Isolation of Enzyme-Bound Peptide Intermediates in Tyrocidine Biosynthesis*

Robert Roskoski, Jr.,† Horst Kleinkauf, Wieland Gevers, and Fritz Lipmann‡

ABSTRACT: The tyrocidines are cyclic decapeptide antibiotics produced by specific strains of *Bacillus brevis*. Enzyme-bound intermediate peptides, including Phe-Pro-, Phe-Pro-Phe-, Phe-Pro-Phe-Phe-, and others, up to the linear decapeptidyl Phe-Pro-Phe-Phe-Asn-Gln-Phe-Val-Orn-Leu-, can be isolated from appropriately constituted mixtures by precipitation with trichloroacetic acid, and then identified on two-dimensional thin-layer chromatograms after liberation by treatment with alkali. The growing peptide chains are bound to the enzymes by thio ester linkages. The requisite amino acids must be added in the proper order for polypeptide synthesis; if phenylalanine or proline are omitted, no polypeptides are formed. Omission of asparagine stops synthesis at the tetrapeptide stage, even when the succeeding amino acids are present; if ornithine is omitted, synthesis stops at the octapeptide stage. These results indicate that chain growth begins at the D-phenylalanine residue next to proline, and proceeds in order, from amino to carboxyl terminus, until the addition of leucine. Finally, the carboxyl group of this residue, activated as thio ester, reacts with the free amino group of phenylalanine causing cyclization and release of the final product, a relatively slow reaction in the case of tyrocidine.

The development of cell-free systems active in tyrocidine formation has furthered our understanding of the mechanism of its biosynthesis. We report, in the preceding paper, that extracts of tyrocidine-producing strains of *Bacillus brevis* (ATCC 8185), on filtration through Sephadex G-200, yield three complementary fractions which synthesize tyrocidine when supplied with ATP, Mg^{2+}, and the corresponding amino acids (Roskoski *et al.*, 1970). The light component (mol wt 100,000) and the intermediate component (mol wt 230,000) activate phenylalanine and proline, respectively. The heavy component (mol wt 460,000) activates the remaining tyrocidine constituent amino acids. After activation, the aminoacyl residue is transferred from the aminoacyl adenylate–enzyme complex into a thio ester linkage to an enzymic sulfhydryl group, from which it enters into the chain elongation step. The identification of intermediate thio ester-linked peptides in tyrocidine biosynthesis will be the main subject of the present paper.

The isolation from the gramicidin S synthesizing system of Phe-Pro-diketopiperazine (Kurahashi, 1961), Phe-Pro-Val (Tomino and Kurahashi, 1964), and Phe-Pro-Val-Orn (Tsuji, 1966; Holm *et al.*, 1966) had indicated that in this process the direction of chain growth appeared to be from phenylalanine to leucine. In gramicidin S biosynthesis we have reported the identification of tri-, tetra-, and pentapeptidyl hydroxamates from the corresponding peptidylthioenzymes (Gevers *et al.*, 1969; Kleinkauf and Gevers, 1969), and did not find intermediates containing more than five residues, results confirmed by Frøshov *et al.* (1970). We concluded that cyclization to the decapeptide occurred by head-to-tail condensation of two pentapeptidyl thio esters. Similarly, Kurahashi *et al.* (1969) have reported that Phe-Pro-diketopiperazine is formed in extracts which synthesize tyrocidine, suggesting that chain growth begins at the same residue as in the gramicidin S system. Our present data indicate that chain growth in tyrocidine biosynthesis begins at D-phenylalanine and proceeds in order, from amino to carboxyl terminus, to form the linear decapeptide ending with thio ester-linked leucine. This peptide cyclizes relatively slowly to form the final product. The cyclization step in the two decapeptide-synthesizing systems thus appears to differ in a rather interesting way.

Experimental Section

Enzyme Preparation and Formation of Protein-Bound Nascent Peptide Chains. The light, intermediate, and heavy enzyme fractions were prepared from extracts of *B. brevis* (ATCC 8185) by ammonium sulfate fractionation and Sephadex G-200 gel filtration as described in the preceding paper (Roskoski *et al.*, 1970). The enzyme-bound peptide chains were separated from reaction mixtures by Sephadex G-50 gel filtration and trichloroacetic acid precipitation, all as described previously (Roskoski *et al.*, 1970). Radioactivity in the trichloroacetic acid precipitable material was collected on filter disks and determined by the method of Mans and Novelli (1961).

