MACBECINS I AND II, NEW ANTITUMOR ANTIBIOTICS

I. PRODUCING ORGANISM, FERMENTATION AND ANTIMICROBIAL ACTIVITIES

SEIICHI TANIDA, TŌRU HASEGAWA and EIJI HIGASHIDE

Microbiological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd.,
17-85, Josohonmachi 2-chome, Yodogawa-ku, Osaka 532, Japan

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New antibiotics, macbecins I and II, have been found in the culture fluid of an actinomycete, which has the following properties: delayed fragmentation of vegetative mycelia, formation of coremia on solid media, the occurrence of *meso*-diaminopimelic acid in the cell wall, lysozyme resistance, and guanine-cytosine content of 71 ± 1 mol%. The organism has been designated *Nocardia* sp. No. C-14919 (N-2001). A marked enhancement of the production of macbecins I and II was observed in cultures containing L-tyrosine.

The antibiotics are moderately active against several Gram-positive bacteria and fungi. The antibiotics also inhibit the growth of *Tetrahymena pyriformis* W at 2 μg/ml but show no activity against the regeneration of cilia in partially deciliated *Tetrahymena* at 10 μg/ml.

In screening for new antibiotics, antifungal and antiprotozoal activities were detected in a culture fluid of actinomycete strain No. C-14919 (N-2001). The active materials were found to be new antibiotics belonging to the group of benzenoid ansamycins, and have been named macbecins I and II. In this paper the properties of the producing organism, fermentation studies and the antimicrobial activities of macbecins I and II are presented. The mode of action of the antibiotics is also discussed.

Properties of the Producing Organism

Actinomycete strain No. C-14919 (N-2001) was isolated from the leaf surface of grass collected in Shiga Prefecture, Japan. Mycelia were grown on yeast extract-malt extract agar (ISP 2) at 28°C[1]. Cultural and physiological characterizations were carried out by methods described previously[1]. The organism has the following properties: Coremia formation on the surface of various solid media, release of motile spores upon immersion of the matured aerial hyphae into liquid media, fragmentation of some brached mycelia at a later stage of growth in liquid culture (with some of the fragments showing motility), lysozyme resistance, the occurrence of *meso*-diaminopimelic acid and galactose in the cell wall, and a guanine-cytosine content of 71 ± 1 mol %.

These characteristics place the organism in the genus *Nocardia* and the designation *Nocardia* sp. No. C-14919 (N-2001) is proposed. Detailed taxonomic studies will be presented elsewhere.

Fermentation Studies

The vegetative medium contained glycerol, 1%; glucose, 1%; peptone, 1%; yeast extract, 0.5% at pH 7.0. Forty milliliters of this medium in a 250-ml Erlenmeyer flask was inoculated with 1 ml of the glycerol suspension of the organism and incubated on a rotary shaker at 28°C for 3 days. A 2-

ml portion of the vegetative culture was transferred to a 250-ml flask containing 40 ml of fermentation medium consisting of glycerol, 1%; yeast extract, 1%; $NaNO_3$, 0.2%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; KCl, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.001% at pH 7.0, and was incubated at 28°C for 144 hours. In large scale fermentations 1 liter of the vegetative culture was transferred to a 200-liter fermenter containing 100 liters of vegetative medium and cultured at 28°C for 2 days. A 5-liter portion of this culture was transferred to a 200-liter fermenter containing 100 liters of the fermentation medium and cultured at 28°C for 144 hours. Potency was determined by a paper disk method using *Candida albicans* IFO 0583 with trypticase soy agar (BBL) at 37°C for 24 hours. Growth was measured as packed cell volume (PCV) from 10 ml of broth centrifuged at 2,000 *g* for 10 minutes. Glycerol in the broth was determined by the method of IWAI *et al.*[2]

The time course of a typical fermentation is shown in Fig. 1. Antibiotic activity appeared in the culture supernatant at about 96 hours and reached a maximum at about 144 hours. The active materials, extracted with ethyl acetate and purified by silica-gel column chromatography, were new benzenoid ansamycin antibiotics and were named macbecins I and II. Details of the isolation and their physico-chemical properties will be given in a separate report[3].

