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Studies on Amino Acid Substitution in the Biosynthesis of the Antibiotic Polypeptide Tyrocidine*

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ABSTRACT: The tyrocidines, a series of antibiotic cyclic decapeptides synthesized by *Bacillus brevis*, have been found to consist of three homologous peptides containing both L- and D-amino acid residues. These peptides, tyrocidines A–C [Battersby, A. R., and Craig, L. C. (1952), *J. Am. Chem. Soc. 74*, 4019] differ by a single amino acid replacement of tryptophan for phenylalanine. By introducing large amounts of tryptophan into the growth medium, it has been possible to alter the relative amounts of these three homologs and also to induce the incorporation of L-tryptophan in place of L-tyrosine, thereby producing a hitherto unobserved peptide which has been designated tyrocidine D. Moreover, it has been possible to induce the incorporation of alloisoleucine and isoleucine, amino acids not normally found in tyrocidine, by adding them to the growth medium. These amino acids have been found to replace nonselectively the residues of valine and leucine. These observations on the lack of specificity in tyrocidine biosynthesis give added support to the earlier hypothesis that tyrocidine biosynthesis proceeds by a mechanism different from that for proteins [Mach, B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 263; Mach, B., and Tatum, E. L. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 876]. The procedures for the induced biosynthesis and chemical characterization of these new tyrocidine peptides are given in this report.

Although tyrocidine was discovered more than 25 years ago (Dubos, 1939; Hotchkiss and Dubos, 1940) it was not until the development of countercurrent distribution that this substance was shown to be a mixture of three closely related polypeptides designated tyrocidines A–C (Battersby and Craig, 1952). Chemical degradation studies elucidated the amino acid sequences for the three peptides (Paladini and Craig, 1954; King and Craig, 1955; Ruttenberg *et al.*, 1965) and revealed that they were cyclic decapeptides which differed by a single amino acid replacement. Such heterogeneity has subsequently been found with other small peptides (Craig, 1956) to an extent which led to the suggestion that a biosynthetic mechanism different from that for proteins might be involved. In view of the current interest in protein biosynthesis, it seemed appropriate to investigate the biosynthesis of tyrocidine. Recent observations in this regard have confirmed the hypothesis that the synthetic mechanism is different from that for proteins (Mach, 1963; Mach and Tatum, 1964). Extension of these studies in the present work has shown that the introduction of large amounts of various natural amino acids into the growth medium can result in their incorporation into tyrocidine. Since such amino acid replacements are unknown in protein biosynthesis, this result reinforces the conviction that tyrocidine biosynthesis proceeds by a mechanism different from protein biosynthesis.

Experimental Section

Methods. The *Bacillus brevis* cultures were grown as previously described (Mach and Tatum, 1964). The reagents used were of the highest purity obtainable. All solvents were redistilled. The dialysis experiments were performed in cells described by Craig and Konigsberg (1961). Amino acid analyses were

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performed as described by Spackman *et al.* (1958). The optical rotations were measured on a Bendix polarimeter using the D line of sodium, and the concentration of the DNP-amino acids which were used for these measurements were determined spectrophotometrically at 350 mμ. The values used as standards for the specific rotations and extinctions were those reported by Rao and Sober (1954).

Isolation of Tyrocidine D. The *B. brevis* cultures were grown in the presence of an excess of L-tryptophan, under conditions which resulted in the almost exclusive synthesis of tyrocidine D (Mach and Tatum, 1964). L-Tryptophan at a concentration of 0.5 μM/ml was added at the beginning of tyrocidine production, when the A_{600}^{1cm} of the culture was 1.3. The tyrothricin was isolated 5 hr later from the culture by ethanol extraction and passage through a column of acid alumina as described earlier by Mach (1963). The crude peptide extract (tyrothricin) was submitted to fractionation by countercurrent distribution in the system chloroform–methanol–0.01 N HCl as described by Ruttenberg *et al.* (1965). Analysis after 500 transfers gave the pattern shown in Figure 1. Peak 1 was the gramicidin component with a distribution coefficient of 0.11. This material was not further characterized. Peak 2 with a distribution coefficient of 0.96 was the tyrocidine component. This material was further fractionated by means of adsorption chromatography on Sephadex G-25. Fractionation by this method separates the various tyrocidines according to their degree of aromaticity (Ruttenberg *et al.*, 1965). As shown in Figure 2, the tyrocidine from the countercurrent distribution emerged as a single peak in a position