Liberation of the Enzyme-Bound Peptide Chains. To break the thio ester link by performic acid oxidation, the method of Frøshov *et al.* (1970) was used. Alkaline cleavage was carried out as follows: washed precipitates were transferred to tubes and macerated with 50 μl of water and 2.5 μl of 1.0 N KOH, using a steel spatula. The pH was adjusted to 9–10 if necessary. The tubes were stoppered and placed in a water bath at 90° for 20 min. Then 1 ml of 90% aqueous methanol was added to each tube before centrifugation at 1500*g* for 10 min. The supernatants were removed with Pasteur pipets and dried in a stream of air at 37°. The residues were taken up in 80

* From The Rockefeller University, New York, New York 10021. *Received August 20, 1970.* Supported by Grant GM-13972 from the U. S. Public Health Service.

† Recipient of a special fellowship (F03 CA22798) from the U. S. Public Health Service.

‡ To whom to address correspondence.

TABLE I: Formation of Protein-Bound Nascent Peptide Chains.[a]

Added Amino Acids	Bound Radioactivity ($\mu\mu$moles)
1. [^{14}C]Phe	25.1
2. [^{14}C]Phe,Pro	49.4
3. [^{14}C]Phe,Pro,Asn	67.2
4. [^{14}C]Phe,Pro,Asn,Gln	101.0
5. [^{14}C]Phe,Pro,Asn,Gln,Val	132.0
6. [^{14}C]Phe,Pro,Asn,Gln,Val,Orn	148.0
7. [^{14}C]Phe,Pro,Asn,Gln,Val,Orn,Leu	182.0
8. [^{14}C]Phe,—,Asn,Gln,Val,Orn,Leu	25.1
9. [^{14}C]Phe,Pro,—,Gln,Val,Orn,Leu	50.5
10. [^{14}C]Phe,Pro,Asn,Gln,Val,—,Leu	120.0

[a] The light (40 μg), intermediate (100 μg), and heavy (150 μg) fractions were incubated with medium M and the specified amino acids (0.1 mM) for 30 min at 37° (final volume 60 μl). Then 50-μl aliquots were transferred to filter disks and the radioactivity was determined as described in the Experimental Section.

μl of 90% methanol and chromatographed on silica gel plates (20 × 20 cm). The solvents were: solvent 1, ethyl acetate–pyridine–acetic acid–water (60:20:6:11, v/v); and solvent 2, 1-butanol–acetic acid–water (100:10:30, v/v, upper phase). The chromatograms were developed in solvent 1, dried, and then developed in the second dimension with solvent 2. Autoradiography was carried out by exposing the chromatograms to Royal Blue Medical X-Ray films (Eastman Kodak) for about 10 days.

Results

Formation of Protein-Bound Nascent Peptide Chains. Incubation of the three enzyme fractions, ATP, Mg^{2+}, and L-[^{14}C]phenylalanine, together with the specified amino acids, led to increases in the amount of radioactivity isolated with the trichloroacetic acid precipitable material (Table I). To obtain the increased incorporation, indicating formation of new peptide chains, it was required that the amino acids were added in such an order as to permit elongation in sequence, namely, proline, asparagine, glutamine, valine, ornithine, and leucine. When all constituent amino acids, except proline, were added, [^{14}C]phenylalanine incorporation was no greater than that found in the absence of the other amino acids. Similarly, when asparagine or ornithine were omitted, the level of incorporation was the value to be expected if interruption of peptide synthesis had taken place at that point in an obligatory-order type of polymerization. [^{14}C]Phenylalanine, in addition to being starter, also adds twice between proline and asparagine, and later between glutamine and valine. The high phenylalanine binding in the absence of other amino acids reflects its binding to four independent enzyme sites corresponding to positions one, three, four, and seven in the antibiotic (see Figure 1). Since the number of phenylalanine

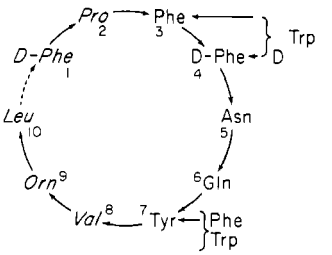


FIGURE 1: Primary structure of the tyrocidines. Residues are numbered arbitrarily.

residues in the growing chains differs (from one to four), increases in phenylalanine incorporation with addition of further amino acids were not regular because the different chains have different specific activities.

