RECENT CHEMICAL STUDIES OF BIOACTIVE MICROBIAL PRODUCTS: GENETICS, ACTIVE STRUCTURES, DEVELOPMENT OF EFFECTIVE AGENTS WITH POTENTIAL USEFULNESS

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Abstract — Antibiotics can be divided into various groups, each containing a characteristic structural part common to antibiotics of the same group. A gene and gene set, involved in the biosynthesis of a characteristic structural part is shown by chemical structures of antibiotics to be widely distributed among various strains of microorganisms. On the basis of this fact, genetics of microbial secondary metabolites was studied and the reason why various compounds were produced by microorganisms was elucidated. On the basis of the fact that microorganisms produce various compounds, the author searched for compounds which bound to the cell surface, that is, he searched for inhibitors of enzymes on the cellular surface and was successful in finding immunomodulators. Mechanisms of actions and toxicities of useful antibiotics and enzyme inhibitors such as aminoglycoside antibiotics, bleomycins, bestatin etc. were studied in relation to their structures, and on the basis of their results, more effective useful derivatives of them were synthesized and successful results were obtained. Microbial products were shown to be one of the most important areas of application in organic chemistry to develop bioactive compounds useful in the treatment of diseases.

The screening of bioactive microbial products provided chemists with interesting materials for the structure studies and the design of new chemical syntheses, and the results of these studies promoted the progress of the study on structure-activity relationships. Moreover, the study of genetics and biosyntheses of microbial

products began with their structures. In these studies, the author endeavoured to open up new research areas where new useful bioactive compounds were discovered and new principles were explored. In 1953, the author extended the study of antimicrobial antibiotics to the study of antitumor antibiotics, reporting the findings of two new microbial products which inhibited the growth of experimental animal tumors[1,2] and in 1967, the author extended the study of antibiotics to small molecular enzyme inhibitors.[3] Up to now, the author has found more than 40 new enzyme inhibitors. These small molecular enzyme inhibitors have no antimicrobial activity. Findings of enzyme inhibitors and antibiotics indicate that microorganisms produce various compounds which have widely varied structures. This led the author to the study of their genetics. Microbial secondary metabolites have been defined to be the microbial products which have no function in the growth of microbial cells. Recently the author extended the study of enzyme inhibitors to those which enhanced immune responses. These compounds have potential usefulness in the treatment of cancer and resistant infections. On the other hand, the author was successful in the elucidation of enzymic mechanisms of resistance to aminoglycosidic antibiotics in 1967 which opened up a fruitful research area where the derivatives active against resistant infections were developed.

The study of bioactive microbial products is one of the most important areas of application in organic chemistry. In other words, chemistry is the fundamental science of biological and medical sciences and it may be expressed in the future completely in chemical terms. At present, chemical studies of bioactive microbial products still continue to discover the active agents useful in the treatment of diseases difficult to cure. It is also interesting that almost all bioactive microbial secondary metabolites contain oxygen or nitrogen, or both of them.

In this paper, the author describes his recent studies on genetics of bioactive microbial products, active structures and development of compounds with potential usefulness.

GENETICS OF A CHARACTERISTIC STRUCTURAL PART COMMON TO A GROUP OF MICROBIAL SECONDARY METABOLITES

On the basis of chemical structures, antibiotics can be divided into various groups and each group of antibiotics have a characteristic structural part common to them. If kanamycin is taken as an example, a kanamycin-producing strain and its mutant produce not only kanamycins A, B and C but also four other 2-deoxystreptamine-

containing compounds as shown in Fig. 1.[4] Moreover, more than sixty 2-deoxystreptamine-containing compounds have been found in cultured broths of streptomyces, micromonospora, nocardia and bacteria.[5] This indicates that a characteristic gene involved in the biosynthesis of 2-deoxystreptamine has been generated

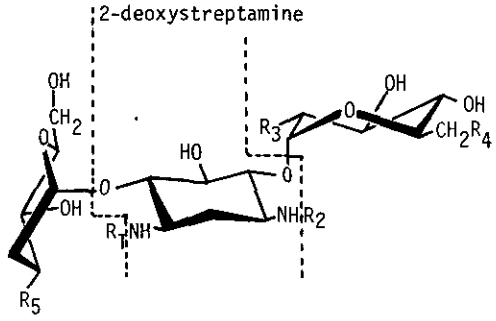


Fig. 1. 2-Deoxystreptamine-containing compounds produced by a kanamycin-producing strain and its mutant: Kanamycin A (R_1,R_2=H, R_3=OH, R_4=NH_2, R_5=NH_2), Kanamycin B (R_1,R_2=H, R_3,R_4=NH_2, R_5=NH_2), Kanamycin C (R_1,R_2=H, R_3=NH_2, R_4=OH, R_5=NH_2), NK1001 (R_1,R_2=H, R_3,R_4,R_5=OH), NK1012-1 (R_1,R_2=H, R_3,R_4=NH_2, R_5=OH), NK1013-1 (R_1=CH_3CO and R_2=H or R_1=H and R_2=CH_3CO, R_3,R_4=NH_2, R_5=CH_3CONH), NK1013-2 (R_1=CH_3CO and R_2=H or R_1=H and R_2=CH_3CO, R_3,R_4=NH_2, R_5=NH_2)

and widely distributed among various strains, species, genera, families and orders of microorganisms. In fact, the presence of such a gene has been confirmed: the ability of a strain to produce kanamycin was eliminated by acriflavine treatment and most kanamycin-nonproducing mutants thus obtained produced kanamycin in culture media to which 2-deoxystreptamine was added.[6]

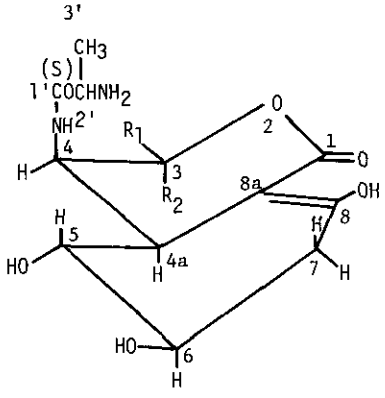


Fig. 2. Actinobolin (R_1=CH_3, R_2=H) and bactobolin (R_1=$CHCl_2$, R_2=CH_3)

Actinobolin is an antitumor antibiotic which was found by Haskel et al.[7] in cultured broths of streptomyces. Recently the author found a new antibiotic of a

similar type in a strain of bacterium classified as Pseudomonas sp. and named it bactobolin.[8] It inhibits the growth of various bacteria and exhibits an antitumor action against mouse leukemia L-1210. As shown in Fig. 2, its structure may be called 3-epi-3-dichloromethylactinobolin. The structure relationships between these two antibiotics suggest the possible involvement of a same gene in the biosynthesis of their characteristic dicyclic part.

