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The Chemistry of Tyrocidine. III. The Structure of Tyrocidine A

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In order to determine the order of the amino acids in tyrocidine A it has been partially hydrolyzed and the products fractionated by countercurrent distribution, ion exchange and paper chromatography. A number of peptides have been isolated and the order of their amino acids determined. This has permitted the structure of tyrocidine A to be deduced.

Introduction

In previous publications data have been presented which show conclusively that tyrocidine A, one of the polypeptides present in the mixture called "tyrocidine," has the empirical formula $C_{66}H_{86}O_{13}N_{13}$. This formula agrees with that derived by connecting valine, tyrosine, leucine, proline, ornithine, glutamine, asparagine and three phenylalanine molecules through simple amide linkages to give a cyclic polypeptide with no free carboxyl groups. The corresponding amino acids have all been isolated following complete hydrolysis and found to be present in the expected amount. The presence of only two functional groups, the hydroxyl group of the single tyrosine residue and the δ-amino group of the single ornithine has been demonstrated.

The next step in the study of the structure of the polypeptide is logically that of the determination of the sequence of the amino acids present. This paper reports a number of the experiments designed to reveal the sequence by partial hydrolysis with hydrochloric acid.

The over-all procedure used was similar to that developed by Consden, *et al.*,[2] for polypeptides and used with such striking success by Sanger[3] and collaborators for studying the amino acid sequences present in insulin from beef. The major difference in procedure in the study reported here is to be found in the fractionation tools used for separation of individual polypeptides from the hydrolysis mixtures.

A search for the optimum hydrolysis conditions was first made by hydrolysis of a number of small samples under different conditions. The hydrolysates were then compared by paper chromatography and the conditions which appeared to give the best defined spots other than those expected from the known amino acids of the total hydrolysate were chosen for a more extended study. Appropriate areas in the paper assumed to contain peptides were cut out, eluted and the residues hydrolyzed. The hydrolysate from each area was then studied again by paper chromatography in order to reveal the amino acids present in the peptide. A tentative sequence was derived in this way even though there was some doubt as to whether it was the only sequence which alone could explain the experimental results.

Following this preliminary experience a 4-g. sample of tyrocidine A was hydrolyzed and extensive fractionation was made by the use of countercurrent distribution and ion exchange chromatography. Each step of this preparative fractionation was controlled by two dimensional paper chromatography. In some cases where paper chromatography indicated a simple mixture of two peptides to be present, the mixture was treated with 1-fluoro-2,4-dinitrobenzene (FDNB) and the resulting peptide derivatives then fractionated by countercurrent distribution.

Experimental

The tyrocidine A used in this study was fractionated from crude crystalline tyrocidine as described in a previous publication.[4] We are indebted to the Wallerstein Company for a generous supply of the starting material.

The preliminary survey of hydrolysis conditions included experiments with concentrated hydrochloric acid for various times of heating at temperatures of 37°, 60° and 80°. The action of 50% formic acid at 100° for various times also was studied. From these experiments a temperature of 80° was chosen for a more extended study of the hydrolysis.

Six-hour Hydrolysis.—A sample of 390 mg. of tyrocidine A dissolved in 1 ml. of glacial acetic acid and 39 ml. of concentrated hydrochloric acid was heated in a flask at 80° for six hours. The acid was then removed by evaporation in the rotating evaporator[5] under reduced pressure and at a water-bath temperature not higher than 25°. The residue was taken up in water and evaporated a second time. It was then subjected to countercurrent distribution in the system made by equilibrating equal volumes of 2-butanol and 0.1 *N* hydrochloric acid. Equal phase volumes in a 220-tube automatic apparatus[6] were used.

At 423 transfers an analysis on the lower phases of the equilibration tubes was made by weight.[7] The effluent upper phases also were analyzed. The result for the latter is shown by the right segment of the pattern in Fig. 1. The pattern for the equilibration tubes indicated two families of overlapping bands with a good minimum at tube 110. The contents of tubes 110 to 219 were then withdrawn separately and set aside. The weight pattern for these tubes is given in the middle segment of Fig. 1. The empty tubes of the machine were then filled with fresh phases of the system and 940 transfers applied by the recycling procedure.[6] Weight analysis then gave the pattern in the left-hand segment of Fig. 1.

