STUDIES ON MYCOTRIENIN ANTIBIOTICS, A NOVEL CLASS OF ANSAMYCINS

I. TAXONOMY, FERMENTATION, ISOLATION AND PROPERTIES OF MYCOTRIENINS I AND II

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Mycotrienins I and II have been isolated from the cultured broth of *Streptomyces rishiriensis*. Taxonomical studies of the producing organism, fermentation, isolation and characterization of the antibiotics are described.

In the course of our screening program for antitumor antibiotics, two active substances which were interconvertible *via* a redox reaction have been isolated from the culture broth of a strain of *Streptomyces* sp. T-23 newly isolated from a soil sample.

After careful characterization, the reduced form was identified by comparison with mycotrienin previously reported by CORONELLI *et al*[1], as an antifungal antibiotic and the other was characterized as its oxido-form, hence, the name mycotrienin I was given to the latter and mycotrienin II to the former.

This paper presents a detailed description of taxonomic studies of the producing organism, fermentation, isolation, physicochemical and biological properties of mycotrienins I and II (MTN-I and -II). The structures of MTN-I and -II have been established as described in the following report.

Materials and Methods

General

Melting points were determined by a Yanaco micro apparatus and are uncorrected. UV spectra were recorded using a Hitachi 320 spectrophotometer. IR spectra were taken with a JASCO A-102 infrared spectrophotometer. Mass spectra were run on a Hitachi M-80 spectrometer. ^{1}H and ^{13}C NMR spectra were measured by a JEOL FX-400 spectrometer operating at 400 MHz and 100 MHz, respectively.

Analysis of Mycotrienins

Mycotrienins were detected by UV absorption on thin-layer chromatography plates (Merck, Kieselgel 60 F254). The solvent system used for TLC analysis was a mixture of chloroform - ethanol (15: 1, v/v). Amounts of mycotrienins were determined by the UV spectrophotometric measurement of thin-layer plates with a Shimadzu dual wavelength TLC scanner CS-900.

Bioassay of Mycotrienins

Bioassay of mycotrienins was carried out by using mouse leukemia L-5178Y cells. The cells grown in Eagle minimum essential medium with 10% fetal calf serum, 0.01% asparagine and 0.01% glutamine were harvested by centrifugation and resuspended in fresh medium at $2 \sim 3 \times 10^4$ cells/ml. The cell suspension (2 ml) and the ethanol solution of a test sample (20 or 40 μl) were pipetted into a test tube with a

fitted rubber cap. After 3 days incubation, the number of cells was determined by a Nihon Koden MEK-1200 cell counter.

Redox Reaction of Mycotrienins

MTN-I (5 mg) was dissolved in 2 ml of MeOH. To this solution was added 2~3 mg of $Na_2S_2O_4$ and it was stirred for 30 minutes at room temperature. The reaction mixture was filtered and diluted with 10 ml of ethyl acetate. After washing with water, the solution was dried over Na_2SO_4 and evaporated to dryness. MTN-II was obtained as a white powder (4.5 mg).

MTN-II (5 mg) was dissolved in 2 ml of a 1% methanolic $FeCl_3$ solution and stirred for 30 minutes at room temperature. The reaction mixture was diluted with ethyl acetate and washed with water. After drying over Na_2SO_4, the solution was concentrated *in vacuo* to give 4.1 mg of MTN-I as a yellow powder.

Acid Hydrolysis of Mycotrienins

MTN-I or -II (100 mg) was suspended in 10 ml of 6 N HCl and was heated at 120°C for 16 hours in a sealed tube. The reaction mixture was diluted with water and evaporated several times to remove HCl. The residue was applied to a column of Dowex 50W×2 (H^+ form), which was washed with water and developed with 1/2 N NH_4OH. The eluate was concentrated to dryness. The residue was dissolved in distilled water and passed through a Sephadex G-15 column to afford an amino acid (3 mg), which was identified as D-alanine by TLC and ORD: $[\alpha]_{300}^{20}$ −177°, $[\alpha]_{275}^{20}$ −233°, $[\alpha]_{250}^{20}$ −433° and $[\alpha]_{225}^{20}$ −1230° (*c* 0.6, 1/10 N HCl).

