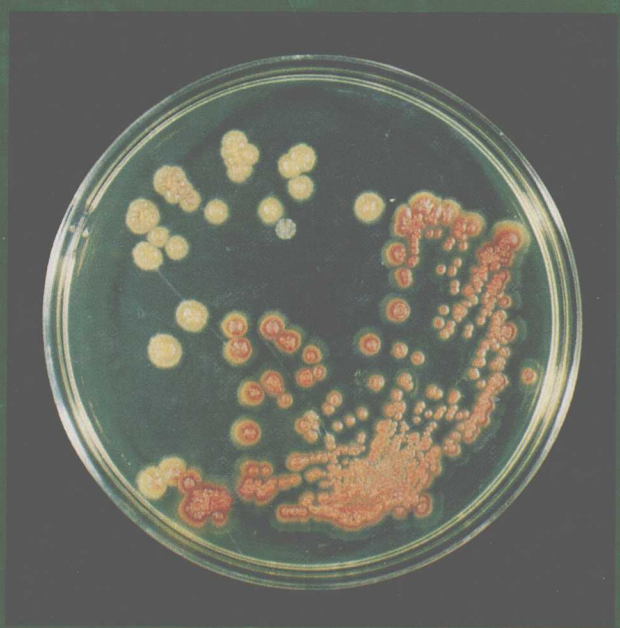
Satoshi Ōmura

Editor

The Search for Bioactive Compounds from Microorganisms



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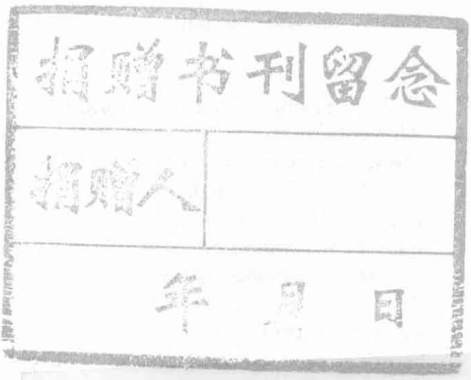
The Search for Bioactive Compounds from Microorganisms

With 74 Figures



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Cover: Colonies of *Streptomyces* sp. SK-1894, which produces the acyl-CoA synthetase inhibitors triacsins discovered by S. Ōmura and colleagues.

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Preface

In the days in 1973 when I had my own research group at the Kitasato Institute, it was said that most of the useful antibiotics had already been discovered and that the approaches, which are still in use today, had been exhausted. Some researchers who were engaged in the development of antibiotics were rather pessimistic about the discovery of any further new antibiotic. Some industrial research groups decided to withdraw from screening for new antibiotics. Actually, however, important antibiotics such as the avermectins, monobactams, and lactivicin were discovered later. This was the result of enthusiastic efforts aimed at development of new methods for the isolation of producing strains, modification of production conditions, and improvement of assay methods using unique test organisms such as hypersensitive mutants.

As of today more than 6000 antibiotics have been discovered. Although many antibiotics have unpleasant side effects, some show interesting modes of action on the basis of which they can be utilized clinically or as agrochemical agents.

For example, J.F. Borel and his co-workers observed an interesting side effect of cyclosporin A, an antifungal compound found by G. Thiel and his co-workers in 1970. Cyclosporin A showed specific toxicity to lymphocytes. Borel et al. then developed the antibiotic as an immunosuppressant. The new application of this antibiotic, in combination with other broad-spectrum antibiotics such as semisynthetic cephalosporins and penicillins, showed a good prophylactic effect and helped to make organ transplantation possible. In another study concerning side effects of erythromycin, Z. Itoh found that this antibiotic acts to mimic motilin, a gastrointestinal peptide hormone. Recently, we obtained an erythromycin derivative, EM-536, that exhibits gastrointestinal motor-stimulating activity 2860-fold more active than erythromycin and which has no antimicrobial activity. The motilides, named for this series of macrolide compounds with gastrointestinal motor-stimulating activity, are

expected to be very useful not only in therapy for digestive disorders but also as reagents in studies of gastrointestinal motility. In another example, bialaphos, originally discovered as an antifungal agent, was later found to be a potent herbicide. These examples indicate that reevaluation and redevelopment of known antibiotics as pharmacological or agrobiological drugs may be highly promising.

