NOVEL ANTIFUNGAL ANTIBIOTICS OCTACOSAMICINS A AND B

I. TAXONOMY, FERMENTATION AND ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

KAZUYUKI DOBASHI, NAOKO MATSUDA, MASA HAMADA, HIROSHI NAGANAWA, TOMOHISA TAKITA and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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Two antifungal antibiotics octacosamicins A and B were isolated from the culture broth of a strain of actinomycetes, which was identified as a strain of *Amycolatopsis*. These antibiotics were isolated by resin adsorption and purified by column chromatography and preparative HPLC. Both antibiotics were found to be new substances from their physico-chemical properties. They showed broad antifungal spectra.

Novel antifungal antibiotics were discovered in our screening of antibiotics from actinomycetes and were designated as octacosamicins A and B. This paper reports the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties and biological activities of these antibiotics.

Taxonomy of the Producing Organism

Strain MG398-hF9 was isolated from a soil sample collected in the premises of Institute of Microbial Chemistry, Tokyo, Japan.

Morphological and physiological characteristics of strain MG398-hF9 were examined according to the methods described by SHIRLING and GOTTLIEB[1], and WAKSMAN[2]. Detailed observation of mycelial morphologies was performed with the use of a light microscope and a scanning electron microscope. Diaminopimelic acid and sugars in whole cell hydrolysate were determined by the methods of BECKER *et al.*[3] and LECHEVALIER[4], respectively. Phospholipids, menaquinones and mycolic acid were analyzed by the procedures of LECHEVALIER *et al.*[5], COLLINS *et al.*[6], and MINNIKIN *et al.*[7], respectively.

Taxonomic features of strain MG398-hF9 are shown in Table 1. The aerial mycelium was relatively long. Mature spores showed bamboo-like appearance (Fig. 1) and occurred generally in chain of more than twenty spores. The spores were $0.4 \times 1.0 \sim 3.0$ μm in size with smooth surface. The substrate mycelium was often observed to be zig-zag shaped (Fig. 2). Sporangia and flagellated spores were not observed. The strain was not acid-fast.

Whole cell hydrolysates contained *meso*-2,6-diaminopimelic acid, galactose and arabinose. This indicated that the strain was an actinomycete cell-wall type IV A according to the classification of LECHEVALIER and LECHEVALIER[8]. The strain had phospholipids of type P II and contained MK-9(H_4) as main component and small amount of MK-9(H_2).

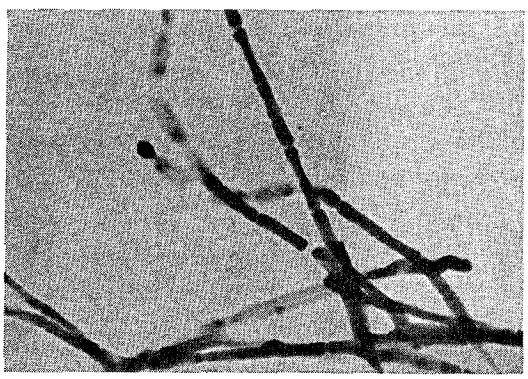
Based on the above features, strain MG398-hF9 was considered to belong to the genus *Amycolatopsis*[9]. The comparative examination revealed that the taxonomic features of *Amycolatopsis*

