PRODUCTION AND BIOLOGICAL ACTIVITIES OF A NEW ANTIFUNGAL ANTIBIOTIC, TAN-950 A

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(Received for publication October 24, 1991)

A novel antifungal antibiotic, TAN-950 complex, was isolated from the culture filtrate of *Streptomyces platensis* A-136 (IFO 14603, FERM BP-1786). The water-soluble amphoteric substances in this complex were purified by chromatography using ion-exchange resins, QAE-Sephadex and adsorptive resins and were designated TAN-950 A and TAN-950 A~E mixture. The molecular formula of TAN-950 A was determined to be $C_6H_7N_2O_4Na$ for the sodium salt. This new amino acid antibiotic showed antifungal activity against *Candida albicans in vitro* and *in vivo*, and had low toxicity in mice.

In the course of screening for new antifungal antibiotics, a strain of *Syreptomyces platensis* was selected for isolation of its antifungal metabolite. From the broth filtrate, a fat-soluble component having wide antifungal activity was firstly isolated and identified as a known antifungal antibiotic, trichostatin A[1]. The aqueous layer after extraction with ethyl acetate (EtOAc) also showed antifungal activity especially against *Candida albicans* in the diffusion assay. Thus, an amphoteric substance, TAN-950 A, was isolated as a new amino acid antibiotic. The chemical structure of TAN-950 A was determined to be (*S*)-2-amino-3-(2,5-dihydro-5-oxo-4-isoxazolyl)propanoic acid as is shown in Fig. 1[2].

This paper deals with the taxonomy of the producing organism and the production and antifungal activities of TAN-950 A.

Materials and Methods

Taxonomic Studies on the Antibiotic-producing Strain

Methods adopted by the International Streptomyces Project (ISP) were used for taxonomic characterization and carbohydrate utilization studies. The color notations are those of the Color Harmony Manual, 4th Ed.[3]. Observations were made after incubation at 28°C for 21 days. The procedure of LECHEVALIER was used to prepare the cells and chromatographically detect the isomers of diaminopimelic acid.

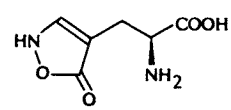
In Vitro Antifungal Acivity

The antifungal activity of TAN-950 A was determined on yeast nitrogen base (Difco) supplemented with 2% glucose and 1.5% agar. A conventional agar dilution method was used. The MIC for filamentous fungi and yeasts was expressed in terms of μg/ml after incubation for 48~72 hours at 28°C.

Amino Acid Antagonism

Antagonism of amino acids was determined on yeast nitrogen base (Difco) supplemented with 2% glucose and 1.5% agar. A testing amino acid was included in an assay medium. A conventional agar diffusion method was used.

Fig. 1. Structure of TAN-950 A.



Protective Effect in Mice

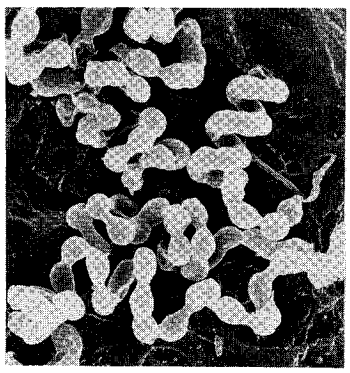
Five-week old female CF#1 mice were given an intravenous injection of 1×10^6 cfu of *C. albicans* TA, and antibiotics were administered orally or subcutaneously 0 and 2 hours later. ED_{50} (total dose giving 50% survival) values were calculated from the survival rate 7 days after infection.

Results

Taxonomy of the Producing Strain

The producing organism, strain A-136, was isolated from a soil sample collected in West Germany. The vegatative mycelia were well developed. Aerial mycelia were branched and formed spiral chains of spores. Mature spore chains had 10 or more spores per chain. The spores were elliptical and measured $0.8 \sim 1.2 \times 0.9 \sim 1.3\ \mu m$, with a smooth surface as shown in Fig. 2. Sporangia, flagellated spores and sclerotic granules were not observed.

Fig. 2. Scanning electron micrograph of spore chains of strain A-136.



Yeast extract-malt extract agar, 10 days (×8,000).

The culture characteristics of strain A-136 grown on various media at 28°C for 21 days are shown in Table 1. The aerial mass color was in the gray color series. The color of the reverse side of the colonies was pearl gray to light yellowish brown.

The physiological characteristics of strain A-136 are presented in Table 2. Strain A-136 grew at 10~36°C with an optimum temperature of 28~32°C on yeast extract agar. No melanoid pigment was formed in peptone yeast extract-iron agar or

Table 1. Cultural characteristics of strain A-136.

