Irrespective of the problem of the loss of ammonia it appeared likely that the material in tubes 390 to 420 of pattern C of Fig. 1 represented material of high purity. Evidence supporting this view was obtained in the experiment designed to establish the molecular weight by the method of partial substitution.[2]

This experiment gave only two major yellow bands in addition to the colorless band of the unchanged peptide. All three of the bands were in close agreement with their respective calculated curves. A small amount of material, only a little over 2% of the whole, was to be found in the minor band in the region of tubes 20–35 of Fig. 3. Total hydrolysis of this latter material and two dimensional paper chromatography showed the same spots as a similar experiment with tyrocidine B, except for tyrosine and ornithine. Spots corresponding to δ-DNP-ornithine and O-DNP-tyrosine were obtained. The labile amide could again explain the minor band.

The band occurring in the region of tubes 178–188 was found to contain unchanged peptide. The solute in the one next to it in the region of tubes 145 to 165 was shown by hydrolysis and paper chromatography to be a mono-DNP derivative with the DNP group substituted on the δ-amino group of the single ornithine residue. A yellow spot corresponding to the position of δ-DNP ornithine was obtained instead of the ornithine spot shown by the hydrolysate of the unchanged peptide. All other spots were the same in both cases.

The solute in the band, tubes 50 to 75, was found to be a di-DNP derivative. Hydrolysis and paper chromatography showed spots in the region of δ-DNP-ornithine and O-DNP-tyrosine.

When the molecular weight was calculated from the data given in Fig. 3 for the mono-DNP derivative using a molecular extinction coefficient of 14500[2] for the wave length of 350 mμ a value of 1660 was obtained. Similarly, calculation of the molecular weight from the di-DNP derivative using a molecular extinction of 17000[2] gave a figure of 1890.

When a sample of the hydrolysate of tyrocidine B was studied by two dimensional paper chromatography, spots corresponding to ornithine, leucine, phenylalanine, proline, tryptophan, aspartic acid, glutamic acid, tyrosine and valine were found. No other spots were found. In this study the solvent systems aqueous ammonia, 2-butanol and aqueous formic acid, 2-butanol were used.[10]

Following this study each of the amino acids was isolated in a state of analytical purity by a combination of C.C.D., fractional crystallization and sublimation. From the data obtained it was possible to calculate the molar ratios to within about 15% as given in Table II. All were present in equimolar proportions except phenylalanine which was present in double the molar amount.

Tyrocidine B was found not to contain a free carboxyl group. When studied by the Sanger DNP procedure[11] only two functional groups were found which would react with 2,4-dinitrofluorobenzene. These were found by hydrolysis and paper chromatography to be the δ-amino group of the single ornithine and the hydroxyl group of the tyrosine. Tyrocidine B, like tyrocidine A, is therefore probably a cyclic ten amino acid residue polypeptide.

When the empirical formulas of the ten amino acid residues mentioned above together with two ammonia molecules to form the amides are added with the loss of 12 molecules of water, a formula $C_{68}H_{85}O_{13}N_{14}$ is obtained. The over-all analytical results obtained with the hydrochloride are in good agreement with this formula.

The molecular weight of the proposed formula is 1346. The mono-DNP derivative would have the molecular weight 1512 and the di-DNP derivative 1678. The molecular weights calculated for these two derivatives from weight–optical density ratios are 1660 and 1890, respectively. These values come within a figure believed to be the experimental error of the method.[2]

From the data given in Table I it can be seen that hydrolysis of tyrocidine B gives amino acids of the levo configuration except for the two residues of phenylalanine. These are of the dextro configuration. The tryptophan, proline and aspartic acid residues appear from the data in Table II to emerge partly racemized under the hydrolysis conditions used.

Summary.—Tyrocidine B has been purified and more accurately characterized by determination of its amino acid composition. The molecular weight has been determined by the method of partial substitution.

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NEW YORK 21, N. Y.

