TETRAFUNGIN, A NEW POLYENE MACROLIDE ANTIBIOTIC

II. TAXONOMY OF THE PRODUCING ORGANISM AND COMPARISON WITH NYSTATIN BY MEANS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Tetrafungin, a new polyene macrolide antibiotic, is produced by a Streptomyces strain identified as a new subspecies of *Streptomyces albulus* and named *Streptomyces albulus* subsp. *tetrafungini*. Tetrafungin and nystatin have been investigated and compared by HPLC. It has been demonstrated that tetrafungin and nystatin differ qualitatively in, at least, one component, and quantitatively in their relative amounts of common components.

As described in the preceding paper[1], tetrafungin is a new antifungal antibiotic produced by a Streptomyces strain. Its UV absorption spectrum and its physico-chemical characteristics place this antibiotic in the tetraene group of polyene macrolide (2.2.2.3 of the BERDY[2] classification). Since most polyene antibiotics are found to be mixtures of closely related homologs, an efficient analytical procedure is necessary to obtain a good characterization of these compounds. Several procedures have been used for this purpose and HPLC has previously been shown to be the most useful[3~6]. The present report describes the taxonomic characterization of the producing strain of tetrafungin as well as a comparative study between tetrafungin and nystatin by HPLC.

Material and Methods

Taxonomy

The methods and media recommended by the International Streptomyces Project[7] were used primarily, along with several supplementary tests. The procedure of BECKER *et al.*[8] was used for the preparation of cells and chromatographic detection of the isomers of diaminopimelic acid.

High Performance Liquid Chromatography

Apparatus: The experiments presented here were carried out in two commercially available liquid chromatographs:

A. Hewlett-Packard model HP-1081A Liquid Chromatograph consisting of:
Fixed wave-length 254 nm UV detector, Valve loop injector (20 μl), LiChrosorb RP-8 column for reverse phase (300×4.6 mm internal diameter), Hewlett-Packard model 3380 integrator.

B. Varian Aerograph model 5020 Liquid Chromatograph consisting of:
Variable wave-length UV detector (UV 50 Varian Aerograph), Valve loop injector (10 μl), MicroPak MCH-10 column for reverse phase (300×4 mm internal diameter), Varian Aerograph 9176 register.

Solvent System: Methanol (E. Merck, Darmstadt, G. F. R.) was mixed with bidistilled water in different proportions to produce various solvent systems useful in the separation. Prior to use, all solvent systems were degassed by a vacuum technique.

When apparatus A was employed, all separations were made under isocratic conditions, and formic

acid (Merck) was added to the degassed mobile phase to a final concentration of 0.3% (v/v).

When apparatus B was employed, different gradient elutions were made.

Antibiotic Preparations: Tetrafungin ($E^{1\%}_{1cm}$ 825 at 305 nm) obtained as described previously[1] and two different samples of nystatin, nystatin 1 (Squibb lot 55317-098, E. R. Squibb, New Brunswick, N. J.), and nystatin 2 (Sigma lot 95C-0209, Sigma Chem. Co., Saint Louis, Missouri), were used as pure samples. Standards of these antibiotics for HPLC were prepared by dissolving 10 mg of each sample in 1 ml of dimethyl sulfoxide (Merck) and diluting the solution with 9 ml of methanol (Merck) to a final concentration of 1 mg/ml. Higher dilutions were made by adding more methanol.

The non-purified tetrafungin samples for HPLC were obtained as follows: the Streptomyces strain No. 67 was grown in a 50-ml Erlenmeyer flask containing 15 ml of fermentation medium[1] for 72 hours at 28°C on a rotary shaker running at 300 rpm. After incubation, the cells were collected by centrifugation from 10 ml of fermented culture. Tetrafungin was extracted from the pellet with 10 ml of methanol (Merck) as described previously[1]. This methanolic extract without further treatment was used as a non-purified sample of tetrafungin.

Before injection, all samples were filtered throught a Millipore membrane filter (pore size: 0.5 μm; Millipore Co., Bedford, Mass.,). Injected volumes were 10 and 20 μl containing 2.5~10 μg of the antibiotic.

Instrument Operation: After achieving equilibrium in the instruments, demonstrated by a constant base line on the recorder, the samples were applied. The chart speed in both recorders was constant at 0.5 cm/minute. During chromatography, the columns were operated at room temperature. In apparatus B the detector wavelength was selected to be 305 nm. Other operating conditions are shown in the results.

At the end of each working day, the columns should be rinsed with methanol for about 30 minutes.

Results and Discussion

Taxonomy

The producing organism, strain No. 67, produces a vegetative mycelium which does not fragment into coccoid or bacillary elements, and an aerial mycelium, which later forms spore chains. Sporophores are compact spirals (Plate 1) classified in the Spiral Section. Spores were oblong to cylindrical with a spiny surface (Plate 2). This morphology was observed in ISP No. 3 medium, ISP No. 4, and mainly in ISP No. 9 basal medium with fructose as a sole carbon source.