On the other hand, the increases in radioactivity were in regular progression when L-[^{14}C]proline binding was measured, since the proline residues occur only once in each of the nascent peptide chains except for initiating D-phenylalanine. Increased incorporation of [^{14}C]proline did not occur unless phenylalanine was added (Table II), and the amino acids had also to be added in the same order as in the experiment with labeled phenylalanine. These data suggested that it might be possible to isolate the individual bound intermediates.

Isolation of Enzyme-Bound Phe-Pro, Phe-Pro-Phe, and Phe-Pro-Phe-Phe. When the light and intermediate components were incubated with proline and labeled phenylalanine, [^{14}C]phenylalanine, and [^{14}C]Phe-Pro could be isolated as protein-bound intermediates and identified, after liberation in mild alkali, by thin-layer chromatography (Figure 2). The dipeptide was quantitatively converted into Phe-Pro-diketopiperazine under the conditions used (Tomino and Kurahashi, 1964). This product was also labeled if [^{14}C]proline was incubated with unlabeled phenylalanine. When the light, intermediate, or heavy fractions were incubated alone, or the heavy and intermediate components only were combined, no diketopiperazine was formed, and addition of the heavy fraction to the two others did not increase the formation of

TABLE II: Formation of Protein-Bound Nascent Peptide Chains.[a]

Added Amino Acids	Bound Radioactivity ($\mu\mu$moles)
1. —,[^{14}C]Pro	6.0
2. Phe,[^{14}C]Pro	20.5
3. Phe,[^{14}C]Pro,Asn	27.6
4. Phe,[^{14}C]Pro,Asn,Gln	39.0
5. Phe,[^{14}C]Pro,Asn,Gln,Val	43.5
6. Phe,[^{14}C]Pro,Asn,Gln,Val,Orn	49.3
7. Phe,[^{14}C]Pro,Asn,Gln,Val,Orn,Leu	54.7
8. —,[^{14}C]Pro,Asn,Gln,Val,Orn,Leu	7.1
9. Phe,[^{14}C]Pro,—,Gln,Val,Orn,Leu	21.0
10. Phe,[^{14}C]Pro,Asn,Gln,Val,—,Leu	43.7

[a] Incorporation was measured as described in Table I.

