ATPENINS, NEW ANTIFUNGAL ANTIBIOTICS PRODUCED BY *PENICILLIUM* SP.

PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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Penicillium sp. FO-125, a soil isolate, was found to produce a new antifungal antibiotic complex named atpenin. Three components A4, A5 and B were isolated from the fermentation broth of the producing strain by solvent extraction, silica gel column chromatography and HPLC. The molecular formula of atpenins A4, A5 and B were determined to be $C_{15}H_{22}NO_5Cl$, $C_{15}H_{21}NO_5Cl_2$ and $C_{15}H_{23}NO_5$, respectively, on the basis of high resolution electron impact mass spectrometry and elemental analysis. They are active against filamentous fungi, especially, *Trichophyton* sp.

Drugs affecting lipid metabolism are expected to be useful for the control of hypercholesterolemia, obesity, diabetes and so on. During the course of our screening for new compounds affecting lipid metabolisms from microorganisms, triacsins A, B, C and D were isolated from the cultured broth of *Streptomyces* sp. SK-1894, a soil isolate. We also reported that triacsins are new inhibitors of acyl-CoA synthetase and have a *N*-hydroxytriazene moiety in their structure, and *N*-hydroxytriazene is essential for the activity.[1,2]

In our continual screening for new lipid metabolism effectors from microorganisms, we discovered a new antibiotic complex termed atpenin in a cultured broth of a fungal stain FO-125. Atpenins A4, A5 and B were isolated from the cultured broth, and characterized. This paper discribes the producing organism, fermentation, isolation, physico-chemical and biological properties of atpenins.

Taxonomy of Producing Organism

Strain FO-125 was isolated from a soil sample collected in Sagamihara-shi, Kanagawa Prefecture, Japan. It is a filamentous fungus belonging to the Fungi Imperfecti. For the identification of the fungus, CZAPEK's agar, malt-extract agar, potato agar, oatmeal agar and YpSs agar were used. YpSs agar contains soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.005% and agar 1.2%, pH 6.0. Morphological observation was done under a scanning electron microscope (Hitachi, model S-430).

The colony was ivory on CZAPEK's agar and was green on malt-extract agar, potato agar and oatmeal agar in color. On YpSs agar, sporulation was abundant and the spore was green. As shown in Fig. 1, spores occurred in chains from phialides arranged in verticils borne terminally on conidio-

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phores. The spore was oval and 2.2~3.1 μm in diameter. The spore bearing phialides had a mean length of 9.5 μm and mean diameter of 2~4 μm.

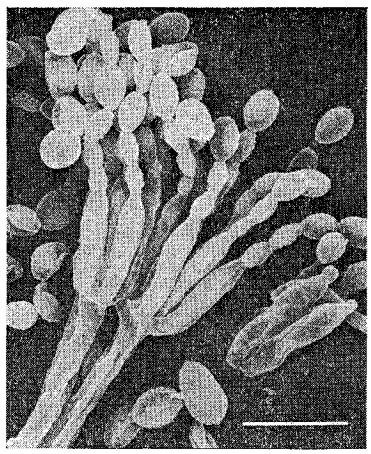
The above characteristics are consistent with the generic designation of *Penicillium*.

Fermentation

A slant culture of strain FO-125 grown on an potato - glucose agar was inoculated into 500-ml Sakaguchi flasks containing 100 ml of a seed medium (glucose 1.0%, Trypton 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 3 days at 27°C. Seven hundred ml of the seed culture were transferred into 70 liters of a production medium in a 100-liter jar fermentor and the fermentation was carried out at 27°C for 67 hours with aeration of 20 liters per minute and agitation of 250 rpm. The composition of production medium was the same as seed medium. The antibiotic production was monitored by a paper-disk agar diffusion method using *Candida lipolytica* L7 KF236 as a test organism, which is used for this screening. A typical time course of the fermentation is shown in Fig. 2. The maximum antibiotic titer was reached after 3 days of fermentation.

Fig. 1. Scanning electronmicrograph of penicillia and spores of the strain FO-125 on YpSs agar for 7 days at 27°C.