Adriamycin (Fig. 3) is an antitumor antibiotic which has been widely used in the treatment of various types of human cancer and its strong anticancer activity

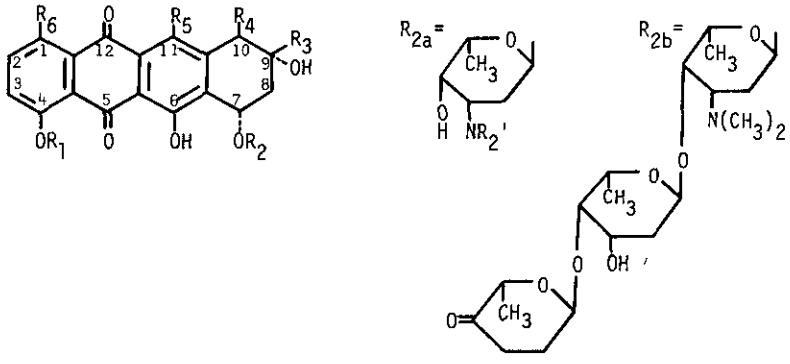


Fig. 3. Aklavinone (R_1=H, R_2=H, R_3=C_2H_5, R_4=CH_3OOC, R_5=H, R_6=H), Aclacinomycin A (R_1=H, R_2=R_{2b}, R_3=C_2H_5, R_4=CH_3OOC, R_5=H, R_6=H), Adriamycin (R_1=CH_3, R_2=R_{2a} (R_2'=H_2), R_3=$HOCH_2CO$, R_4=H, R_5=OH, R_6=H), Daunomycin (R_1=CH_3, R_2=R_{2a} (R_2'=H_2), R_3=CH_3CO, R_4=H, R_5=OH, R_6=H), Cinerubin A (R_1=H, R_2=R_{2b}, R_3=C_2H_5, R_4=CH_3OOC, R_5=H, R_6=OH), Pyrromycin (R_1=H, R_2=R_{2a} (R_2'=$(CH_3)_2$), R_3=C_2H_5, R_4=CH_3OOC, R_5=H, R_6=OH)

stimulates the study of anthracycline antibiotics in detail. The study of the biosynthesis of anthracycline antibiotics[9] has shown that aklavinone or its precursor (9-demethoxycarbonyl-9-carboxyaklavinone) is an biosynthetic intermediate common to pyrromycin, aclacinomycin, cinerubin, daunomycin, adriamycin etc. (Fig. 3). The addition of aklavinone to culture media caused the production of these antibiotics by their nonproducing mutants, although Blumauerová et al.[10] reported that aklavinone was not the intermediate to daunomycin. The results of these biosynthetic studies indicate a wide distribution of a gene involved in the biosynthesis of aklavinone or its precursor among various species of streptomyces.

On the basis of the structures of each group of antibiotics and recent studies on their

genetics, it can be proposed as follows: (1) genes involved in the biosynthesis of characteristic structural parts or characteristic biosynthetic intermediates of each group of secondary metabolites have been generated and the products like 2-deoxystreptamine etc. are synthesized; (2) these products produced in cells have no cytotoxicity and are transformed or modified to final products by reactions of enzymes which are produced by following the control of other genes and the final products are released extracellularly; (3) in cells of different strains, the products are modified to different final products; (4) the wide distribution of these genes involved in the biosynthesis of characteristic structural parts among microorganisms suggests that in some or many cases these genes generated in a strain have been transferred to cells of other strains. In the case that they lie on plasmids, it is possible that they have been transferred into other cells of other strains or different species where the products are further differently modified.[9]

The author[11] first noticed a possible involvement of plasmids in the biosynthesis of some antibiotics, and Hopwood[12,13] proved the involvement of a plasmid in the biosynthesis of methylenomycin. The structure of methylenomycin A, 2-methylene-cyclopentane-3-one-4,5-epoxy-4,5-dimethyl-1-carboxylic acid,[14,15] suggests its possible biosynthesis by a multienzyme system. Leupeptin (Fig. 4), that is, acetyl(or propionyl)-leucyl-leucyl-argininal found by the author,[16] inhibits trypsin, plasmin, papain and cathepsin B. Trypsin hydrolyzes the arginyl bond, and leupeptin which contains argininal inhibits such endopeptidases. This aldehyde structure of inhibiting serine-thiol proteases was first found in leupeptin.[17] Leupeptin was found in culture filtrates of many strains belonging to more than 18 species of streptomyces. This indicates that a characteristic gene involved in leupeptin biosynthesis is widely distributed among various strains of various different species of streptomyces. It suggests that this gene might lie on a plasmid and have been transferred to other strains. In fact, this was confirmed.[18] Leupeptin is biosynthesized by the reduction of leupeptin acid (acetylleucylleucyl-arginine), and an enzyme complex which catalyzes the synthesis of leupeptin acid can be extracted from homogenates of a leupeptin-producing strain. In a reaction mixture containing ATP, acetate, leucine and arginine, the enzyme complex catalyzes the synthesis of leupeptin acid starting from the synthesis of acetyl-leucine.[19] On the other hand, the ability to produce leupeptin is eliminated by acriflavine treatment of a leupeptin-producing strain, and most leupeptin-

nonproducing mutants thus obtained do not produce the enzyme complex which catalyzes the synthesis of leupeptin acid. The ability to produce leupeptin can be transferred from a leupeptin-producing strain to a leupeptin-nonproducing mutant by conjugation. This indicates that the gene involved in the biosynthesis of the enzyme complex lies on a plasmid.