It was the late Dr. H. Umezawa and his co-workers who initiated research and development of microbial enzyme inhibitors as therapeutic agents for the control of abnormalities of homeostasis. This approach led his and other research groups to discover a number of interesting pharmacologically active microbial metabolites such as bestatin (aminopeptidase inhibitor, immunostimulant), FK-506 (immunosuppressant), mutastein (glycosyltransferase inhibitor), and pravastatin (HMG-CoA reductase inhibitor). There is the greatest possibility of finding many more substances with interesting bioactivities (such as immunomodulative, antiulcer, and hypotensive) among microbial metabolites.

It is an enjoyable and rewarding task to find new physiologically active substances. Toward this goal, it is very important to set the direction and the methods of searching in proper paths to avoid any waste of time and money. The first step toward the successful discovery of new substances requires both natural science and technology. In this book the principles, processes, and examples of finding physiologically active substances, including antibiotics, are described with emphasis on the strategy and the methods of research.

I have been engaged for a long time in educating and encouraging the researchers of my group at Kitasato. From our laboratory, 31 researchers have obtained the Ph.D. degree to date. They have learned their ways of thinking and details of proceeding studies with us, and today these people participate actively in research at various places in Japan and overseas. In commemoration of the twentieth anniversary of my laboratory, those who belong to my group have helped me with the preparation of this book. I sincerely hope that it will be helpful not only to researchers but also to students who are interested in bioactive compounds at their microbial origins.

I wish to thank the late Dr. Shigenobu Okuda for his helpful advice and assistance in editing this book, for which he was also responsible for preparing some chapters.

Satoshi Ōmura, Ph.D.

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Contents

1

Antibacterial Agents

Ruiko Ōiwa

1.1 Introduction

Penicillin, discovered by Fleming in 1928, was rediscovered by Chain et al. (1940) as a chemotherapeutic agent. In the same year, Waksman started the screening of antibacterial substances produced by a soil actinomycete, and discovered the actinomycins, some of which are used as antitumor agents. After many clinically useful antibiotics—streptomycin, chloramphenicol (chloromycetin), chlortetracycline (aureomycin), neomycin, oxytetracycline (terramycin), erythromycin, etc.—were discovered, most bacterial infections seemed to be conquered. However, about 10 years after the spread of antibiotic therapy, a number of species of *Staphylococcus*, *Mycobacterium*, and Gram-negative enteric bacteria had developed resistance to antibiotics. Then, the resistant strains of bacteria were used as test organisms to obtain new useful antibiotics. After the mechanisms of resistance to antibiotics in bacteria, such as enzymatic transformation, decrease in cell permeability, or decrease in ribosomal affinity had been elucidated, many derivatives of antibiotics were prepared chemically or biologically on the basis of information about the resistance mechanisms.

Strategies of screening methods for useful antibiotics were also modified. Results of the basic studies of mechanisms of action of antibiotics were applied to screening methods. The selective toxicity of a therapeutic agent should be superior to avoid the problem of side effects. From this point of view, some types of β-lactam antibiotic and inhibitors of bacterial cell wall biosynthesis were detected by various screening methods. A penicillinase inhibitor itself was also detected.

With antibiotic therapy, various septicemia caused by *Streptococcus* or *Staphylococcus* infection or violent enteric infections caused by Gram-negative bacteria decreased remarkably, and the average span of a human life has been gradually increased.

However, in hospitals, there is a problem of opportunistic infections of patients after operations, partly because of a decrease of immunological activity against various pathogens, even against weak ones. These infectious

diseases have been mainly caused by various Gram-negative bacteria such as *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Proteus* sp., and *Serratia marcescens.* Nowadays, some major efforts have been made to obtain new types of antibacterial antibiotics, which are superior in selective toxicity, in pharmacokinetic properties, and in treating complex infections including opportunistic ones.

To discover new effective antibiotics, there are two subjects that cannot be considered separately. One is "production" of metabolites and the other is "detection" of activities of the metabolites. The former includes how to discover novel microorganisms and how to have the microorganisms produce various new metabolites. The latter problem includes how to sensitively and effectively detect activities of the metabolites. The latter subject, the "detection," is mainly described in this chapter, while the "production" is mentioned in Chapter 16.

Screening methods for antibacterial antibiotics have been modified by the changes of target in pathogens and on the basis of the elucidation of mechanisms of action of antibiotics. Traditionally, antibacterial activities were detected by the agar diffusion method using some Gram-positive and Gram-negative bacteria as test organisms. From the 1970s, these methods have been successfully replaced by detection of morphological changes of the test organisms, use of supersensitive mutants, inhibition of enzyme activities of bacteria, or application of monoclonal antibodies to an enzyme-linked immunosorbent assay.