Plate 1. Sporophores of strain No. 67.
Basal medium (ISP No. 9) with fructose. 14 days. ×400.

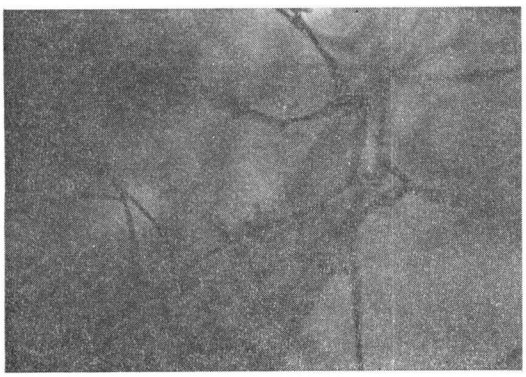
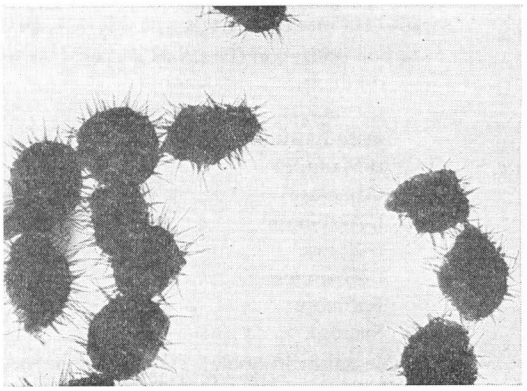


Plate 2. Electromicrograph of spores of strain No. 67.
Basal medium (ISP No. 9) with fructose. ×14,000.



The cultural and summarized physiological properties are shown in Tables 1 and 2 respectively. The results were recorded after 7, 14 and 21 days of incubation. LL-Diaminopimelic acid is detected in whole-cell hydrolysates of the culture.

Microscopic studies and cell wall pattern indicated that strain No. 67 belongs to the genus Streptomyces. Accordingly, comparison of this strain was made with published descriptions[9~18] of the *Streptomyces* species. From the above-mentioned information, strain No. 67 may be considered to be closely related to *Streptomyces albulus*. However, they can be differentiated by the existence or otherwise of melanine production (negative for *S. albulus* but weakly positive for strain No. 67). As a result of the above comparison, the only difference observed between strain No. 67 and *S. albulus* does not seem to us sufficient to make a distinction between both organisms. Consequently, strain No. 67 has been designated as *Streptomyces albulus* subsp. *tetrafungini* subsp. nova. The culture has been deposited in the Colección Española de Cultivos Tipo, where it has been assigned the number CECT 3238.

Table 1. Cultural characteristics of strain No. 67.

Medium	Growth	Aerial mycelium	Vegetative mycelium	Soluble pigment
Glucose - asparagine agar	Poor	None	Pale yellow	None
Glycerol - asparagine agar (ISP No. 5)	Moderate	White	Reddish brown	None
Inorganic salts - starch agar (ISP No. 4)	Good	Gray to black	Pale yellow	None
Tyrosine agar (ISP No. 7)	Good	White	Yellow-brown	Brown (weak)
Yeast extract - malt extract agar (ISP No. 2)	Good	White	Reddish brown	None
Oatmeal agar (ISP No. 3)	Good	Brown	Pale yellow	None
Nutrient agar	Moderate	White	Pale yellow	None

Table 2. Physiological characteristics of strain No. 67.

