Bioactive Peptides Produced by Microorganisms

Edited by
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Preface

The growing interest in biologically active peptides appears to be based on the importance of the spectrum of unique activities which has been found. Indeed, this is continuously expanding even in unexpected directions, and the trend promises entirely novel areas for future exploitation in the field of peptide chemistry. In particular, microorganisms have been shown to produce numerous peptides with diverse biological activities. Compared to peptide hormones, they exhibit special characteristics in their component structures, such as unusual or D-amino acids. This has encouraged deep and far-reaching research in this special field.

In view of the above situation, a cumulative review concerning the bioactive peptides produced by microorganisms was published in 1976 in Japan. Peptides which were found for the first time in Japan, or had been studied most extensively in Japan, were selected for the purpose of providing a summary of the important activities in the field of microbial or peptide chemistry in Japan.

However, since this review was in Japanese only, many requests were subsequently received by the editors for the preparation of a new, revised edition in English. Although space limitations prevented the inclusion of translations of all articles in the original publication, a new plan was developed with articles in review style which covered the biosynthesis, structural determination, partial or total synthesis, and conformational analysis of various important peptides, mainly with antibacterial activity. Moreover, we were fortunate in being able to add a contribution from overseas, specifically on the subject of the biosynthesis of antibiotic peptides.

Special mention should be made of the tabular list which appears at the end of the book. This includes details, so far as possible, of all bioactive peptides produced by microorganisms which were reported in the literature up to 1977. It is hoped that the content and format will render this list a most useful reference for peptide and other researchers wishing to locate the details of bioactive peptides originating from microorganisms.

We are deeply indebted to the various authors for their contribution and careful preparation of material for this book. Furthermore, we wish to express our sincere thanks to the staff of Kodansha Scientific, particularly Mr. M. Takahatake, for their kind cooperation throughout the project.

April, 1978

H. UMEZAWA
T. SHIBA
T. TAKITA

Contents

CHAPTER 1

New Trends in Studies on Bioactive Microbial Peptides

Hamao UMEZAWA

Numerous peptides which can be regarded as hormones have been found in physiological and pharmacological studies of animal functions. They are necessary for the life of such multicellular organisms but may not be required for the growth of cells producing them. Bacterial toxins which are macromolecular peptides have been found in studies on the mechanisms of virulence in pathogenic bacteria, and small molecular peptides have been found through screening of microbial products which inhibit the growth of microbial or animal cells or enzyme reactions, or which exhibit pharmacological action. These peptides have no obvious function in the growth of microbial cells and can be regarded as secondary metabolites of microbes. The reason why so many and varied microbial secondary metabolites are produced by microorganisms, or why microorganisms in nature have acquired the ability to produce so many compounds with varying chemical structures, represents an interesting subject of recent study in microbiology.

The ability of streptomycete strains to produce many antibiotics is eliminated by cultivation in media containing acriflavine, or mutagenic agents, or by UV irradiation, and strains which have lost this ability often produce the antibiotics in media which contain biosynthetic intermediates. This suggests that a set of genes involved in the biosynthesis of an antibiotic is contained in cells producing this antibiotic. In most cases, more than one antibiotic of related chemical structure is produced by a single strain, and the biosynthesis of the structural part common to all of the antibiotics is thought to be controlled by a set of genes. Kanamycin-producing strains produce the following seven 2-deoxystreptamine-containing compounds: kanamycin A[1] [4-(6-amino-6-deoxy-D-α-glucopyranosyl)-6-(3-amino-3-deoxy-D-α-glucopyranosyl)-2-deoxystreptamine]; kanamycin B [4-(2,6-

