Screening of New Antibiotics Produced by Actinomycetes and Their Production

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I have studied actinomycetes and their antibiotics over thirty-eight years except eight years for which I had been engaged in the research on the quality control for sterilization of microorganisms. My colleagues and I were successful to discover twelve new species of actinomycetes and fourteen new group antibiotics. In this review, I would like to describe rufomycins, enduracidins, validamycins, maridomycins, T-2636 antibiotics and ansamitocins in terms of their discovery, producers and/or industrial development.

Rufomycins (cyclic peptides) with a specific activity to mycobacteria was discovered from *Streptomyces atratus*, a new species, in our screening program targeting anti-tuberculosis antibiotics. Enduracidins (Enramycins; cyclic peptides with a fatty acid side chain) were screened as antibiotics with low toxicity and strong bactericidal spectrum including multiple drug-resistant streptococci. As to aminocyclitol antibiotics, validamycins, new assay methods (reversed layer method and dendroid-test method) were established in order to develop an industrial fermentation for their agricultural use. As to T-2636 antibiotics with macrolacton ring, the esterase catalyzing the transformation of T-2636 C to T-2636 A was identified in the producing organism, *S. rochei* subsp. *volubillis* and then the industrial enzymatic transformation was established. Maridomycins, new macrolide antibiotics, were discovered as a complex of about twenty components from *S. hygrosopicus* No. B-5050. Selective and improved production of maridomycin III was established by strain improvement by mutation on the basis of the regulation of amino acid metabolism involved in maridomycin biosynthesis. Ansamitocins, new antitumor antibiotics, were discovered from a new species of *Actinosynnema* and turned out to be similar in structure to maytansine with a strong mitosis-arrest activity from tropical plants.

1. *Streptomyces atratus*, rufomycins and their biosynthesis.

In the search for novel actinomycetes and their antibiotics with unique antibacterial spectra, a *Streptomyces* strain No. 46408 was found in 1959. The strain showed the following morphology; vegetative mycelium with simple branching, aerial mycelium forming mainly loops and rarely spirals with spherical to ellipsoidal spores of which surface were smooth. The aerial mass color was gray to black and the reverse side color became black at mature phase. No soluble pigment was produced on various synthetic and natural media. Thus the properties of this strain fell into *lectus apertum*, gray series and non-chromogenic type. Since there was no known species whose taxonomic properties were identical to those of the strain, a

new species named *S. atratus* sp. nov.¹⁾ was established for the strain No. 46408. In modern Latin, *atratus* means adj. clothed in black color of the aerial mass color and the reverse side.

The antibiotics produced by the strain No. 46408 was extracted with ethyl acetate from both cultured-broth filtrate and mycelium. Their anti-bacterial activity was so unique that they showed a specific activity to *Mycobacterium* spp. with MICs of 0.1 to 5 µg/ml. They were not active to Gram-positive and negative bacteria, fungi and yeasts even at the concentration of 100 µg/ml or higher. No cross-resistance was observed with the other antibiotics tested. The antibiotics are neutral and lipophylic and turned out to be novel polycyclic peptides composed of seven L-amino acids; L-alanine, L-N-methyl-leucine (2 moles), L-3-nitrotyrosine,

As shown in Table 1, the antibiotics showed a strong bactericidal activity to Gram-positive bacteria⁹). Even at 0.1 µg/ml, no colony gener-

Fig. 3. Enduracidins A and B

ated from staphylococcal cells ($10^5/\text{ml}$). In addition, the drug resistance hardly appeared, compared with the cases of chlortetracycline and penicillin G. The characteristics of strong bactericidal activity, a little absorption through the intestine membrane and very low occurrence of drug resistant led us to develop the antibiotics (commercial name: enramycin) as the feed additive after the fermentation and purification process in the industrial scale were

Table 1. Antibacterial spectrum of enduracidin.

Test organisms	M.I.C. ($\mu\text{g}/\text{ml}$)
<i>Staphylococcus aureus</i> FDA 209P	0.1
<i>S. aureus</i> (multiple drug resistant)	0.1
<i>Mycobacterium avium</i> IFO 3153	5
<i>Escherichia coli</i> IFO 3044	100
<i>Neisseria gonorrhoeae</i>	6.25
<i>Aspergillus oryzae</i> IFO 4626	100

established. When broilers chickens were fed with the additive, their body weight were increased by 2% with 1 ppm and by 5% with 20 ppm of the additive, respectively. The feed efficiency were 96.5% and 95.5% with the addition of 3 ppm and 6 ppm, respectively, as shown in Fig. 4¹⁰⁾. In addition, chickens suffering from cannibalism decreased dramatically by use of enramycin.