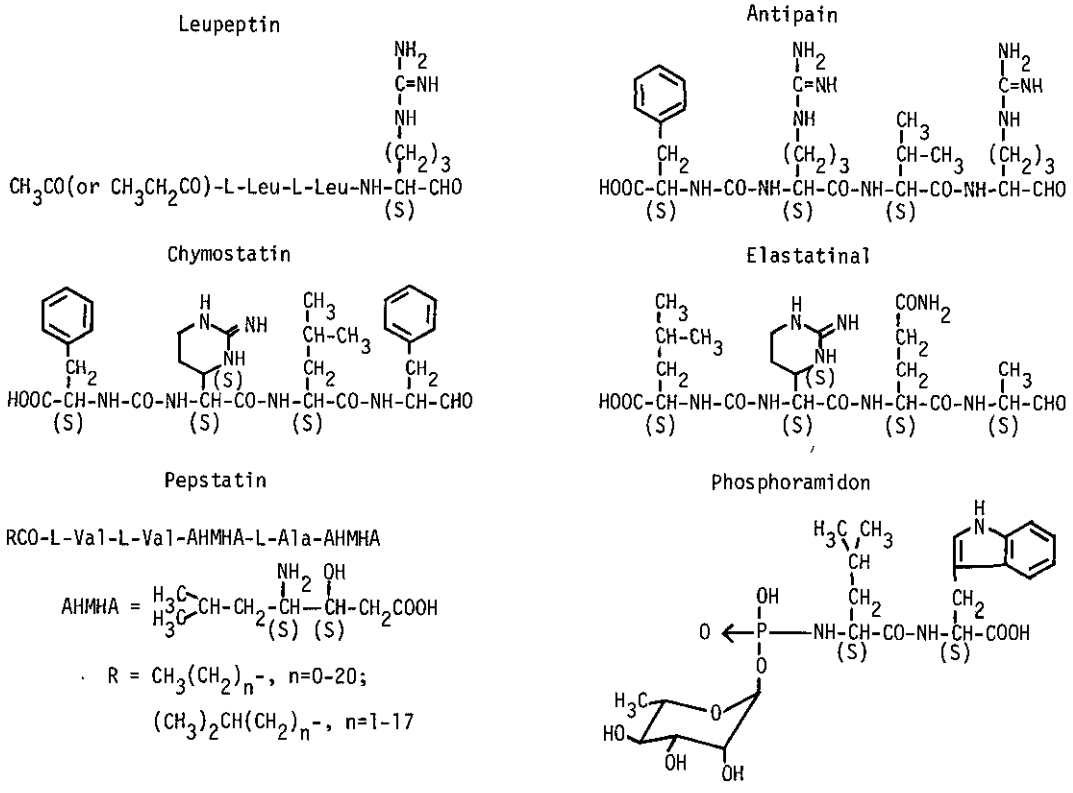


Fig. 4. Protease inhibitors found in streptomyces culture filtrates

Various protease inhibitors which the author found in culture filtrates of streptomyces strains[16] are shown in Fig. 4. Antipain which contains argininal inhibits trypsin, papain and cathepsins A and B. Chymostatin which contains phenylalaninal inhibits chymotrypsins strongly and papain weakly, and elastatinal which contains alaninal inhibits pancreas elastase. Chymotrypsins hydrolyzes phenylalanyl bonds in peptides and elastase hydrolyzes glycyl or alanyl bonds. Corresponding to the bonds hydrolyzed by these enzymes, their inhibitors contain phenylalaninal or alaninal moiety. These structures suggest possible involvements of enzyme complexes in the biosyntheses of their main peptide parts as in the case of leupeptin. Pepstatin inhibits pepsin, cathepsin D and renin. It contains two 3S,4S-4-amino-

3-hydroxy-6-methylheptanoyl groups. This amino acid added to the fermentation medium is not incorporated into this amino acid moiety of pepstatin molecule. This amino acid moiety has been shown to be biosynthesized from L-leucine and malonate or acetate.[20] Pepstanone in Fig. 4 is produced by pepstatin-producing strains and is different from pepstatin in the C-terminal amino acid moiety. Instead of 4-amino-3-hydroxy-6-methylheptanoic acid, pepstanone contains 3-amino-5-methylhexanone-2.[21] These observations suggest the possible involvement of an enzyme complex in the biosynthesis of pepstatin and pepstanone. It is easily understood that the decarboxylation of the C-terminal leucylacetyl group of the biosynthesis intermediate binding to the enzyme complex can give pepstanone.

Phosphoramidone inhibits thermolysin and other metalloendopeptidases. As later described, inhibitors of aminopeptidases have also been found in streptomyces culture filtrates.

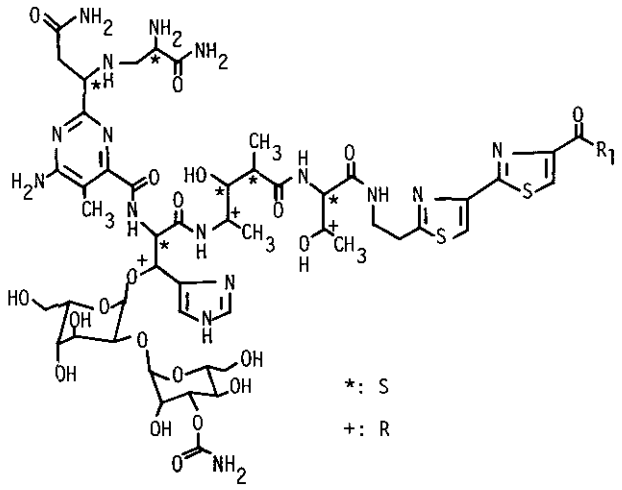


Fig. 5. Various bleomycins A1 (R_1=NH-$(CH_2)_3$-SO-CH_3), Demethyl-A2 (R_1=NH-$(CH_2)_3$-S-CH_3), A2 (R_1=NH-$(CH_2)_3$-$S^+(CH_3)_2$), A2'-a (R_1=NH-$(CH_2)_4$-NH_2), A2'-b (R_1=NH-$(CH_2)_3$-NH_2), A2'-c (R_1=NH-$(CH_2)_2$-imidazole), A5 (R_1=NH-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2), A6 (R_1=NH-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2), B1' (R_1=NH_2), B2 (R_1=NH-$(CH_2)_4$-NH-C(=NH)-NH_2), B4 (R_1=NH-$(CH_2)_4$-NH-C(=NH)-NH-$(CH_2)_4$-NH-C(=NH)-NH_2) and Bleomycinic acid (R_1=OH)

The structure of bleomycins are shown in Fig. 5.[22] They are very complicated glycopeptides and are different from one another in the terminal amine moiety.