In this section, some traditional methods and screening processes are described in the former part, and some target directed methods are shown in the latter part.

1.2 Conventional Assay Methods

The agar diffusion assay methods using some bacteria as test organisms is simple and easy to use in finding antibacterial activity in cultured broths or test materials. This method gives only limited information, for example, "one or more substances contained in the test sample inhibit the growth of the test organism." This method, however, is still useful in the improved screening procedures.

Use of antibiotic-resistant mutants After various pathogens that acquired resistance to antibiotics appeared in the clinical field, efforts to find new types of antibiotics active against such resistant bacteria were accelerated. Then resistant mutants of the assay organisms were also employed in the screening procedures, and mechanisms of resistance such as enzyme inhibition or blocking of transport systems also have been taken into consideration.

In recent screening programs to find new antibiotics active against the

bacterial cell surface, an antibiotic selectively active against antibiotic resistant mutant *Staphylococcus aureus* 4R was sought (Higashide et al., 1985). The mutant strain which is resistant to tetracycline, erythromycin, chloramphenicol, and streptomycin was derived from *S. aureus* FDA 209P in vitro. In this screening process, about 2,000 actinomycete strains were tested, and a new dipeptide antibiotic named alahopcin was found. The antibiotic was assumed to be an inhibitor of bacterial cell wall synthesis, since it inhibited the incorporation of [^{3}H]diaminopimelic acid into *Escherichia coli.* Alahopcin also showed synergistic activity with erythromycin against drug-resistant bacteria, especially constitutive types of macrolide resistant strains.

In another screening program to detect oligopeptide antibiotics (Yoshida et al., 1986), a resistant mutant *Bacillus subtilis* SRY-7, was used as an assay organism. The mutant strain, which was selected by its resistance to a tripeptide antibiotic SF-1293, seemed to be an oligopeptide transport deficient mutant.

SF-1293 (L-phosphinothricyl-alanyl-alanine) is known to be taken up by *E. coli* K-12 *via* an oligopeptide transport system. On the other hand, a triornithine resistant mutant of *E. coli* K-12 was known to be an oligopeptide transport deficient strain. Therefore, a mutant of *B. subtilis* resistant to SF-1293 was isolated from *B. subtilis* M8193 (met^-, trp^-) as an oligopeptide transport deficient mutant. Growth response of the mutant and the parent strain to di- and tripeptides was tested. When the di- and tripeptides contained methionine or methionine plus alanine the mutant did not grow, although the parent utilized both types.

In this screening system, antibiotics that are active against the parent strain M8193 and inactive against the mutant strain SRY-Y were selected. A new antibiotic SF-2339, 2-(valylvalylaminoxy)malic acid, was discovered.

Use of mutant supersensitive to antibiotics To detect directly small amounts of antibiotic produced in a culture broth by the traditional agar diffusion method, a supersensitive mutant of a conventional assay organism was used. Sometimes a multiple drug sensitive mutant was used, and sometimes a supersensitive mutant to a specific antibiotic was used. It was also useful to employ a larger paper disc and a thin-layered agar medium for an assay plate. In the screening programs to find new β-lactam antibiotics, supersensitive mutants of various test organisms were found to be useful, as mentioned in the latter part in this section.

In a recent example of a screening program using a multiple drug sensitive strain of *Micrococcus flavus,* a new pyrrole-amidine antibiotic TAN-868 A, which is active against bacteria, fungi, and protozoa, was found to be produced by *Streptomyces idiomorphus* (Takizawa et al., 1987).

Assay under anaerobic condition It was found that the antibacterial activity of fosfomycin is more potent under anaerobic condition than under aerobic condition. Based on this fact, screening for antibiotics that are more

active under anaerobic than under aerobic conditions was carried out using *Escherichia coli* NIHJ as assay organism.

To examine the screening system, whether the phenomenon is specific or not, inhibitory activities of various known antibiotics against *E. coli* NIHJ were assayed under aerobic and anaerobic conditions. As a result, inhibitory activities of most types of antibiotics—except for penicillin G and cycloserine—were not found to be enhanced under anaerobic conditions.

Among several antibiotics selected in this screening system, a new antibiotic SF-2312, 1,5-dihydroxy-2-oxopyrrolidin-3-yl-phosphonic acid, was found (Watanabe et al., 1986).