Medium	Growth	Aerial mycelium	Reverse side of colony	Soluble pigment
Sucrose-nitrate agar	Moderate, spreading	Poor Pearl (3ba) to light gray (3dc)	Pearl (3ba)	None
Glucose-asparagine agar	Moderate, restricted	Poor Pearl (3ba)	Pale yellow (2ca) to yellow (2ea)	None
Glycerol-asparagine agar	Good, spreading	Abundant White to light gray (3dc)	Pale yellow (2ca) to beige (3ec)	None
Inorganic salts-starch agar	Good	Abundant White to light gray (3dc) to pale brownish gray (3fe) with black moist patches	Yellow (2ea) to yellowish brown (2gc) to black	None
Nutrient agar	Moderate, restricted	Poor Pearl (3ba)	Pearl (3ba) to beige (3ec)	None
Yeast-malt agar	Good, spreading	Abundant White to pale brownish gray (3fe) to gray (5fe) with black moist patches	Pale yellow (2ca) to yellowish brown (2ng)	Light brown
Oatmeal agar	Good, spreading	Abundant Pearl (3ba) to pale brownish gray (3fe) with black moist patches	Pearl (3ba) to pale yellow (2ca)	None

Table 2. Physiological properties of strain A-136.

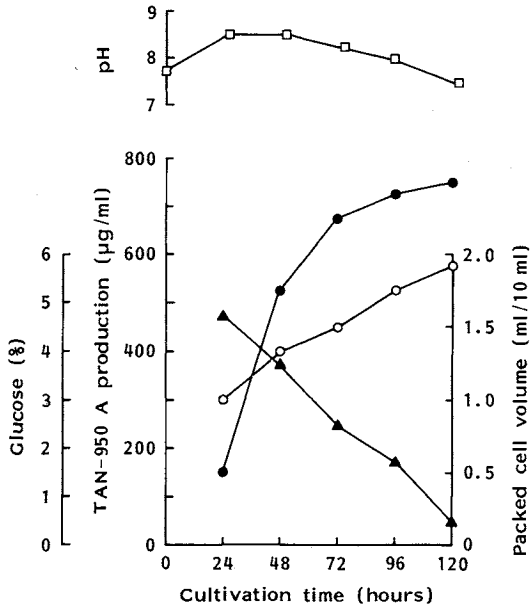
Temperature range for growth	10~36°C
Optimum temperature for growth	28~32°C
Melanoid pigment	
Peptone-iron agar	Negative
Tyrosine agar	Negative
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Milk peptonization	Negative
Nitrate reduction	Negative
Type of diaminopimelic acid	LL
Carbon source utilization of	
Good utilization:	D-Glucose, D-fructose, raffinose, inositol, mannitol
Fair utilization:	L-Arabinose,
No utilization:	Sucrose, L-rhamnose

tyrosine agar.

Carbon source utilization by strain A-136 was examined on ISP medium 9. The pattern of carbon utilization by strain A-136 is shown in Table 2. Inositol, D-mannitol, D-glucose, D-fructose, raffinose, and L-arabinose were utilized, but rhamnose and sucrose were not. LL-Diaminopimelic acid was detected in whole cell hydrolysates of the culture.

Fig. 3. Time course of TAN-950 A production.

Glucose (▲), potency (●), packed cell volume (○) and pH (□).



Based on the taxonomic properties described above, strain A-136 was considered to belong to the genus *Streptomyces*. Therefore, the characteristics of the strain were compared with published descriptions of various *Streptomyces*[4,5]. Consequently, strain A-136 was designated *Streptomyces platensis* A-136.

Fermentation

A slant culture of strain A-136 was inoculated into a 2-liter flask containing 500 ml of the seed culture medium consisting of 2% glucose, 3% soluble starch, 1% soybean flour, 1% corn steep liquor, 0.5% Polypeptone (Daigo Nutritive Chem., Ltd.), 0.3% NaCl and 0.5% $CaCO_3$ and adjusted to pH 7.0 before sterilization. The flask was incubated on a reciprocal shaker at 28°C for 48 hours. Five hundred ml of the seed culture were transferred to a 50-liter fermenter containing 30 liters of the seed culture medium supplemented with 0.05% Actocol (Takeda Chem. Inc.). The seed culture was carried out at 28°C for 48 hours under aeration of 30 liters/minute and agitation of 280 rpm.