As the weight analysis was being made the solid residue in each shell after weighing was taken up in sufficient 50% ethanol to make approximately a 1% solution. A 5-microliter sample of each solution was then spotted on a suitable paper (No. 1 Whatman) for 2 dimensional paper chromatography. A large cabinet permitting 16 papers to be run simultaneously was constructed for this purpose. Here the systems used were the 2-butanol–formic acid and 2-butanol–ammonia systems used by Hausmann.[8]

By comparing the chromatograms of those tubes designated by the experimental points on the curve of the pattern in their order from left to right, it was noted as had been expected that a particular spot on the paper, for instance a spot known to be caused by ornithine, would be given only

(1) Rockefeller Foundation Fellow 1951–1953.

(2) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **41**, 590 (1947).

(3) F. Sanger and H. Tuppy, *ibid.*, **49**, 463 (1951); F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353 (1953).

(4) A. R. Battersby and L. C. Craig, THIS JOURNAL, **74**, 4019 (1952).

(5) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1426 (1950).

(6) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *ibid.*, **23**, 1236 (1951).

(7) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *ibid.*, **23**, 1326 (1951).

(8) W. Hausmann, *ibid.*, **74**, 3181 (1952).

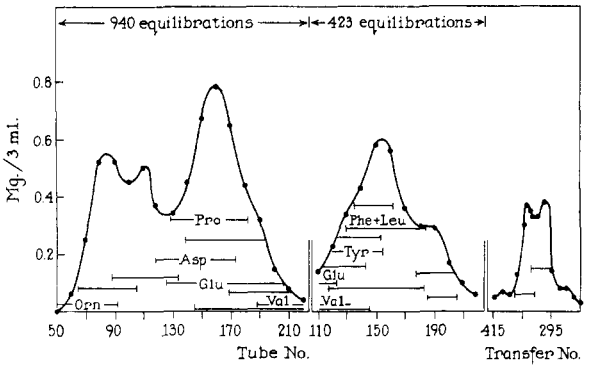


Fig. 1.—Countercurrent distribution pattern of partial hydrolysate of tyrocidine A.

by those tubes in the series nearest each other. Such a spot was strongest from those tubes in the middle of the region. Such a region in the distribution pattern is designated on Fig. 1 by a solid line and where the position of the spot on the paper corresponded with one of the amino acids known to be present in tyrocidine A, the line is so designated. Lines not named therefore represent bands of peptide material.

Following construction of the pattern of Fig. 1, recombination of small aliquots from selected tubes of the pattern exclusive of the smaller right-hand effluent section, gave a mixture which was studied by two dimensional paper chromatography for comparison with the results of Fig. 1. For this purpose four sheets were run simultaneously. The first and last sheets were developed by spraying with ninhydrin (0.2% in ethanol and permitted to develop overnight at room temperature). These sheets gave the map shown in Fig. 2 and served as guides for cutting out spots from the undeveloped middle sheets. The round or elliptical spots in Fig. 2 show the position of the colored spots, but the outer lines around them indicate the area cut out from the undeveloped sheets. The solutes in the cut-out portions were separately eluted with water, evaporated to dryness and hydrolyzed with 6 *N* hydrochloric acid at 108° for 24 hours. An indication of the amino acids in the hydrolysates was then obtained by paper chromatography in the systems already mentioned.

In Table I the results of this study are given. Here the way of indicating the result is essentially the same as that employed by Sanger, *et al.*, in their work with insulin.[3] Weak spots are indicated by a single x; strong spots by a triple x. The amino acid composition of a peptide spot is placed in parentheses when the order of the amino acids has not been indicated. However, the order given could be deduced from the more conclusive experiments reported further on as will be shown.

TABLE I

Spot	Amino acid found by paper chromatography after hydrolysis Pro.	Phe.	Asp.	Glu.	Tyr.	Val.	Orn.	Leu.	Amino acid composition indicated
5						x	xxx	xxx	(Orn·Leu)
6						xxx	xxx	xxx	(Val·Orn·Leu)
7						x	x	x	(Val·Orn·Leu)
8		x				x	x	x	(Val·Orn·Leu·Phe)
4					xx	xxx	xxx		(Tyr·Val·Orn)
9		xx	x	xxx	x				(Phe·Asp·Glu·Tyr)
10		xxx	xxx						(Phe·Asp)
1			xxx	xxx					(Asp·Glu)
3						xxx	xxx		(Val·Orn)
2			xxx	xxx		x	x	x	?