The main structure part common to all of them are called bleomycinic acid. The structure of the peptide part of this antibiotic except for the side chain on the pyrimidine ring of an amino acid moiety was proposed in 1971[23] and the complete structure was proposed recently (Fig. 5).[22] All carbon atoms in the bleomycin molecule can be identified by ^{13}C-nmr.[24] Bleomycins shown in Fig. 5 different from one another in the terminal amine moiety are produced by a bleomycin-producing strain. The amino acid which consists of 4-amino-6-carboxy-5-methylpyrimidine and a side chain (Fig. 5) is called pyrimidoblamic acid. It may be interesting to readers that the pK of the imino group of the 1-(2-amino-2-carbamoylethylamino)-2-carbamoylethyl part of the pyrimidoblamyl moiety is 2.7. This low pK was confirmed by the synthesis of model compounds. Recently, biosynthetic intermediate peptides were isolated by the collaboration of Fujii et al., Research Institute, Nihon Kayaku Co. The results suggest the biosynthesis of the peptide part in the following sequence: demethylpyrimidoblamylhistidine, demethylpyrimidoblamylhistidylalanine, demethylpyrimidoblamylhistidyl-(4-amino-3-hydroxy-2-methyl)pentanoic acid, demethylpyrimidoblamylhistidyl-(4-amino-3-hydroxy-2-methyl)pentanoylthreonine, pyrimidoblamylhistidyl-(4-amino-3-hydroxy-2-methyl)pentanoylthreonine (in this step, the methylation of the pyrimidine ring occurs), pyrimidoblamylhistidyl-(4-amino-3-hydroxy-2-methyl)pentanoyl-(2-aminoethyl)-2',4-bithiazole-4-carboxylic acid, pyrimidoblamyl-β-hydroxyhistidyl-(4-amino-3-hydroxy-2-methyl)pentanoylthreonyl-(2-aminoethyl)-2,4'-bithiazole-4-carboxylic acid (in this step, the histidyl part is hydroxylated), deglycobleomycin. The equimolar copper complex of demethylpyrimidoblamylhistidylalanine was crystallized. On the basis of the X-ray crystal analysis of this intermediate,[25] ^{13}C-nmr and chemical synthesis of model compounds, the structure of the pyrimidoblamic acid moiety was conclusively determined. The isolation of the biosynthetic intermediate peptides described above suggests a possible synthesis of the peptide part by a multienzyme system. It can be imagined that the peptide part synthesized binds to the sugar [2-O-(3-O-carbamoyl)-D-α-mannopyranosyl)-L-gulose] during passing through the cell membrane and is released extracellularly.

As already described, leupeptin acid which is a characteristic biosynthetic intermediate to leupeptin has been confirmed to be synthesized by a multienzyme system. Gramicidins and bacitracin are known to be synthesized by a multienzyme system specific to each of them. As described above, all specific multienzyme systems

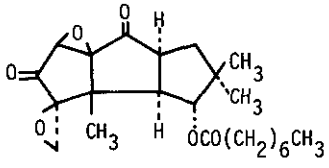
were suggested to be involved in the biosynthesis of almost all protease inhibitors and bleomycin. Extending these to the biosynthesis of other types of microbial products, it seems to be interesting to examine whether a multienzyme system is involved in the biosynthesis of a characteristic structural part of a secondary metabolite or its precursor. For instance, it may be an interesting study to examine whether 2-deoxystreptamine or its precursor is synthesized by an enzyme complex, that is, by a multienzyme system.

As described in this paragraph, it has been shown that secondary metabolites which have widely varied structures are produced by microorganisms, and the reason why so many compounds are produced has been analyzed up to the understandable step. Therefore, if an exact screening method is established, it is reasonable to search for microbial products which are fit to the purpose. The results of this study will supply new bioactive chemical structures.

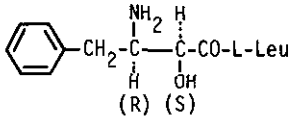
SMALL MOLECULAR IMMUNOMODULATORS PRODUCED BY MICROORGANISMS

In cancer patients the cell immunity is decreased. Therefore, it is thought that compounds enhancing cell immunity should enhance the effect of any cancer treatment. It is also thought that immunity-enhancing agents will be useful in the treatment of resistant infections in immunodeficient patients. Therefore, the author extended the study of enzyme inhibitors to the screening of microbial products which enhanced or decreased immune responses.

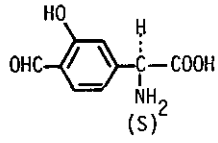
Diketocoriolin B



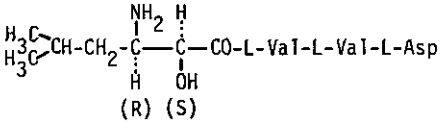
Bestatin



Forphenicine



Amastatin



Esterastin

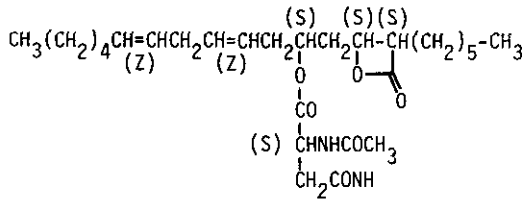


Fig. 6. Microbial products affecting immune responses

In this study, first, the author found that coriolin group antibiotics[26,27] and its derivative (diketocoriolin B) as shown in Fig. 6 increased the number of antibody-forming cells in mouse spleen.[28] For instance, a very low dose of diketocoriolin B such as 0.01-0.1 mcg/mouse increased the number of mouse spleen lymphocytes producing antibody against sheep red blood cells. This effect was also observed in cultured mouse spleen cells and this compound was shown to act on B lymphocytes and increase the number of antibody-forming cells. On the other hand, coriolin group antibiotics and diketocoriolin B inhibited Na^+-K^+-ATPase[29] which was located in cell membrane. It suggests that coriolin or diketocoriolin B molecules bind to the membrane of B lymphocytes and this binding causes mitogenesis, that is, division of these cells.

It is known that glycopeptides such as concanavalin A, phytohemagglutinin etc. bind to the surface of lymphocytes and cause mitogenesis. Therefore, the author thought that even small molecular compounds which bound to the cellular surface or cell membrane might cause mitogenesis and enhance immune responses, and the author initiated the screening of inhibitors of enzymes which were located on the cell surface, because such inhibitors should bind to the cell surface or membrane.