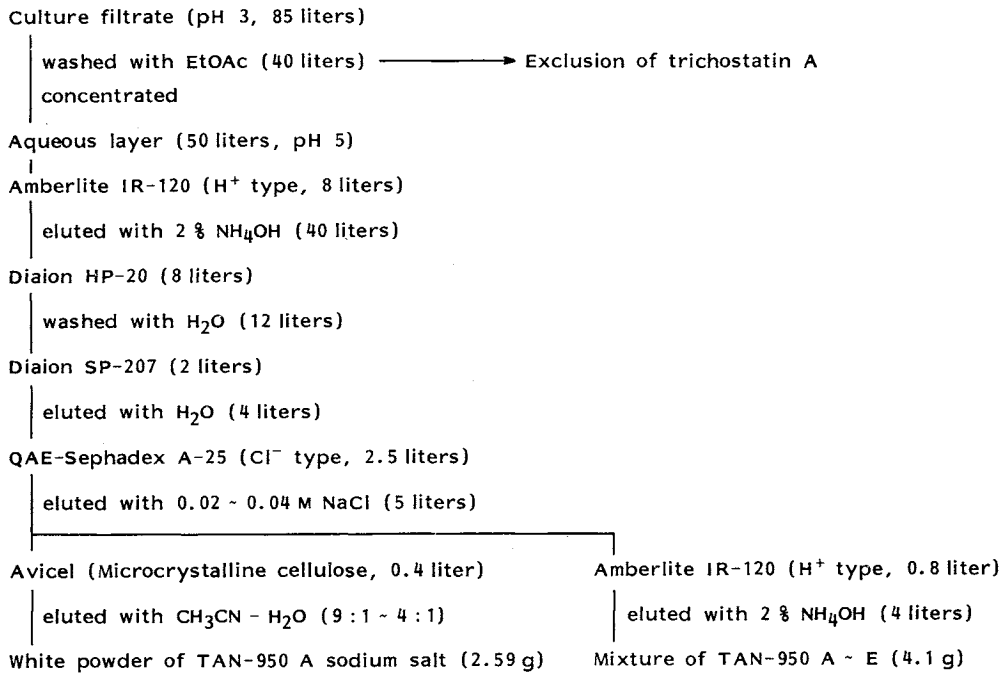
Six liters of the seed culture were transferred to a 200-liter fermenter containing 120 liters of the fermentation medium consisting of 0.5% glucose, 5% dextrin, 3.5% soybean meal, 0.7% $CaCO_3$, and 0.05% Actocol and adjusted to pH 7.0 before sterilization. The fermentation was carried out at 28°C for 90 hours under aeration of 120 liters/minute and agitation of 200 rpm. Glucose was determined by the glucose oxidase system. The growth was determined by packed cell volume. The progress of fermentation was monitored by checking the size of the inhibition zone using *Candida albicans*.

The time course of antibiotic production in a 200-liter fermenter is shown in Fig. 3. Antibiotic production reached about 750 μg/ml at 120 hours after inoculation.

Isolation

Fig. 4 summarizes the procedure for the isolation of the component of TAN-950 complex. The culture

Fig. 4. Isolation procedure for TAN-950 A.



broth (100 liters) was adjusted to pH 8 and filtered using a Hyflo Super Cel. The filtrate (85 liters) adjusted to pH 3 was washed with EtOAc. The concentrate of the aqueous layer was applied at pH 5 to a column of Amberlite IR-120. An antibiotic was eluted with 2% aqueous ammonia, and the eluate was concentrated. The concentrate was passed through a column of Diaion HP-20 washed with water. The concentrate of the effluent was chromatographed on a column of Diaion SP-207 eluting with water. The bioactive fractions were chromatographed on a column of QAE-Sephadex A-25 eluting with 0.02~0.04 M NaCl. The pure fractions detected by HPLC were combined, concentrated and chromatographed on microcrystalline cellulose eluting with acetonitrile-water (9:1 to 4:1) to afford a white powder of TAN-950 A as the monosodium salt (2.59 g).