(10) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).
(11) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

The Chemistry of Tyrocidine. V. The Amino Acid Sequence of Tyrocidine B

BY T. P. KING AND L. C. CRAIG

RECEIVED JULY 11, 1955

A preceding paper[1] has shown that tyrocidine B, one of the bactericidal polypeptides produced by *B. brevis*, has the empirical formula $C_{68}H_{88}N_{14}O_{13}$. This formula can be derived by joining in amide linkages the amino acids and two moles of ammonia obtained on complete hydrolysis. These amino acids were L-aspartic acid, L-glutamic acid, L-tyrosine, L-valine, L-ornithine, L-leucine, L-proline, L-tryptophan and two moles of D-phenylalanine. The formulation indicates a cyclic peptide, a conclusion supported by the absence of a free carboxyl group or α-amino group. The only functional groups present are the δ-amino group of the ornithine and the hydroxyl group of the tyrosine. These groups were shown to be present by the preparation of DNP derivatives. Two moles of ammonia liberated in the amide determination would account for the two extra carboxyl groups of aspartic and glutamic acids.

This paper will describe experiments which have

(1) T. P. King and L. C. Craig, THIS JOURNAL, **77**, 6624 (1955).

led to the establishment of the amino acid sequence. Since this had already been done for tyrocidine A it was thought that the same hydrolysis conditions would give the same proportions of familiar peptides except those involving the L-phenylalanine residue present in A. In B this residue was thought to be replaced by L-tryptophan. However, less similarity was found than had been expected.

In A a reasonably good yield of the crystalline peptide D-Phe.L-Pro.D-Phe was obtained. With B not only was it difficult to isolate a significant amount of any peptide containing tryptophan but the relative proportions of the other peptides not directly connected to the tryptophan were different. Yet as will be seen the amino acid sequence is identical with A except for the tryptophan.

Experimental

The tyrocidine B used in this work was purified as described in the previous paper. A 2.0-g. sample was taken up in 185 ml. of concentrated hydrochloric acid and heated for 3 hr. in an evacuated sealed tube at a temperature of 80°. These conditions were chosen after dialysis studies of partial hydrolysates as described in an accompanying paper.[2]

The hydrochloric acid was removed by evaporation under reduced pressure in the rotary evaporator[3] at a temperature never higher than 30°. The residue was separated by partial dialysis as described[2] into a dialysate and a residual fraction. The residual fraction containing the larger peptide material which dialyzed more slowly was then hydrolyzed again for 3 hr. and put through the same dialysis process. This gave a total of 2.019 g. of dialysate and a residual fraction of 0.180 g. which had not passed through the cellophane. The latter was colored and contained all the humin formed during the hydrolysis.

The nearly colorless dialysate fractions were further fractionated by countercurrent distribution in the system 0.01 *N* hydrochloric acid–phenol. The phenol was freshly distilled as a mixture containing about 10% water. The distribution was made in a 420-tube automatic apparatus of the type described.[4] The upper phase volumes were 5 ml. and the lower were 10 ml. The initial charge was scattered in 10 tubes. After 960 transfers had been accomplished addition of upper phase was discontinued but the apparatus was permitted to operate until no more upper phases were in the machine, 1405 transfers.

After weight analysis the top pattern A shown in Fig. 1 was constructed. The left-hand part of the pattern represents the phenol phases while the right-hand represents the aqueous phases. After preliminary study of the various residues by paper chromatography and paper electrophoresis 12 cuts were taken as shown on the pattern.

Two dimensional paper chromatography studies were made by the ascending technique in the systems made by equilibrating 2-butanol with 3% aqueous ammonia and 2-butanol with formic acid. The paper used was Whatman No. 1. Paper electrophoresis was performed according to the technique described by Kunkel.[5] The buffer at *p*H 5.6 contained 0.8% pyridine and 0.2% of acetic acid.

The solutes were recovered from the various cuts by extraction of the phenol by chloroform using a three-stage diamond pattern.[6] Even this was not sufficient to prevent loss of material from cuts 1, 2, and 3 which were the most phenol soluble. With these the phenol was mostly removed by sublimation at 0.1 mm. pressure and 25°.

Where paper chromatography (P.C.) and paper electrophoresis (P.E.) showed a given cut to be a single peptide a portion of it was hydrolyzed and the amino acids identified by two-dimensional P.C. Then part or all of the remainder was converted to the DNP derivative. For this purpose the fraction (20 mg.) dissolved in a solution of 0.1 ml. of triethylamine and 0.9 ml. of 65% ethanol was treated with 200 mg. of 2,4-dinitrofluorobenzene at 40° for 15 minutes. The solution was then evaporated to dryness and the residue was dissolved in water containing 0.1% triethylamine. Extraction with ether removed the excess reagent. After fractionation by countercurrent distribution (C.C.D) the DNP derivative was recovered. The DNP-amino acid was identified following hydrolysis by comparison of the partition ratios determined in the systems given by Hausmann, Weisiger and Craig.[7]

In Fig. 1 the letters P. E. by an arrow indicate that a separation was made by preparative paper electrophoresis. Cuts 2 and 3 were further fractionated by redistribution in the system indicated before conversion to DNP derivatives.