Aminopeptidases are one group of proteases which hydrolyze the N-terminal peptide bonds. The activities of these aminopeptidases can be exactly determined by measuring β-aminonaphthalene produced by the hydrolysis of N-aminoacyl-β-aminonaphthalenes. The author first found that all kinds of aminopeptidases were not only located in cells but also on the surface of all kinds of animal cells.[30] All intact cells including mouse lymphocytes and macrophages hydrolyzed N-aminoacyl-β-aminonaphthalene, but the enzymes were not released extracellularly. Intact cells hydrolyzed the N-terminal leucyl bond of leucyl-glycyl-glycyl-alkylamine attached to glass beads which did not penetrate into cells. Moreover, the author and Aoyagi found that alkaline phosphatase and esterase were also located on the cell surface.[9]

The author searched for microbial culture filtrates which inhibited the hydrolysis of N-arginyl-β-aminonaphthalene by aminopeptidase B prepared from rat liver in a reaction mixture containing 0.50 ml of pH 7.0, 0.1 M Tris-HCl, 0.25 ml of 2 mM arginyl-β-aminonaphthalene, 0.24 ml of distilled water with or without a diluted culture filtrate which was heated for 3 minutes at 100°C and 0.01 ml of the enzyme solution. The hydrolyzed β-aminonaphthalene was determined by reading the optical

density at 525 nm after addition of 1.0 ml of Fast Granet GBC [2-methyl-4-[(2-methylphenyl)azo]benzene diazonium chloride solution]. In this study, we discovered bestatin which inhibited aminopeptidase B and leucine aminopeptidase.[31] The structure of bestatin (Fig. 6) was determined to be 3-amino-2-hydroxy-4-phenylbutyryl-L-leucine. The 2S,3R stereochemistry of the unusual amino acid was determined by X-ray crystal analysis of the hydrobromide of the methyl ester of 3-amino-2-hydroxy-4-phenylbutyric acid.[32] The oxazolidone of this amino acid prepared by alkali treatment of its N-benzyloxycarbonyl derivative showed 4.0 Hz as the coupling constant of vicinal protons which conformed to the threo-structure.[33] Thus, it was shown that Shiba's oxazolidone method for determination of stereochemistry of α-amino-β-hydroxy acids could be applied also to the determination of β-amino-α-hydroxy acids.

All 8 stereoisomers of bestatin were synthesized and the 2S configuration was found to be the key structure for the activity to inhibit aminopeptidases. Four 2R isomers showed none or only very weak activity and all three 2S isomers showed a similar degree of activity as bestatin.[34] Bestatin enhanced delayed-type hypersensitivity in a footpad test using CDF_1 mice older than 8 weeks.[35] In this test, sheep red blood cells were injected into a footpad and at the same time bestatin was given orally or intraperitoneally and 4 days thereafter sheep red blood cells were injected into the other footpad and the swelling of this footpad was measured 24 hours thereafter. 0.1-100 μg/mouse of bestatin enhanced this hypersensitivity, indicating the enhancement of cell immunity. All 2S stereoisomers showed the same degree of activity as bestatin in enhancing the hypersensitivity but all 2R isomers showed much weaker or no activity. It suggests that the action to enhance the hypersensitivity might be related to the binding of bestatin to aminopeptidases on cells involved in immune responses. In experiments of mouse spleen cells cultured in media containing fetal calf serum, bestatin increased thymidine incorporation into T cells in the presence of macrophages.

Bestatin given orally is well absorbed and excreted into urine. About 5-15% of bestatin orally given to human was metabolized to p-hydroxybestatin. Modification of the benzene ring of bestatin can give derivatives which have stronger activity to inhibit aminopeptidases[36] than bestatin. p-Hydroxybestatin shows about 5-10 times stronger activity than bestatin in inhibiting aminopeptidases and stronger activity in enhancing the hypersensitivity to sheep red blood cells or the hypersensitivity to small molecular allergic agents such as oxazolone (4-

ethoxymethylene-2-phenyl-2-oxazolin-5-one). p-Hydroxybestatin increased also the number of antibody-forming cells in mouse spleen.

Bestatin inhibits the growth of slowly growing subcutaneous mouse tumors such as Gardner lymphosarcoma and IMC carcinoma. Bestatin has an extremely low toxicity and has been studied clinically in the last 3 years. Before the clinical study, it was not certain whether the compound which enhanced mouse immune system might produce the same action on the human immune system. From the clinical study in Japan and Sweden, bestatin was confirmed to increase T lymphocytes and the activity of natural killer lymphocytes. During clinical studies doctors felt that the frequency of bacterial infections might be lower in patients treated with bestatin than those without bestatin. In animal experiments bestatin was suggested to suppress a light infection of Pseudomonas in mice in experiments where the immune resistance of mice had been lowered by the administration of cyclophosphamide, an alkylating anticancer agent which suppressed immune responses.

As already described, aminopeptidase A which hydrolyzes the N-terminal aspartyl or glutamyl bond is located on the cellular surface. The author found an inhibitor of this enzyme in a culture filtrate of streptomyces and named it amastatin.[37] It contains an unusual β-amino-α-hydroxy acid as well as bestatin (Fig. 6). The 2S,3R configuration of this amino acid was elucidated by the application of Shiba's rule to its oxazolidone derivative and by the chemical synthesis of this amino acid from D-leucine. Amastatin inhibits aminopeptidase A and leucine aminopeptidase. The activity of these enzymes on cultured human thymocytes and lymphocytes is inhibited by amastatin, indicating the binding of amastatin to the cellular surface. Intraperitoneal injection of 10 or 100 μg/mouse increased the number of antibody-forming cells in mouse spleen. The finding of bestatin and amastatin demonstrated new type of active structures such as αS-β-amino-α-hydroxy acids in inhibiting aminopeptidases and enhancing immune responses.

As already described, alkaline phosphatase is located on the cellular surface. By the screening of its inhibitors, a new amino acid named forphenicine was discovered (Fig. 6).[38] It inhibits chicken intestine alkaline phosphatase very strongly but other alkaline phosphatase only weakly. The kinetics of the inhibition of chicken intestine alkaline phosphatase indicates that the type of inhibition is uncompetitive, that is, allosteric. Therefore, its derivative which has no or only weak activity to inhibit the enzyme can bind to the surface of cells. Forphenicine exhibits a similar action as bestatin in enhancing immune

responses and in inhibiting experimental animal tumors. Intraperitoneal injection of 1, 10 or 100 μg/mouse of forphenicine enhanced delayed-type hypersensitivity against sheep red blood cells in footpad test and 10, 100, 1,000 μg/mouse increased the number of lymphocytes producing antibody against sheep red blood cells in spleen. In the mouse spleen cell culture, 0.1 μg/ml of forphenicine increased the number of cells producing antibody to sheep red blood cells. In this case, it was shown that forphenicine first acted on macrophages in the presence of an antigen such as sheep red blood cell and this action on macrophages resulted in the increase of B lymphocytes producing the antibody.

Esterase is located on the cell surface and an inhibitor named esterastin (Fig. 6) was found in a culture filtrate of streptomyces.[39] The intraperitoneal injection of 62 μg/mouse or more suppressed both hypersensitivity and antibody formation. It contains a β-lactone group which is found only very rarely among natural products. Immune suppressive agents may be useful in the treatment of autoimmune diseases.