Table 1. Cultural characteristics of strain MG398-hF9.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose - nitrate agar	Pale yellow ~ pale yellowish brown [2 gc, bamboo]	Thin, white	Faint, brownish
Sucrose - nitrate agar +Casamino acid 0.1% +peptone 0.2%	Pale yellow ~ olive gray [1½ ig, olive gray]	White ~ light bluish gray [13½ ig, fog blue]	None
Glucose - nitrate agar	Pale yellow [2 ic, honey gold]	White	None
Glucose - asparagine agar	Pale yellow	White	None
Glycerol - asparagine agar (ISP medium 5)	Pale yellow ~ pale yellowish brown [2 le, mustard ~ 2 ne, mustard gold]	White ~ light bluish gray [13 fe, dusk ~ 15 ih, slate]	None
Inorganic salts - starch agar (ISP medium 4)	Colorless ~ pale yellowish brown [2 le, mustard]	White	None
Tyrosine agar (ISP medium 7)	Pale yellow ~ pale yellowish brown	White ~ light bluish gray [13 fe, dusk]	None
Nutrient agar	Pale yellow	Thin, white	None
Yeast extract - malt extract agar (ISP medium 2)	Pale yellowish orange ~ pale yellowish brown [2 gc, bamboo ~ 3 ne, topaz]	White	None
Oatmeal agar (ISP medium 3)	Colorless ~ pale yellow	Thin, white	None
Glycerol - nitrate agar	Colorless ~ pale yellowish brown [3 ne, topaz]	White	Faint, brownish

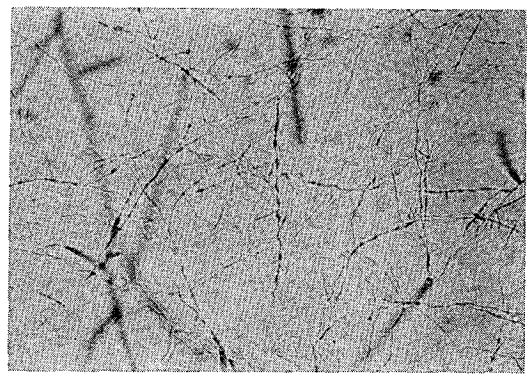
[ ]: Color names and numbers are designated from Color Harmony Manual, 4th Ed., Container Corporation of America, Chicago, Illinois, U.S.A., 1958.

Fig. 1. Electron micrograph of spore chains.



Inorganic salts - starch agar (ISP medium 4), 9 days at 27°C. Bar represents 1 μm.

Fig. 2. Substrate mycelium of strain MG398-hF9.



Glycerol - asparagine agar (ISP medium 5), 19 days at 27°C. Bar represents 10 μm.

azurea IMC A-0138 (JCM 3275) were very similar to those of strain MG398-hF9 (Table 2). Therefore, strain MG398-hF9 was classified and designated as *A. azurea* MG398-hF9. The strain MG398-hF9 was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. of FERM P-8865.

Table 2. Comparison of taxonomic characterization of strain MG398-hF9 and *Amycolatopsis azurea*.

Characteristic	MG398-hF9	*A. azurea* IMC A-0138
Substrate mycelium morphology	Zig-zag	Zig-zag
Spore shape	Bamboo-like	Bamboo-like
Spore surface	Smooth	Smooth
Aerial mycelium color	White, white~bluish gray	White, white~bluish gray
Growth color	Pale yellow~pale yellowish brown	Pale yellow~pale yellowish brown [colorless~pale yellowish brown (reverse)]*
Soluble pigment	—	—
Growth color on glucose - nitrate agar medium	Pale yellow	Pale yellow~bluish gray~olive gray [blue (reverse)]*
Soluble pigment on glucose - nitrate agar medium	—	Dark bluish gray~olive gray [blue]*
Melanin formation on ISP 1, 6, 7	—	—
Nitrate reduction	+	— [+]*
Hydrolysis of starch	+	+
Coagulation of skim milk	+	+
Peptonization of skim milk	+	+
Gelatin liquefaction:		
Simple gelatin	+	±
Glucose - peptone gelatin	+	+
Carbon utilization:		
D-Glucose	+	+
L-Arabinose	+	+
D-Xylose	+	+
D-Fructose	+	+
Sucrose	(−)**	(−)**
Inositol	+	+
L-Rhamnose	—	—
Raffinose	+	(+)**
D-Mannitol	+	+
Temperature range of growth	20~37°C	NT [20~36°C]*
Mycolic acid	—	—
Phospholipid type	P II	P II
Menaquinones	MK-9 (H_2) MK-9 (H_4)	MK-9 (H_2) MK-9 (H_4)

* Cited from ref 10.
** Doubtful.
NT: Not tested.