Cut 12 was found to contain mostly ornithine but a small amount of the dipeptide Val. Orn was present.

Cut 11 was found to contain this dipeptide, aspartic acid and a small amount of glutamic acid. The peptide was separated by preparative paper electrophoresis as described by Weisiger, Hausmann and Craig[8] and characterized by conversion to the DNP derivative. The main band in pattern H gave a weight-optical density ratio at 350 mμ[9] indicating a molecular weight of 570 ± 50; calculated 563.

Cut 10 was separated by P.E. into two basic peptides, two acidic peptides and glutamic acid. This was accomplished on Whatman No. 3 paper 9 × 30 cm. with 1 mg. of sample spread evenly as a band at the origin. The buffer again was *p*H 5.6 pyridine acetate. The zones were revealed by spraying an indicator strip cut from the paper after the run. The solute in the separate bands was eluted and hydrolyzed. The hydrolysates were studied by paper chromatography.

One fraction showed only two spots of equal intensity corresponding to ornithine and leucine. A second showed only three spots of equal intensity corresponding to tyrosine, valine and ornithine. Both acidic fractions showed only spots of aspartic and glutamic acid. However, before hydrolysis they were well separated with a zone shown by P.C. to be glutamic acid occurring between them. It was accordingly thought that one of the two peptides still had an amide group on one of the carboxyl groups.

In order to support this view a sample of cut 10 was converted to the DNP derivatives. The DNP derivatives of the basic peptides had very different partition ratios in the benzene, acetic acid, 0.1 *N* HCl system from the acidic peptides which were practically 0. A preliminary separation in this system was accordingly made and the acidic peptides then distributed in the chloroform, acetic acid, 0.1 *N* HCl system to give pattern G.

After recovery of the cuts indicated, hydrolysis and P.C. gave in each case only one blue spot in the position of glutamic acid. A yellow spot also in each case was observed in the position of DNP-aspartic acid. The identity of the DNP amino acid in the yellow spot was substantiated by *K* determination. From these data the sequence Asp. Glu has been established and the two bands, $K = 3.0$ and 8.3, respectively, could arise by the presence of an amide group on the solute in the band on the left.

P.C. and P.E. indicated that cut 9 was a mixture of tyrosine, valine, a basic peptide and an acidic peptide. Preparative P.E. permitted easy separation of the acidic and basic peptides.

Following complete hydrolysis two clear spots were obtained for the acidic peptide which corresponded to glutamic acid and tyrosine, while for the basic peptide three clear spots indicating valine, ornithine and leucine were obtained.

A similar investigation of cut 8 indicated that it had the same four solutes as did 9 but with much less peptide material and much more tyrosine and valine.

Cut 7 was found by P.C. and P.E. to be a mixture of leucine, three acidic peptides and the basic peptide which had a lower mobility than the di- or tripeptides studied above. The basic peptide was easily isolated by preparative P.E. Following hydrolysis and study by P.C. four spots

(2) L. C. Craig and T. P. King, THIS JOURNAL, **77**, 6620 (1955).

(3) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

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

(5) H. G. Kunkel, Zone Electrophoresis, in "Methods of Biochemical Analysis," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1953, p. 141.

(6) M. T. Bush and P. Densen, *Anal. Chem.*, **20**, 121 (1948).

(7) W. Hausmann, J. R. Weisiger and L. C. Craig, THIS JOURNAL, **77**, 723 (1955).

(8) J. R. Weisiger, W. Hausmann and L. C. Craig, *ibid.*, **77**, 731 (1955).

(9) A. R. Battersby and L. C. Craig, *ibid.*, **74**, 4023 (1952).

corresponding to tyrosine, valine, ornithine and leucine were obtained. The major acidic peptide was found to contain phenylalanine and aspartic acid.

