TETRAFUNGIN, A NEW POLYENE MACROLIDE ANTIBIOTIC

I. FERMENTATION, ISOLATION, CHARACTERIZATION, AND BIOLOGICAL PROPERTIES

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A new polyene macrolide antifungal antibiotic, named tetrafungin, was isolated from the cultured mycelium of a Streptomyces strain. Fermentation, isolation, physico-chemical and biological properties of the antibiotic are described. Its UV absorption spectrum and its physico-chemical characteristics place this antibiotic in the group 2.2.2.3 of the BERDY classification.

In the course of screening for new antifungal antibiotics, a culture of a Streptomyces isolated from a Santiago (Spain) soil sample[1] produced an antifungal antibiotic which inhibited the yeast *Candida albicans* and several fungal dermatophytes. Following detailed taxonomic studies, the producing strain was found to be related to *Streptomyces albulus*. The characteristic UV spectrum places this antibiotic in the tetraene group of polyene macrolide, and it was thus named tetrafungin.

In this report, we are describing the fermentation of this antibiotic, its isolation, and its physico-chemical and biological characteristics. In the following paper[2] we describe the taxonomy of the producing organism and a comparative study of this antibiotic with nystatin by means of high performance liquid chromatography.

Organism

The producing organism, *Streptomyces albulus* subsp. *tetrafungini*, was cultivated at 28°C for 5 days on a Petri plate of yeast extract agar (yeast extract 0.4%, malt extract 1%, glucose 0.4%, agar 2%, pH 7.3). A well grown colony was transferred into 10 ml of V medium (glucose 0.5%, beef extract 0.5%, peptone 1%, KH_2PO_4 0.005%, $MgSO_4$ 0.005%, NaCl 0.3%, pH 7.8) in a 50-ml flask and incubated at 28°C and 300 rpm for 72 hours on a rotary shaker. The inoculum was developed further by transferring the entire culture mentioned above into 70 ml of V medium in a 250-ml Erlenmeyer flask. The culture was grown for 24 hours at 28°C on a rotary shaker at 300 rpm. Five percent (v/v) of this culture was used to inoculate the fermentation medium.

Fermentation

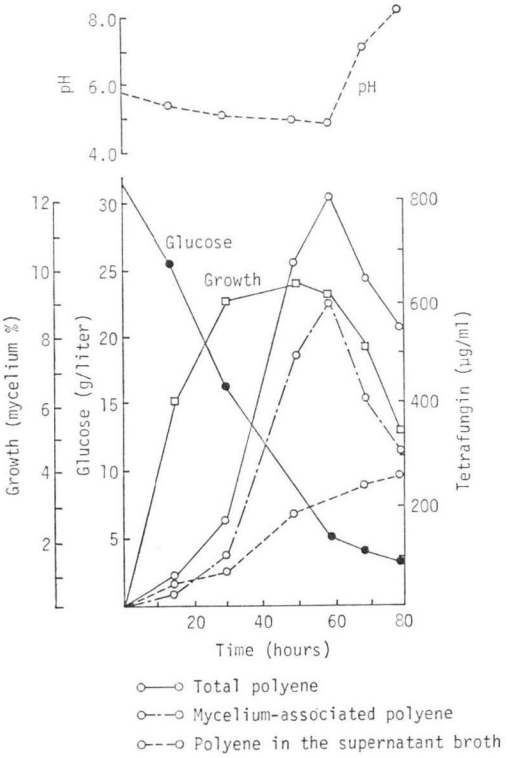
The fermentation was carried out in a fermenter (model MF-214, New Brunswick Scientific Co., New Brunswick, N. J.) of 14 liter capacity, equipped with an automatic antifoam addition system, filled with 5 liters of the production medium consisting of: glucose 3%, soybean meal 2%, $ZnSO_4 \cdot 7H_2O$ 5×10^{-4} M. The pH was adjusted to 5.5 by addition of 20% NaOH before sterilization. Silicone antifoam AF emulsion (Analema, Vorquímica, Vigo, Spain) was used as the antifoaming agent diluted in distilled water at a ratio of 1 : 3. The fermenter was sterilized at 121°C for 1 hour. Glucose and antifoam were sterilized separately for 20 minutes. Glucose was then added to the medium, and the anti-

foam added as required. The fermentation was run at 28°C under agitation at 600 rpm and aeration of 1 v/v/minute.