Effluent fractions 304 and 343 (transfer numbers 304 and 343 from Fig. 1) were evaporated and studied by paper chromatography. Each gave essentially single spots which after hydrolysis showed the amino acid spots, respectively, Phe +++, Pro ++ and Leu +++, Phe +++. These were interpreted as indicating (Phe_2Pro) and (LeuPhe) peptides.

Preparative Hydrolysis.—Following the preliminary experience a more extensive experiment was planned. Four grams of tyrocidine A was hydrolyzed for 1 hour at 80° in 10 *N* hydrochloric acid. After evaporation of the hydrochloric

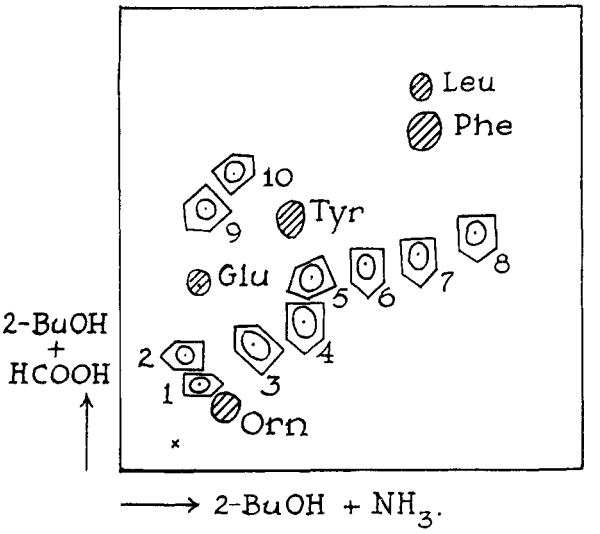


Fig. 2.—Paper chromatogram map of partial hydrolysate of tyrocidine A.

acid the residue was given a preliminary distribution of 151 transfers in the system 2-butanol–0.1 *N* hydrochloric acid. Pattern a of Fig. 3 was obtained by weight analysis.

The fractionation procedure used to separate the various polypeptides resulting from the partial hydrolysis is given schematically in Fig. 3. Here the actual distribution patterns or effluent patterns from the ion exchange chromatography have been condensed to small size and show only the minimum of essentials. BuOH on the pattern means the system used was the 2-butanol–0.1 *N* HCl system mentioned earlier; $CHCl_3$ refers to a system made by equilibrating chloroform, methanol, 0.1 *N* HCl in the volume proportions of 2, 2, 1; Bz refers to the system made by equilibrating benzene, glacial acetic acid and 0.1 *N* HCl in the volume proportions 2, 2, 1; and HAc refers to the system made from glacial acetic acid, chloroform, 0.1 *N* HCl in the volume proportions of 2, 2, 1.

Separations were studied at each of the stages shown in the patterns by two dimensional paper chromatography. Only the two systems used for obtaining the map of Fig. 2 were used for this purpose.

Paper chromatography studies indicated material in the right hand peak of pattern a to be a mixture of larger peptides only slightly smaller than intact tyrocidine A itself. A cut was therefore taken as indicated by the bracket and the solutes were hydrolyzed again at 80° in 10 *N* hydrochloric acid for a 2-hour period. This hydrolysate was then distributed again in the 2-butanol system to 403 transfers in a 420-tube apparatus. Weight analysis gave pattern b.

The solutes in the two left-hand bands from both patterns were combined for fractionation by ion exchange chromatography as given below. However, after removal of these two bands the second distribution was continued by recycling until 732 transfers after which the triplet 10, 11 and 12 was permitted to emerge from the machine as effluent. Pattern c was obtained from this effluent.

The intermediate doublet of pattern b was left in the train until 1415 transfers had been reached. Pattern d was obtained at this point, and shows almost complete separation of the doublet. Two dimensional paper chromatography of samples from each of the bands gave only a single spot for material from the right-hand band but three definite spots with material from the left-hand band.