Fermentation and Isolation

A loopful of mycelial suspension from slant culture of MG398-hF9 was inoculated into a 500-ml flask containing 110 ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, Bacto-Soyton (Difco) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The seed flask was incubated on a rotary shaker at 30°C for 72 hours and the seed culture thus obtained was inoculated into two 30-liter jar fermentors containing 15 liters of production medium each. The production medium consisted of galactose 1.2%, glucose 1.2%, dextrin 2.4%, Bacto-Soyton (Difco) 1.2%, corn steep liquor 0.6%, spent hops 0.25%, $(NH_4)_2SO_4$ 0.24%, and $CaCO_3$ 0.24% (pH 7.4 before sterilization). The fermentation was carried out at 27°C for 65 hours under agitation of 250 rpm, aeration of 7.5 liters per minute.

Antibiotics production began 48 hours after inoculation and reached the maximum after 64 hours. The broth was harvested after 65 hours and filtered to give 23 liters of clarified filtrate (pH 7.3). It was passed through a column of Diaion HP-20 (1.2 liters). The column was washed with 3 liters of water and 1 liter of 30% aqueous acetone followed by elution with 60% aqueous acetone. The bioactive fraction against *Candida albicans* 3147 by disk agar diffusion method was collected. The fraction was evaporated to give a brown oily substance, which was washed with ethyl acetate (200 ml). The antibiotic activity remained in the residual part (9.8 g). The residue was dissolved in 100 ml of 0.1 M NaOH and was charged onto a column of Diaion CHP-20P (75~150 μ, 110 ml). After washing with water the bioactive material was eluted with a linear gradient solution between 30% acetone and 60% acetone (each 1,000 ml). The evaporation of the bioactive fractions gave 351 mg of crude octacosamicin A (43~48%, acetone) and 1,455 mg of crude octacosamicin B (48~53%, acetone).

The crude antibiotics thus obtained were subjected to the preparative reversed phase HPLC. Senshupak-Nucleosil $5C_{18}$ column, which has 20 mm of diameter and 300 mm of length (Senshu Kagaku Co., Ltd.), was used with the mobile phase of 0.1 M phosphate buffer (pH 3.0) containing 35% acetonitrile in 12 ml/minute of flow rate. The crude antibiotics were dissolved in 0.1 M NaOH (*ca.* 100 mg of crude powder/ml) and applied to the HPLC column at a 1-ml fraction. The antibiotics eluted from the HPLC were allowed to stand for 3 days at 10°C to give a white amorphous precipitate. They were filtered and washed with water to give 114.7 mg of purified octacosamicin A and 411.5 mg of purified octacosamicin B.

In order to get the acetate form of each antibiotic for elemental analysis, the following treatment was conducted. Ten mg of octacosamicin A was dissolved in 1 ml of acetic acid, this solution was then diluted with 10 ml of water and adsorbed onto a column of Diaion CHP-20P (3 ml). The column was washed with water and eluted with 60% aqueous acetone. After evaporation of acetone, a white precipitate was obtained. Thus, 4.5 mg of octacosamicin A acetate was obtained by filtration. A 5.6-mg of octacosamicin B acetate was obtained from 12 mg of octacosamicin B in the same manner. These acetates were subjected to elemental analysis, because the elemental analyses of the free forms did not give satisfactory results.

Physico-chemical Properties

The physico-chemical properties of octacosamicins A and B are summerized in Table 3. These two antibiotics are colorless amorphous powders. They are soluble in dimethylsulfoxide,

Table 3. Physico-chemical properties of octacosamicins A and B.