Concentrations of tetrafungin were assayed in samples of the fermentation broth as well as in the corresponding pellet of mycelia. Ten ml of supernatant or the mycelial cake from the same volume of broth, were extracted with a same volume of 1-butanol and methanol respectively in a 50-ml flask by shaking at 300 rpm during 1 hour at 28°C. After centrifugation at 3,000 rpm, the organic extracts were properly diluted and optical densities were measured on a spectrophotometer at 305 nm. In the same way as with other polyenes[3,4], the concentrations of tetrafungin in the original samples were calculated assuming a value of $E^{1\%}_{1cm}$ 1,000 for pure antibiotic.

The concentration of tetrafungin reached a maximum at 60~72 hours after inoculation. The results of a typical fermentation are shown in Fig. 1. A typical fermentation in the above conditions produced about 800 μg/ml. The antibiotic activity was found mainly (80%) in the mycelium, and partly (20%) in the culture filtrate.

Fig. 1. Pattern of batch fermentation.



Isolation and Purification

The antibiotic was only recovered from cultured mycelia by extraction with methanol in the same fermentation. The isolation and purification of the tetrafungin are summarized in Fig. 2. Precautions against light and thermal inactivations were taken. The antibiotic was obtained as a yellow powder with $E^{1\%}_{1cm}$ value of 825 in methanol at 305 nm. It showed a single spot on silica gel G thin-layer plates developed with several kinds of solvent systems (Plate 1).