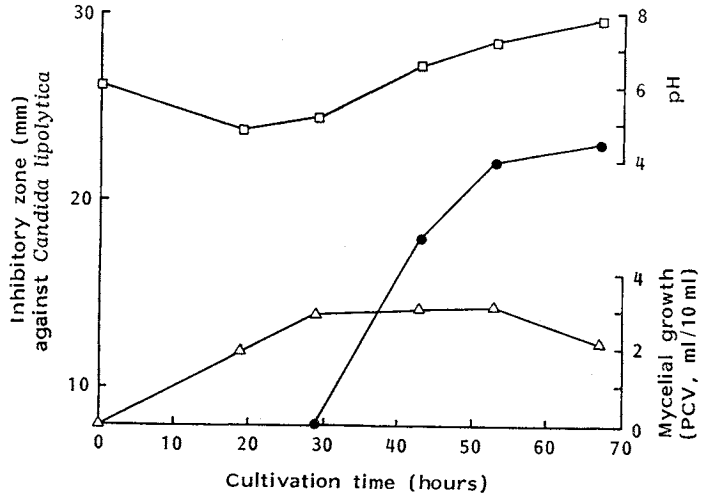
The bar represents 10 μm.



Isolation

The whole cultured broth (140 liters) was extracted with 108 liters of *n*-hexane. The extracts were concentrated *in vacuo* to dryness to yield a oily material (37.5 g). The material was

Fig. 2. Time course of atpenins production in a 100-liter jar fermentor.



Seed and production medium: Glucose 1.0%, Tryptone 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 0.1% and pH 6.0. PCV: Packed cell voluue.

Fig. 3. Isolation procedures for atpenins and HPLC chromatogram of fraction A of atpenins.

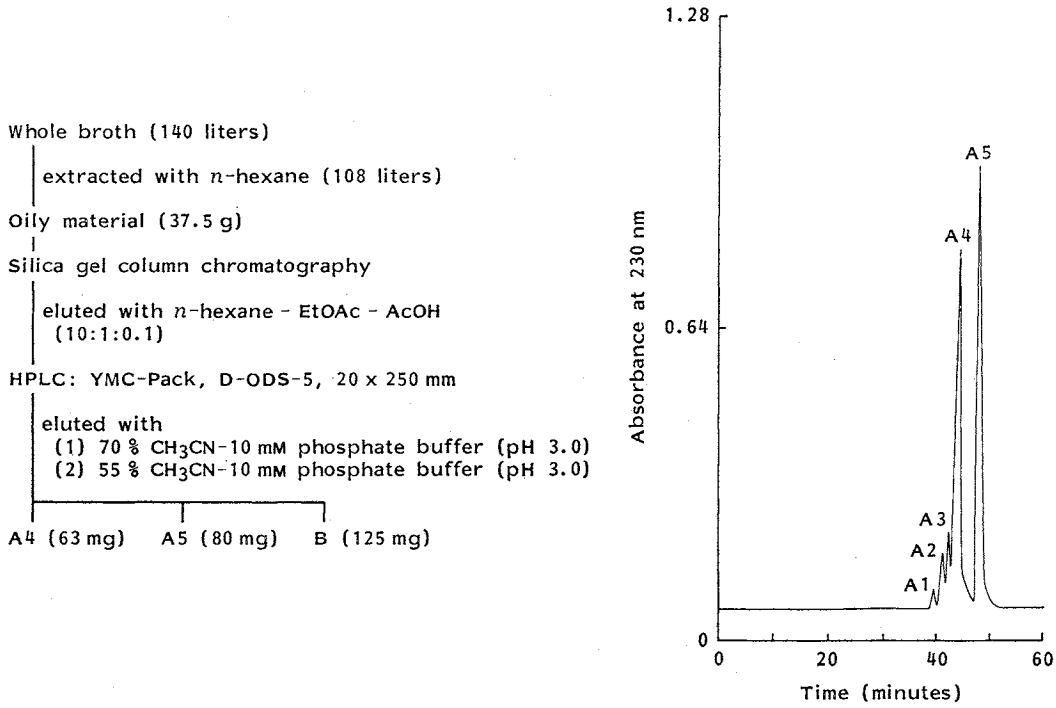


Table 1. Physico-chemical properties of atpenins.