Use of anaerobic bacteria Opportunistic infections caused by some anaerobes are sometimes a clinical problem with associated high mortality rates. For instance, pseudomembranous colitis, which tends to occur in association with antibiotic therapy, is one of such serious infectious diseases.

To search for new antianaerobic antibiotics, *Bacteroides, Clostridium, Fusobacterium, Peptococcus,* and other anaerobic bacteria are used as assay organisms. One of them, *Clostridium difficile,* the major causative pathogen of pseudomembranous colitis, is extremely sensitive to oxygen. Even after being exposed to air once, it cannot grow any further. Strict anaerobic conditions are necessary for its growth. Such anaerobic bacteria are too difficult to use as assay organisms for a routine process in a conventional assay system.

Masuma et al. (1987) reported a simple and convenient agar medium for the growth of *C. difficile* as assay organism. It can be handled under air for several hours before the assay of test materials, and incubation in an anaerobic chamber.

To protect *C. difficile* cells from exposure to oxygen, a double-layered agar plate was prepared. *C. difficile* was seeded in the bottom agar layer and an upper agar layer was used as an oxygen barrier and nutrient reservoir. This is the reverse phase of the double layer of the conventional agar plate for paper disc or cup assay method.

Using this assay technique, thiotetromycin (Ōmura et al., 1983), luminamicin (Ōmura et al., 1985), lustromycin (Tomoda et al., 1986), and clostomicin A, B_1, B_2, C, and D (Ōmura et al., 1986) were found.

In other recent screening systems from 1987 to 1988, although the methods were not described, discovery of abbeymycin, coloradocin (identified with luminamicin), and tirandalydigin were reported.

Detection of synergistic activity In the clinical field, the use of two or more antimicrobial agents in combination for the treatment of serious bacterial infections is practical. Such an approach is founded on the premise that the agents may act synergistically in vivo. To improve an antimicrobial spectrum or activity of a clinically useful antibiotic that acts as a protein or cell wall synthesis inhibitor etc., a bacterial membrane affecting antibiotic may be useful.

Ichimura et al. (1987) tried to screen for new antibiotics that showed antibacterial activity against *E. coli* when tested together with spiramycin, which itself is not effective against Gram-negative bacteria. Antibiotic activity in the fermentation broth was determined by the paper disc agar diffusion method using *E. coli* ATCC 26 as assay organism in the presence of 40 μg/ml of spiramycin.

Using this screening system, a new antibiotic, CV-1, was discovered. Although the antibacterial activity of CV-1 is very weak, it showed a cooperative effect with spiramycin against *E. coli.* The mode of action of CV-1, which is 1,2-diamino-1,2-*N*,*N*′-carbonyl-1,2-dideoxy-α-D-glucose hydrate, seemed to be the inhibition of lipopolysaccharide synthesis. The lipopolysaccharide of the outer membrane seems to have an important role as a barrier to transport. The cooperative effect of CV-1 seems due to the increased entry of spiramycin into cells of *E. coli.*

Radioimmune and enzyme-linked immunosorbent assays Immunoassays of various types have been widely used in clinical laboratories for both the detection and the quantitative analysis of antibodies, hormones, and drugs in body fluids. In microbiological or agricultural laboratories, enzyme-linked immunosorbent assays (ELISA) have been applied to the rapid detection and quantification of mycotoxins (Pestka et al., 1981). Yao and Mahoney (1984) developed an ELISA for detecting aminoglycoside antibiotics in screening for novel fermentation products. In other studies, radioimmune assays and the ELISA technique were successfully applied to erythromycin derivatives (Tanaka et al., 1988) and antichlamydial agents (Cervenini et al., 1987).

Identification of known antibiotics Since the discovery of new antibiotics has become more and more difficult, it is necessary to increase assay sensitivity with shorter assay times. Moreover, rapid and accurate identification of new antibiotics is an absolute necessity to prevent wasteful duplication of effort. In most laboratories, accumulated personal data about antimicrobial spectra and paper or thin-layer chromatographic properties of known antibiotics have been utilized for initial antibiotic identification and classification in crude culture broths.

Although there are few reviews mentioning early identification of antibiotics, Mitscher and Omoto (1977) published a brief report with 158 references about physical methods, X-ray methods, mass-spectrometry, nuclear magnetic resonance, and circular dichroism and their application to identification of antibiotics.