3. *Streptomyces hygroscopicus* subsp. *limoneus*, validamycin and its activity

At almost the same time of enramycin discovery, Shibata/Iwasa group found a new antibiotic useful for the control of the sheath blight of rice plants. It suppressed the growth of *Pellicularia oryzae* *in vivo*. The producer showed the following morphological properties; simply branching vegetative mycelia, spiral aerial mycelia forming oval or cylindrical spores ($1.0\sim 1.3 \times 1.0\sim 1.5 \mu\text{m}$) with smooth surface. Aerial mass color was gray series and the organ-

Fig. 4. Effect of enramycin on body weight (I) and feed efficacy (II) of broiler chickens.

Fig. 5. Validamycins A and B

ism was non-chromogenic. Based on these properties, *S. hygroscopicus* subsp. *limoneus* was established for this organism¹¹⁾. In modern Latin *limoneus* means lemon yellow, which is the color of vegetative mycelium of the organism and faint soluble pigment.

Fig. 5 shows the chemical structures of validamycins A and B¹³⁾. They were found to be strongly active *in vivo*, but not active *in vitro* when tested by the usual dilution method. Therefore, we developed new microbiological assay methods; the reversed layer method and

Table 2. Effect of validamycins on fungi.

Test organisms	MIC (μ g/ml) causing abnormal fungi	
	VM-A	VM-B
<i>Pellicularia sasakii</i>	0.01	0.5
<i>Pellicularia praticola</i>	0.02	50
<i>Glomella cingulata</i>	5	-
<i>Alternaria kikuchiana</i>	100	100
<i>Fusarium oxysporum</i>	100	100
<i>Sclerotinia sclerotiorum</i>	100	100

the dendroid-test method. The former permitted the inhibition zone formation and the latter the detection of the end point of abnormal growth of *Pellicularia sasakii* as a test organism. Minimum inhibitory concentrations of validamycins A and B determined by the dendroid-test method¹²⁾ were shown in Table 2. The strong effect *in vivo* of validamycin A was expressed as the average length of the lesion per stem. As shown in Table 3, the expanding rate after treating with 30 ppm of validamycin A was 0%, while the rate at day 7 was 9 % when treated with 0.4% iron ammonium-methane arsenic acid. Toxicity to mice was very weak; acute toxicity was over 2,000 mg/kg upon

Table 3. Effect of validamycin against the sheath blight of rice plants.

	Concn. (ppm)	Expanding rate* (%)			
		7d.	14d.	21d.	28d.
Untreated	0	100	100	100	100
VM-A	15	4	5	47	76
	30	0	0	0.1	0.2
	60	0	0	0	0
Monkit dust**		9	4	0.1	0.3

* Expanding rate = Average length of lesion per stem treated with drugs / Average length of lesion per stem untreated

** Containing 0.4% of iron ammonium-methane arsonic acid.

intravenous injection and over 10 g/kg upon oral administration.

Subsequently, we established the fermentation¹⁴⁾, purification¹⁵⁾ procedures and the quality control in the early and final stages on a commercial basis. The antibiotic showed a specific activity against the growth of *Pellicularia orizae* *in vivo*¹⁶⁾ resulting in a distinctive cure of the disease. In addition, it turned out that a soil bacterium, *Flavobacterium saccharophilum* was capable of hydrolyze the antibiotic rapidly to valienamine¹⁷⁾ and validamine, which show a strong glycosidase inhibiting activity¹⁸⁾.

4. *Streptomyces rochei* subsp. *volubilis*, T-2636 antibiotics, esterase and industrial production of Sedecamycin.