diamino-2,6-dideoxy-D-α-glucopyranosyl)-6-(3-amino-3-deoxy-D-α-glucopyranosyl)-2-deoxystreptamine]; kanamycin C [4-(2-amino-2-deoxy-D-α-glucopyranosyl)-6-(3-amino-3-deoxy-D-α-glucopyranosyl)-2-deoxystreptamine]; 4,6-di(D-α-glucopyranosyl)-2-deoxystreptamine; 4-(2,6-diamino-dideoxy-D-α-glucopyranosyl)-6-(D-α-glucopyranosyl)-2-deoxystreptamine; 4-(2,6-diamino-2,6-dideoxy-D-α-glucopyranosyl)-6-(D-α-glucopyranosyl)-1,3-*N*-diacytyl-2-deoxystreptamine; and 4-(2,6-di-amino-2,6-dideoxy-D-α-glucopyranosyl)-6-(D-α-glucopyranosyl)-1(or 3)-*N*-acetyl-2-deoxystreptamine. The ability to produce kanamycin is eliminated by arcriflavine treatment and strains which have lost this ability have been found to produce kanamycins in media to which 2-deoxystreptamine was added.[2] The elimination of the ability to produce antibiotics by acriflavine treatment suggests that plasmids specific to each of the antibiotics may be involved in the biosynthesis of the characteristic structural parts of many antibiotics or their characteristic biosynthetic intermediates.

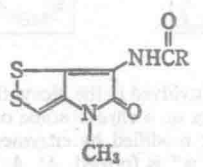
As described by Aoyagi in this book, leupeptins which inhibit trypsin, plasmin, papain and cathepsin B are produced by many streptomycete strains. The ability to produce leupeptin is eliminated by acriflavine treatment. Since this compound is produced by many strains belonging to various streptomycete species, if a plasmid is involved in its biosynthesis, this plasmid can be thought to be transferred from leupeptin-producing strains to leupeptin-nonproducing strains more easily than other plasmids involved in the biosynthesis of other microbial products. As reported by Umezawa *et al.*[3] the ability to produce leupeptin is transferred from a leupeptin-producing methionine-requiring mutant to a leupeptin-nonproducing arginine-requiring mutant. This finding provides a proof for plasmid involvement in leupeptin biosynthesis. Furthermore, it has been demonstrated that the plasmid is involved in the biosynthesis of its biosynthetic intermediate.

As described in this book, great progress has been made in the last 10 years in the study of the biosynthesis of peptide antibiotics produced by bacteria. They are synthesized on multienzyme systems specific to each of them. One of the leupeptins is acetyl-L-leucyl-L-leucyl-L-argininal and its oxidation product (acetyl-L-leucyl-L-leucyl-L-arginine) is called leupeptin acid. Hori *et al.*[4] of the author's institute have succeeded in extracting and purifying a multienzyme which catalyzes the synthesis of leupeptin acid in reaction mixtures containing acetate, L-leucine, L-arginine and ATP. The synthesis is begun from the synthesis of acetylleucine, and acetylleucylleucine and leupeptin acid are then synthesized successively on the multienzyme. Strains which have lost the ability to produce leupeptin lack the ability to produce this multienzyme.[3] It has thus been shown that a plasmid is involved in the biosynthesis of a multienzyme which catalyzes the synthesis

of leupeptin acid. Leupeptin acid produced in leupeptin-producing cells is reduced to leupeptin by an enzyme which can be found in cell homogenates. These studies of leupeptin biosynthesis suggest that other serine thiol protease inhibitors such as antipain, chymostatin and elastatinal which all have *C*-terminal aldehyde groups, may be biosynthesized by means similar to the case of leupeptin. It is thought that various sets of genes involved in the biosynthesis of various peptides may be distributed in actinomycete strains, and those containing the *C*-terminal argininal, phenylalaninal or alaninal may have been found by the screening of microbial products inhibiting proteases which hydrolyze the peptide bonds at the carboxyl side of arginine, phenylalanine or alanine.