Table 1. Km and Ki of amastatin, bestatin, forphenicine and esterastin and the types of their inhibition

Inhibitors	Enzymes[1)]	Substrates	Km($\times10^{-4}$M)	Ki($\times10^{-8}$M)	Type of inhibition
Amastatin	AP-A	L-Glutamic acid NA[2)]	1.0	15	Competitive
	Leu-AP	L-Leucine NA[2)]	37	160	Competitive
Bestatin	AP-B	L-Arginine NA[2)]	1.0	6.0	Competitive
	Leu-AP	L-Leucine NA[2)]	5.8	2.0	Competitive
Forphenicine	Alkaline phosphatase	PNPP[3)]	4.6	16.4	Uncompetitive
Esterastin	Esterase	PNPA[4)]	4.0	0.016	Competitive

1) AP-A=aminopeptidase A; Leu-AP=leucine aminopeptidase; AP-B=aminopeptidase B
2) L-N-glutamyl, L-N-leucyl or L-N-arginyl-β-aminonaphthalene
3) p-Nitrophenyl phosphate 4) p-Nitrophenyl acetate

In Table 1, kinetic constants of bestatin, amastatin, forphenicine and esterastin are described. Ki value of esterastin is very small compared with those of others and indicates a very strong binding of esterastin with esterase. Although it has not yet been studied, the action of esterastin in suppressing immune responses may be related to its strong binding to the cell surface. As described above, the

microbial compounds found by the screening of culture filtrates which inhibited the enzymes on the cell surface has given those which enhance or decrease immune responses, and new bioactive structures were thus discovered.

DEVELOPMENT OF EFFECTIVE DERIVATIVES AND ANALOGS

Following the isolation and the structure determination of a useful bioactive compound, it is natural for chemists to attempt to synthesize this compound and its derivatives or analogs. These studies are promoted by practical necessity. Kanamycin (Fig. 1) which the author discovered in 1957[40] in the screening of low toxic water soluble basic antibiotics was evaluated in its effect on resistant infections. In about 1965, after 8 years of its wide clinical use, Gram negative organisms and staphylococci resistant to this antibiotic appeared in hospital patients. In 1967, the author was successful in the elucidation of the enzymic mechanism of resistance of these resistant strains and it became possible to predict the active structures.[41,42] Resistant strains produced enzymes which inactivated kanamycin, and the enzyme which was the cause of the resistance of most strains resistant to kanamycin was kanamycin 3'-O-phosphotransferase. It transfered the terminal phosphate of ATP to the 3'-hydroxyl group (the 3-hydroxyl group of 6-amino-6-deoxy-, 2,6-diamino-2,6-dideoxy- or 2-amino-2-deoxy-D-glucose moiety) of kanamycins (Fig. 1). On the basis of this mechanism of resistance, the author thought that 3'-deoxykanamycin A and 3'-O-methylkanamycin A would be effective against resistant strains and S. Umezawa *et al.*[43,44] synthesized these derivatives. Then, the former inhibited both sensitive and resistant strains including *Pseudomonas aeruginosa* which was resistant to kanamycin since the discovery of this antibiotic, but the latter was not active. 3',4'-Dideoxykanamycin B exhibited a strong activity to inhibit both sensitive and resistant strains[45] and was shown to have low toxicity. This derivative has been widely used for the treatment of resistant infections. The analysis of the reaction of 3'-O-phosphotransferase suggested the involvement of 1-NH_2 (or 3-NH_2), 2'-OH (or 2'-NH_2), 3'-OH, 6'-NH_2 (or 6'-OH) of kanamycins A and B (Fig. 1) in their binding with the enzyme.[46] It also suggests that the modification of these binding groups might give derivatives active against resistant strains. Butirosin[47] produced by *Bacillus circulans* which contained a 2S-2-hydroxy-4-aminobutyryl group on 1-NH_2 of the deoxystreptamine moiety inhibited resistant strains and a kanamycin derivative of this type, 1-N-(2S-2-hydroxy-4-aminobutyryl)kanamycin A[48] inhibited

resistant strains and has been used clinically. As described above, the chemical studies of the active derivatives of aminoglycosidic antibiotics were initiated by the author and have been continued to develop effective agents against present and future resistant strains.

There are other enzymes involved in resistance: 4'-O-phosphotransferase, 2"-O-adenylyltransferase, 3-N-acetyltransferase, 2'-N-acetyltransferase and 6'-N-acetyltransferase.[42] Derivatives which are effective against resistant strains due to these enzymes have also been synthesized. In such derivative studies, it may be important to clarify the most simple structure for the activity. Recently, the author confirmed that 3',4',4",6"-tetradeoxyamikacin had a strong activity to inhibit Gram positive and negative bacteria.[49]

S. Umezawa et al.[50,51] were successful in the total chemical syntheses of streptomycin and dihydrostreptomycin. The most common enzyme which causes the resistance to streptomycin is 3"-O-adenylyl or 3"-O-phosphotransferase which transfers AMP or phosphate to the 3-hydroxyl group of the N-methyl-L-glucosamine moiety.[52,42] S. Umezawa[53] synthesized 3"-deoxydihydrostreptomycin. This derivative inhibits the growth of resistant strains except for Pseudomonas aeruginosa. Based on this result, the resistance of Pseudomonas aeruginosa to this derivative was found to be due to the 6-O-phosphotransferase which transferred phosphate to the 6-hydroxyl group of the streptamine moiety.[54] It is now possible to synthesize streptomycin derivatives active against resistant strains.

If the mechanism of action is elucidated on the structural level, as in the case of aminoglycoside antibiotics, active structures can be predicted and the compounds which have the expected activity can be synthesized. From this aspect, the author is trying to develop bleomycin derivatives. Bleomycin found by the author[55,56] is used in the treatment of squamous cell carcinoma, Hodgkin's lymphoma and testis tumors.

In the presence of ferrous ion, bleomycin reacts with DNA. The reaction results in strand scissions of DNA. ESR study of bleomycin metal complexes has elucidated the stereochemical structure of the bleomycin-ferrous complex (Fig. 7). On the basis of this structure and the quenching the fluorescence of bleomycin by the addition of polynucleotides,[57] it has been proposed that the bithiazole moiety of bleomycin binds to the guanine moiety of DNA and the oxygen molecule which binds to the ferrous ion of bleomycin-ferrous complex is activated and reacts with the deoxyribose moiety of DNA.[58] Cupric ion binds very strongly to bleomycin.

