THIRTY-FIVE YEARS WITH BIOACTIVE MICROBIAL PRODUCTS

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It is a great pleasure and honor for me that outstanding professors of organic chemistry for whom I have great respect have taken the time to contribute papers in this book which has been published to celebrate my 65th birthday. Time passes very quickly, and about 35 years have passed since I first entered the research of antibiotics. It is in 1941 that the first paper by professors Florey, Chain, Abraham and Heatley on the discovery of the effective penicillin appeared in Lancet and inaugurated the modern antibiotic era. Boys who were born at about that time have now grown to become active scientists, and they may feel this discovery is an ancient one. However, even today, I feel like this is a recent great finding. It is in 1933, in the course of microbiology in Medical School of the University of Tokyo that I learned for the first time, the development of effective arsenic compounds by Prof. Ehrlich in 1910, and at that time I felt this study was like one of the very old famous events. In fact, only 25 years had passed since Prof. Ehrlich's discovery up to that time. Therefore, it may be interesting to young readers to hear about my research career on antibiotics briefly.

During the four years from 1933 to 1937 when I was in the course of Medical School of the University of Tokyo, almost everyday after school, I used to stay over at the Nezu Chemical Institute belonging to Musashi High School and learned chemistry, particularly reaction kinetics from Prof. Bunichi Tamamushi who was an authority on colloidal chemistry. I believe, this was a great education for my research thereafter. During the summer vacation periods of these four years, I stayed in the Department of Biochemistry of Medical School and received a preparatory education of biochemistry from Prof. Saburo Kakiuchi. After graduating from Medical School in 1937, I entered the Department of Bacteriology and joined in Professor Matsujiro Takeuchi's study on the vaccination of Japanese encephalitis. During this study I was interested in immunochemistry, because at that time structure-

specificity relationships of haptene antigens had already been studied in detail and the amount of antibody had been determined by its nitrogen contents. In April, 1939, I was drafted to Narashino Army Hospital. This hospital was located not far from Tokyo City and I was able to continue research in the testing laboratory of this hospital and also go to the laboratory of the University almost every evening. At about that time, I read Prof. Dubos' paper on his discovery of tyrothricin, a mixture of tyrocidin and gramicidin. It was to my great surprise that soil bacteria produced antibacterial substances which were crystallized. I repeated this study. Although in this experiment I could not find soil bacteria which caused the lysis of staphylococci as reported by Prof. Dubos, I learned that all soil samples contained actinomycetes, and many of them produced antibacterial substances. Moreover, Professor Waksman reported his discovery of actinomycin in the Journal of Bacteriology of 1940 which arrived at the library of University Hospital before the start of the second world war. In April, 1943, I was released from army service and came back to the University. In July, I was promoted to Associate Professor of University of Tokyo and moved to the department of the Institute of Infectious Diseases of the University of Tokyo where I could continue the research independently. The other happening which determined my research direction thereafter is the arrival of a new volume of Klinische Wochenschrift of 1943 to Tokyo in November of 1943. It is said that this is one of the books carried by a Japanese Navy submarine from Germany. I was awfully impressed by an article where the progress of penicillin research after 1941 was reviewed thoroughly for its chemistry, action and clinical effects. At the end of 1943, I was asked by Army Medical School to translate this article into Japanese, and in order to obtain penicillin quickly, the Army Medical School in Tokyo organized a joint study of microbiologists and chemists in February of 1944. I joined in this study. I extracted penicillin as a crude yellow powder at the end of September of this year (1944). At the time, there was no incubator with a cooling accessory, the summer temperature was always higher than 25°C, and during the summer no penicillin was obtained. The first Japanese penicillin obtained from cultured bottles was placed on my desk in September. A small scale industrial production was started in two factories soon thereafter. The second world war finished on August 15th, 1945, and I was asked by the Japanese Army Medical School to summarize and report the penicillin study in Japan to officers of the U.S. Army who came to the School in the middle of September, 1945. I remember that it was during this meeting that I heard a

little from one of the medical officers about U.S. studies of nitrogen mustard for the treatment of cancer.