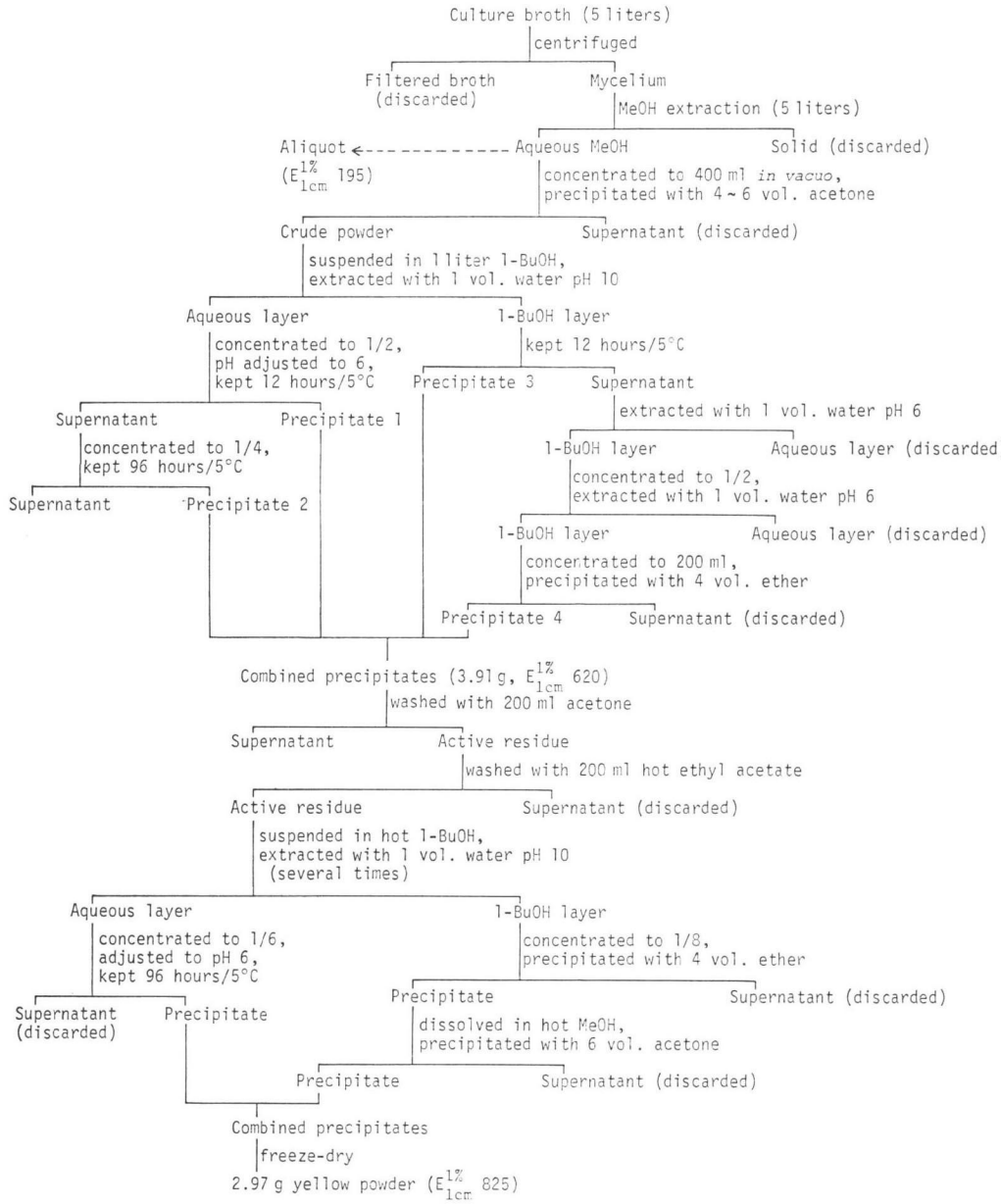
Physical and Chemical Properties

Tetrafungin has no definite melting point. The yellow powder turns black at 155~160°C, without melting at 270°C. Its specific rotation is $[\alpha]_D^{23}$ −23.6° (*c* 0.5, dimethylformamide), and $[\alpha]_D^{23}$ −16.5° (*c* 0.2, anhydrous methanol). An elemental analysis gave the following values: C 55.27, H 8.05, N 1.37; $O_{diff.}$ 35.31; corresponding to an empirical formula $C_{47}H_{82}O_{23}N$ (molecular weight 1,029).

Positive results were obtained for the following chemical test: Schiff, Tollens and concentrated sulfuric acid (black-brown coloration). The Molisch test was uncertain, and ferric chloride, Millon and Fehling tests were negative.

The UV spectrum in methanol shows a characteristic polyene spectrum very similar to that of nystatin (Fig. 3) with maxima at 234 ($E^{1\%}_{1cm}$ 280), 292 ($E^{1\%}_{1cm}$ 560), 305 ($E^{1\%}_{1cm}$ 825) and 320 nm ($E^{1\%}_{1cm}$ 735), and a shoulder at 280 nm ($E^{1\%}_{1cm}$ 267), corresponding to a tetraene antibiotic. The infrared spectrum, determined in a potassium bromide tablet, was of the usual type characteristic of polyene antibiotics[5,6], showing the presence of several hydroxyl groups (strong bands at 3400 and 1070 cm^{-1}). A distinct

Fig. 2. Isolation and purification of tetrafungin.

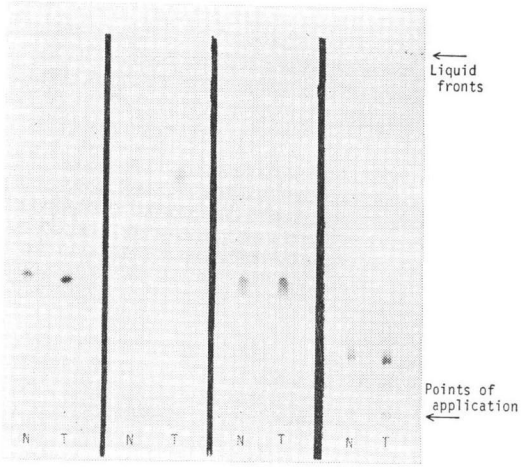


band at 1715 cm^{-1} due to the carbonyl group of the lactone group is indicative of the macrolide nature of the antibiotic. The spectrum also shows the presence of an ionized carboxyl group by another band at 1580 cm^{-1}, and a polyene system by the band at 1640 cm^{-1}.