Cut 6 was indicated by P.C. and P.E. to contain proline, leucine and two peptides. Following hydrolysis of the mixture strong spots corresponding to tyrosine and valine appeared, but an attempt to isolate the dipeptide has not thus far been made.

P.C. and P.E. indicated three solutes to be present in cut 5. These corresponded to a small amount of phenylalanine, a basic peptide and an acidic peptide. These were easily separated by zone electrophoresis.

Hydrolysis of the basic peptide and paper chromatography gave spots corresponding to ornithine, leucine and phenylalanine. The sequence must be Orn. Leu. Phe since valylornithine and ornithylleucine have already been isolated.

Hydrolysis and paper chromatography of the acidic peptide gave satisfactory spots corresponding to phenylalanine, aspartic acid and glutamic acid. The intact peptide was converted to the DNP derivative and distributed as in pattern F. Only a single band was obtained which gave a weight–optical density ratio indicating a molecular weight of 640 ± 60. The calculated molecular weight of DNP Phe.Asp.Glu is 575. That phenylalanine is the N-terminal amino acid was shown by hydrolysis of the DNP-derivative and identification of DNP-phenylalanine by a combination of P.C. and partition ratios in different systems.

Cut 4 was a mixture similar to 5 but with phenylalanine as the major component.

Cut 3 was directly fractionated again by C.C.D. to 580 transfers in the methanol, chloroform, 0.1 *N* HCl system. This gave pattern C. When a portion of a cut as shown on the pattern was hydrolyzed and studied by P.C., strong spots of phenylalanine and leucine were obtained and a weaker spot of proline. Since this indicated a possible mixture, a second portion of the cut was converted to DNP derivatives and distributed to 180 transfers in the benzene, acetic acid, 0.1 *N* HCl system to give pattern E.