The solute in the right-hand band was recovered and redistributed to 219 transfers in the $CHCl_3$ system. Pattern e of Fig. 3 was obtained. The main band agreed well with a theoretical distribution curve and gave only a single spot on paper. It was therefore assumed to represent a single peptide and the solute was recovered by evaporation. This gave a crystalline residue weighing approximately 300 mg.

Total hydrolysis of a sample of this peptide and paper chromatography showed only two spots, a strong one in the position of phenylalanine and the characteristic yellow spot of proline. Ultimate analysis indicated the peptide to be

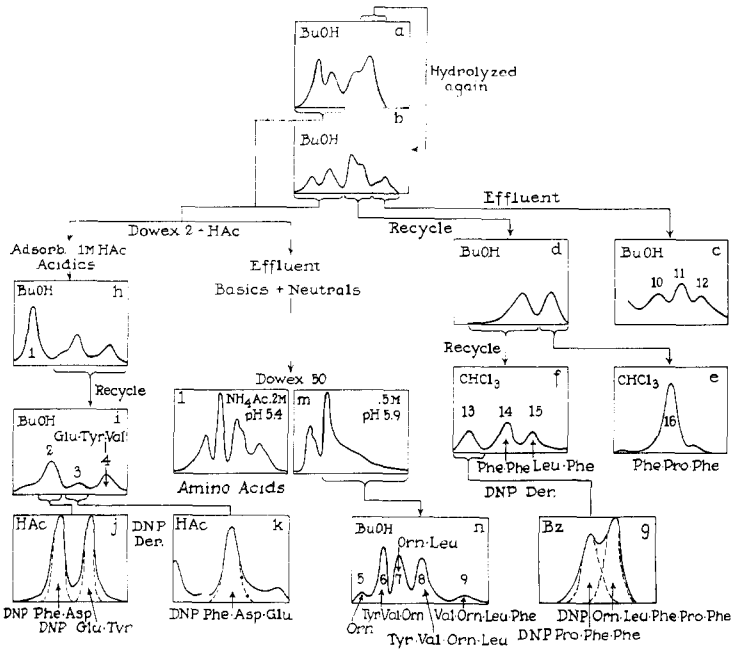


Fig. 3.—Fractionation flow sheet for peptides from the partial hydrolysis of tyrocidine A.

a tripeptide containing two phenylalanine residues and a single proline.

Anal. Calcd. for $C_{23}H_{27}O_4N_3 \cdot HCl$: C, 61.9; H, 6.37; Cl, 7.95; N (Van Slyke), 3.14. Found: C, 62.09; H, 6.48; Cl, 7.53; N (Van Slyke), 3.16.

The solute in the band on the left in pattern d of Fig. 3 was recovered and distributed to 1886 transfers in the $CHCl_3$ system. Pattern f was obtained. At this point each of the three bands agreed fairly well with theoretical curves. Two dimensional paper chromatography gave only single spots with the solute from bands 14 and 15, but two spots of about equal intensity were found with that from 13.

The solute was recovered from band 15 by evaporation and since the residue was crystalline it was analyzed without further purification. Complete hydrolysis and paper chromatography gave two spots of about equal intensity which agreed in position with leucine and phenylalanine.

Anal. Calcd. for $C_{15}H_{22}O_3N_2 \cdot HCl$: C, 57.3; H, 7.38; N (Van Slyke), 4.45. Found: C, 57.7; H, 7.06; N (Van Slyke), 4.05.

A crystalline residue was obtained when the solvent was evaporated from band 14. On complete hydrolysis and paper chromatography only a single strong spot was obtained which corresponded in position to that of phenylalanine. Analysis indicated it to be a dipeptide.

Anal. Calcd. for $C_{18}H_{20}O_3N_2 \cdot HCl$: C, 62.1; H, 6.1; N (Van Slyke), 4.01. Found: C, 62.06; H, 6.08; N (Van Slyke), 3.97.

A 50-mg. sample of the dipeptide was hydrolyzed completely in 6 *N* HCl. The excess hydrochloric acid was removed by evaporation and the phenylalanine crystallized from aqueous ethanol and pyridine. The crystalline material showed no optical activity.