	Octacosamicin A		Octacosamicin B	
SI-MS (M+H) (*m/z*)	625		639	
Analysis	Found	Calcd for $C_{31}H_{52}N_4O_9 \cdot CH_3COOH$	Found	Calcd for $C_{32}H_{54}N_4O_9 \cdot CH_3COOH$
C:	57.44,	57.88,	58.68,	58.43,
H:	8.42,	8.24,	8.58,	8.36,
N:	7.93	8.18	8.07	8.02
UV $\lambda_{max}^{0.1N\ HCl}$ (ε)	228 (26,600), 283 (21,900)		228 (25,600), 283 (20,800)	
$\lambda_{max}^{0.1N\ NaOH}$ (ε)	212 (76,300), 228 (34,400), 283 (22,600)		212 (79,000), 228 (32,400), 283 (20,900)	
$[\alpha]_D^{24}$	+0.56° (*c* 0.549, DMSO)		−9.18° (*c* 0.512, DMSO)	
MP (°C, dec)	152~162		92~107	

SI-MS: Secondary ion mass spectra.

Fig. 3. IR spectra of octacosamicins A (A) and B (B) (KBr).

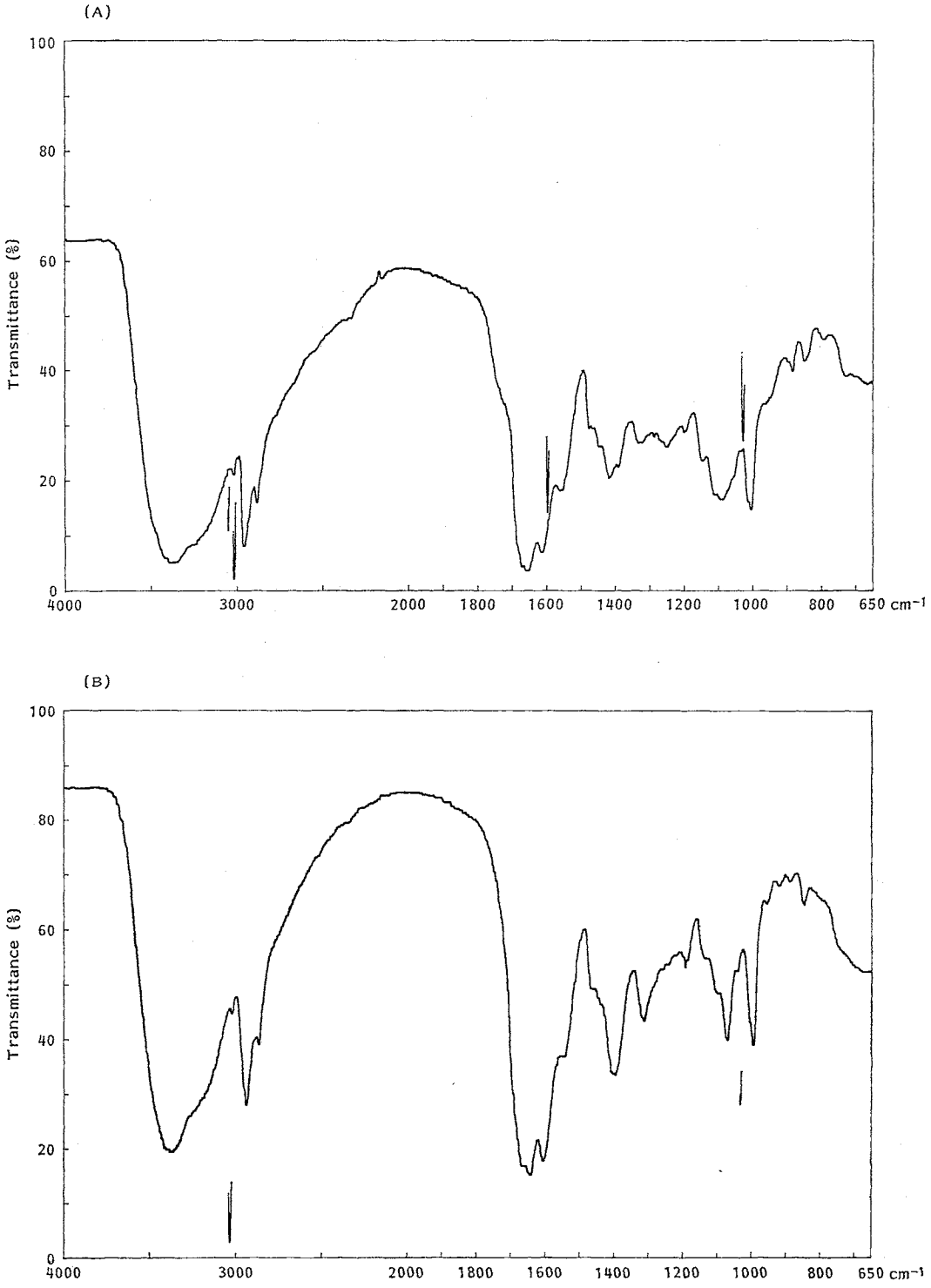


Fig. 4. ^{1}H NMR spectra of octacosamicins A (A) and B (B) (400 MHz, CD_3COOD).