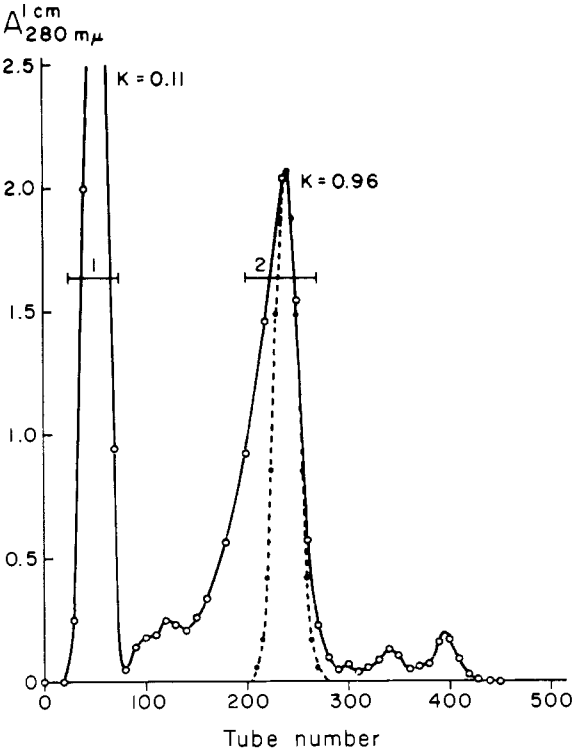

FIGURE 1: Countercurrent distribution of crude peptide extract (tyrothricin) from a culture of *B. brevis* grown in a tryptophan-rich medium. The solvent system was composed of chloroform–methanol–0.01 N HCl (2:2:1). The analysis was made after 500 transfers in a 1000-tube automatic machine employing 2 ml of each phase. The solid curve is the experimental result; the dotted curve represents the theoretical Gaussian distribution.

TABLE I: Amino Acid Composition of Tyrocidine D Compared to Tyrocidines A–C and Gramicidin S.[a]

Amino Acid	Tyrocidine D	Tyrocidine A	Tyrocidine B	Tyrocidine C	Gramicidin S
Valine	0.99	1	1	1	2
Ornithine	1.00	1	1	1	2
Leucine	1.00	1	1	1	2
Phenylalanine	1.02	3	2	1	2
Proline	1.01	1	1	1	2
Aspartic	1.01	1	1	1	0
Glutamic	1.01	1	1	1	0
Tyrosine	0.00	1	1	1	0
Tryptophan	2.9	0	1	2	0
Total number of residues	9.94	10	10	10	10

[a] The values refer to the number of residues per molecule of peptide. The tryptophan was determined both by amino acid analysis and by spectrophotometric measurement. In all cases, the aspartic and glutamic acids are present in the peptides as asparagine and glutamine.

which suggested that it had a more aromatic character than tyrocidine C. The elution position of this tyrocidine from Sephadex G-25 led to its designation as tyrocidine D. As shown in Table I, amino acid analysis of the tyrocidine D confirmed the assumption of high aromatic content; with respect to composition, it is a homolog of tyrocidine C in which a residue of tyrosine is replaced by a tryptophan residue.[1]

Characterization of Tyrocidine D. The least ambiguous technique to show that tyrocidine D is a cyclic decapeptide homologous in structure to tyrocidines A–C would be to determine its amino acid sequence. Because of the scarcity of the peptide, this direct approach was not taken, but, instead, several properties of the tyrocidine D were compared to those of the other tyrocidines whose primary structures have already been determined. In these studies, all of the properties of tyrocidine D which were examined were con-

[1] Preliminary studies had indicated earlier (Mach and Tatum, 1964) that the tryptophan in tyrocidine D was replacing the phenylalanine of tyrocidine C. This observation was in error, as indicated in this report.