After penicillin, it is natural that I started the screening of new antibacterial substances produced by soil actinomycetes. The first one obtained from penicillium was patulin which had a strong toxicity. Thereafter, I engaged in the study of antibacterial substances produced by actinomycetes, because I did not have enough training for the classification of fungi. The first one obtained was deep red crystals identified to be a member of actinomycin. The second one was similar to streptothricin. Up to this step, I followed completely the same course of Prof. Waksman's study on new antibiotics. I remember that when I visited Prof. Waksman in Rutgers University in 1950, he said "this is resembling to the principle, the individual evolution follows the systematic evolution". After obtaining these products, I added steps in the screening to differentiate the products from known ones as quickly as possible and succeeded in finding new antibiotics. The term "antibiotic" proposed by Prof. Waksman became popular after about 1946 in Japan. Up to now, I have discovered more than 70 new antimicrobial antibiotics. One of them is kanamycin. At about 1956, it was not easy to differentiate various aminoglycosides chemically and therefore, I endeavoured to find aminoglycoside antibiotics which have no delayed toxicity and discovered kanamycin. This antibiotic exhibited strong therapeutic effects on infections resistant to all other drugs at that time. During carbon chromatography, this antibiotic crystallized incidentally. The analysis showed that this crystal consists of kanamycin and one mole of sulfuric acid. The crystal in solution showed pH 8.0. Thus, an easy method of crystallization was established: pH was adjusted to 8.0 and ethanol or acetone was added. The structure of kanamycin was elucidated by the study from 1957 to 1958. I remember that the structure determination was much more difficult at that time than at present. At that time, structure elucidation was dependent on hydrolysis, determination of hydrolysis products and the application of periodate oxidation. There was no nmr. In 1963, I discovered kasugamycin which was a member of aminoglycosides and exhibited a strong action in preventing the most terrible rice plant disease. In this case, nmr of 60 Mz equipment was available, and we reached the structure very rapidly, including the stereochemistry. The absolute structure was rapidly solved by X-ray crystal analysis of its hydrobromide. At that time, I felt there would be a great progress in the chemistry of structure determination within five years since 1958. In 1967, the structure of kanamycin

monosulfate was also determined by X-ray crystal analysis. Of course, the result confirmed the structure proposed by chemical studies. Moreover, a strong hydrogen bonding between 5-OH and 2'-OH shown by X-ray analysis was useful information for development of active derivatives. Prof. Iitaka who has collaborated with me in the structure determination of many new microbial products by X-ray crystal analysis has often said, "it is interesting that the studies dependent on experienced rules can reach the correct structures".

In 1947, Japanese National Institute of Health was established and I moved to become the director of Department of Antibiotics of this institute. It is in this department that I discovered many new antibiotics including kanamycin. Besides this position, from 1954 to 1975, I had a professor chair in the Institute of Applied Microbiology of the University of Tokyo. Up until about 1960, it was not a easy job to direct the students in the graduate course. Screening was not a good subject for the students. Structure determination was not so easy and the molecular biology of the mechanism of action was in a very early stage of development. There has been a great progress since then, for presently, there are many fundamental subjects of studies in antibiotics and other microbial secondary metabolites. In 1962, Institute of Microbial Chemistry was founded by a nonprofit foundation called Microbial Chemistry Research Foundation which was established in 1958, on the basis of the royalty from kanamycin. Besides the Department of Antibiotics of Japanese N.I.H. and the department in the Institute of Applied Microbiology, I was able to use the big research facility of the Institute of Microbial Chemistry which was equipped strongly enough. A year after the start of the research in this institute, I discovered kasugamycin as described above. This is an ideal agricultural product to prevent rice blast. More than 100 tons of its pure compound are used in Japan and foreign countries. After this was introduced, there has been no crop decrease caused by this rice plant disease.