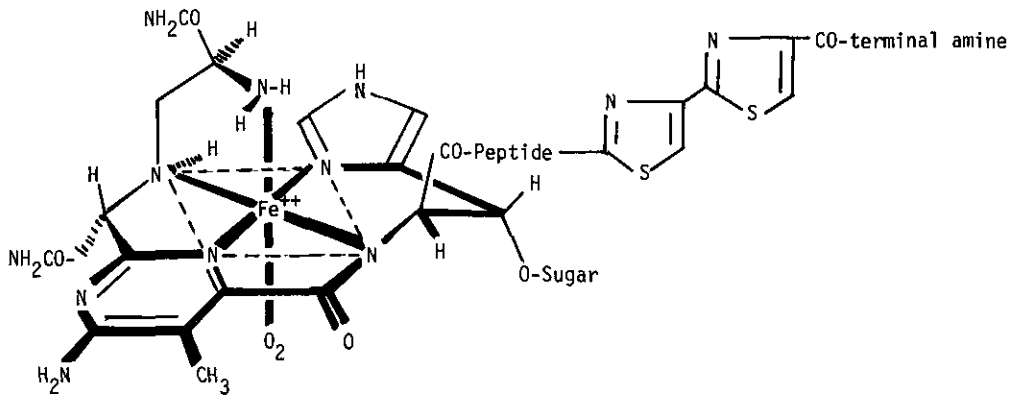


Fig. 7. Bleomycin ferrous complex binding to oxygen molecule

Although bleomycin copper complex binds to DNA, but neither causes DNA fragmentation nor the inhibition of DNA synthesis. On the other hand, the behavior of bleomycin injected into humans or animals has been elucidated as follows: bleomycin injected binds with cupric ion in the body fluid or blood; after penetrating into cells the cupric ion of bleomycin copper complex is reduced by intracellular reducing agents such as cysteine etc. and the cuprous ion thus formed is transferred to a cellular protein which binds to cuprous ion; copper-free bleomycin thus produced in cells undergoes the action of bleomycin hydrolase which hydrolyzes the α-aminocarboxamide group of the pyrimidoblamyl moiety; pK of the α-amino group of bleomycin shifts from 7.4 to 9.3 after the hydrolysis by this enzyme and this amino group of pK 9.3 cannot be involved in the coordination to produce ferrous complex at neutral pH and thus bleomycin is inactivated by this enzyme reaction; copper-free bleomycin which escaped from the action of bleomycin hydrolase reaches the nucleus and binds with DNA and ferrous ion and causes DNA strand scission, or bleomycin-copper complex which escaped from the action of reducing agents reaches the nucleus, binds with DNA, the cupric ion is reduced and replaced with ferrous ion and DNA is fragmented.[59]

Therapeutic effect of bleomycin on squamous cell carcinoma is due to a low content of bleomycin hydrolase and a high concentration of this antibiotic in this tumor.

On the basis of this behavior in vivo and the action mechanism of bleomycin, it is possible to predict the structure of the derivatives which will show a different antitumor spectrum. Derivatives which are resistant to bleomycin hydrolase may show a wider antitumor spectrum. The modification of the α-amino group of

the α-aminocarboxamide group or the modification of the amide group of the α-aminocarboxamide moiety gives derivatives which are resistant to bleomycin hydrolase and inhibit experimental animal tumors.

In the case of antitumor antibiotics, chemical derivation or the screening by fermentation is successfully developing, affording derivatives and analogs which have lower toxicity than the parent compounds.

Bleomycins which contain two or more guanidine groups or an amidine group in the terminal amine have a strong renal toxicity (Fig. 5). Therefore, the mixture of bleomycins consisting mainly of 70% A2 and 25% B2 has been used clinically. The mixture showed the strongest action against Ehrlich carcinoma compared with any single bleomycin shown in Fig. 5. The side effect of bleomycin used at present appears in the lung after the administration of it in large amounts. The degree of pulmonary toxicity was found to be different among various bleomycins which were different in their terminal amine moiety.

The main structural part common to all bleomycins is called bleomycinic acid. By searching for enzymes which hydrolyzed bleomycin to bleomycinic acid, an enzyme which hydrolyzed bleomycin B2 to bleomycinic acid and agmatine was found in a fungus belonging to Fusarium species.[60] This enzyme is specific to acylagmatine and hydrolyzes not only bleomycin B2 but also acetyl, propionyl and benzoylagmatine. A chemical method has also been developed to give bleomycinic acid.[61] The treatment of the copper complex of bleomycin demethyl-A2 with cyanogen bromide gives bleomycinic acid as follows:

$$\text{RCO-NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-S-CH}_3 \xrightarrow{\text{BrCN}} \text{RCO-O-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH}_3^+\text{Br}$$

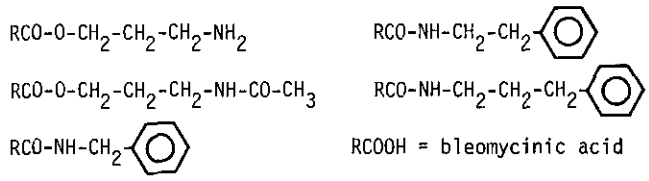
$$\xrightarrow{\text{PhCOCl}} \text{RCO-O-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH-CO-Ph}$$

$$\xrightarrow{\text{OH}^-} \text{RCOOH (bleomycinic acid)}$$

$$\xrightarrow{\text{R'RNH}_2} \text{RCONHR'}$$

In this reaction the α-amino group of the pyrimidoblamyl moiety is protected by copper chelation. An amine or an alcohol in the reaction mixture replaces the 3-benzoylaminopropanol group and the bleomycin containing this amine or alcohol is obtained.

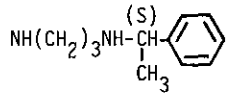
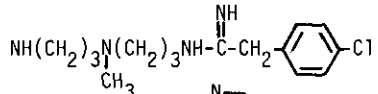
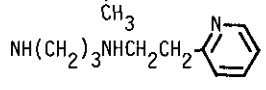
By the chemical method, the following bleomycins which cannot be prepared by fermentation have been prepared:



As already described, the side effect of bleomycin which limits the use of its large dosage is pulmonary toxicity. Therefore, a bleomycin which has lower pulmonary toxicity than the present bleomycin and which has the same or a stronger activity can exhibit a stronger therapeutic effect on human cancer sensitive to bleomycin treatment. A method of testing pulmonary toxicity has been established by using aged mice and bleomycins which have low pulmonary toxicity has been selected. Some examples are shown in Table 2. Among these bleomycins shown in Table 2, the pulmonary toxicity of A5033 was first tested clinically and was confirmed to have a lower pulmonary toxicity than the present bleomycin. It suggested

Table 2. Degree of pulmonary toxicity of new bleomycins

Bleomycin = $RCOR^1$

Bleomycins	R^1	Lung fibrosis index
Present BLM	Amines of A2, B2	1.0
BAPP	$NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3CH_3$	0.22
PEP		0.25
A5033	$NH(CH_2)_3NH(CH_2)_3NHCOCH_2CH_2COOH$	0.21
M5196		0.05
PYD		1.24

that the pulmonary toxicity test using aged mice gave a right selection of useful bleomycins. Among bleomycins shown in Table 2, bleomycin PEP which is called pepleomycin has been studied in most detail: pepleomycin shows a stronger therapeutic effect against head, neck and skin carcinoma than the present bleomycin and has a lower pulmonary toxicity. This stimulates further studies of various bleomycins in the purpose to find more effective and useful ones.