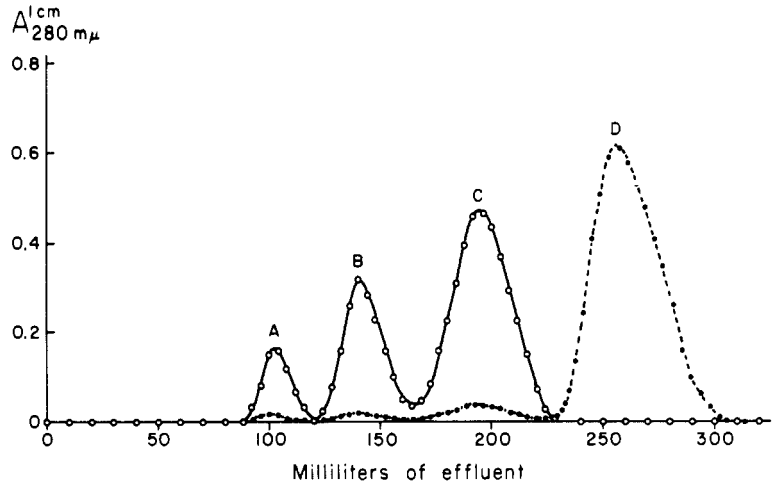

FIGURE 2: Sephadex chromatography of tyrocidine on a 0.9 × 150 cm column of Sephadex G-25 in 10% acetic acid. Fractions (2 ml) were collected at a flow rate of 10 ml/hr. The solid curve is the pattern given by a mixture of tyrocidines A–C. The dotted curve was obtained by chromatography of the tyrocidine peak (cut 2) from the countercurrent distribution (Figure 1). This separation was obtained using the crushed Sephadex. The beaded Sephadex does not give as good a resolution.

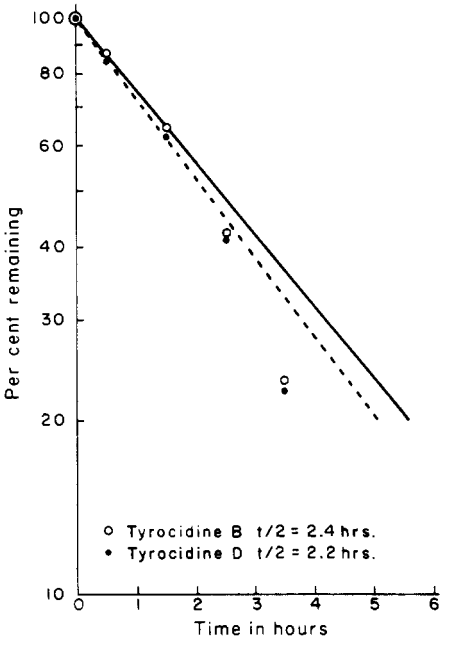

FIGURE 3: Thin film dialysis of tyrocidine B, O———O, and tyrocidine D, ●-----●, in 10% acetic acid.

sistent with its being a homolog in the tyrocidine series.

The first experiment was to look for amino-terminal groups in the molecule. Accordingly, a sample of tyrocidine D was treated with an excess of fluorodinitrobenzene[2] in 50% methanol containing sufficient triethylamine to maintain the pH at 8.5. The dinitrophenyltyrocidine D was then hydrolyzed for 24 hr in an evacuated sealed tube which had been flushed with nitrogen. The hydrolysate was diluted with water, extracted with ether, and the ether extract was subjected to paper chromatography in the toluene system as described by Fraenkel-Conrat *et al.* (1955). The only DNP-amino acid detected was δ-DNP-ornithine. This indicated the absence of an amino-terminal residue.

If the replacement of the residue of L-tyrosine in tyrocidine C by tryptophan in tyrocidine D were stereospecific, then tyrocidine D should contain one residue of D-tryptophan and two residues of the L enantiomorph. In order to show that this was the case, the tryptophan from tyrocidine D was isolated and its specific rotation determined.

As shown previously (King and Craig, 1955; Ruttenberg *et al.*, 1965), good recovery of tryptophan may be obtained with direct acid hydrolysis of tyrocidine. This tryptophan could be separated easily from the other amino acids by means of adsorption chromatography on Sephadex G-25 in 0.2 M acetic acid (Ruttenberg *et al.*, 1965). In the case of tyrocidine D, the tryptophan from 1 μmole of hydrolysate was isolated by this method and it was converted to the dinitrophenyl (DNP) derivative. The rotation of the DNP-tryptophan was measured in 1 N NaOH and the value for $[\alpha]_D^{25}$ was found to be $-530 \pm 120°$. The reported value for $[\alpha]_D$ of DNP-L-tryptophan in 1 N NaOH is $-1291°$ (Rao and Sober, 1954). Since it is unlikely that specific racemization of one of the three tryptophans in tyrocidine D would occur during hydrolysis, the conclusion was that two of the tryptophan residues in tyrocidine D are of the L configuration and one is of the D configuration. Although the experimental error in the rotation was rather high due to the low

[2] Abbreviation used: FDNB, fluorodinitrobenzene.

TABLE II: Incorporation of Isoleucine and Alloisoleucine into Tyrocidine.