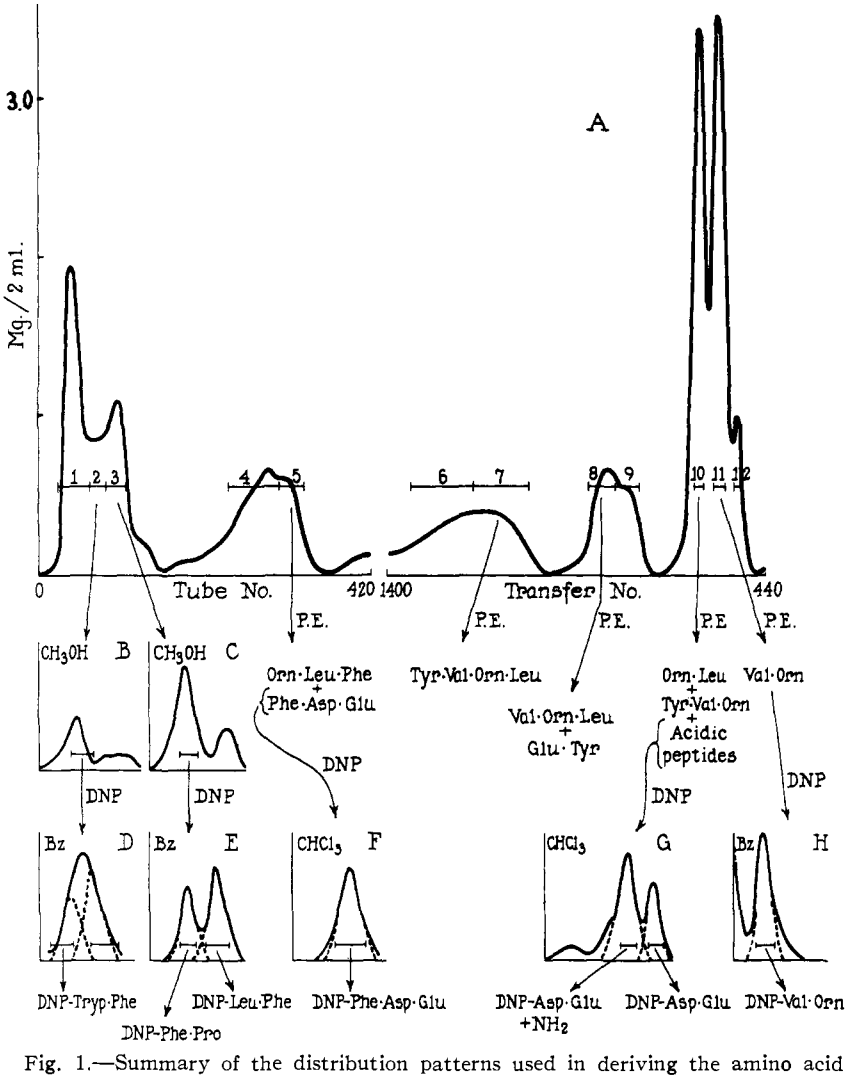

Fig. 1.—Summary of the distribution patterns used in deriving the amino acid sequence of tyrocidine B: CH_3OH = methanol, chloroform and 0.1 *N* HCl (2:2:1); $CHCl_3$ = chloroform, glacial acetic acid and 0.1 *N* HCl (2:2:1); Bz = benzene, glacial acetic acid and 0.1 *N* HCl (2:2:1).

The solute in the band on the right with a K of 1.6 after hydrolysis and P.C. gave two spots corresponding to DNP-leucine and phenylalanine. The identity of DNP-leucine was confirmed by determining its partition ratio in several systems. Weight–optical density measurements on the intact DNP derivative indicated a molecular weight of 460 ± 40. The derivative is DNP-leucylphenylalanine, mol. wt. 444.

A similar study with the band on the left, K = 0.92, gave spots corresponding to DNP-phenylalanine (confirmed by K determinations), proline and a spot in the position of phenylalanine. Weight–optical density measurements indicated a molecular weight of 510 ± 50. These data would indicate a tripeptide with two phenylalanine residues. However, such a peptide would complicate the sequence interpretations which are otherwise clear cut thus far. A closer identification of the spot corresponding to free phenylalanine in the hydrolysate of the DNP peptide accordingly was desired. Therefore, the hydrolysate was extracted with ether to remove the DNP-phenylalanine and the remaining free amino acids were converted to DNP derivatives. Distribution of the reaction mixture gave a band in the position of DNP-proline but nothing in the position of DNP-phenylalanine. The spot corresponding to phenylalanine is therefore an artifact or impurity and the peptide is DNP-phenylalanylproline, mol. wt. 428.

This conclusion was confirmed by the synthesis of DNP-D-phenylalanyl-L-proline as given further on. The synthetic material had the same K in the system used in pattern E and gave a similar optical rotation, $[\alpha]^{25}D$ +48° as compared to 44° for the peptide from tyrocidine B.

A sample of cut 2 was found to contain tryptophan after hydrolysis. The remainder of the cut was distributed to 650 transfers in the methanol, chloroform system as given in pattern B. Investigation by P.C. of a hydrolysate of the cut as indicated suggested that it could still be a mixture.

The cut was therefore converted to DNP derivatives and distributed to 440 transfers in the benzene, acetic acid, 0.1 *N* HCl system. This gave pattern D. Preliminary investigation of the band indicated it to be an overlapping doublet. The residue from a cut of pattern D on the left (K = 0.82) as shown on the pattern gave a strong *p*-dimethylaminobenzaldehyde test for tryptophan while the material from the right-hand cut (K = 0.92) gave a negative test and proved to be DNP-phenylalanylproline already found in the solute from pattern E.

When the tryptophan-containing material was hydrolyzed and studied by P.C. only a single spot corresponding to phenylalanine was obtained. The DNP-amino acid was completely destroyed as would have been expected if it

were DNP-tryptophan. However, weight–optical density measurements indicated the DNP-peptide to have a molecular weight of 580 ± 60. It was concluded that this peptide derivative is DNP-tryptophanylphenylalanine, mol. wt. 517.

Cut 1 gave rather confusing results. These will be treated in the discussion.

DNP-D-Phenylalanyl-L-proline.—The dipeptide ester was prepared by condensation of DNP-D-phenylalanine (66 mg.) with L-proline methyl ester (27 mg.) using the mixed anhydride procedure of Vaughn and Osato.[10] The ester was then hydrolyzed at 100° for 75 minutes in a mixture of 4 ml. of acetic acid and 2 ml. of 2.9 *N* HCl. The solvent was removed and the residue taken up in ether. The desired derivative was extracted by sodium bicarbonate. It was recovered from the bicarbonate and further purified by C.C.D. to 100 transfers in the benzene, acetic acid, 0.1*N* HCl system (K = 0.90). It was recovered as a yellow powder (26 mg.) by lyophilization from an acetic acid solution; $[\alpha]^{25}D$ +48° (*c* 0.46 in $CHCl_3$).