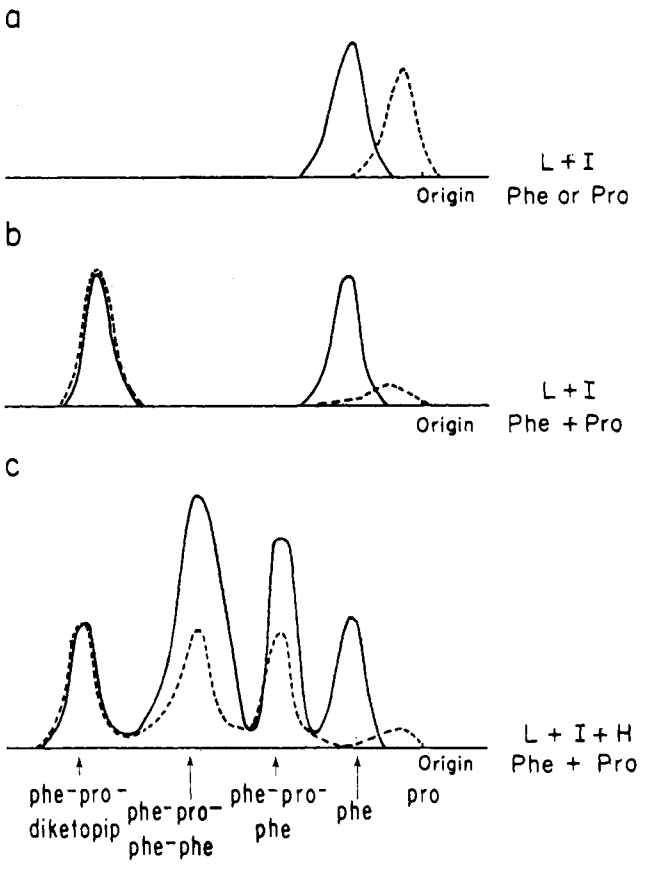


FIGURE 2: Thin-layer chromatography of protein-bound peptides after alkaline liberation. Enzyme fractions were incubated in 100 μl with medium M and the specified amino acids (0.1 mM) for 30 min at 37°. (a) Light (20 μg) and intermediate (50 μg) fractions were incubated with labeled phenylalanine or proline. (b) The same, except that both amino acids were present, one of them labeled. (c) The same as part b, except that 75 μg of heavy fraction was added. Sephadex G-50 gel filtration, alkaline liberation of bound peptides, and thin-layer chromatography in solvent 1 were carried out by the methods given in the Experimental Section. (——) Incorporated [^{14}C]phenylalanine; (----) [^{14}C]proline.

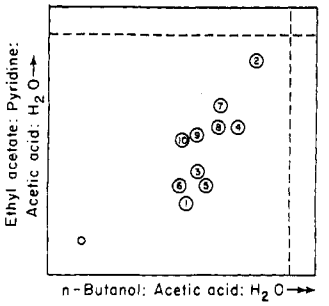


FIGURE 3: Two-dimensional thin-layer chromatography of protein-bound peptides after alkaline liberation. The conditions of the experiment were the same as in Figure 2c. The final mixture contained L-[^{14}C]phenylalanine, L-proline, L-asparagine, L-glutamine, L-valine, L-ornithine, and L-leucine at 0.1 mM. Chromatography and radioautography were carried out as described in the Experimental Section. The encircled figures indicate the number of residues in each peptide.

the Phe-Pro-adduct. Two additional labeled products, however, were now formed, with R_F's on thin layers of 0.36 and 0.54. Their radioactivity was the same when [^{14}C]proline was supplied as precursor, but the product with higher mobility contained more label when [^{14}C]phenylalanine was incorporated; it was therefore concluded that this was Phe-Pro-Phe-Phe, and the product with lower mobility, Phe-Pro-Phe. Development of the chromatograms in the second direction enabled us to fix the positions of these four products (1–4) which helped in the analysis of the products of other incubations as described below (Figure 3). We conclude that only the light and intermediate fractions are required for the synthesis of the first peptide bond.

Identification of Longer Protein-Bound Peptides. When the light, intermediate, and heavy components were incubated with L-[^{14}C]phenylalanine, proline, and asparagine, products in positions 1–5 were obtained (Figure 3). Using these same amino acids, labeled proline was incorporated into products 2–5, and labeled asparagine into product 5, which was taken to be Phe-Pro-Phe-Phe-Asx. We are not sure if amide groups of amino acid residues were removed during alkaline treatment. By use of L-[^{14}C]phenylalanine, proline, asparagine, and glutamine, products in positions 1–7 were found (Figure 3). In parallel experiments L-[^{14}C]proline was incorporated into products 2–7, and L-[^{14}C]glutamine appeared in products 6 and 7. The specific activities of products 6 and 7 were the same when glutamine was used as precursor, but product 7 had a higher specific activity when phenylalanine was used; thus, product 6 is Phe-Pro-Phe-Phe-Asx-Glx, and 7 is Phe-Pro-Phe-Phe-Asx-Glx-Phe.

When [^{14}C]phenylalanine, proline, asparagine, glutamine, and valine were incubated with the combined tyrocidine fractions, products in positions 1–8 were isolated (Figure 3). In similar experiments, [^{14}C]proline occurred in products 2–8, and [^{14}C]valine occurred in product 8. Product 8 corresponds to Phe-Pro-Phe-Phe-Asx-Glx-Phe-Val. Products 1–9 were obtained with labeled phenylalanine and proline, asparagine, glutamine, valine, and ornithine. In parallel experiments, proline occurred in products 2–9, and labeled ornithine occurred in product 9 which represents Phe-Pro-Phe-Phe-Asx-Glx-Phe-Val-Orn. Finally, when L-[^{14}C]phenylalanine, proline, asparagine, glutamine, valine, ornithine, and leucine were incubated with the three enzyme fractions, products 1–10 were obtained. Labeled proline occurred in products 2–10, and labeled leucine occurred in product 10, which derives from the linear decapeptidyl Phe-Pro-Phe-Phe-Asx-Glx-Phe-Val-Orn-Leu thio ester.