Results and Discussion

Taxonomy of the Mycotrienin Producer

The mycotrienin producing strain T-23 was isolated from a soil sample collected at Iwamizawa, Hokkaido in Japan. Characterization of the strain was performed by the methods of the International Streptomyces Project (ISP)[2] and of WAKSMAN[3].

Morphological Properties

The cultural and physiological characteristics of the strain are listed in Tables 1 and 2 together with the utilization of carbon sources. The vegetative mycelium of the strain T-23 develops well without fragmentation on most of the media used. The aerial mycelium monopodially branches with short sporophores forming spore chains with 10 to 50 or sometimes more than 50 spores per chain. The spore chains are usually loose coils (*Spira*), and chains with terminal hooks or loops and primitive spirals (*Rectus-Flexibilis*) are also common (Plate 1). The spores are oval to cylindrical (1.0~1.5×0.5~0.7 μm) with a smooth surface under the electron microscope (Plate 2). Sporangia, motile spores, sclerotia and other special morphology are not observed.

Cultural and Physiological Properties

The cultural properties of strain T-23 grown on various media at 27°C for 2 to 3 weeks, and the physiological properties of the strain are shown in Tables 1 and 2, respectively. These properties of the strain could be summarized as follows: aerial mass color is Gray color-series, brownish gray or reddish gray to dark purplish gray; reverse side of colony shows no distinctive pigments, brownish white to light yellow on synthetic media and pale yellowish brown on organic media; melanoid pigments are formed on the ISP's test media, and other soluble pigments are not found in most media; the strain is mesophilic, and possesses diastatic and proteolitic natures; and all tested sugars except D-mannitol are utilized for growth as carbon sources.

Comparison with Other Related Species

On the basis of its morphological features, strain T-23 seemed to belong to the genus *Streptomyces*.

Table 1. Cultural properties of *Streptomyces* sp. T-23.

Media	Growth	Sporulation	Color of colony	Reverse side of colony	Color in media
Sucrose-nitrate agar (Waksman, No. 1)	Moderate	Poor	White	Pale yellow	None
Glucose-asparagine agar (Waksman, No. 2)	Moderate	Moderate	Gray color series (brownish gray to reddish gray)	Brownish white to light yellow	None
Glycerol-asparagine agar (ISP No. 5)	Poor	Poor	White to light gray	Brownish white to pale yellow	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	Moderate	Gray color-series (reddish gray to purplish gray)	Light gray	None
Tyrosine agar (ISP No. 7)	Poor	Poor	White to grayish white	Brownish white to pale yellow	Light brownish gray
Nutrient agar (Waksman, No. 14)	Poor	None		Brownish white or pale yellow to pale yellowish brown	Pale yellowish brown
Yeast extract - malt extract agar (ISP No. 2)	Moderate	Moderate	Gray color-series (reddish gray to dark purplish gray)	Pale yellow orange to pale yellowish brown	Pale yellowish orange
Oatmeal agar (ISP No. 3)	Moderate	Moderate	Gray color-series (reddish gray to purplish gray)	Pale yellow to pale yellowish brown	None or trace of brownish

Table 2. Physiological properties of *Streptomyces* sp. T-23.

Temperature for growth	10~37°C
Optimum	20~30°C
Production of melanoid pigments	
Tyrosine agar	Positive
Peptone - yeast extract iron agar	Positive
Tryptone - yeast extract broth	Positive
Other organic media	Positive
Hydrolysis of starch	Positive
Liquefaction of gelatin	Positive
Peptonization of milk	Positive
Coagulation of milk	Negative
Nitrate reduction	Positive
Utilization of carbon sources	
Positive utilization	D-Glucose, L-arabinose, D-fructose, L-rhamnose D-xylose, sucrose, raffinose, *i*-inositol
Negative utilization	D-Mannitol

Among the species of *Streptomyces* described in the 8th edition of BERGEY's manual[4], SHIRLING's ISP reports[5], and the other species listed on

Plate 1. Spore chains of strain T-23. Loose coils, primitive spirals and loops morphology on glucose-asparagine agar, 21 days.