Anal. Calcd. for $C_{20}H_{20}N_4O_7$: C, 56.07; H, 4.71. Found: C, 56.01; H, 4.90.

Discussion

In the experimental part of this paper the isolation experiments were summed up in the charts of Fig. 1. The isolation of 12 peptides was described in Table I and arranged in such order that the sequence in tyrocidine B is obvious. DNP derivatives of peptides 1, 2, 3, 7, 11 and 12 were also reported. Hydrolysis of these DNP-peptides revealed the N-terminal amino acid of each peptide. These data are sufficient to permit the assignment of a unique sequence for tyrocidine B as shown in formula 1.

TABLE I

1—Tryp.Phe
2—Phe.Asp.Glu
3—Asp.Glu
4—Glu.Tyr
5—Tyr.Val.Orn.Leu
6—Tyr.Val.Orn
7—Val.Orn
8—Orn.Leu
9—Val.Orn.Leu.Phe
10—Orn.Leu.Phe
11—Leu.Phe
12—Phe.Pro

L-Orn	
L-Val	L-Leu
L-Tyr	D-Phe
L-Glu	L-Pro
L-Asp	L-Tryp
D-Phe	

Formula 1, tyrocidine B

L-Orn	
L-Val	L-Leu
L-Tyr	D-Phe
L-Glu	L-Pro
L-Asp	L-Phe
D-Phe	

Formula 2 tyrocidine A

Comparison of the sequence so derived with that suggested for tyrocidine A,[11] formula 2, shows an identical sequence except for the tryptophan. Even the optical configuration of the amino acid residues is the same in both peptides.

Direct evidence for the presence of every sequence given in formula 1 can be derived from Table I except the prolyl–tryptophan sequence. Although an indication of this was obtained from investigations of the solutes in cut 1 of Fig. 1 there were certain complications which appeared to result from the transformations of tryptophan.

Hydrolysis of a portion of the material from cut 1 and P.C. indicated leucine, phenylalanine, proline and tryptophan to be present. However, redistribution in another solvent system indicated inhomogeneity. Conversion of a portion to DNP derivatives and C.C.D. gave a series of bands one of which appeared to approach rather closely the criteria of purity used throughout this paper. It gave good spots in the positions expected for DNP-phenylalanine, proline and phenylalanine after hydrolysis and P.C. The presence of tryptophan was shown by a *p*-dimethylaminobenzaldehyde test on the intact DNP derivative. However, the absorption spectrum curve of the DNP derivative showed an extra maximum at 310 mμ in addition to the one at 350 mμ.

Another tryptophan containing band obtained by C.C.D. of the DNP mixture showed the presence of DNP-leucine, proline and phenylalanine on hydrolysis. This band would have been satisfactory from every standpoint except that it was definitely wider than a calculated band.

In general the type of behavior indicated some subtle reaction taking place, perhaps cyclization. In connection with the dipeptide phenylalanylproline an indication of this type of behavior was noted.

After the conversion of the peptides to DNP derivatives it was usually the practice to extract the alkaline solution with ether to remove the excess FDNB. If this extraction step was omitted during conversion of cuts 2 and 3 a yellow band appeared during C.C.D. in the benzene system in addition to those in Fig. 1. The yellow solute in this band had a K of about 20 and was not soluble in a buffer at *p*H 8. On redistribution in a system made from benzene, isoöctane, acetic acid and 0.1 *N* HCl (volume proportions 12, 8, 20, 10) a single band of K 2.7 was obtained. On hydrolysis and P.C. only spots corresponding to DNP-phenylalanine and proline were obtained. Since it is known that proline-containing peptides cyclize easily to diketopiperazines[12] it is likely that this neutral derivative is a diketopiperazine derivative.

If so it must have formed in the alkaline reaction mixture immediately after reaction with FDNB had taken place. The diketopiperazine would not be expected to react with FDNB; nor would the diketopiperazine occur in the same band during C.C.D. with the free peptide.