To know whether a newly detected antibiotic is new or identified, it is necessary to compare the physical and chemical characteristics of the unknown compound with those of known compounds. When the number of antibiotics to be compared is not large, edge-punched cards were used. Today, however, more than 6,000 antibiotics and a similar number of inactive metabolites have been reported to be produced by microorganisms. Two com-

puter systems have been designed to assist in the identification of known antibiotics. One is by NIC, Frederick Cancer Research Center in Hungary (Bostian et al., 1977), and the other is by the Microbial Chemistry Laboratories of the Kitasato Institute in Japan (unpublished).

The former, BERDY data base, was reported to contain physical, chemical, and biological data except for structural and IR spectra. The latter, KMC (Kitasato Microbial Chemistry) data base, contains similar items of physical, chemical, and biological characteristics of microbial products including low molecular weight metabolites. In the near future, structures of the compounds will also be covered by this data base system.

1.3 Target-Directed Screening

The screening target is usually focused on a mechanism of action of an antibiotic that shows highly selective toxicity. Usually, on the basis of various findings of mechanisms of action of useful known antibiotics, some congeners or alternative structures of these are sought. Sometimes rationally selected enzymes or receptor sites, for which there are no known inhibitors, are noted and used as the screening target.

Inhibitors of bacterial cell wall synthesis Many efforts were made in searching for inhibitors of bacterial cell wall synthesis, because their specific inhibitory activity on the biosynthesis of bacterial cell wall, which animal cells lack, provides a guarantee of their low toxicity to human cells. Therefore, the bacterial cell wall received great emphasis as the target of antibiotic action, and many inhibitors have been sought. However, only a few inhibitors of cell wall synthesis were found for clinical use, because some of them lack activity *in vivo*, and some of them show cross-resistance with the clinically used antibiotics. However, various inhibitors acting on the different sites of the pathway of cell wall peptidoglycan synthesis were used as important tools for elucidating details of bacterial cell wall biosynthesis. Inhibitors of cell wall biosynthesis which were found in recent target-directed screening are shown in Table 1.1.

Observation of morphological changes Spheroplast or bulge formation by bacterial cells is one proof that an inhibitor of cell wall biosynthesis is present in the test material. For the purpose of screening for inhibitors of bacterial cell wall synthesis, spheroplast or bulge formation was observed microscopically. Sometimes the screening procedure is effectively carried out in combination with other detection methods, such as the use of supersensitive mutants and the observation of synergistic activity with other inhibitors of bacterial cell wall synthesis.

In recent work, L-cycloserine, which does not show antimicrobial activity, was detected by spheroplast formation of *E. coli* LS-1, a supersensitive mutant

Table 1.1 Screening methods for inhibitors of bacterial cell wall formation

Name of antibiotic	Group	Producing organism	Screening method	Reference
Penicillin N (identified)		*Streptomyces* sp.		Nagarajan, 1971
Metabolite 2	β-Lactam	*Streptomyces lipmanii*		
Metabolite 3	β-Lactam	*S. clavuligerus*		
Metabolite 4 (= cephamycin C)	β-Lactam			
Cephamycins A and B	Cephem	*S. griseus* and other *Streptomyces* strains	Using *Proteus vulgaris* and *Vibrio percolans*	Stapley, 1972
Cephamycin C	Cephem	*S. lactamdurans*		
Thienamycin	Carbapenem	*S. cattleya*	Using large size paper disc and thin layer agar medium for assay plate	Kahan, 1979
Epithienamycin	Carbapenem	*S. flavogriseus*		Stapley, 1981
Penicillins and cephalosporines (identified)		80 fungal and 30 actinomycete strains out of 30,000 strains	Using *Pseudomonas aeruginosa* PsC^{ss} (hypersensitive to β-lactams)	Kitano, 1975b
C-19393 S_2 and H_2 (= Carpetimycins B and A)	Carbapenem	*S. griseus*		Imada, 1980
Sulfazecin	Monocyclic β-lactam (monobactam)	*Pseudomonas acidophilla*	Using *P. aeruginosa* PsC^{ss} and *Escherichia coli* PG8 (supersensitive to β-lactams, observation of spheroplast formation, and inactivation by β-lactamase	Imada, 1981
Isosulfazecin		*P. mesoacidophilla*		Imada, 1982
Bulgecins A, B, and C	Sugar	*P. acidophilla* and *P. mesoacidophilla*		
Cephabacins F_{1-9} and H_{1-6}	Cephem	*Lysobacter lactamgenus* and *Xanthomonas lactamgena*	Using *P. aeruginosa* C141 and *E. coli* (sensitive to β-lactams) and observation of spheroplast formation	Ono, 1984

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