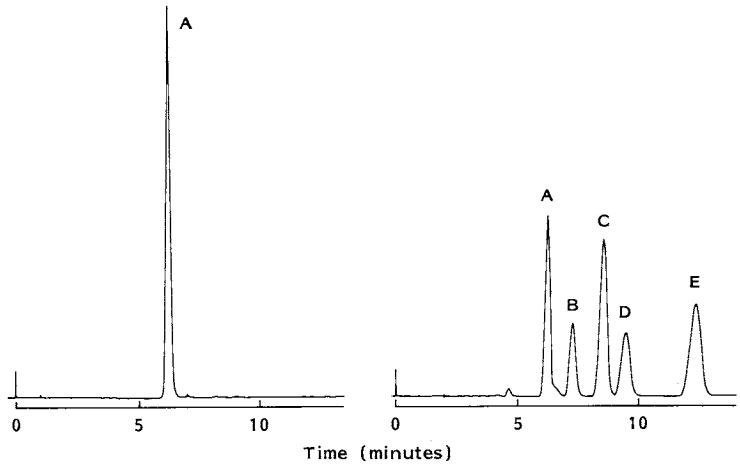
Upon QAE-Sephadex A-25 chromatography, fractions containing mainly TAN-950 B, C, D and E were combined and applied to a column of Amberlite IR-120 (H^+ type, 0.8 liter) eluting with 2% aqueous ammonia. The eluate was concentrated and lyophilized to give a white powder of a mixture of TAN-950 A~E (4.1 g).

Fig. 5 shows HPLC patterns of these antibiotics whose retention times (minutes) were TAN-950 A, 6.2; B, 7.3; C, 8.5; D, 9.5 and E, 12.3 under these conditions. The ratio of the peak areas estimated by HPLC analysis was A:B:C:D:E=2:1:2:1:2. We tried to separate TAN-950 B, C, D and E by preparative HPLC, but could not because isomerization proceeded rapidly. Table 3 shows the stability of TAN-950 A in phosphate buffer of various pHs. TAN-950 A reached an equilibrium state after about one hour. The chemical conversion is described in the previous paper[2].

Chemical Characterization of TAN-950 A

TAN-950 A was isolated as the sodium salt. It is soluble in water, dimethyl sulfoxide and methonal,

Fig. 5. HPLC pattern of TAN-950 A and A~E mixture.



Column, ODS, YMC-Pack AQ-312 (Yamamura Chem. Lab.); mobile phase, 0.25 mM n-$Bu_4N^+OH^-$/0.02 M phosphate buffer (pH 6.0); flow rate, 2 ml/minute; detection, absorbance at 214 nm.

and sparingly soluble in acetone, ethyl acetate and chloroform. It gave positive color reactions with ninhydrin and Ehrlich reagents, and negative color reactions with Dragendorff and Greig-Leaback reagents.

The physico-chemical properties of TAN-950 A are summarized in Table 4. The specific rotation of TAN-950 A in water is $-69.5°$. The UV spectrum indicated a maximum at 253 nm, suggesting the presence of an α,β-unsaturated carbonyl group. The molecular formula of TAN-950 A was determined to be $C_6H_7N_2O_4Na$ on the basis of elemental analysis, secondary ion mass spectrum (SI-MS) and ^{13}C NMR data. The structure determination of TAN-950 A is described in another paper[2].

Table 3. Stability of TAN-950 A in 0.05 M phosphate buffer (1 mg/ml, 60°C).

Time (hours)	Residual amount (%)		
	pH 3	pH 7	pH 9
0	100	100	100
0.5	69	75	73
1	64	73	69
2	57	66	67
4	49	63	67
6	44	60	67

Biological Activity

MICs for TAN-950 A against 10 different molds and yeasts are shown in Table 5. TAN-950 A showed strong activity against several yeasts with MIC values of 0.78~3.13 μg/ml. However, growths of molds tested were not inhibited at the concentration of 100 μg/ml.

It was found also that the activity against yeasts was not observed in Sabouraud dextrose agar. The effect of amino acids on the activity of TAN-950 A was examined. Of the amino acids tested, glutamic acid, aspartic acid and leucine greatly reduced the antifungal activity of TAN-950 A as shown in Table 6.

In vivo antifungal activity of subcutaneously (sc) and orally (po) administrated TAN-950 A was examined in mice with a systemic *Candida albicans* TA infection. The activity of miconazole was used for comparison, and the date are shown in Table 7. TAN-950 A showed 50% effective doses against systemic candidiasis at 35.5 mg/kg (sc) and 100 mg/kg (po). The efficacy was slightly weaker than that of miconazole.

TAN-950 A did not cause any toxic changes in mice during a 2-week observation period following

Table 4. Physico-chemical properties of TAN-950 A sodium salt.