Peptide intermediates were not formed when phenylalanine or proline were omitted from the otherwise complete reaction mixture. Chain growth was stopped at the tetrapeptidyl stage when asparagine was omitted, and at the octapeptidyl peptide stage when ornithine was omitted, even though the succeeding amino acids were present. Likewise, the peptide intermediates were quantitatively liberated from the enzyme proteins by performic acid oxidation. Cleavage by this procedure, which permits one to distinguish between oxygen esters and thio esters (Harris *et al.*, 1963), supports the notion that all the intermediate peptides are bound to the enzyme proteins exclusively by thio ester linkages, as in the case of gramicidin S biosynthesis (Kleinkauf and Gevers, 1969; Frøshov *et al.*, 1970).

Interaction of the Light and Intermediate Components in

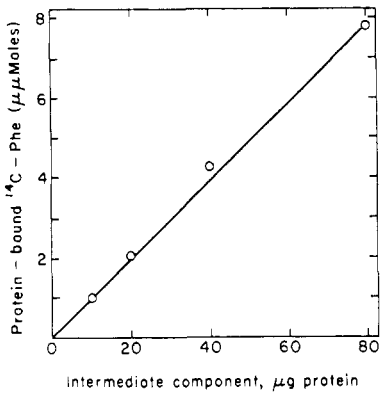


FIGURE 4: Interaction of the light and intermediate fractions in the synthesis of enzyme-bound [^{14}C]Phe-Pro. A constant amount of light fraction (5 μg, which bound 1.0 $\mu\mu$mole of [^{14}C]phenylalanine as thio ester determined by the filter disk method described previously, Roskoski *et al.*, 1970), and the specified amount of the intermediate fraction (12.5 μg, which was capable of binding 1.0 $\mu\mu$mole of [^{14}C]proline) were incubated in medium M with L-[^{14}C]-phenylalanine (0.1 mM) and proline (0.1 mM) for 30 min at 37° in a total volume of 60 μl. Then 50-μl aliquots were transferred to Whatman No. 3MM disks, which were immersed in 10% trichloroacetic acid, and their radioactivity was determined. The 1.0 $\mu\mu$mole of [^{14}C]phenylalanine bound by the light component was subtracted in each case. Alkaline liberation of the bound peptides, followed by thin-layer chromatography in solvent 1, showed that phenylalanine and Phe-Pro (isolated as the diketopiperazine) were the only products; this procedure would have detected 5% contaminating substances.

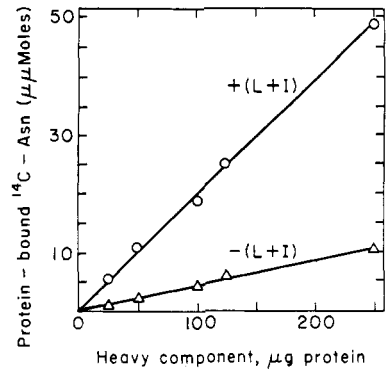


FIGURE 5: Interaction of the light and intermediate fractions with the heavy fraction. The light (5 μg, which bound 1.0 $\mu\mu$mole of [^{14}C]phenylalanine) and intermediate (12.5 μg, which bound 1.0 $\mu\mu$mole of [^{14}C]proline) fractions were incubated with the specified amount of heavy component in medium M with L-phenylalanine, L-proline, L-[^{14}C]asparagine, L-glutamine, L-valine, and L-ornithine (each at 0.1 mM) for 30 min at 37° in a total volume of 60 μl. Then 50-μl aliquots were transferred to filter disks, which were immersed in 10% trichloroacetic acid before radioactive incorporation. The light and intermediate fractions did not bind L-[^{14}C]asparagine, but 20 μg of the heavy component bound 1.0 $\mu\mu$mole.

Phe-Pro-Thioenzyme Formation. The formation of protein-bound [^{14}C]Phe-Pro was measured by incubating a constant amount of light fraction with varying amounts of intermediate fraction in the presence of ATP, Mg^{2+}, labeled phenylalanine, and proline, followed by trichloroacetic acid precipitation to determine the bound radioactivity (Figure 4); 1 equiv of the light component catalyzed the formation of 8 equiv of the Phe-Pro-thioenzyme. This shows that the light component, after peptide synthesis, dissociates from the intermediate fraction, at least under the conditions of the incubation.