	A4		A5		B	
Nature	White powder		White powder		White powder	
MP (°C)	98		86		78	
$[\alpha]_D^{22}$ (c 1.0, EtOH)	−8.6°		−0.8°		−27.0°	
Elemental *Anal* (%)	Found:	Calcd:	Found:	Calcd:	Found:	Calcd:
C	54.15	54.30	49.68	49.19	59.86	60.59
H	6.71	6.68	5.93	5.78	7.70	7.80
N	4.11	4.22	3.70	3.82	4.39	4.71
Cl	11.29	10.69	18.92	19.36		
HR-MS Calcd:	331.1185		365.0795		297.1575	
Found:	331.1183		365.0777		297.1575	
Molecular formula (MW)	$C_{15}H_{22}NO_5Cl$ (331.5)		$C_{15}H_{21}NO_5Cl_2$ (366)		$C_{15}H_{23}NO_5$ (297)	
UV λ_{max}^{EtOH} nm (ε)	235 (12,200), 267 (8,400), 320 (5,800)		237 (6,400), 272 (4,400), 320 (3,500)		237 (5,600), 270 (3,600), 318 (3,100)	
IR ν_{max}^{KBr} cm^{-1}	1645, 1600, 1440, 1320, 1200, 1160, 995		1645, 1600, 1440, 1320, 1200, 1160, 995		1645, 1600, 1440, 1320, 1200, 1160, 995	

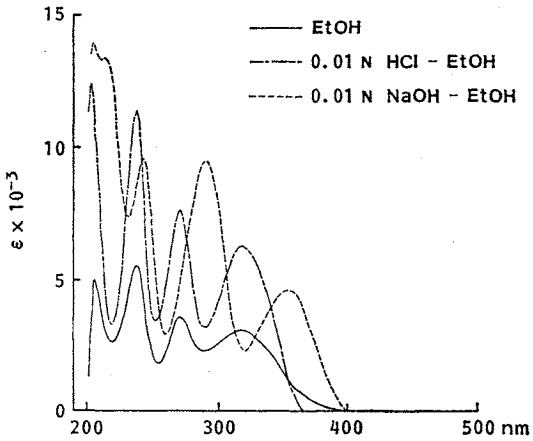
applied onto a column of silica gel (Merck, Kiesel gel 60, 1.5 liters), and then the active components were eluted with *n*-hexane - ethyl acetate - acetic acid (10 : 1 : 0.1). The combined active fractions were concentrated *in vacuo* to give an orange powder (3.2 g). The powder was purified by HPLC (Jasco TriRotar V, column: YMC-Pack D-ODS-5, 20×250 mm, solvent: 70% CH_3CN in 10 mM phosphate buffer (pH 3.0), flow rate: 8.0 ml/minute, detection: UV at 230 nm). A typical choromatographic run yielded two active fractions A and B with retention times at 16 and 20 minutes, respectively. Pure

atpenin B (125 mg) was obtained from fraction B by extraction with ethyl acetate. The fraction A yielded pure atpenins A4 (63 mg) and A5 (80 mg) after further purification by HPLC with 55% CH_3CN in 10 mM phosphate buffer (pH 3.0) as eluent. Fig. 3 shows the chromatographic profile, where A4 and A5 were eluted at 43 and 47 minutes, respectively. Atpenins A1, A2 and A3 showed antifungal activity, but were not obtained in pure form because of their low amounts.

Physico-chemical Properties

The physico-chemical properties of atpenins A4, A5 and B are summarized in Table 1. They are soluble in most organic solvents tested, but are insoluble in water. The molecular formula of atpenins A4, A5 and B were determined as $C_{15}H_{22}NO_5Cl$, $C_{15}H_{21}NO_5Cl_2$ and $C_{15}H_{23}NO_5$ on the basis of elemental analysis and high resolution-electron impact mass spectrometry (HREI-MS). The UV and IR spectra of atpenin B are shown in Figs. 4 and 5, respectively.

Fig. 4. UV spectra of atpenin B.



They gave positive reactions to H_2SO_4, I_2 and ferric chloride, but were negative to ninhydrin, Dragendorff, and Ehrlich reagents.

Biological Properties

Antimicrobial activities were assayed by a conventional agar dilution method using Sensi-

Fig. 5. IR spectrum of atpenin B (KBr).