Appearance	White powder
Nature	Water-soluble amphoteric substance
Optical rotation	$[\alpha]_D^{23}$ −69.5° (*c* 0.52, H_2O)
SI-MS: *m/z*	195 $(M+H)^+$, 217 $(M+Na)^+$
Molecular formula	$C_6H_7N_2O_4Na(H_2O)$
Elemental analysis	Found: C 33.64, H 4.31, N 12.72, Na 11.0
	Calcd: C 33.97, H 4.28, N 13.21, Na 10.66
UV $\lambda_{max}^{H_2O}$ nm (ε)	253 (8,060)
IR ν_{max}^{KBr} cm^{-1}	3430, 1640, 1500, 1410, 1350
^{13}C NMR: δ ppm (D_2O)	180.58 (s), 177.09 (s), 155.54 (d), 82.85 (s), 58.49 (d), 26.51 (t)

Table 5. Antifungal activity of TAN-950 A.

Test organism		MIC (μg/ml)
Candida albicans	IFO 0583	3.13
C. parakrusei	IFO 0640	3.13
C. parapsilosis	IFO 1396	1.56
C. tropicalis	IFO 0006	0.78
Torulopsis glabrata	IFO 1085	>100
Cryptococcus neoformans	IFO 0410	>100
Saccharomyces cerevisiae	IFO 0209	1.56
Aspergillus fumigatus	IFO 6344	>100
Trichophyton rubrum	IFO 5467	>100
Microsporum gypseum	IFO 6075	>100

Medium: Yeast nitrogen base (supplemented with 2% glucose and 1.5% agar)
Incubation temperature and time: 28°C, 2~3 days.

Table 7. Protective effects of TAN-950 A against systemic infection with *Candida albicans* TA in mice.

Antibiotic	ED_{50} (mg/kg)	
	sc	po
TAN-950 A	35.5	100
Miconazole	25.0	63.1

Mice (n=5) infected with *Candida albicans* TA (iv, 1×10^6 cells).

Table 6. Effect of amino acids on the antifungal activity of TAN-950 A.

Amino acid (10 mM)	Paper disc assay (mm)	
	100 μg/ml	1,000 μg/ml
Ala	13	24
Val	13	22
Leu	0	9
Ile	12.5	23
Ser	13	25
Thr	13	23
Met	13	23
Phe	15	24
Trp	13	23
Pro	14	22.5
Asp	0	9
Asn	12	25
Glu	0	0
Gln	13	24
Lys	14	23
None	13	22.5

Test organism: *Saccharomyces cerevisiae* IFO 0209.
Medium: Yeast nitrogen base (supplemented with 2% glucose and 1.5% agar).

intravenous, intraperitoneal, subcutaneous or oral administration of single dose of 4,000 mg/kg.

Discussion

TAN-950 A is a novel antifungal antibiotic produced by *Streptomyces platensis* A-136. Other TAN-950 A producing strains have been found among known microorganisms: *Streptomyces hygroscopicus* A-300 (FERM P-1312)[1], *Streptomyces hygroscopicus* subsp. *angustmyceticus* IFO 3934 and *Streptomyces hygroscopicus* subsp. *hygroscopicus* IFO 14012.

Glutamic acid, aspartic acid and leucine greatly reduced the antifungal activity of TAN-950 A against *Saccharomyces cerevisiae*. TAN-950 A had no effect on the activity of transaminase from *C. albicans* or glutamate dehydrogenase from *S. cerevisiae*. As leucine is an essential amino acid for mammals, some relationship may exist between biological activity of TAN-950 A and the antagonism by amino acids. Two amino acid antibiotics have recently been reported. One is (*S*)-2-amino-5-hydroxy-4-oxo pentanoic acid (HON[6] or RI-331[7]), which was active against yeasts *in vitro* and *in vivo*. We found that this compound

was antagonized by methionine (data not shown). The other is (−)-(1*R*,2*S*)-2-amino-cyclopentane-1-carboxylic acid (ACPC)[8,9], which had already been isolated as a new cyclic β-amino acid[10] which is a part of aminoacyl nucleoside antibiotic, amipurimycin[11]. ACPC was antagonized by valine and isoleucine (data not shown). It may be that these types of antibiotics will become useful drugs for systemic fungal infections because of their selective toxicity.

Antagonism of TAN-950 A by glutamic acid and aspartic acid and its glutamate-analogous structure led to the idea that this compound shows affinity for excitatory amino acid receptors (kainate, quisqulate and *N*-methyl-D-aspartate subtypes) in the central nervous system. It was found to bind strongly to these receptors and also to elicit the firing of rat hippocampal CA1 neurons *in vitro*[12,13].

Acknowledgments

The authors wish to thank Dr. H. OKAZAKI of this division for his encouragement during this work. Thanks are also due to Dr. S. CHIBA for conducting the toxicity test, and the excellent technical assistance of K. SASABE and Y. NOHARA is gratefully acknowledged.

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