Interaction of the Light and Intermediate Components with the Heavy Fraction. An analogous experiment was carried out with a combination of the three enzyme fractions, measuring the incorporation of L-[^{14}C]asparagine into enzyme-bound peptides. Constant amounts of the light and intermediate fractions were incubated in the presence of increasing amounts of the heavy component while the tyrocidine-constituent amino acids, except leucine, were present. Under these conditions, chain growth to the nonapeptide occurs, beginning with N-terminal phenylalanine and ending with C-terminal ornithine, labeled asparagine being present in the penta- through nonapeptides. One equivalent of the light and intermediate components catalyzed the formation of 20 times as many equivalents of chains bound to the heavy fraction (Figure 5), showing that the two smaller components also dissociate from the heavy fraction despite the fact that the overall synthesis of antibiotic is not possible; this means that the synthesis is probably not carried out by a stabilized aggregate of the three fractions.

Replacement of Phenylalanine by Tryptophan and/or Tyrosine. As shown in Table III (lines 1–3) addition first of phenylalanine and then of phenylalanine + asparagine, led to increased acid-stable binding of [^{14}C]proline to the three tyrocidine enzyme fractions incubated with ATP and Mg^{2+}. Nascent peptide chains composed of up to five residues long are formed under these conditions (Figure 3). Substitution of tryptophan or tyrosine for phenylalanine in these mixtures led to approximately the same increases in incorporation (Table III). This suggested that these amino acids could also initiate synthesis of the antibiotic. In confirmation, it was found with the systems for both tyrocidine and gramicidin S, that the synthesis was more than 97% dependent on addition of phenylalanine or tyrosine; tryptophan could replace both these amino acids in tyrocidine formation only (Table IV). At concentrations of 100 μM, tryptophan and tyrosine were 33 and 95% as effective, respectively, as phenylalanine in supporting tyrocidine biosynthesis. The products of such incubations, extracted with organic solvents, cochromatographed with standard tyrocidine and gramicidin S on thin

TABLE III: Chain Initiation by Aromatic Amino Acids.[a]

Added Amino Acids	Bound Radioactivity ($\mu\mu$moles)
1. —,[^{14}C]Pro	5.8
2. Phe,[^{14}C]Pro	20.1
3. Phe,[^{14}C]Pro,Asn	26.7
4. Trp,[^{14}C]Pro	21.2
5. Trp,[^{14}C]Pro,Asn	26.1
6. Tyr,[^{14}C]Pro	20.7
7. Tyr,[^{14}C]Pro,Asn	26.4
8. —,[^{14}C]Pro,Asn	6.1

[a] Incorporations were measured by the procedures given in Table I.

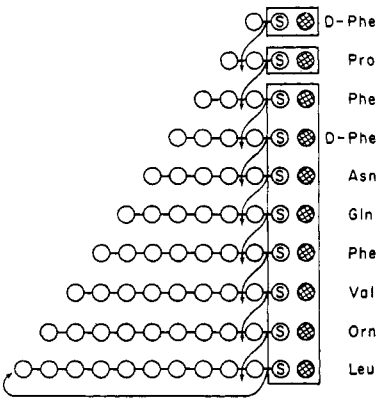


FIGURE 6: Summary of reactions involved in tyrocidine biosynthesis. The cross-hatched areas indicate individual sites in the three enzyme fractions for binding of the aminoacyl adenylates, each adjacent to sulfhydryl groups responsible for thio ester binding of amino acids and peptides as indicated. Chain growth occurs by a vectorial series of peptidyl transfers involving thio ester bound intermediates until a decapeptidyl thioenzyme is formed prior to cyclization. This scheme very likely will need modification when the function of phosphopantotheine can be fully appreciated.

layers, suggesting that the reaction products were in fact cyclic decapeptides and not smaller by-products. It thus appears that tyrocidine molecules containing tryptophan and/or tyrosine and/or phenylalanine in positions 1, 3, 4, and 7 (Figure 1) can be synthesized *in vitro*.

One might suspect that contaminant phenylalanine in the tryptophan and tyrosine was responsible for these results. However, analysis of the compounds in the amino acid analyzer showed that phenylalanine was absent (less than 0.5%). The different response to tryptophan of the gramicidin S and tyrocidine systems argues against such contamination. Nevertheless, definite proof of the existence of tyrocidine containing tyrosine or tryptophan at four positions, and gramicidin S containing tyrosine at two positions, requires primary structure determinations, for which the quantities of antibiotic produced in these incubations were not sufficient.