We searched antibiotics with strong activity against *Staphylococcus aureus* without cross-resistance to known lipophylic antibiotics. As a result, antibiotics T-2636 A, B and C were discovered from the organism named *S. rochei* subsp. *volubilis* subsp. nov¹⁹⁾. Antibiotic T-2636 C showed a strong antibacterial activity against multi-drug resistant *S. aureus*, a low toxicity and the protective effect by oral administration against the experimental *S. aureus* infection in mice. Structure determination

revealed 17 macrolacton ring (Fig. 6)²⁰⁾ that was later found to be identical to that of lankacidin A (bundlin B) and lankacidin C (bundlin A). It was subsequently turned out that the producer contained a novel esterase catalyzing esterification at the 14 position carbon of the antibiotics T-2636 A and C. The antibacterial spectrum²¹⁾ of antibiotic T-2636 C was as follows (MIC, $\mu\text{g/ml}$); *S. aureus* 6.25, *Streptococcus pyogenes* 0.39, *Diplococcus pneumoniae* 3.13, *Neisseria gonorrhoeae* 0.39, *Vibrio cholerae* 0.2, *Treponema hydrohysenteriae* 3.13. Acute toxicity (LD_{50} in mice) was over 10 g/kg (p.o.) and 8~10 g/kg (i.p.) for T-2636 A, and over 10 g/kg (p.o.) and 4.5 g/kg (i.p.) for T-2636 C. Only A showed the protective effect²²⁾ against the experimental *T. hydrohysenteriae* DJ70P infection²³⁾ in mice as follows; drugs, MIC ($\mu\text{g/ml}$), per oral administration (mg/kg/day), gross cecal lesion, % and No. of positive mice/examine: T-2636 A, 6.25, 5, 0 and 0/35, Carbadox 0.006, 10, 0 and 0/35, Tiamulin 0.1, 20, 0 and 0/30, Lincomycin 25, 25, 0 and 0/25. Antibiotic T-2636 C is esterified with ethyl acetate as the ester donor at the 14 position carbon and its deacylation occurred at the same position. The esterification reaction was used for industrial production of T-2636 A as the commercial name of Sedecamycin²³⁾.

Fig. 6. T-2636 A, B and C

5. *Streptomyces hygroscopicus* No. B-5050, maridomycins and the selective production of maridomycin III.

New 16 member macrolide antibiotics named maridomycins (ML) were discovered from a *Streptomyces* strain identified to *S. hygroscopicus* No. B-5050²⁴). The chemical structures of ML are shown in Fig. 7²⁵). The microorganism produced over ten chemically similar antibiotics (maridomycins, leucomycins and carbomycins). For industrial fermentation, we were required to breed strains with improved ML productivity of over 4 mg/ml from a strain with productivity of 100 µg/ml. In addition, a high producing strain with preferential accumulation of one component (ML-III), the most desirable component, was required. Feeding experiment revealed that the addition of aspartic acid family amino acids (homoserine, threonine, methionine, isoleucine, α-amino-*n*-butyrate and α-ketobutyrate) resulted in the preferential increase in ML-III accumulation²⁶). This result led us to breed a valine resistant with an expectation of that it may produce ML-III preferentially. As expected, we were successful to obtain the mutant AV²⁷) able to produce markedly and selectively increased

Fig. 7. Maridomycins

amount of ML-III without addition of isoleucine or the other amino acids.

6. Genus *Actinosynnema*, ansamitocins, and other antibiotics

★Producing plant: *Maytanus serrata*

Fig. 8. Ansamitocins and maytansine

Around 1970, we were eager to find a new antitumor antibiotic with novel mechanism of action. We found a unique actinomycete strain from the leaf surface of a plant of the family *Cyperaceae*. The strain formed many unusual coremia and motile spores that made us difficult to identify the organism in the early stage of study. However, the producer was found to belong to a newly established genus *Actinosynema*³⁰⁾.

In the early stage of our study, it turned out that the organism produced a compound (identified as nocardicin) active to Gram-negative bacteria and an antiphage compound (identified as tomaymycin). Subsequently, the organism was found to produce novel compounds active against only eukaryotes but not against prokaryotes in a producing medium. The antibiotics were composed of six components exhibiting a strong antitumor activity due to antimitotic mechanism and named ansamitocins²⁸⁾ (Fig. 8). Ansamitocins have similar structure to maytansine that was discovered from plant sources by Kapchan *et al.*²⁹⁾. Among the antibiotics ansamitocin P-3 showed the strongest antitumor activity against mouse leukemia P338, showing 123% increase in life span over controls (T/C%-100) with the dose of 25 µg/kg/injection to mice.

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