A second 50-mg. sample was converted to the DNP derivative by treatment with fluoro-2,4-dinitrobenzene as given by Sanger.[9] Without attempting to purify the derivative by recrystallization it was hydrolyzed with 6 *N* hydrochloric acid for 24 hours in a sealed tube at 108°. Most of the excess acid was evaporated and after dilution with water the DNP-phenylalanine was extracted with ether. It was recrystallized from glacial acetic acid–water.

Anal. Calcd. for $C_{15}H_{13}O_6N_3$: C, 54.4; H, 3.95. Found: C, 54.4; H, 3.94; $[\alpha]^{25}D$ −115° (*c* 0.914 in methanol).

A sample of the authentic levo derivative prepared directly from L-phenylalanine gave a rotation of −110°.

(9) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

The acid aqueous layer remaining from the hydrolysis above was evaporated to dryness and the remaining phenylalanine was converted to the dinitrophenyl derivative. It was crystallized from glacial acetic acid–water.

Anal. Found: C, 54.2; H, 4.11; $[\alpha]^{25}D$ +76° (*c* 0.714 in methyl alcohol).

When the solute from band 13 of pattern f was studied by paper chromatography two distinct spots were formed. Part of the material (20 mg.) was converted to the mixed DNP derivatives and distributed to 66 transfers in the benzene system described above. Analysis by optical density at a wave length of 350 mμ with the Beckman quartz spectrophotometer gave pattern g of Fig. 3.

A sample taken from the two tubes to the right of the maximum of the right-hand band was hydrolyzed completely and studied by paper chromatography. Spots corresponding to leucine, phenylalanine, proline and a yellow spot in the position of di-DNP-ornithine were found. The yellow spot did not turn blue when sprayed with ninhydrin solution. The phenylalanine spot was more intense than the others, suggesting two residues. That the yellow spot was indeed di-DNP-ornithine was confirmed by extracting the dye from the hydrolysate with ether and determining its partition ratio in the chloroform system given above, $K = 0.4$. Under similar conditions the value of a known sample of di-DNP-ornithine was found to be 0.38. The other amino acids present in tyrocidine A give DNP derivatives with quite different K's from this in the system except valine ($K = 0.34$). However, paper chromatography of the hydrolysate of band 13 in pattern f showed valine to be absent.

Weight analysis[7] of the solution in one of the above tubes which had an optical density of 2.98 gave a residue weighing 0.100 mg. per ml. If the suggested[10] value of 14,500 for the molar extinction of the DNP radical be used, a molecular weight of 974 can be calculated for a di-DNP peptide. The calculated value for such a derivative of a peptide containing ornithine, leucine, proline and two residues of phenylalanine is 968.

In a similar manner a sample of material from the tubes slightly to the left of the left peak in pattern g of Fig. 3 was hydrolyzed and studied by paper chromatography. Most of the yellow color of the peptide was destroyed during hydrolysis.

A solution of the peptide from one of these tubes which had an optical density of 2.26 at 350 mμ was found to contain 0.086 mg. per ml. A molecular weight of 551 could thus be calculated for a mono-DNP derivative. The calculated value for a tripeptide with two residues of phenylalanine and one residue of proline is 545. DNP-proline was found to have a molecular extinction coefficient not significantly different from the aliphatic amino acid derivatives in these solvents.

Separation of Acidic Peptides.—The solutes in the two left-hand bands of both patterns a and b were combined for fractionation by ion exchange chromatography as shown by the brackets in Fig. 3.

Sufficient Dowex 2, 200–400 mesh in the hydrochloride form to fill a 5-cm. diameter column to a height of 10 cm. was washed on a buchner funnel with 4 liters of 2 *N* sodium hydroxide. It was then washed with 3 liters of 1 *N* acetic acid and finally with water until the *p*H of the rinsings was approximately 5. The resin was transferred to the column as a slurry.

The mixed solutes which were still in the chloride form were dissolved in 45 ml. of water and the *p*H adjusted to 5.3 ± 0.2 with 1 *N* ammonium hydroxide. This solution was transferred to the column and was followed by water for development. The rate of flow was 1–1.5 ml. per min. Fifty fractions were collected with an automatic fraction collector. Analysis on the effluent was made by weight. No clear-cut bands of single solutes were indicated. The first 20 fractions contained 3.12 g. of solute in 352 ml. of solvent. This

(10) A. R. Battersby and L. C. Craig, THIS JOURNAL, **74**, 4023 (1952).

is the neutral and basic material considered below. The next 212 ml. contained only 0.013 g. and the next 363 ml. contained only 0.011 g.