The pepstatins described by Aoyagi in this book contain (3*S*, 4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid. The labeled amino acid when added to fermentation media is not incorporated into the pepstatin molecule. This amino acid is biosynthesized from L-leucine and malonate (or acetate), which suggests that a biosynthetic intermediate peptide containing the leucylacetic acid moieties might be synthesized on a multienzyme system and reduced to pepstatin thereafter. Pepstanone which contains the *C*-terminal 3-amino-5-methylhexanone-2 instead of the *C*-terminal (3*S*, 4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid of pepstatin is also produced by pepstain-producing strains. This structure of pepstanone suggests that in the last step of its biosynthesis the *C*-terminal leucylacetic acid may be decarboxylated to yield pepstanone.

In 1970, Umezawa *et al.*[5] suggested the possible involvement of plasmids in the production of kasugamycin and aureothricin. Aureothricin and thiolutein, which are closely related in structure, are produced by kasugamycin-producing strains and the ability to produce these antibiotics is eliminated by acriflavine treatment or high temperature cultivation.



Aureothricin: R = CH_3CH_2
Thiolutein: R = CH_3

These antibiotics may be regarded as peptide antibiotics. Labeled cysteine or cystine is incorporated into these antibiotics and their structures suggest that they are biosynthesized from one molecule of cystine, propionic acid or acetic acid and the methyl group of methionine. It has been shown that

aureothricin-producing strains contain a genetic set which is involved in the biosynthesis of depropionylaureothricin or demethyldepropionylaureothricin, which is the structural part common to aureothricin and thiolutein. Strains which have lost the ability to produce these antibiotics produce aureothricin and thiolutein in media containing depropionylaureothricin. Moreover, these antibiotics are produced by chloramphenicol-producing strains in media containing depropionylaureothricin.

As described above, studies on microbial secondary metabolites indicate that numerous genetic sets which control the biosynthesis of various multienzymes involved in the synthesis of microbial secondary metabolites, are distributed in microorganisms. Many or some of them may lie on plasmids. It is thought that the evolution and transfer of such genetic units have not killed cells, and microorganisms containing such genetic sets have acquired the ability to produce various microbial secondary metabolites (Fig. 1.1). It is possible therefore that not only antimicrobial antibiotics but also other microbial peptides which have various kinds of bioactivity can be found in microorganisms.

Rectins which bind to the surface of animal cells are known to enhance certain immune responses. One of the methods for finding such

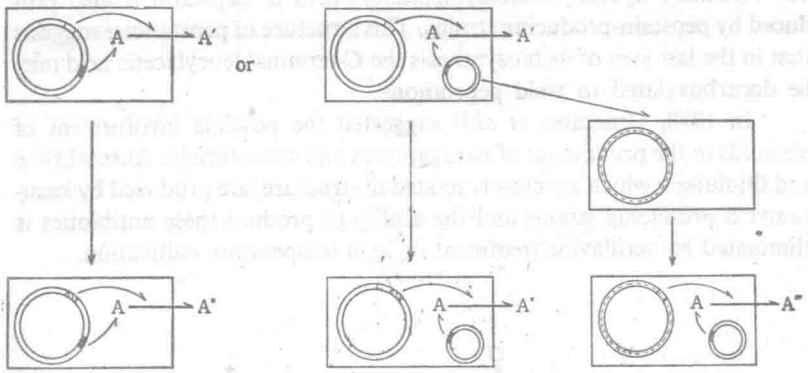
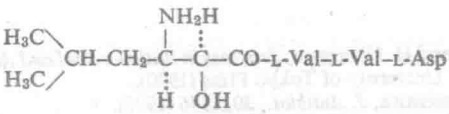
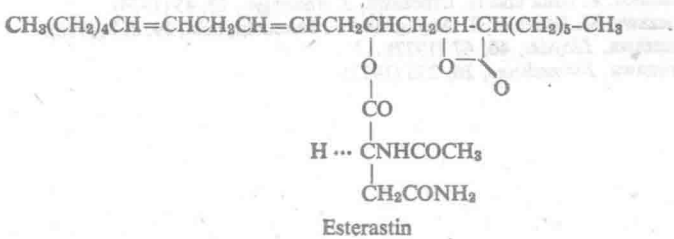