The only uncertainty remaining with tyrocidine B concerns the asparagine and glutamine residues. Direct proof that isoasparagine and isoglutamine are not involved is not available. However, Battersby[13] has ruled out isoasparagine and isoglutamine in the case of tyrocidine A. It is unlikely that B is different in this regard.

Summary.—The amino acid sequence in tyrocidine B has been studied by partial hydrolysis with hydrochloric acid. Various fractionation proce-

(10) J. R. Vaughn and R. Osato, THIS JOURNAL, **74**, 676 (1952).
(11) A. Paladini and L. C. Craig, *ibid.*, **76**, 688 (1954).
(12) J. S. Fruton, "Advances in Protein Chemistry," Academic Press, Inc., New York, N. Y., 1949, Vol. V, p. 68.
(13) Personal communication to be published by A. R. Battersby.

dures have given a series of peptides. All the data obtained can be rationalized by a unique sequence which forms a ring containing ten amino acids.

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NEW YORK 21, N. Y.

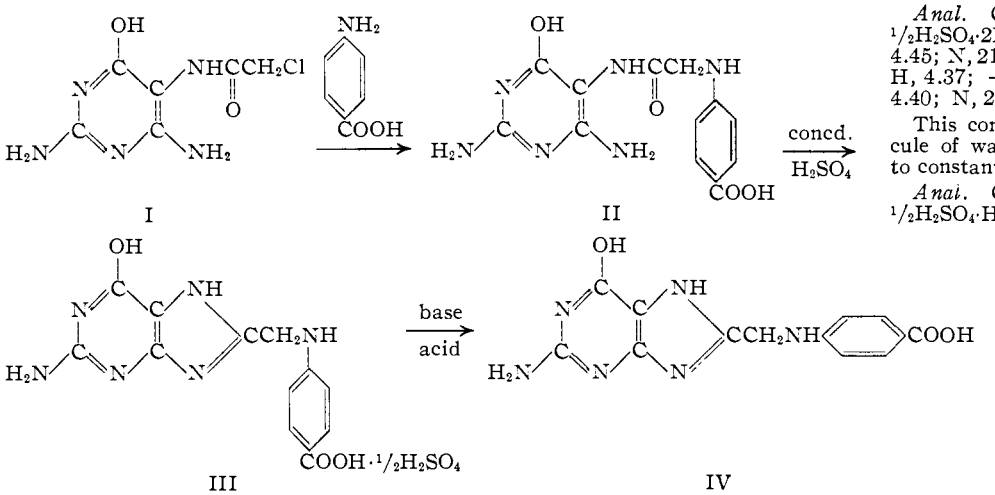
Preparation of *p*-[(2-Amino-6-hydroxy-8-purinemethyl)-amino]-benzoic Acid[1]

BY WILLIAM T. CALDWELL AND CHAO-SHING CHENG

RECEIVED JUNE 20, 1955

Pteroic acid, *p*-[(2-amino-4-hydroxy-6-pteridylmethyl)-amino]-benzoic acid, is a part of folic acid and is itself active for *S. faecalis R*[2]. Since simple modifications of the purines that occur in nucleic acids produce compounds with striking physiological properties, such as, for example, 6-mercaptopurine and azaguanine, it seemed to us to be interesting to prepare an analog of pteroic acid in which the pyrazine moiety is replaced by an imidazole ring: *p*-[(2-amino-6-hydroxy-8-purinemethyl)-amino]-benzoic acid (IV).

IV was therefore prepared according to the following scheme:



Experimental[3]

p-[(2,4-Diamino-6-hydroxy-5-pyrimidyl)-carbamylmethyl-amino]-benzoic Acid (II).—A solution of 4.50 g. (0.019 mole) of 2,4-diamino-6-hydroxy-5-chloroacetamidopyrimidine[4] (I) and 5.75 g. (0.042 mole) of *p*-aminobenzoic acid in 240 ml. of boiling water was refluxed over a small flame for 6 hours. During this time, a brown precipitate slowly formed. This was separated by filtration, washed with some water and dissolved in 1 *N* sodium hydroxide. The solution was treated with charcoal and then filtered. The product was reprecipitated by adding to the filtrate 1 *N* hydrochloric acid to a *p*H of about 7. The precipitate was collected by filtration and washed with water. Weight of this crude product was 3.2 g. (51.6%). After a second solution by alkali and precipitation by acid, the precipitate was ready for analysis after drying at 90–95° for 19 hours. The compound began to discolor at about 260° and did not melt up to 360°. The analysis showed that it was a hemihydrate, which was confirmed by subsequent reactions and analysis. By heating the hemi-hydrate at 180° for 15 hours to constant weight, the loss was 2.34%; calculated, 2.75%.