At this point the developing solvent was changed to 1 *N* acetic acid. After this solvent began to emerge the solute concentration rose sharply but again no clear-cut resolution to single substances was indicated; 373 ml. of effluent contained a total of 0.817 g. The next 210 ml. contained 0.151 g. These two fractions were combined and the solute was recovered by evaporation in the rotary evaporator.

The residue was distributed to 1026 transfers in the butanol system. Analysis gave pattern h of Fig. 3. A band apparently containing solute arising from the resin itself is not shown on this pattern.

Band 1 was removed from the apparatus at this point. It was almost entirely made up of aspartic and glutamic acids but a search for the presence of a dipeptide containing these two amino acids was nonetheless made by conversion of the whole to DNP derivatives and distribution in the HAc system. Two clear bands of the derivatives of aspartic and glutamic acid derivatives were obtained and a third smaller band, which, however, was considerably wider than a calculated band. Since a mixture was indicated the solutes in the right and left limbs of this band were compared by hydrolysis and paper chromatography. The solute from the right limb gave a number of spots beside those of glutamic and aspartic acid, but that in the left limb gave only three spots, a strong spot in the glutamic acid region, a faint one in the aspartic acid region and a yellow spot in the DNP aspartic acid region.

The solute in that portion of pattern h designated by brackets was recycled to 1803 transfers to give pattern i. Paper chromatography indicated the solutes in bands 2 and 3 to be mixtures still, but only a single spot was obtained with the solute from band 4. Hydrolysis of the latter and paper chromatography gave three spots in the region of glutamic acid, tyrosine and valine. The intensities of the spots were those expected from equal amounts of these three acids.

The solutes in those tubes of band 2 of pattern i designated by the bracket were recovered and converted to DNP derivatives. The mixed derivatives were distributed to 116 transfers in the HAc system. Analysis by optical density gave pattern j of Fig. 3.

Hydrolysis of the solute from the left band and paper chromatography gave only a single spot corresponding to the position of aspartic acid and a yellow spot which traveled with a high R_f value in both solvents as DNP-phenylalanine has been found to do. Further confirmation of its identity was obtained by extracting the DNP derivative with ether and determination of the partition ratio of the extracted yellow dye in the HAc system (K = 0.195) and in a buffer system made by equilibrating ethyl acetate with a buffer solution made from 2 *M* NaH_2PO_4, 2 *M* K_2HPO_4 and water in the volume proportions 1, 9, 5, respectively. The K of DNP-phenylalanine in the first system is 0.19 and in the buffer system is 10.4 (found 10.7) as contrasted to the other neutral amino acids in tyrocidine A, *e.g.*, leucine 4.1, valine 1.15 in the buffer system.

A solution of the DNP derivative containing 0.081 mg. per ml. gave an optical density at 350 mμ of 2.46; calcd. mol. wt. 478. The DNP derivative of the peptide Phe·Asp has a mol. wt. of 444.

The solute from the right band of pattern j was hydrolyzed and studied by paper chromatography. A strong yellow spot in the position of DNP-glutamic acid was obtained. After spraying with ninhydrin a faint blue spot in the position of glutamic acid developed as well as two other spots in the positions[10] given by tyrosine and O-DNP-tyrosine.

A solution of the DNP derivative containing 0.098 mg. per ml. gave an optical density of 2.44 at 350 mμ; calcd. mol. wt. 669 (assuming the absorption of the O-DNP radical to be 15% that of the N-DNP radical). The N,O-di-DNP derivative of Glu·Tyr has a mol. wt. of 642.

The solute from band 3 of pattern i was recovered and converted to DNP derivatives. This mixture was distributed to 90 transfers in the HAc system. Analysis by optical density gave pattern k of Fig. 3. Hydrolysis and paper chromatography of the main band gave spots in the positions of aspartic and glutamic acids and a yellow fast moving spot which would be expected from DNP-phenylalanine.

A solution of the DNP-peptide from the main band which contained 0.105 mg. per ml. had an optical density of 2.58 at 350 mμ; calcd. mol. wt. 590. The mol. wt. of the DNP derivative of Phe·Asp·Glu is 575.