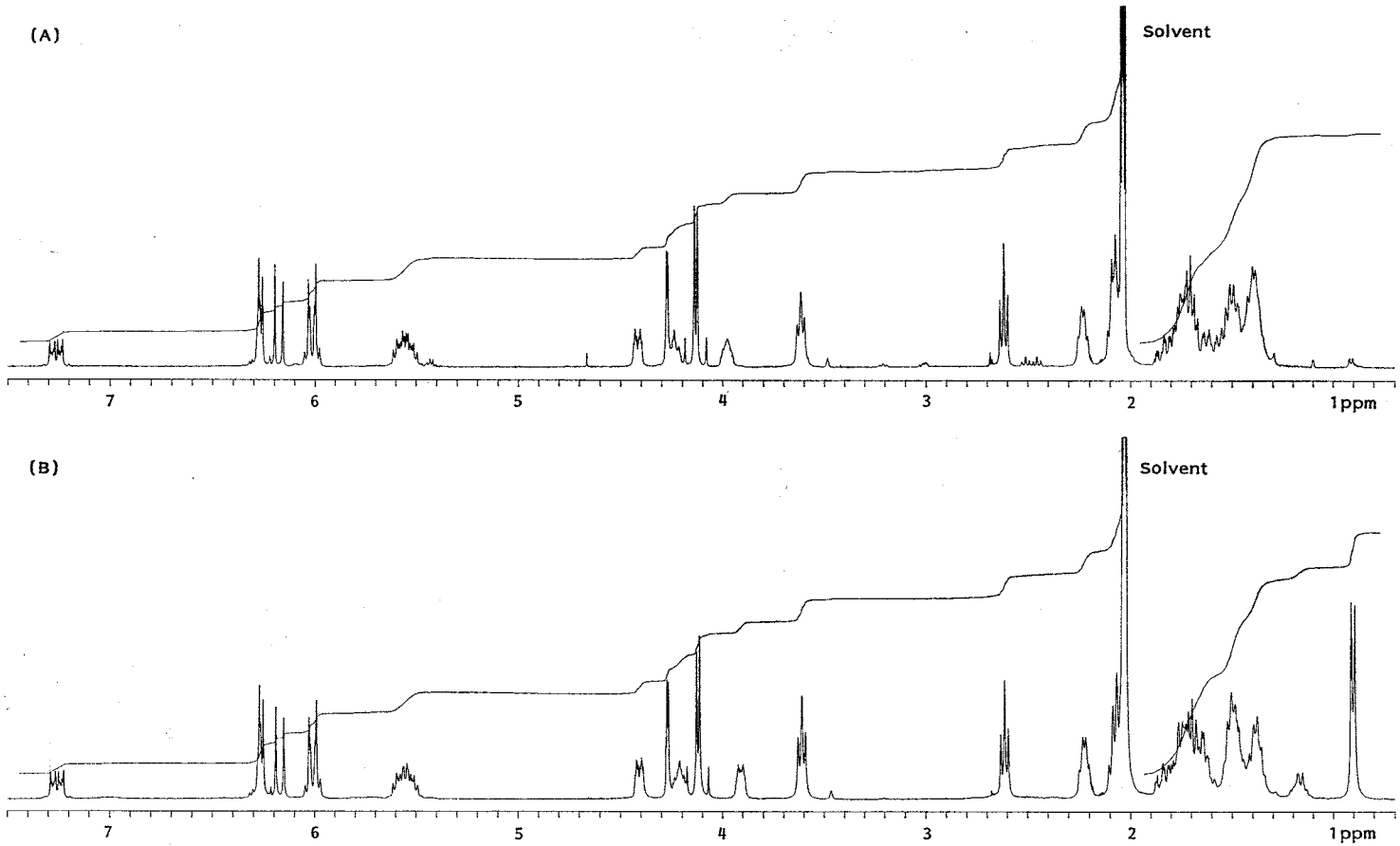


Table 4. The antimicrobial spectra of octacosamicins A and B.

Test organisms	Octacosamicin A	Octacosamicin B
Staphylococcus aureus FDA 209P	50	25
S. aureus Smith	50	25
Micrococcus luteus PCI 1001	100	50
Bacillus anthracis	>50	50
B. subtilis PCI 219	>100	50
B. cereus ATCC 10702	100	50
Corynebacterium bovis 1810	25	50
Escherichia coli NIHJ	100	100
E. coli K-12	>100	>100
Shigella dysenteriae JS11910	>50	50
Salmonella typhi T-63	>100	>100
Proteus vulgaris OX19	>100	>100
Serratia marcescens	>50	>100
Pseudomonas aeruginosa A3	>50	100
Klebsiella pneumoniae PCI 602	100	100
Mycobacterium smegmatis ATCC 607	100	100
Candida tropicalis F-1	6.25	6.25
C. pseudotropicalis F-2	12.5	25
C. albicans 3147	25	12.5
Candida Yu-1200	25	12.5
C. krusei F-5	25	12.5
Saccharomyces cerevisiae F-7	12.5	12.5
Cryptococcus neoformans F-10	6.25	6.25
Helminthosporium oryzae	12.5	25
Pyricularia oryzae	>25	25
Pellicularia sasakii	>25	50
Xanthomonas citri	>100	>100
X. oryzae	>100	>100
Aspergillus niger F-16	12.5	12.5
Trichophyton asteroides 429	>25	>50
T. asteroides 833	>25	>50

acetic acid and 0.1 M NaOH. Octacosamicin A is hardly soluble (*ca.* 0.5 mg/ml) in MeOH, while octacosamicin B is soluble in MeOH (*ca.* 5 mg/ml) at room temperature. Both antibiotics are insoluble in EtOH, $CHCl_3$, EtOAc and water. These antibiotics gave positive color reactions to Rydon-Smith, Ehrlich, diacetyl, TTC, sulfuric acid and 2,4-dinitrophenylhydrazine reagents. They also show a weak positive reaction to Sakaguchi reagent.

The IR spectra, and ^1H NMR spectra of octacosamicins A and B are shown in Figs. 3 and 4. All these physico-chemical properties show that both substances are a new type of antibiotic.

Biological Properties

The antimicrobial activities of octacosamicins A and B against bacteria, yeast and filamentous fungi by the agar dilution method are presented in Table 4. Although both antibiotics exhibited very weak or no activity against Gram-positive and Gram-negative bacteria, they showed stronger activity against fungi and yeast. The toxicity (LD_{50}, ip) of octacosamicins A and B in mice are >140 mg/kg and 52.5~105 mg/kg, respectively.

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