Macbecins I and II could be detected in

Fig. 1. Time course of the fermentation.

A 10-ml portion of the culture was centrifuged at $3,000 \times g$ for 10 minutes at room temperature. Potency in the supernatant was determined by the paper-disk method using *Candida albicans* IFO 0583 with trypticase soy agar (BBL) at 37°C for 24 hours.

Growth is reported as packed cell volume (PCV) after the centrifugation, and represents the percentage of the maximum volume attained.

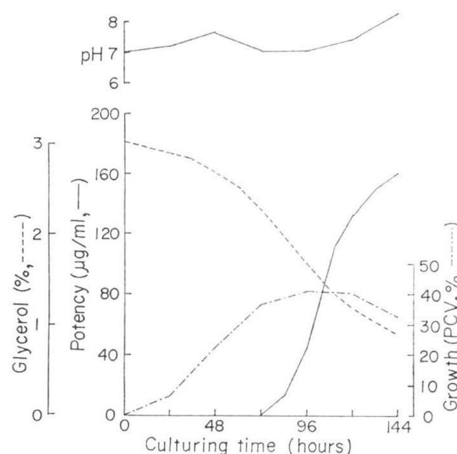
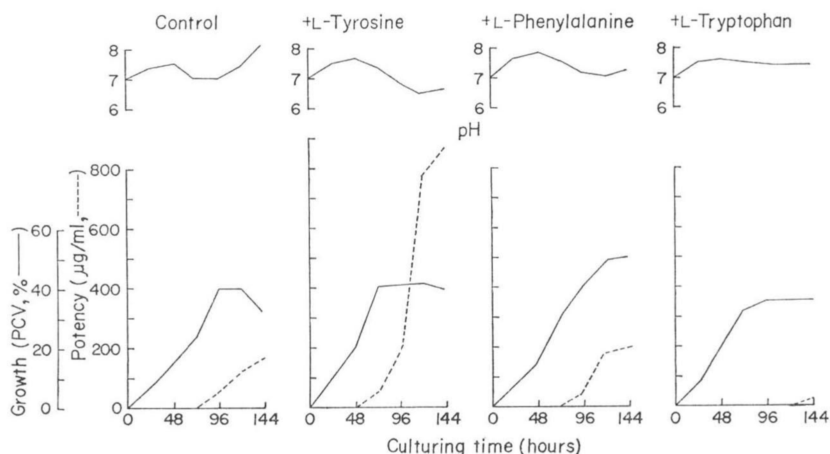


Fig. 2. Effect of aromatic amino acids on the fermentation.

Aromatic amino acids were added aseptically at final concentrations of 5 μg/ml. All assays were performed as in Fig. 1.



culture extracts by thin-layer chromatography on silica gel (Merck, 60 F254) with water-saturated ethyl acetate. They were visualized by exposing the plate to UV light (2527Å) and gave absorption zones of the same size.

Effect of Aromatic Amino Acids and Related Compounds on the Fermentation

On the assumption that the metabolism of aromatic amino acids would be involved in controlling the production of macbecins I and II, since the aromatic nuclei in ansamycins are derived from an intermediate of the shikimic acid pathway[4~7], the effect of aromatic amino acids and metabolically related compounds on antibiotic production was investigated. As shown in Table 1, 5 mg/ml of L-tyrosine increased the amounts of the antibiotics in the supernatant of the culture to about five times those of the control. On the other hand, L-phenylalanine showed no stimulative effect. In the culture containing 5 mg/ml of L-tyrosine or L-phenylalanine, macbecin II was the major product. L-Tryptophan, anthranilic acid and *p*-aminobenzoic acid suppressed production of the antibiotics whereas shikimic acid showed no effect. The time course of the fermentations in the presence of aromatic amino acids is shown in Fig. 2.