Separation of the Neutral and Basic Solutes.—Dowex-50, 200–400 mesh, in the sodium form was washed with 2 *N* hydrochloric acid, then with water until the filtrate was nearly neutral. It was suspended in 4 *N* NH_4OH, filtered and washed with water. Sufficient of this material was transferred as a slurry to the 5-cm. diameter chromatographic tube to make a column 11 cm. in height.

The solutes in the effluent from the Dowex 2 column mentioned above were recovered and dissolved in 80 ml. of a 0.2 *M* ammonium acetate buffer at *p*H 5.4. It was found necessary to add a few drops of acetic acid to dissolve all the residue. This lowered the *p*H to about 4–4.5. The solution was passed through the column at a flow rate of 0.5 ml. per min. and followed with the *p*H 5.4 buffer. After 700 ml. had passed through the column, weight analysis gave pattern 1 of Fig. 3. Paper chromatography indicated the solutes in the different bands to be almost entirely the free neutral amino acids. Therefore they were not studied further.

The buffer entering the column was changed to 0.5 *M* ammonium acetate at *p*H 5.9. After 100 ml. of effluent had emerged the weight pattern m shown in Fig. 3 was obtained.

The solute in the left band of this pattern has not been studied thus far beyond the paper chromatography stage. A mixture of peptides was indicated. However, a broad cut from the right band, as shown by the bracket leading to the next chart, was recovered and distributed to 950 transfers in the BuOH system. At this point the weight pattern n was obtained.

Paper chromatography indicated the solute in band 5 to be ornithine. The solutes in bands 6, 7, 8 and 9 gave single spots by paper chromatography. After hydrolysis each was again studied by paper chromatography. The solute from 6 gave spots corresponding to tyrosine, valine and ornithine. That from 7 gave spots corresponding to ornithine and leucine. That from 8 gave spots corresponding to tyrosine, valine, ornithine and leucine. That from 9 gave spots corresponding to valine, ornithine, leucine and phenylalanine. In every case the relative intensities of the spots were those expected from equimolecular amounts of the amino acids.

Discussion

If tyrocidine A is a simple cyclic decapeptide with two amide groups as the experimental evidence to date indicates[4] then the most complex hydrolysis mixture which could result from equal rates of hydrolysis of all amide bonds including the amide groups, would be of the order of 150 substances. However, aspartic and glutamic acids have been found to emerge[11] from hydrolyses of peptide linkages before other amino acids and it was hoped that this would prove to be a weak point in the cycle.

The preliminary study with paper chromatography gave some support to this probability but when larger amounts were fractionated by countercurrent distribution it became apparent that other peptide bonds were splitting at a rate almost as great. A very complex mixture was encountered as the flow sheet in Fig. 3 would indicate. Nonetheless it was possible to isolate peptides in a clear-cut manner. No attempt has been made to isolate all the peptides possible by the fractionation techniques employed. The work was discontinued when a sufficient number of peptides had been isolated and studied to permit an unambiguous sequence for the amino acids in tyrocidine to be deduced.

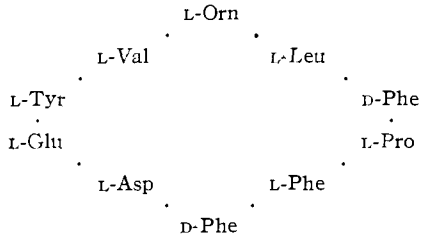
In relating the various peptides the dipeptide Phe·Phe, 14 in Fig. 3 can be used as a starting point. The tripeptide Pro·Phe·Phe whose structure was

(11) S. M. Partridge and H. F. Davies, *Nature*, **165**, 62 (1950).

indicated by the behavior of its DNP derivative on hydrolysis and its quantitative light absorption, builds the sequence up to 3. Peptide 16 gave good analytical data for a tripeptide also containing proline and two phenylalanine residues, but the amino nitrogen analysis indicated a free $-NH_2$ and showed the N-terminal amino acid to be phenylalanine. This leaves only two possibilities for the sequence in peptide 16. Tyrocidine A contains three phenylalanine residues and would permit the formation of two isomeric tripeptides, Pro·Phe·Phe and Phe·Pro·Phe by direct hydrolysis from the sequence Phe·Pro·Phe·Phe, but not Phe·Phe·Pro if Pro·Phe·Phe were one of the tripeptides formed.