Amino Acid	No Isoleucine Added	L-Isoleucine	D-Allo-isoleucine	L-Allo-isoleucine	L-Alloisoleucine after Chro-matography[a]
Valine	1.00	0.80	0.96	0.38	0.36
Ornithine	1.00	1.00	1.01	0.98	0.99
Leucine	1.05	0.92	0.97	0.40	0.34
Phenylalanine	1.25	1.47	1.20	1.24	1.00
Proline	0.94	1.07	0.99	1.00	0.97
Aspartic	0.92	1.00	1.00	1.00	0.93
Glutamic	0.96	1.03	1.00	1.02	1.03
Tyrosine	0.54	0.47	0.49	0.52	0.80
Tryptophan	2.6	1.7	2.5	2.5	2.4
Isoleucine	0.00	0.10	0.00	0.00	0.39
Alloisoleucine	0.00	0.30	0.00	1.05	0.77
Total number of residues	10.16	9.86	10.12	10.09	9.98

[a] See Figure 4.

concentration of DNP-tryptophan, the value obtained was consistent only with this interpretation. This result demonstrated that the replacement of the L-tyrosine in tyrocidine C by a tryptophan residue in tyrocidine D was stereospecific and gave additional support to the hypothesis that tyrocidine D is homologous to tyrocidine C.

A pertinent result was obtained from the comparison of the dialysis rates for tyrocidine D and tyrocidine B. The technique of thin film dialysis has been useful in studying the tyrocidines, since these peptides tend to associate very strongly in aqueous solution. These dialysis experiments are discussed in detail elsewhere (Ruttenberg *et al.*, 1966). It has been shown that gramicidin S, a cyclic decapeptide of very similar primary structure to that of tyrocidines A–C, does not aggregate in 0.01 M acetic acid and dialyzes with a half-time of 0.3 hr. Furthermore, linear tyrocidine B, derived from cyclic tyrocidine B by means of reductive cleavage at the phenylalanylproline linkage according to the method of Ruttenberg *et al.* (1964), dialyzes with the same half-time. In contrast, intact tyrocidine B dialyzes under the same conditions with a half-time of 6.1 hr. Therefore, it appears that both the proper amino acid sequence as well as the cyclic structure are necessary for the aggregation of tyrocidine, and this fact suggested that dialysis would be a useful tool to aid in the characterization of tyrocidine D. The dialysis rates for tyrocidine B and tyrocidine D were compared in 10% acetic acid. Although aggregation is somewhat less in this solvent than in 0.01 M acetic acid, the typical concentration dependence (Craig, 1965) of the aggregation can be seen more clearly in a single experiment. The results are given in Figure 3. It can be seen that for tyrocidine B, the dialysis rate increases as the concentration of the peptide inside the membrane decreases. The pattern for the tyrocidine D is almost identical. Until the primary structure of tyrocidine D is confirmed by sequential analysis or chemical synthesis, the foregoing experimental results may be used as strong evidence in favor of the hypothesis that tyrocidine D is a homolog of the tyrocidine A–C series in which the L-tyrosine residue of tyrocidine C is replaced by a residue of L-tryptophan.

Studies on the Incorporation of Other Amino Acids into Tyrocidine. Since it had been possible to induce the synthesis of a new form of tyrocidine resulting from a substitution between two structurally related amino acids, it was hoped that the same principle might apply in the case of other amino acids which are not normally utilized for tyrocidine biosynthesis and that their incorporation into the decapeptide could be demonstrated. The experimental conditions and the extraction and purification of the tyrocidine were as described above for the production of tyrocidine D, with the exception that the fractionation by countercurrent distribution was omitted. In this case, the gramicidin component was removed by electrophoresis (Mach, 1963). The studies were begun with isoleucine, and amino acid analysis of the resulting tyrocidine revealed that isoleucine and alloisoleucine were incorporated. These results are summarized in Table II. The commercial isoleucine which was used was subjected to amino acid analysis and it was found to contain about 2% of alloisoleucine. It appeared, therefore, that *B. brevis* incorporated alloisoleucine in preference to isoleucine, and further studies showed that it was L-alloisoleucine and not the D isomer which was incorporated.

The tyrocidine which was analyzed for isoleucine and alloisoleucine incorporation was not fractionated with respect to the A–D homologs, and as may be seen in Table II, was a mixture of some or all of these peptides. This is apparent since the sum of the aromatic