The stimulation by L-tyrosine and suppression by L-tryptophan, anthranilic acid or *p*-aminobenzoic acid strongly suggests that metabolism of aromatic amino acids in the organism influences the synthesis of macbecins I and II. However, it is not known whether the strong stimulation by exogenous L-tyrosine is due to regulation of the production of macbecins I and II or to L-tyrosine acting as a biosynthetic precursor of the antibiotics.

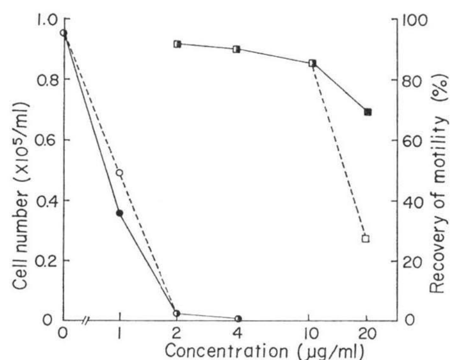
Table 1. Effect of aromatic compounds on the fermentation.

Aromatic compound	Concn. (mg/ml)	Growth (PCV %)	Potency (μg/ml)
L-Tyrosine	5	25	870
	1	28	210
	0.1	32	155
L-Phenylalanine	5	32	195
	1	33	196
	0.1	33	142
L-Tryptophan	5	27	25
	1	32	68
	0.1	35	90
Anthranilic acid	0.5	20	<15
p-Aminobenzoic acid	0.5	31	<15
Shikimic acid	0.5	32	165
None		31	180

Aromatic compounds were added aseptically at time zero. Potency and growth were determined after 144 hours cultivation at 28°C, using the method described in Fig. 1.

Fig. 3. Effect of macbecins on growth and cilia regeneration of *Tetraphymena*.

Growth inhibition of *T. pyriformis* W was determined by the broth dilution method.[8] Cell density was measured electronically (○, ●). The cilia regeneration system was established by the method described previously.[8] Recovery of motility after 90-minutes incubation was observed with a phase contrast microscope (×40 magnification) (□, ■). Dotted and solid lines indicate the addition of macbecins I and II, respectively.



Antimicrobial Activity

Antibacterial and antifungal activities were determined by the agar-dilution method. Inhibition

of the growth of *T. pyriformis* W was determined by the broth-dilution method.[8] The regeneration of cilia in *Tetrahymena* was assayed by observing recovery of motility of partially deciliated cells. Details have been described previously[8].

As shown in Table 2, macbecins I and II had moderate activities against several Gram-positive bacteria and fungi. Their activity against murine leukemia P388 and melanoma B16 is described in a separate publication[3]. The antibiotics strongly inhibited the growth of *T. pyriformis*. As shown in Fig. 3, complete inhibition was obtained at 2 μg/ml of the antibiotics. In the assay system for regeneration of cilia in partially deciliated *Tetrahymena*[8], macbecins I and II did not inhibit at 10 μg/ml which is 5 times higher than the minimum inhibitory concentration for growth. Since this assay system is one of the simple indicators of antitubulinic activity in various agents[8], inhibition of *Tetrahymena* by the antibiotics is thought to be due to interference with cellular targets distinct from the microtubule system. Since macbecin I at 20 μg/ml affected the recovery of motility in partially deciliated *Tetrahymena*, the possibility that the antibiotics have antitubulinic properties cannot be excluded, although effects at high drug levels may be due to nonspecific actions. Because of the similarity of their benzenoid ansa structures and antimicrobial activities, geldanamycin[9] and herbimycin[10] were also tested. As shown in Fig. 4, inhibition of the growth of *Tetrahymena* was observed at 0.2 μg/ml of geldanamycin and at 2 μg/ml of herbimycin. On the other hand, neither of the antibiotics showed activity against regeneration of cilia in *Tetrahymena* at levels up to 10 μg/ml. The results are thus consistent with those found for the macbecins.