The isolation of the DNP derivative of Phe·Asp permits assignment of the aspartic residue to the terminal Phe·carboxyl of the above tetrapeptide. This leaves only the N-terminal position for the leucine in the dipeptide containing leucine and phenylalanine. Aspartic and glutamic acids are shown to be connected by isolation of the DNP derivative of the tripeptide containing phenylalanine, aspartic and glutamic acid residues. This is supported by attempts to isolate the dipeptide containing aspartic and glutamic acid by countercurrent distribution and by the hydrolysis and paper chromatography of a spot from a paper chromatogram as given in Table I.

The result with the DNP peptide from pattern j of Fig. 3 together with the foregoing indicates clearly the sequence from leucine to tyrosine which is given in Fig. 4.



Structural formula of tyrocidine A.

Fig. 4.

The position of ornithine can be deduced from the fact that a dipeptide containing ornithine and leucine is found in band 7 of pattern n. It is Orn·Leu since the sequence Leu·Phe has already been shown. It then follows that spot 3 in Fig. 2 contains the dipeptide Val·Orn and the tripeptide from band 6 of pattern n in Fig. 3 indicates the Tyr·Val sequence. The sequences in the tetrapeptides from the bands 8 and 9 are then indicated and the ring is closed as given in Fig. 4. No sequences have been found which could not arise from such a structure.

All the amino acids present in tyrocidine A have the levo configuration except phenylalanine.[4] The phenylalanine isolated by hydrolysis of the peptide showed a rotation which would be that formed by two moles of D-phenylalanine and one of L-phenylalanine. Upon investigation of the optical activity of the phenylalanine arising from hydrolysis of peptide 14, Fig. 3 (Phe·Phe), it was found to be optically inactive.

When, however, the DNP derivative of this dipeptide was hydrolyzed, and the DNP derivative of the N-terminal phenylalanine isolated, it was found to give a rotation corresponding to the derivative of L-phenylalanine.

The C-terminal phenylalanine of the dipeptide which remained after extraction of the above DNP derivative, was also converted to the DNP derivative and its rotation studied. This preparation was strongly dextrorotatory but did not show a dextrorotation as high as the levo-DNP isomer. It is known that DNP derivatives hydrolyze partly[12] under the conditions of hydrolysis used here. This could explain the lower value found for the dextro isomer. Regardless of this point the rotation data reported previously[4] together with the above results clearly show that the sequence –D-Phe·L-Pro·L-Phe·D-Phe– is the one present in tyrocidine A.

The rotation of the first phenylalanine in the above series was also studied directly by conversion of the peptide Phe·Pro·Phe to its DNP derivative, hydrolysis and isolation of the resulting DNP phenylalanine. It was found to be dextrorotatory but of a lower rotation than that expected (+75° for a crystalline fraction obtained from the hydrolysate). This could indicate either partial racemization during preparation of the DNP derivative or that this particular phenylalanine in the peptide is not entirely of the dextro configuration. The first possibility appears much more likely.

Only one unsettled point remains, namely, the way in which the aspartic and glutamic acid residues are attached in the ring. Each has two carboxyl groups. It would appear more likely that the carboxyls nearest the amino groups are involved in the amide linkages of the ring with the γ- and δ-carboxyls of the aspartic and glutamic acid residues covered by the two amide groups. However, at present there is no direct evidence for this and the reverse is possible in either or both cases. Work is under way to settle this point.

A few years ago Consden, Gordon, Martin and Synge[13] determined the sequence of the amino acids present in gramicidin S. Their experiments suggested the sequence –L-Val·L-Orn·L-Leu·D-Phe·L-Pro–. Later work from this Laboratory[14] gave clear evidence that gramicidin S is not a pentapeptide but is instead a decapeptide. The cyclic nature of the peptide seems clear from the experimental evidence and therefore, the sequence above must be repeated in the cycle.

It is of considerable interest that this sequence is now found in tyrocidine A as half of the polypeptide ring. The other half, however, is entirely different being –L-Phe·D-Phe·L-Asp·L-Glu·L-Tyr–.

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