Fig. 1.1. Evolution and transfer of a set of genes involved in the biosynthesis of microbial secondary metabolites. A genetic set which lies on a chromosome or plasmid controls the biosynthesis of product A. A is further modified by enzymes produced by following the control of other genes, and A′, A″ or A‴ is formed. A′, A″ or A‴ means a group of compounds which are related in structure. Cells containing the genetic set controlling the biosynthesis of A are mutated and in the mutated cells, A is modified to A″. The plasmid carrying the genetic set controlling the biosynthesis of A is transferred to cells of other different species where A is modified differently (to A‴). A′, A″ and A‴ represent secondary metabolites which are not essential for growth, and genesis of the genetic set controlling their biosynthesis does not cause death of the cells. Thus, a limitless number of such genetic sets could have been generated in the natural environment.

compounds which can bind to the cell surface is to find inhibitors of enzymes located on the cell surface. As described by Aoyagi in this bok, aminopeptidases appear on the cell surface but are not released extracellularly. Bestatin, which is also described in detail by Aoyagi in this book, inhibits aminopeptidase B and leucine aminopeptidase. It inhibits the aminopeptidase B activities of various intact cells including rat mononuclear leucocytes, mouse spleen lymphocytes, etc. ^{3}H-Labeled bestatin binds to these cells. As reported by Umezawa *et al.*,[6] it enhances delayed-type hypersensitivity within a wide dose range and produces an immune resistance to experimental animal tumors. It inhibits the growth of subcutaneous mouse tumors for which growth can be examined for more than 30 days. It enhances the effect of anticancer agents.[7,8] Amastatin which was found by Umezawa *et al.* is produced by a streptomycete and inhibits aminopeptidase A. It enhances antibody formation. Esterastin which was found by Umezawa *et al.* is produced by a streptomycete and inhibits esterase. Esterase activity can also be detected on the cell surface. Esterastin suppresses both antibody formation and delayed-type hypersensitivity. Thus, peptides affecting certain immune responses have been found in microorganisms.

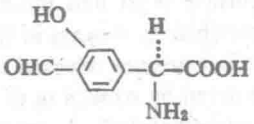


Amastatin



Esterastin

Forphenicine which was found by Umezawa *et al.* inhibits chicken intestine alkaline phosphatase. The type of inhibition is uncompetitive with the substrate. This compound is an unusual amino acid produced by a streptomycete. It enhances delayed-type hypersensitivity and increases antibody formation. It inhibits the growth of experimental animal tumors for which growth can be examined for more than 30 days. For example, it inhibits Gardner lymphosarcoma, if it is administered after day 8 from the inoculation.



Forphenicine

As described in this book, various peptides are produced by microorganisms and those which have various useful bioactivities have been found by precise screening studies. At present, the structures of peptides can be determined rapidly. Bioactive peptides are utilized for the analysis of biological functions, and their structures provide valuable information for studies of the mechanisms of action and possible biosynthetic pathways. Moreover, research on the genetic sets involved in their biosynthesis may provide useful information for studies of the evolutionary relationships among various microorganisms. A thorough review of microbial peptides will undoubtedly contribute greatly to progress in the life sciences.

REFERENCES

1. M. Murase, T. Ito, S. Fukatsu and H. Umezawa, *Progress in Antimicrobial and Anticancer Chemotherapy*, p. 1098, University of Tokyo Press (1970).
2. K. Hotta, Y. Okami and H. Umezawa, *J. Antibiot.*, **30**, 1146 (1977).
3. H. Umezawa, Y. Okami and K. Hotta, *ibid.*, **31**, 99 (1978).
4. M. Hori, H. Hayashi and H. Hemmi, Ann. Mtg. Japan. Biochem. Soc. (1976).
5. M. Okanishi, T. Ohta and H. Umezawa, *J. Antiobiot.*, **23**, 45 (1970).
6. H. Umezawa, M. Ishizuka, T. Aoyagi and T. Takeuchi, *ibid.*, **29**, 857 (1976).
7. H. Umezawa, *Lloydia*, **40**, 67 (1977).
8. H. Umezawa, *Biomedicine*, **26**, 236 (1977).