Table 2. Antimicrobial activities of macbecins I and II.

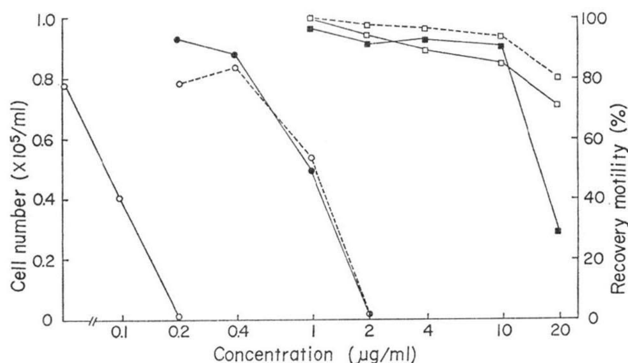
Test organism	MIC (μg/ml)	
	I	II
Escherichia coli K-12	>100	>100
Proteus vulgaris IFO 3045	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100
Salmonella typhimurium IFO 12529	>100	>100
Alcaligenes faecalis IFO 13111	>100	>100
Serratia marcescens IFO 3046	>100	>100
Bacillus subtilis ATCC 6633	50	100
B. pumilus IFO 3810	50	100
B. cereus IFO 3514	100	>100
B. megaterium IFO 12180	50	50
B. brevis IFO 3331	50	50
Staphylococcus aureus FDA 209P	>100	>100
Micrococcus luteus IFO 12708	50	50
Mycobacterium smegmatis ATCC 607	>100	>100
Myc. vaccae ATCC 15483	>100	>100
Candida tropicalis IFO 1400	100	100
C. utilis IFO 0619	100	100
C. parapsilosis IFO 1396	50	25
C. albicans IFO 0583	50	25
Cryptococcus neoformans IFO 0410	>100	>100
Saccharomyces cerevisiae IFO 0209	100	100
Aspergillus niger IFO 4066	100	100
Trychophyton rubrum IFO 5467	>100	>100
Penicillium chrysogenum IFO 4626	50	25
Tetrahymena pyriformis W	2	2

Antibacterial and antifungal activities were determined by the agar dilution method. Trypticase soy agar (BBL) was used as the assay medium for common bacteria. For acid-fast bacteria and fungi, the medium was supplemented with glycerol, 1% and glucose, 1%, respectively. The activity against *T. pyriformis* W was determined by the broth dilution method with proteose-peptone medium[8].

Discussion

Ansamycin antibiotics are an interesting group of natural products. They are known to cause powerful and selective effects on prokaryotes and eukaryotes *e.g.* the specific inhibition of prokaryo-

Fig. 4. Effect of macbecin-related antibiotics on growth and cilia regeneration in *Tetrahymena*.
Procedures are the same as in Fig. 3. Points are means of three experiments. Circles and squares indicate cell numbers and recovery of motility, respectively.
-●- or -■-, macbecin I added; -○- or -□-, geldanamycin added; --○-- or --□--, herbimycin added.



tic DNA-dependent RNA polymerase by naphthalenoid ansamycins such as rifampicin[11] and the specific inhibition of the function of microtubules in eukaryotic cells by benzenoid ansamycins such as ansamitocins[12]. In spite of possessing the benzenoid ansa structure, macbecins I and II do not show any specific activity against eukaryotic microorganisms. Although they inhibit the growth of *Tetrahymena* at 2 μg/ml, they do not show any marked effect on the regeneration of cilia in this organism at 10 μg/ml. In this they are very different from the ansamitocins which inhibit the regeneration of cilia at very low concentrations, probably due to specific interaction with ciliary microtubules[1]. Macbecins I and II are therefore distinguishable from ansamitocins and other related compounds such as maytansine[13~15] in their biological activities. Geldanamycin and herbimycin, which have similar benzenoid ansa structures and antimicrobial activities to the macbecins, may have the same kind of biochemical actions.

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