. of an unsaturated fatty acid[4] also seems to be present.

Further, investigation was made into the free amino group of glumamycin. As is shown in Fig. 2, the water-soluble DNP-amino acid in the hydrolysate of DNP-glumamycin was identified as mono-substituted DNP-α,β-diaminobutyric acid. The results of the paper chromatography are as follows;

1) Paper chromatogram I is the antibiogram of glumamycin (microorganism; *B. subtilis*), 2) Paper chromatogram II shows the yellow spot of DNP-glumamycin, 3) Paper chromatogram III shows the yellow spot at R_f 0.68 of the DNP-amino acid in the DNP-glumamycin hydrolysate; when the chromatogram is sprayed with the ninhydrin reagent, all the amino acids contained in glumamycin are colored and the yellow spot at R_f 0.68 turns purple (paper chromatogram IV), 4) Paper chromatogram V indicates two yellow spots of partially dinitrophenylated α,β-diaminobutyric acid at R_f 0.68 and 0.75; when the paper chromatogram is sprayed with the ninhydrin reagent, the yellow spot at R_f 0.68 turns purple and the spot at R_f 0.75, reddish brown.

Judging from the color reaction paper chromatogram VI, it is assumed that the substance with R_f 0.68 is mono-DNP-α,β-diaminobutyric acid in which the α-amino group is free and that the β-amino group is dinitrophenylated, while the substance with R_f 0.75 is also the same acid, but its α-amino group is dinitrophenylated and β-amino group is free. On the basis of this assumption, the free amino group of glumamycin is assumed to be the β-amino group of α,β-diaminobutyric acid, and the α-amino group is assumed to participate in peptide linkage. In addition, as another spot of α,β-diaminobutyric acid is found at R_f 0.1 on paper chromatogram V, 1 mol. α,β-diaminobutyric acid of each two in glumamycin takes part in the peptide-linkage through both the α- and the β-amino groups.

Summary

Separatory determination was conducted on the seven amino acids contained in glumamycin. By the Moore-Stein method and by paper chromatography of the DNP-derivatives of the amino acids, 4 mol. of aspartic acid (including α(L), β-methylaspartic acid), 2 mol. each of glycine and α,β-diaminobutyric acid, and 1 mol. each of valine, pipecolic acid and proline were detected. Also, investigation was made into the free amino group of this antibiotic.

The hydrolysate of DNP-glumamycin showed one yellow spot at R_f 0.68, which spot turned purple with ninhydrin.

Compared these results with paper chromatogram of partially dinitrophenylated α,β-diaminobutyric acid, it was assumed that the β-amino group of one of the acids, is free, that the α-amino group participates in the peptide linkage in glumamycin, and that both amino groups of another α,β-diaminobutyric acid take part in the peptide linkage.

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