Tetrafungin is an amphoteric substance, as has been demonstrated by pH chromatography in 1-butanol saturated with water (detecting by bioautography with *Rhodotorula* sp.). Tetrafungin behaves as a base from pH 2 to pH 6 (indicating possibly its isoelectric point), and from this point on up to pH 10, it behaves as an acid.

Tetrafungin has an aminosugar moiety which is identical to that of nystatin (mycosamine) as was

Plate 1. TLC behavior of tetrafungin and nystatin*.



Solvent systems	Rf value	
	Tetrafungin	Nystatin
I Butanol - acetic acid - water (3: 1: 1)	0.37	0.385
II Methanol - acetone - acetic acid (8: 1: 1)	0.65	0.69
III Butanol - pyridine - water (3: 2: 1)	0.36	0.36
IV Ethanol - ammonia - water - dioxane (8: 1: 1: 1)	0.15	0.16

5×20 cm, 0.25-mm thick precoated silica gel plates (Silica gel 60 TLC plates, E. Merck, Darmstadt, G.F.R.). Saturated developing chamber.
Detection: Orthophosphoric acid; heat for 5 minutes at 100°C.
* Squibb lot 55317-098 (Squibb, New Brunswick, N.J., USA).

demonstrated by thin-layer chromatography after its acid hydrolysis in the way described by MARTIN and MCDANIEL[6]. Mycosamine (Rf 0.63) was visualized by spraying the plate with 0.2% ninhydrin in acetone.

Tetrafungin is readily soluble in dimethylformamide, dimethyl sulfoxide, pyridine and acetic acid, but moderately soluble in methanol and butanol, and insoluble in water, chloroform, acetone and ether. The solubility in $CaCl_2$ methanolic (3%) is 10 times greater than in anhydrous methanol.

Tetrafungin is stable for indefinite periods of time when stored at 5°C out of contact with light or air. The stabilities of tetrafungin in methanolic solutions in contact with light, various pH and temperatures are given in Fig. 5. The stability of samples were not protected from oxygen.

Fig. 3. UV spectra in methanol.
(a) Nystatin*: nm ($E^{1\%}_{1cm}$) 234 (279), 280 (266), 292 (513), 305 (826), 320 (719).
(b) Tetrafungin: nm ($E^{1\%}_{1cm}$) 234 (280), 280 (267), 292 (560), 305 (825), 320 (735).
* Squibb lot 55317-098 (Squibb, New Brunswick, N.J., U.S.A.).

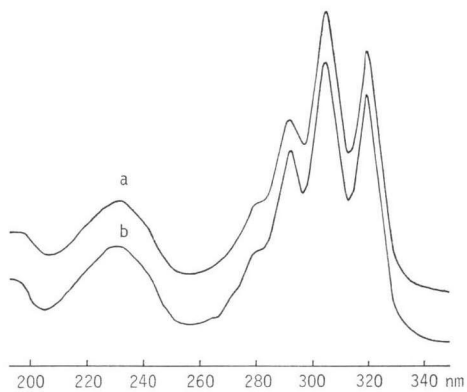


Fig. 4. IR spectrum of tetrafungin (in KBr).

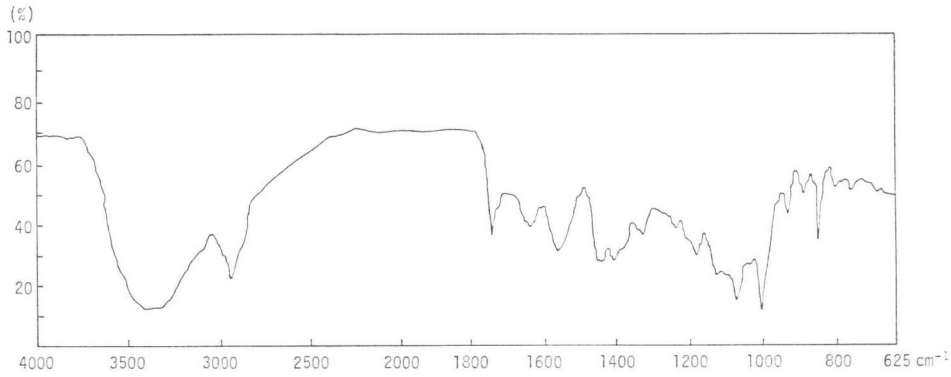


Fig. 5. Stability of tetrafungin in methanolic solutions.