Discussion

The light, intermediate, and heavy fractions may carry charged amino acids without polymerization; for this, the fractions must be combined. The light fraction, carrying D-phenylalanine, initiates peptide synthesis on the intermediate protein by reacting with (bound) proline to form H_2N-Phe-Pro-thioenzyme. This reacts with the amino group of the phenylalanyl thio ester on the heavy component forming a tripeptidyl thioenzyme, the activated carboxyl of which reacts with the next activated, bound amino acid, and so on up to the decapeptidyl stage (Figure 6). Finally, the leucine carboxyl group, activated as thio ester, reacts with the free amino group of D-phenylalanine at the N terminus of the growing peptide chain, causing cyclization and release of the final product. The nature of the sulfhydryl carriers of activated amino acids and peptides is not known. We have previously suggested that 4′-phosphopantetheine might play a role in gramicidin S synthesis (Gevers *et al.*, 1969). Recently, Gilhuus-Moe and coworkers (1970) have reported that their preparation of gramicidin S fraction I (heavy) contains 1 mole of 4′-phosphopantetheine/mole of enzyme, which we have confirmed. They propose that the function of this bound cofactor is to transfer growing peptide chains from one amino acid binding site to the next. Preliminary experiments indicate that 4′-phosphopantetheine is present only in the heavy fraction involved in tyrocidine synthesis. The number of moles of bound cofactor per mole of enzyme remains to be determined in pure preparations. Trichloroacetic acid stability, and cleavage by alkali and by performic acid indicates, in any case, that the nascent peptide chains are bound to their corresponding enzyme fractions as thio esters.

TABLE IV: Dependence of Antibiotic Synthesis on Aromatic Amino Acids.[a]

Aromatic Amino Acid Added	Antibiotic Synthesis	
	Tyrocidine ($\mu\mu$moles)	Gramicidin S ($\mu\mu$moles)
None	0.6	1.5
L-Phenylalanine	33.6	31.5
L-Tryptophan	12.2	1.2
L-Tyrosine	32.1	33.9

[a] Antibiotic synthesis was measured by the Millipore filter assay and confirmed by thin-layer chromatographic analysis as outlined previously (Roskoski *et al.*, 1970). Tyrocidine-constituent amino acids and the specified aromatic amino acids were present.

Our results point to an important and very interesting difference between the tyrocidine biosynthetic process and that described for gramicidin S by Kleinkauf and Gevers (1969). In the former case, chain growth occurs to the decapeptidyl stage before cyclization, and in the latter case growth takes place to the pentapeptidyl stage so that two pentapeptidyl thioenzymes react, head to tail, to form the cyclic peptide gramicidin S. Cyclization in gramicidin S seems to be a rather fast process, but it may be rate limiting in the formation of tyrocidines. Thus, the quantity of the intermediate peptides, isolated by Sephadex G-50 gel filtration and trichloroacetic acid precipitation, decreases to almost nil when the last amino acid, L-leucine, is added to the gramicidin S synthesizing system (Gevers *et al.*, 1969). On the other hand, the addition of leucine to the tyrocidine system does not decrease the quantity of the intermediate peptides.

The light, intermediate, and heavy components associate functionally in tyrocidine biosynthesis. There is no evidence for the formation of stable complexes among them on sucrose density gradients, where they are readily resolved (Roskoski *et al.*, 1970), or during the formation of nascent peptide chains, since the light and intermediate fractions appear to act catalytically (Figures 4 and 5). However, the intermediate and heavy components have a tendency to associate during Sephadex G-200 gel filtration (Roskoski *et al.*, 1970). The three fractions are isolated from the cell-free extracts in approximately equivalent amounts as measured by thio ester formation with the corresponding substrate amino acids. This suggests that peptide elongation involves transient association of these fractions.

Acknowledgments

We thank Mr. B. Fedyniak for help in the growth and harvest of the cells, and Dr. J. M. Manning for the amino acid analysis.

References

Frøshov, O., Zimmer, T. L., and Laland, S. G. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 68.

Gevers, W., Kleinkauf, H., and Lipmann, F. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 269.