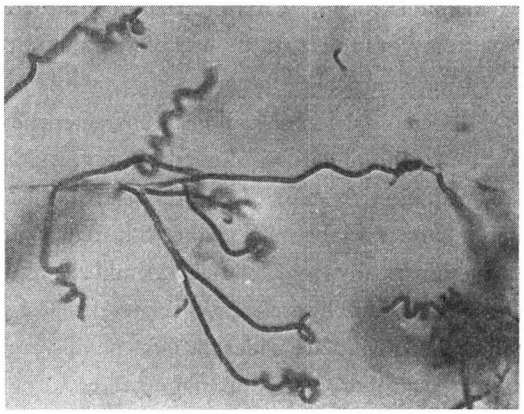
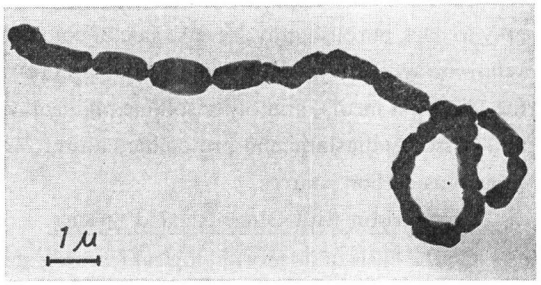


Plate 2. Electron micrograph of spores of strain T-23 from 21 days culture on glycerol-asparagine agar. Smooth surface.

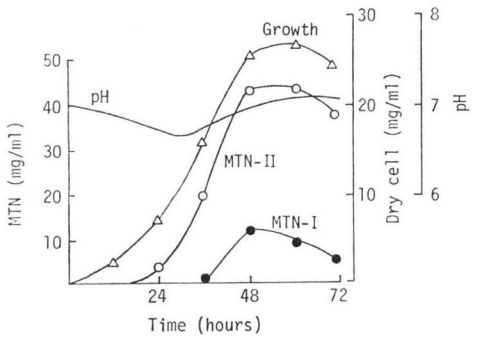


"Approved Lists of Bacterial Names"[6], strain T-23 closely resembles *S. rishiriensis*. The properties of the strain are compared with those of *S. rishiriensis*, and good agreements are obtained except soluble pigments on organic agar media. Therefore, strain T-23 is identified as a strain of *S. rishiriensis*.

Production of Mycotrienins

Seed flasks were inoculated with stock cultures of *Streptomyces rishiriensis* T-23 maintained at −20°C and incubated for 48 hours at 27°C. The seed medium consisted of 1.0% each of starch, molasses, meat extract and Polypeptone (pH 6.8).

Fig. 1. Time course of mycotrienins production.



The fermentation of MTN was carried out in a 30-liter jar fermentor containing 15 liters of a medium consisting of 1.0% glucose, 1.5% starch, 1.5% soybean meal, 0.2% dry yeast, 0.5% NaCl and 0.4% $CaCO_3$ (pH 7.0). The fermentor was inoculated with 300 ml of the seed culture and incubated at 27°C for 48 hours under an equal volume of aeration with agitation at 300 rpm.

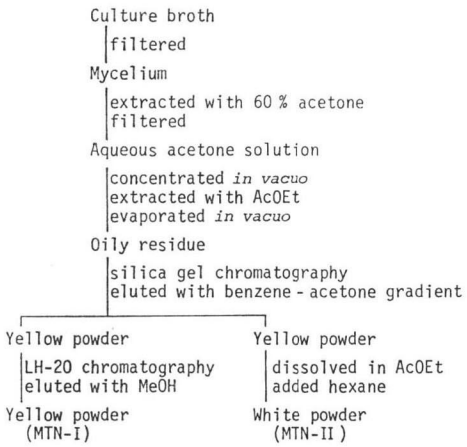
The time course of mycotrienin fermentation is presented in Fig. 1.