(A) In contact with light at pH 7.0 stored at 5°C.

(B) At various temperatures stored in the dark at pH 7.0.

(C) At various pH values stored in the dark at 5°C.

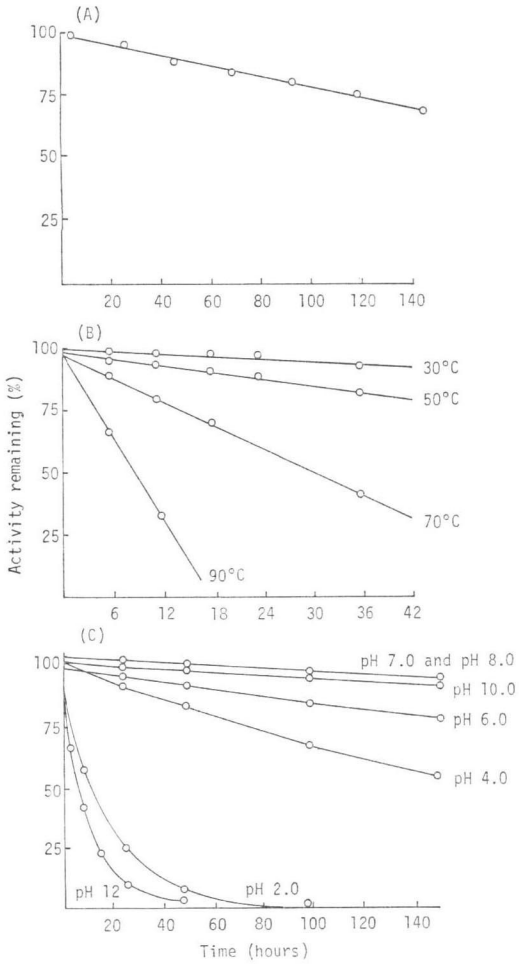


Table 1. Antifungal activity of tetrafungin.

Microorganisms	Minimal inhibitory concentration (μg/ml)
Candida albicans	0.39
C. tropicalis	0.78
C. krusei	0.39
Rhodotorula sp.	0.39
Microsporum gypseum	1.56
M. canis	$\leq$0.048
Trichophyton rubrum	3.12
T. tonsurans	3.12
T. mentagrophytes	1.56
Epidermophyton floccosum	1.56
Aspergillus flavus	0.78
A. fumigatus	3.12
A. niger	1.56
A. luchuensis	0.39

Minimal inhibitory concentrations, given in μg/ml on Sabouraud agar (24 hours for yeasts, 48 hours for Aspergillus and 10 days for other fungi).

Antifungal Activity of Tetrafungin

Minimal inhibitory concentrations of tetrafungin against yeasts and filamentous fungi — strains from our laboratory— are indicated in Table 1.

Conclusions

An antifungal antibiotic has been obtained from a Streptomyces strain isolated from a soil sample.

Its UV absorption spectrum and its physicochemical characteristics place this antibiotic in the tetraene group of polyene macrolides (2.2.2.3 of the BERDY[7] classification) and it has been de-

signated as tetrafungin.

Although tetrafungin differs in its chemical and physical properties from other well-known tetraene antibiotics[8~10], its UV spectrum, melting point and other properties are very similar to those of nystatin. Besides, both antibiotics are produced by strains of *Streptomyces albulus*. It is possible that tetrafungin and nystatin are the same antibiotic and the differences observed between them might be due to small impurities. However, the chromatographic behaviour of both antibiotics on TLC is different, the Rf of the tetrafungin being slightly lower than that of nystatin in 3 of the 4 solvent systems used. Also, as we describe in the following paper[2], tetrafungin and nystatin can be differentiated by means of the HPLC technique.

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