Gevers, W., Kleinkauf, H., and Lipmann, F. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1335.

Gilhuus-Moe, C. C., Kristensen, T., Bredesen, J. E., Zimmer, T. L., and Laland, S. G. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 287.

Harris, J. I., Meriwether, B. P., and Park, J. H. (1963), *Nature (London) 198*, 154.

Holm, H., Frøholm, L. O., and Laland, S. G. (1966), *Biochim. Biophys. Acta 115*, 361.

Kleinkauf, H., and Gevers, W. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 805.

Kurahashi, K. (1961), *Abstracts, 5th Int. Congr. Biochem., Moscow*, 37.

Kurahashi, K., Yamada, M., Mori, K., Fujikawa, K., Kambe, M., Imae, Y., Sato, M., Takahashi, H., and Sakamoto, Y. (1969), *Cold Spring Harbor Symp. Quant Biol. 34*, 815.

Mans, R. J., and Novelli, G. D. (1961), *Biochim. Biophys. Acta 94*, 48.

Roskoski, R., Jr., Gevers, W., Kleinkauf, H., and Lipmann, F. (1970), *Biochemistry 9*, 4839.

Tomino, S., and Kurahashi, K. (1964), *Biochem. Biophys. Res. Commun. 17*, 288.

Tsuji, T. (1966), *J. Osaka City Med. Center 15*, 1

Biosynthetic Studies with Carbon-13: Mollisin*

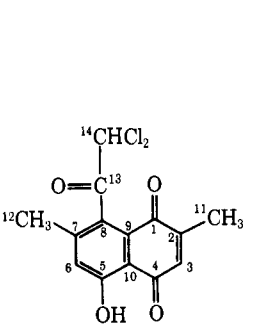
Masato Tanabe† and Haruo Seto‡

ABSTRACT: The biosynthesis of mollisin has been studied using nuclear magnetic resonance spectroscopy techniques. Isotope-enriched mollisin was produced from [2-^{13}C]sodium acetate. Measurement of the position and intensity of the ^{13}C-proton satellite bands and J^{13}C-H in the proton nuclear magnetic resonance spectrum of the labeled mollisin was accomplished with a time-averaging computer. These spectral data show that carbons-3, -6, -11, -12, and -14 of mollisin are derived from the methyl group of acetate. These positions all incorporate acetate at approximately equal intensities, indicating that the biosynthetic pathway of mollisin is from two simultaneously formed polyketide chains which cyclize to mollisin. Confirmation of the level of isotope enrichment in labeled mollisin was achieved by mass spectrometry. The results reported here are in agreement with previous studies of the biosynthesis of mollisin using ^{14}C techniques.

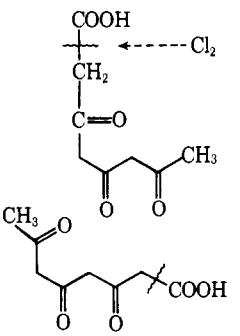
Mollisin (I) is a yellow napthoquinone pigment produced by the mold *Mollisia caesia* grown on solid malt agar medium. Bentley and Gatenbeck (1965) have studied the biosynthesis of mollisin using ^{14}C-labeled substrates. Since radioactive methionine was not incorporated into the pigment, the C_{11} and C_{12} methyl groups are not biosynthetically introduced by methylation of a naphthoquinone unit. The observed incorporation of ^{14}C-labeled acetate and malonate into mollisin can be explained by a biogenetic scheme involving two separate polyketide units.

Bentley and Gatenbeck (1965) obtained evidence for the acetate origin of the C_{11} and C_{12} methyl groups by Kuhn–Roth oxidation of the pigment, which yielded radioactive acetic acid along with nearly the expected level of labeled carbon dioxide from the remaining carbon atoms.

The two hypothetical tetraacetate chains involved in mollisin biosynthesis can be oriented in two ways. Bentley and Gatenbeck prefer the arrangement shown in II since it afforded a rational biochemical route for the introduction of the unique dichloracetyl side chain present in mollisin.



I



II

* From the Department of Pharmaceutical Chemistry, Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025. This work was supported in part by U. S. Public Health Service Research Grant AI 08143 from the National Institute of Allergy and Infectious Diseases.

† To whom to address correspondence.

‡ Present address: The Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.