Purification of Mycotrienins

The cultured broth (15 liters) was filtered and the mycelium was extracted twice with 3 liters of 60% aqueous acetone by stirring for 1 hour. After filtration, the extracts were pooled and concentrated *in vacuo* to about 3 liters. The concentrate was extracted twice with 3 liters of ethyl acetate, and the organic extracts were combined and concentrated to a small volume *in vacuo*, dried over Na_2SO_4 and further concentrated to an oily residue (7 g).

The residue was chromatographed over a column of silica gel (100 g) and eluted with a benzene - acetone gradient (10: 0 ~ 6: 4). Each eluate was monitored by TLC on a silica gel GF_{254} plate developed with chloroform - ethanol (15: 1), and detected by UV absorption. Thus, MTN-I (150 mg) and -II (530 mg) could be separated and obtained as crude yellow powders. The yellow powder of MTN-I was subjected to column chromatography using Sephadex LH-20 and was eluted with methanol. Subsequent concentration of appropriate fractions afforded a yellow amorphous powder of MTN-I (100 mg). The crude powder of MTN-II was dissolved in a small volume of ethyl acetate and the antibiotic was precipitated by the addition of hexane to give a white powder (490 mg).

Chart 1. Flow diagram for the isolation of mycotrienins.



The flow diagram for the isolation of mycotrienins is presented in Chart 1.

Table 3. Physicochemical properties of mycotrienins.

	MTN-I	MTN-II
Appearance	Yellow powder	White powder
Mp	117°C	151°C
$[\alpha]_D$ (*c* 1.0, MeOH)	+92°	+288°
Molecular weight (M^+) *m/z*	636	638
Molecular formula	$C_{36}H_{48}N_2O_8$	$C_{36}H_{50}N_2O_8$
Elemental analyses	Calcd. Found C 67.92 C 67.72 H 7.55 H 7.68 N 4.40 N 4.28	Calcd. Found C 67.71 C 68.06 H 7.84 H 7.86 N 4.39 N 4.39
UV spectrum λ_{max}^{MeOH} nm(ε)	262 (38,500), 272 (49,600), 282 (38,800), 383 (3,400)	260 (40,800), 270 (52,300), 280 (40,500), 310 (5,900)
IR spectrum ν_{max} cm^{-1}	3340 (NH, OH), 1730, 1202 (ester), 1650, 1535 (amide), 1000 (triene) in $CHCl_3$	3340 (NH, OH), 1730, 1200 (ester), 1650, 1535 (amide), 1003 (triene) in Nujol
Rf on silica gel TLC		
Solvent I*	0.61	0.22
Solvent II**	0.78	0.67

* Solvent I: chloroform - ethanol, 15: 1
** Solvent II: benzene - chloroform - methanol, 3: 7: 3

Fig. 2. IR spectra of MTN-I (a) and MTN-II (b).

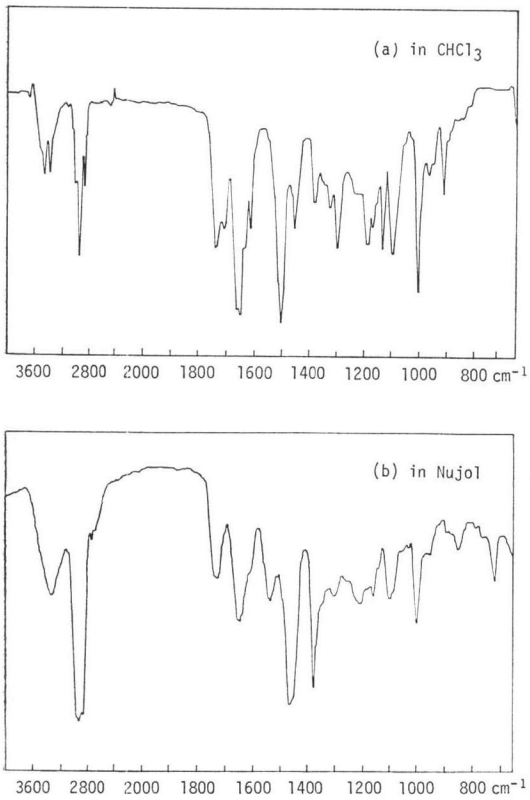


Fig. 3. ^1H NMR spectra of MTN-I (a) and MTN-II (b) in $CDCl_3$.