Kanamycin was effective against resistant infections at the time of its discovery but strains resistant to this antibiotic appeared in hospital patients in 1965. I undertook the study to elucidate the biochemical mechanism of resistance and found that the resistance was due to enzymes in resistant strains which transferred phosphate of ATP or AMP of ATP to some hydroxyl groups of kanamycin or the acetyl group of acetyl CoA to some amino groups. Although there are such various enzymes involved in the resistance mechanism, 3'-O-phosphotransferases were the most frequent cause of the resistance. Substrate and inhibitor specificities to 3'-O-phospho-

transferase which transferred phosphate to the 3'-hydroxyl group of kanamycin suggested which amino and hydroxyl groups of kanamycin molecule are involved in the binding with the enzyme. Thus, deoxylation of the hydroxyl group which undergoes the enzyme reaction or the modification of the group involved in the binding with the enzyme gave the derivatives active against resistant strains. Thus, it became possible to predict the active structures against resistant strains. On the other hand, Dr. S. Umezawa was successful in total syntheses of kanamycins A, B, C, streptomycin, dihydrostreptomycin, neomycin B and C. By application of such chemistry, it became possible to synthesize active derivatives of aminoglycisode antibiotics very rapidly. Moreover, the industrial production of such derivatives also became possible. Thus, the study of chemical mechanisms of resistance and syntheses of active derivatives are contributing to the chemotherapy of resistant infections at the present and in the future.

Here, again, I should like to backtrack to 1951. At that time, resistant organisms except for streptomycin-resistant tubercle bacilli had not yet appeared and the chemotherapy of bacterial diseases seemed to be completed. Therefore, I endeavoured to extend antibiotic research to a new area and initiated the screening of antitumor antibiotics. This study soon became one of the main focus of my research thereafter, and I have since discovered 40 new compounds. One of them, bleomycin was discovered in 1963, although I wrote its first paper in 1966 after the observation of its clinical effect on squamous cell carcinoma. It may be said that the study of antitumor antibiotics promoted the study of microbial products in the last 25 years. Compared with other antibiotics, the longest years were spent on the structure determination of bleomycin. It was difficult to determine its exact molecular weight and all bleomycins were not crystallized. Although almost all the features of the bleomycin molecule became clear in 1971, it took seven more years to reach the conclusive structure. An amino acid consisting of 4-amino-6-carboxy-5-methylpyrimidine and a 2-yl-side chain was a difficult part to elucidate. I cannot forget getting such a low pK as 2.7 for the imino group in the side chain, 1-(2-amino-2-carbamoylethyl)amino-2-carbamoylethyl group.

If compounds which have useful bioactivities are found by a screening study in microbial culture filtrates, then this becomes an important subject of study and will be pursued by many researchers: isolation of new compounds, elucidation of structures, synthesis of their analogs, studies of structure-activity relationships and their uses for analysis of biological functions and for the treatment of diseases.

In 1966, I initiated the screening of small molecular enzyme inhibitors when a new extention building was added to the Institute of Microbial Chemistry. By this study it was shown that microorganisms produced various bioactive compounds which had widely varied structures. They have no antimicrobial activity. I have found about 50 new compounds in this study. Their structures were very rapidly determined and most of them were chemically synthesized. After I reported the isolation of specific inhibitors of various proteases, every week I received more than ten letters from researchers in foreign countries requesting them for their studies. They have been used for identification of proteases, analysis of roles of proteases in biological phenomena, or to prevent the hydrolysis of bioactive peptides during the extraction. Recently, I extended this study of enzyme inhibitors to the compounds which bound to the cell surface and enhanced immune responses. The chemical structures of antibiotics and enzymes are now contributing to the understanding of their biosyntheses and genetics which suggests the reason why so many various compounds are produced by microorganisms. Chemistry of these bioactive microbial secondary metabolites are also contributing to the understanding of the structure-activity relationships. Although I have never been involved, the application of computers for the prediction of drug design has been started. We may be entering a completely new era of organic chemistry and biological and medical sciences. In order to accumulate experimental data, it may be always necessary for us to think about the future goal. Findings of new bioactive compounds in microbial culture filtrates and the study of their action mechanisms at the stereochemical level may be helpful to reach the goal.

In closing this paper, again I wish to express my many thanks to Prof. Kametani for the publication of this book and to all writers for their friendship. I think that organic chemistry is the fundamental science of biology and medicine and in the future all experimental results will be expressed in terms of chemistry.