CHAPTER 2

Biosynthesis of Bioactive Peptides Produced by Microorganisms

S. G. LALAND, T.-L. ZIMMER AND Ø. FRØYSHOV

2.1 INTRODUCTION

Microorganisms produce a variety of bioactive peptides. The biosynthetic mechanism of the formation of some of these peptides has attracted much attention and led to the discovery of new modes for linking amino acids peptidically into specific structures. Their synthesis do not require the participation of ribosomes and *m*-RNA, but are mediated solely by polyenzymes designated the peptide synthetases.[1–4]

The best studied cases are the cyclic decapeptides gramicidin S and tyrocidine, produced by different strains of *Bacillus brevis*. Elucidation of the biosynthesis of these peptides has led to the introduction of the concept, *thiotemplate mechanism*[3] of peptide bond formation, and may be summarized as follows:

The individual amino acids of the peptides are activated by ATP to form aminoacyl-adenylates followed by a transfer of the aminoacyl-moiety to specific thiol groups on the enzymes where they are bound as thioesters. The structural arrangement of these thiol groups in the synthetases specifies the amino acid sequence of the peptide. Consecutive peptide bond formation is mediated by 4′-phosphopantetheine which is an integral part of the multifunctional enzyme(s) of the synthetases. The intermediate peptides are also thioester-bound during synthesis which proceeds in *C*-terminal direction. The linear gramicidins, the bacitracins and the edeines are also synthesized by the thiotemplate mechanism. However, inconclusiveness still prevails concerning the formation of polymyxin, alamethicin and several other peptides. Furthermore, some bioactive peptides, e.g. the penicillins and cephalosporins, are definitely not synthesized by the thiotemplate mechanism.

This chapter will mainly be concerned with the current status of the biosynthetic mechanism of the peptides: gramicidin S, tyrocidine, linear gramicidin, bacitracin, edeine, polymyxin and alamethicin.

2.2 BIOSYNTHESIS OF THE INDIVIDUAL PEPTIDES

To describe the general principles of the thiotemplate mechanism, the biosynthesis of gramicidin S will be used as a model and therefore be given a more detailed treatment than the other peptides. However, it should be realized that the elucidation of the thiotemplate mechanism has been the result of concomitant research on both gramicidin S and tyrocidine.

2.2.1 Biosynthesis of Gramicidin S

Gramicidin S is a cyclic decapeptide with a repeating sequence of five different amino acids[5] (Fig. 2.1). It is synthesized by several strains of *Bacillus brevis*, the most commonly used for basic research being the Nagano strain in Japan and the ATCC 9999 strain in the rest of the world. Originally gramicidin S was isolated by Gause and Brazhnikova from a strain isolated from Russian soil,[6] which was named GB after them.

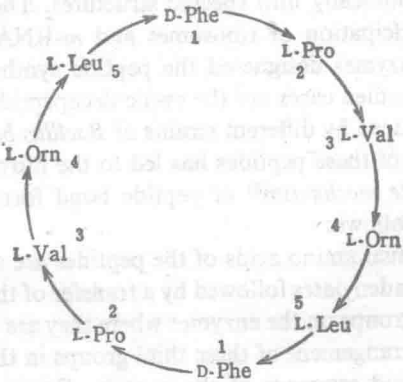


Fig. 2.1. Structure of gramicidin S

Investigation of the biosynthetic mechanism of gramicidin S started some 15–20 years ago,[7,8] and data soon accumulated to indicate the operation of a mode of peptide bond formation different from the ribosomal protein synthesizing system. Well known inhibitors of protein synthesis such as puromycin and chloramphenicol, although inhibiting growth and protein synthesis in the *B. brevis* cells, did not inhibit gramicidin S syn-