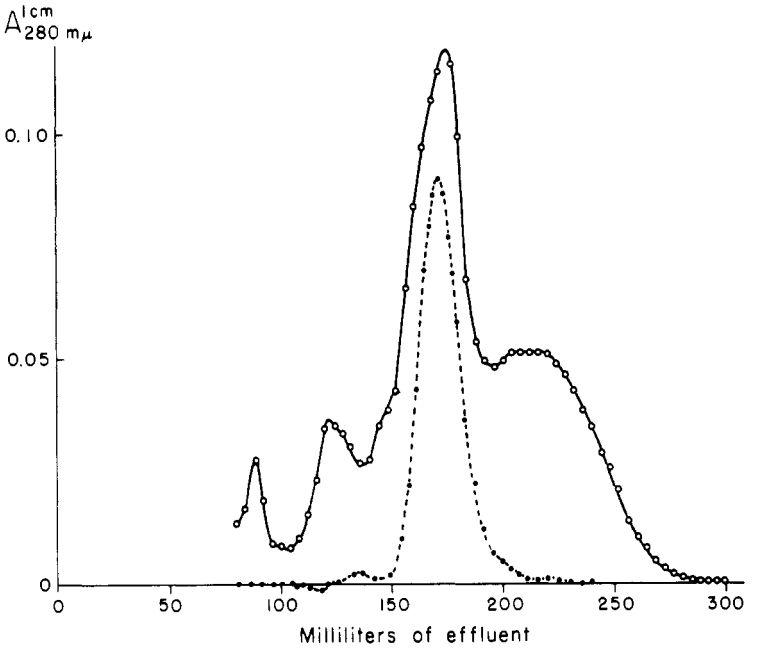

FIGURE 4: Sephadex chromatography of tyrocidine isolated from a culture of *B. brevis* grown in the presence of a large amount of L-alloisoleucine. The same conditions as in Figure 2 were employed. The solid curve was obtained initially. The material in the main peak was removed and rechromatographed to give the dotted curve.

amino acids is always equal to four residues, whereas the relative amounts of these residues are not whole numbers. Likewise, the peptides are heterogeneous with respect to valine, leucine, isoleucine, and alloisoleucine, since the sum of these residues is equal to two, whereas, again, the relative amounts are not whole numbers. An attempt was made to fractionate this complex mixture on Sephadex G-25 in 10% acetic acid. The pattern which was obtained is shown in Figure 4. The solid curve shows the pattern which was obtained initially. The material which appeared in this chromatogram to be the most homogeneous was removed and rechromatographed as shown by the dotted curve. Amino acid analysis of the material in this single peak indicated that it was approximately 80% in tyrocidine C and 20% in tyrocidine D. In addition, the fraction was still heterogeneous with respect to isoleucine, alloisoleucine, valine, and leucine. The values are given in Table II.

The evidence that these "artificial" tyrocidines are still cyclic decapeptides is derived from the observation that all of the observed amino acid compositions indicate a multiple of ten amino acid residues, and treatment of the chromatographed peptide with FDNB failed to show any amino-terminal residue. Furthermore, the experience with tyrocidine D as described earlier indicated that amino acid substitution can occur in tyrocidine with the cyclic decapeptide structure remaining intact.

Since it is likely that *B. brevis* contains inverting enzymes and the commercial samples of isoleucine and alloisoleucine were not isomerically pure to start with, it is not possible to say what the stereochemistry of these amino acids is when they are incorporated into tyrocidine, without actually isolating them from an hydrolysate. This has not yet been possible, because of the very small amounts with which we have been dealing. However, the important fact is that, as can be seen from Table II, alloisoleucine and isoleucine can substitute for valine and leucine in tyrocidine.

Discussion

The results with the tryptophan-enriched medium indicate that this amino acid can substitute for residues of phenylalanine and tyrosine in tyrocidine biosynthesis. If the biosynthesis of tyrocidine is a purely enzymatic process (Mach and Tatum, 1964) then this implies that there is an enzyme or enzymes with receptor sites which can respond to tryptophan as well as phenylalanine or tyrosine. This implication is carried a step further by the results with isoleucine and alloisoleucine. In this case we observed the interchange of amino acids with hydrocarbon side chains. This suggests that the enzyme or enzymes with hydrophobic receptors which recognize valine and leucine can also react with isoleucine and alloisoleucine. By this line of reasoning, it should be possible to substitute homologous amino acids for ornithine or asparagine. We have not yet examined this possibility.

It has been noted earlier (Ruttenberg *et al.*, 1965) that *B. brevis* tends not to modify the pentapeptide half of tyrocidine, Val-Orn-Leu-Phe-Pro, which is common to the cyclic decapeptide gramicidin S (which

is actually a dimer of this pentapeptide). This is apparent in going from tyrocidine B to tyrocidine C; only one of the two phenylalanine residues is observed to be replaced by tryptophan. Again, in the case of tyrocidine D, the biosynthetic system prefers to replace a tyrosine with a tryptophan rather than to replace the phenylalanine residue in the gramicidin S half of the molecule. The reoccurrence of this gramicidin S pentapeptide fragment throughout the tryptophan substitution series (tyrocidines A–D) may be of some significance.

Acknowledgment

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