Anal. Calcd. for $C_{13}H_{14}N_6O_4 \cdot {}^1/_2H_2O$: C, 47.70; H, 4.62; H_2O, 2.75. Found: C, 47.07; H, 4.44; H_2O, 2.34.

This compound formed an unstable hydrochloride of undetermined structure when it was boiled with 4 or 6 *N* hydrochloric acid and then cooled in refrigerator overnight. Shining white crystals were obtained which, on washing with water, immediately hydrolyzed to the original compound II. It also formed an unstable sulfate of undetermined structure when it was dissolved in concentrated sulfuric acid at room temperature, diluted to about 6 *N* strength in cold water and allowed to stand overnight. White crystals were obtained, which on washing with water slowly hydrolyzed to II.

p-[(2-Amino-6-hydroxy-8-purinemethyl)-amino]-benzoic Acid (IV).—Compound II (3.27 g., 0.01 mole) was dissolved in 33 ml. of concentrated sulfuric acid and heated on a steam-bath for 2 hours. The acid solution was then filtered through asbestos fiber and the filtrate run into 750 ml. of water, which was cooled in an ice-bath. After standing in the refrigerator overnight, orange colored needles were obtained which did not hydrolyze to II on washing with water. A yield of 1.53 g. (39.7%) was obtained. After two more recrystallizations from concentrated sulfuric acid and water, it began to decompose at about 225° but did not melt by 290°. Analysis showed that this compound was a sulfate containing two molecules of water of crystallization (III).

Anal. Calcd. for $C_{13}H_{12}N_6O_3 \cdot {}^1/_2H_2SO_4 \cdot 2H_2O$: C, 40.51; H, 4.45; N, 21.81. Found: C, 40.52; H, 4.37; ———. C, 40.61; H, 4.40; N, 21.30.

This compound lost one molecule of water by drying at 110° to constant weight.

Anal. Calcd. for $C_{13}H_{12}N_6O_3 \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 42.50; H, 4.12. Found: C, 42.07; H, 4.16.

When III was boiled with dimethylformamide and the solution run into water, the ring opened and a compound identical with II was obtained. This also was confirmed by analysis.

Anal. Calcd. for $C_{13}H_{14}N_6O_4 \cdot {}^1/_2H_2O$: C, 47.70; H, 4.62. Found: C, 47.89; H, 4.71.

When III was treated under milder conditions—dissolved in 0.5 *N* cold sodium hydroxide and precipitated by 0.5 *N* cold hydrochloric acid—a different compound, *p*-[(2-amino-6-hydroxy-8-purinemethyl)-amino]-benzoic acid (IV), was obtained. It decomposed at 315–317°. Although it had the same total composition as II, analyses proved the presence of one and a half molecules of water of crystallization, indicating that ring formation had occurred to give a purine derivative.

Anal. Calcd. for $C_{13}H_{12}N_6O_3 \cdot 1{}^1/_2H_2O$: C, 47.70; H, 4.62; N, 25.68; H_2O, 8.26. Found: C, 47.20; H, 4.61; N, 25.38; H_2O, 8.54.

The loss of water was determined by heating the compound at 180° to constant weight.

Other methods of ring closure to prepare IV directly from II were tried but without success. II was heated[5] at various temperatures under a vacuum of 15 mm. pressure. At 260–265°, decomposition occurred with the formation of a little brownish, greasy sublimate.

When II was boiled with 20% hydrochloric acid,[6] hydroly-

(1) Taken from a part of the thesis submitted by Chao-Shing Cheng to the Temple University Graduate Council in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) R. B. Angier, *et al.*, *Science*, **102**, 227 (1945); **103**, 667 (1946).

(3) All melting points are uncorrected. Analyses were performed by Micro-Tech. Laboratories, Skokie, Ill., and Huffman Microanalytical Laboratories, Wheatridge, Colo.

(4) G. H. Hitchings and G. B. Elion, THIS JOURNAL, **71**, 467 (1949).

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