As described in the first paragraph of this paper, adriamycin is a very useful

cancer chemotherapeutic agent. The side effect of this drug is the delayed cardiac toxicity due to the degeneration of heart muscles. The degree of this toxicity is measured by electronmicroscopy of heart muscles after the administration of anthracycline antibiotics to hamsters, rats or rabbits. The results of this test indicated that aclacinomycin (Fig. 3)[62] had a much weaker cardiac toxicity than adriamycin and the damage caused by aclacinomycin was reversible. Conforming with the result of this animal test, the low cardiac toxicity of aclacinomycin to cancer patients has been confirmed by clinical studies. At present, adriamycin analogs and derivatives which have lower cardiac toxicity than adriamycin and which have the same or a stronger activity are searched by chemical syntheses and by fermentation. For instance, Arcamone[63] has reported that 4'-epiadriamycin and 4'-deoxyadriamycin have low cardiac toxicity.

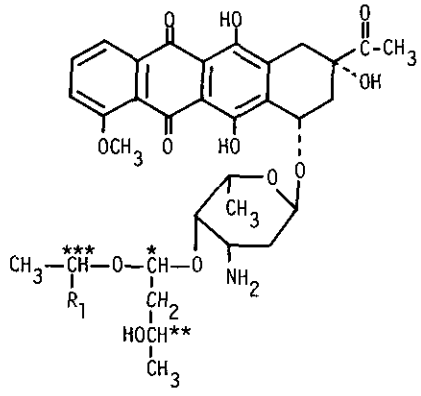


Fig. 8. Baumycins A1, A2 (R_1=CH_2OH, stereochemistry of *, **, *** unknown), B1 (R_1=COOH, *=R, **=S, ***=S), B2 (R_1=COOH, *=R, **=S, ***=R)

The author found eight daunomycinone glycosides in a culture filtrate of a daunomycin-producing strain and elucidated their structures. Four of them were new 4'-O-glycosidic derivatives of daunomycin and named baumycins A1, A2, B1 and B2 (Fig. 8).[64] The stereochemistry of baumycins B1 and B2 was determined by X-ray crystal analysis.[65] Among these baumycins, A1 showed a stronger therapeutic effect than adriamycin against mouse leukemia L-1210. However, the yield of baumycin A1 by fermentation was very small; moreover, the tests using different samples repeated by other laboratories did not give the same result. Therefore, the author decided to synthesize 4'-O-glycosidic derivatives of daunomycin and adriamycin. Among the derivatives thus synthesized, one of the 4'-O-tetrahydropyranyladriamycins showed

a stronger effect than adriamycin in the effect on mouse leukemia L-1210.[66] The author sent this compound to Dr. Mathé in Paris who had proved the low cardiac toxicity of aclacinomycin. He found that this derivative had a significantly lower cardiac toxicity than adriamycin.[67] Its cardiac toxicity was reversible and its grade was similar to aclacinomycin which had never caused delayed cardiac toxicity in clinical study.

As described above, organic chemistry has a predominant role in the development of effective derivatives and analogs. But generally, except for the cases of aminoglycosides active against resistant infections, at present, it is still impossible to predict the effective structures, and the success is dependent upon careful tests of the compounds synthesized randomly or under some hypotheses. Although in the future it may become possible to predict the active structures, at present findings of active structures by screening, chemical studies of their actions and synthesis of their derivatives and analogs are the general approach to the compounds useful in the treatment of diseases difficult to cure.

FUTURE ASPECTS

In concluding this paper, it may be necessary to discuss about the role of organic chemistry in the progress of bioactive compounds or the role of bioactive microbial secondary metabolites in the progress of organic chemistry. As described above, except for bleomycin which is an unusual glycopeptide of about 1,500 molecular weight, and which contains a very unusual amino acid such as pyrimidoblamic acid, the structures of all small molecular microbial products were rapidly elucidated and most of them were chemically synthesized. This indicates a great role of organic chemistry in the study of microbial products and a rapid progress of organic chemistry in the last 20 years. This progress will continue further together with the development of equipments. In the future, the isolation, extraction, purification and structure determination will be completed much faster than at present. It may be possible to establish an organization which elucidate structures rapidly upon the receipt of orders. Moreover, the chemical synthesis of new structure compounds will become easier and easier and the result will give abundant information on structure-activity relationships. Moreover, accumulation of these information and skillfulness of computers will become useful to design the chemical synthesis of active agents. However, it seems to take many years to reach the era where we can predict the bioactive structures exactly. Although

there was a great progress in protein chemistry and the structure of an important molecular part of an inhibitor of kininase was successfully predicted, in general it is almost impossible even to predict all possible structures of a specific inhibitor of an enzyme. Therefore, in the present situation, the screening of microbial products is still one of the most important research routes leading to new bioactive structures. Although it has often been said that microorganisms in nature produce antibiotics to suppress the growth of others, as described in this paper, besides antibiotics various bioactive compounds which have no antimicrobial activity have been obtained from microorganisms. Therefore, in the case that a quantitative simple screening method is established, there is a high possibility to find compounds which have the expected bioactivity. The studies of action mechanisms are now shifting over from the molecular level, that is, to the conformational level, and the results in the future will contribute to the prediction of active derivatives. On the other hand, chemical synthesis of derivatives which lack hydrogen-bonding groups partially or totally may give the most simple active structures. In the case of compounds such as bleomycin, adriamycin etc. which are useful but which have characteristic toxicity, the study of their derivatives and analogs is going to provide us with more effective compounds.

Although it will take many years for biochemistry to clarify the differences between all kinds of cells in human body and their relationships, biochemistry of disease processes is rapidly progressing on the enzymic level, and is helping to design many useful screening methods.

Therefore, also in the future, the screening of bioactive microbial product will dig up new bioactive structures and promote the progress of organic chemistry for structure determination, chemical syntheses and structure-activity relationships.

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