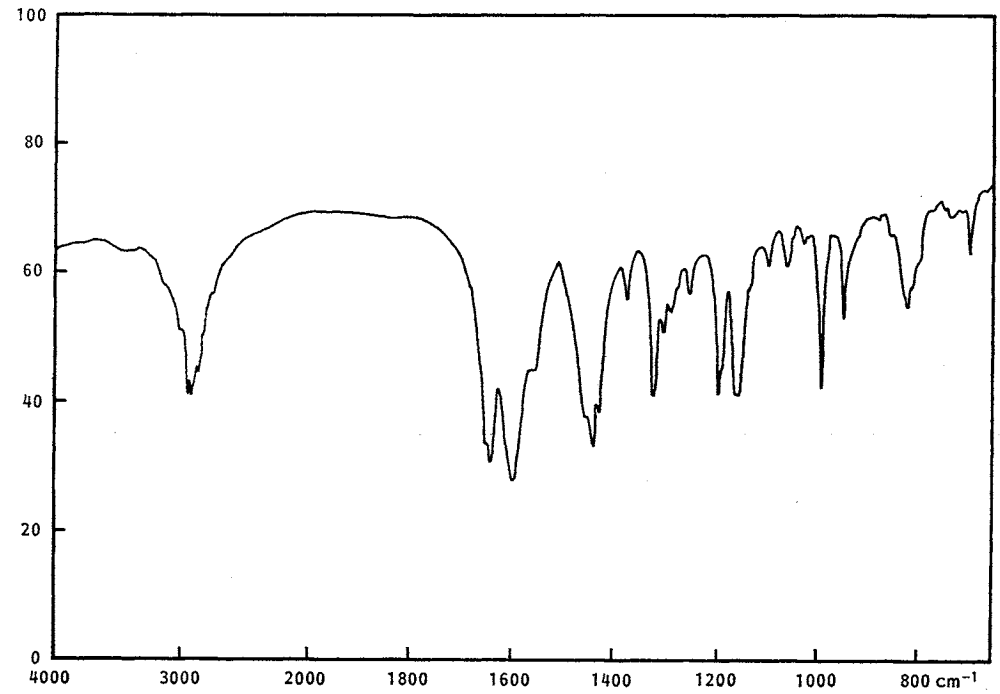


Table 2. Antimicrobial spectra of atpenins.

Test organism	MIC (μg/ml)		
	A4	A5	B
Staphylococcus aureus FDA 209P	>100	>100	>100
Bacillus subtilis PCI 219	>100	>100	>100
Micrococcus luteus PCI 1001	>100	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100	>100
Escherichia coli NIHJ JC-2 IFO 12734	>100	>100	>100
Pseudomonas aeruginosa P-3 KB 105	>100	>100	>100
Candida albicans KF 1	>100	>100	>100
C. lipolytica L7 KF 236[a]	6.25	0.4	1.56
Saccharomyces sake KF 26	>100	>100	>100
Aspergillus niger ATCC 6275	50	12.5	50
Piricularia oryzae KF 180	3.12	3.12	0.78
Mucor racemosus IFO 4581	>100	>100	>100
Trichophyton interdigitale KF 62	6.25	0.05	6.25
T. mentagrophytes KF 213	12.5	0.05	6.25
Microsporum gypseum KF 64	3.12	0.78	6.25

Bacteria: Sensitivity Disc Agar (Nissui); 37°C, 20 hours.
Fungi: Potato - glucose agar; 27°C, 72 hours.
[a] B-medium agar; 27°C, 72 hours.

tivity Disc Agar (Nissui) for bacteria and potato - glucose agar for yeasts and filamentous fungi. The results are shown in Table 2. Atpenins are active against filamentous fungi, especially against *Trichophyton* sp. They showed no activity against Gram-positive and Gram-negative bacteria. Of the three components, atpenin A5 was the most potent. The LD_{50} in mice (ip) of atpenins A4, A5 and B were found to be >50 mg/kg, 10 mg/kg and >50 mg/kg, respectively.

Discussion

Atpenins were compared with other known antibiotics in reference to physico-chemical and biological properties. Atpenin A5 bears some resemblance to fumigachlorin reported by ATSUMI *et al.*[3] in UV and IR spectra and biological properties. However, atpenin A5 is obviously differentiated from fumigachlorin by molecular formula ($C_{15}H_{21}NO_5Cl_2$ vs. $C_{16}H_{25}NO_4Cl_2$). Atpenins A4 and B are different from fumigachlorin with respect to the number of chlorine atom. Therefore, atpenins are reasonably concluded to be new antibiotics. It is considered that atpenins A4, A5 and B are structurally related to each other from the physico-chemical and biological properties described above. Their structures are now under investigation.

The mode of action of atpenins on Raji cell will be reported in near future.

References

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