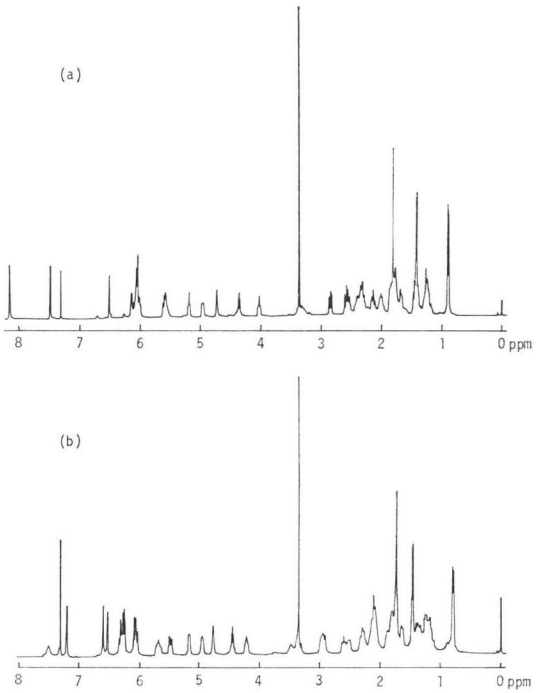


Table 4. ^{13}C NMR chemical shifts of mycotrienins in $CDCl_3$.

No.	MTN-I		MTN-II		No.	MTN-I		MTN-II	
C-1	188.2*	s**	176.9	s	C-19	75.2	d	75.8	d
2	182.5	s	173.3	s	20	68.0	d	68.7	d
3	176.6	s	169.7	s	21	56.6	q	56.6	q
4	172.9	s	149.2	s	22	48.5	d	48.7	d
5	169.4	s	141.1	s	23	44.9	d	45.1	d
6	145.4	s	137.8	s	24	44.8	t	43.1	t
7	139.9	s	134.9	d	25	39.9	d	39.0	d
8	137.9	s	134.4	d	26	33.0	t	33.7	t
9	133.7	d	133.9	d	27	29.4	t	31.7	t
10	133.7	d	132.7	s	28	29.3	t	29.4	t
11	133.2	d	129.6	d	29	29.3	t	29.4	t
12	133.1	d	129.5	d	30	25.6	t	26.6	t
13	131.3	d	129.1	d	31	25.6	t	25.7	t
14	129.5	d	125.5	s	32	25.5	t	25.6	t
15	129.3	d	124.3	d	33	25.5	t	25.6	t
16	122.5	d	115.8	d	34	20.5	q	20.3	q
17	114.5	d	107.5	d	35	17.4	q	17.7	q
18	79.2	d	79.6	d	36	9.7	q	9.6	q

* δ_C relative to TMS.
** Multiplicity in off-resonance spectrum.

Table 5. Antimicrobial activities of mycotrienins.

	M.I.C. (μg/ml)	
	MTN-I	MTN-II
Bacillus subtilis ATCC 6633	>100	>100
Bacillus circulans IAM 1029	>100	>100
Staphylococcus aureus 209P	>100	>100
Pseudomonas aeruginosa T-1	>100	>100
Escherichia coli NIHJ	>100	>100
Serratia marcescens IAM 1022	>100	>100
Aspergillus japonicus IAM 2016	12.5	12.5
Penicillium chrysogenum IAM 7106	8.0	12.5
Mucor pusillus IAM 6122	12.5	12.5
Rhizopus delemar IAM 6015	8.0	8.0
Saccharomyces cerevisiae IFO 0304	4.0	4.0
Candida utilus IFO 0396	4.0	4.0
Candida krusei IFO 0590	8.0	4.0

MIC's were determind by the conventional serial dilution method using a nutrient broth for bacteria (37°C, 24 hours) and a glucose - malt extract broth for yeasts and fungi (30°C, 72 hours). The results were judged after cultivation on a reciprocal shaker.

Physicochemical Properties of Mycotrienins

Physicochemical properties of mycotrienins are listed in Table 3. The IR and ^1H NMR spectra are shown in Figs. 2 and 3, respectively. The ^{13}C NMR spectral data are summarized in Table 4. Mycotrienins are soluble in alcohols, esters, acetone and chloroform; slightly soluble in ethyl ether; insoluble in water, petroleum ether and hexane.

In the UV spectra of mycotrienins, the characteristic absorptions at 260~263 nm, 270~273 nm and 280~282 nm indicate the presence of triene groups in the molecules. Acid hydrolysis of mycotrienins afforded D-alanine.

Physicochemical properties of MTN-II are in good agreement with those of mycotrienin reported by CORONELLI[1]. Furthermore, the ^1H NMR spectra of MTN-II and an authentic sample of mycotrienin* were completely superimposable, thus, MTN-II was identified as mycotrienin isolated by CORONELLI.

* Mycotrienin was a gift from Dr. CORONELLI.

Table 6. Cytotoxicities of mycotrienins against L-5178Y cells.

Concentration (μg/ml)	MTN-I	MTN-II
6.4	−	−
3.2	−	−
1.6	−	−
0.8	−	−
0.4	+	−
0.2	+	+

+, growth; −, no growth

The cytotoxicities of mycotrienins were determined by a procedure identical with that of the bioassay of mycotrienins.

Table 7. Antitumor activities of mycotrienins against P-388 ascites tumor.

Dose (i.p.)	T/C (%)	
	MTN-I	MTN-II
40 mg/kg/day×6	—	58
20	65	138
10	132	127
5	120	120
2.5	106	110
0	100	100

The CDF_1 male mice (20 g±1) were inoculated intraperitoneally with 10^6 cells of mouse leukemia P-388 and were treated once daily by intraperitoneal administration of MTN-I or II for 6 days starting 24 hours after cell inoculation. The prolongation of survival time was observed as criteria of tumor growth.

By reduction with $Na_2S_2O_4$ MTN-I was readily converted to MTN-II and the reverse reaction was accomplished by oxidation with air or $FeCl_3$.

In the ^{13}C NMR spectrum of MTN-I, two carbonyl carbon signals which were observed in the spectrum of MTN-II disappeared and two olefinic quaternary carbon signals were newly observed instead. These data suggest that a quinone nucleus and its hydroxy form are present in the molecules of MTN-I and MTN-II, respectively.

Biological Properties of Mycotrienins

MTN-I and -II are active against fungi and yeasts, but inactive against bacteria. The minimum inhibitory concentrations of these antibiotics are summarized in Table 5.

MTN-I and -II were tested against L-5178Y cells *in vitro*. The results are listed in Table 6. MTN-I and -II possess weak antitumor activities against P-388 ascites tumor as seen in Table 7. The LD_{50} (i.p.) values in mice of MTN-I and -II are 56 mg/kg and 80 mg/kg, respectively.

Acknowledgement

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References

1) CORONELLI, C.; R. C. PASQUALUCCI, J. E. THIEMANN & G. TAMONI: Mycotrienin, a new polyene antibiotic isolated from *Streptomyces*. J. Antibiotics 20: 329~333, 1967
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intl. J. Syst. Bacteriol. 16: 313~340, 1966
3) WAKSMAN, S. A.: The Actinomycetes. Vol. 2, The Williams and Wilkins Co., Baltimore, 1961
4) BUCHANAN, R. E. & N. E. GIBBONS: BERGEY'S Manual of Determinative Bacteriology. 8th ed., The Williams and Wilkins Co., Baltimore, 1974
5) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Intl. J. Syst. Bacteriol. 18: 69~189, 1968; III. Additional species descriptions from first and second studies. Intl. J. Syst. Bacteriol. 18: 279~392, 1968; IV. Species descriptions from the second, third and fourth studies. Intl. J. Syst. Bacteriol. 19: 391~512, 1969; V. Additional descriptions. Intl. J. Syst. Bacteriol. 22: 265~394, 1972
6) SKERMAN, V. B. D.; V. MCGOWAN & P. H. A. SNEATH: Approved lists of bacterial names. Intl. J. Syst. Bacteriol. 30: 225~420, 1980