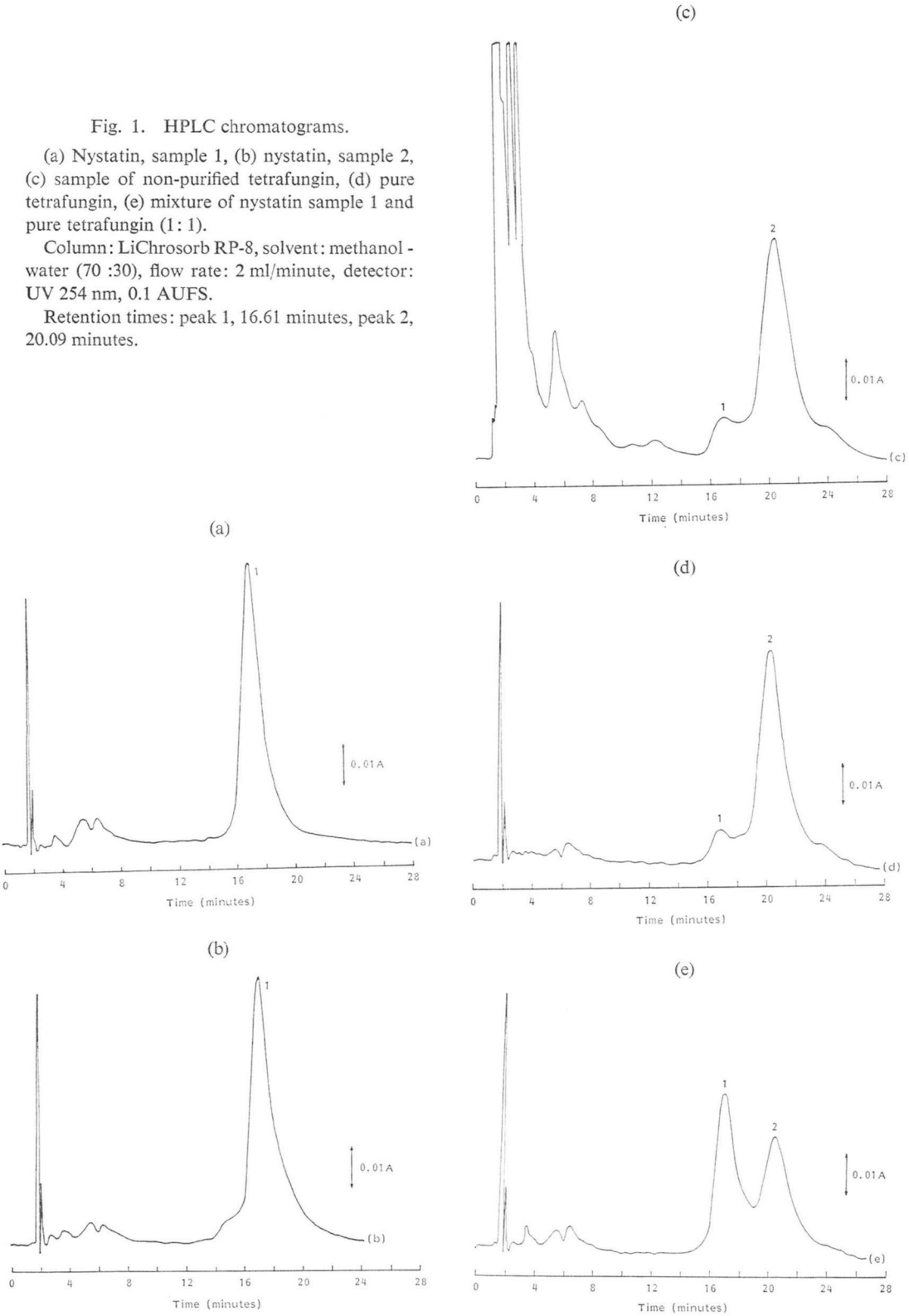
Melanoid production on	
Tyrosine agar (ISP No. 7)	Positive (weak)
Peptone - yeast extract iron agar (ISP No. 6)	Negative
Tryptone - yeast extract broth (ISP No. 1)	Negative
Hydrolysis of	
Starch (Inorganic salts - starch agar, ISP No. 4)	Positive (strong)
Gelatin (Glucose - peptone - gelatin)	Positive
Skim milk	Peptonized but not coagulated
Salt tolerance (BENNETT's agar+0, 4, 7, 10 & 13% NaCl)	4%
Carbon utilization (PRIDHAM and GOTTLIEB basal, ISP No. 9)	
D-Glucose	++
D-Fructose	++
meso-Inositol	++
D-Mannitol	++
Galactose	++
L-Arabinose	−
D-Xylose	−
L-Rhamnose	−
Raffinose	±
Sucrose	−
Whole cell hydrolysate: LL-diaminopimelic acid	

Fig. 1. HPLC chromatograms.

(a) Nystatin, sample 1, (b) nystatin, sample 2, (c) sample of non-purified tetrafungin, (d) pure tetrafungin, (e) mixture of nystatin sample 1 and pure tetrafungin (1 : 1).

Column: LiChrosorb RP-8, solvent: methanol - water (70 :30), flow rate: 2 ml/minute, detector: UV 254 nm, 0.1 AUFS.

Retention times: peak 1, 16.61 minutes, peak 2, 20.09 minutes.



High Performance Liquid Chromatography

Typical chromatograms of HPLC separations obtained in apparatus A and apparatus B are shown in Figs. 1 and 2 respectively. To help in identification, the corresponding principal peaks have been numbered in order of increasing retention time. In order to select the best mobile phase in each apparatus, the criteria involved were the resolution between peaks 1 and 2 and the separation time.

In our studies, when apparatus A was employed, optimal separations were achieved using 70% (v/v) methanol as the mobile phase. An increase in methanol concentration reduced the retention times, making the separation difficult. When the methanol concentration was reduced, the peaks became very broad and the retention times increased. The chromatograms obtained with 70% (v/v) methanol show qualitative and quantitative differences between tetrafungin and nystatin. Peak 2 — the main component of tetrafungin — is absent or in a non-detectable amount in nystatin. On the other hand, peak 1 — the main component of nystatin — appears as a minor component in tetrafungin. The chromatograms of the two commercial nystatin samples from two different producers were found to be practically identical (Fig. 1) and no significative differences are to be seen.

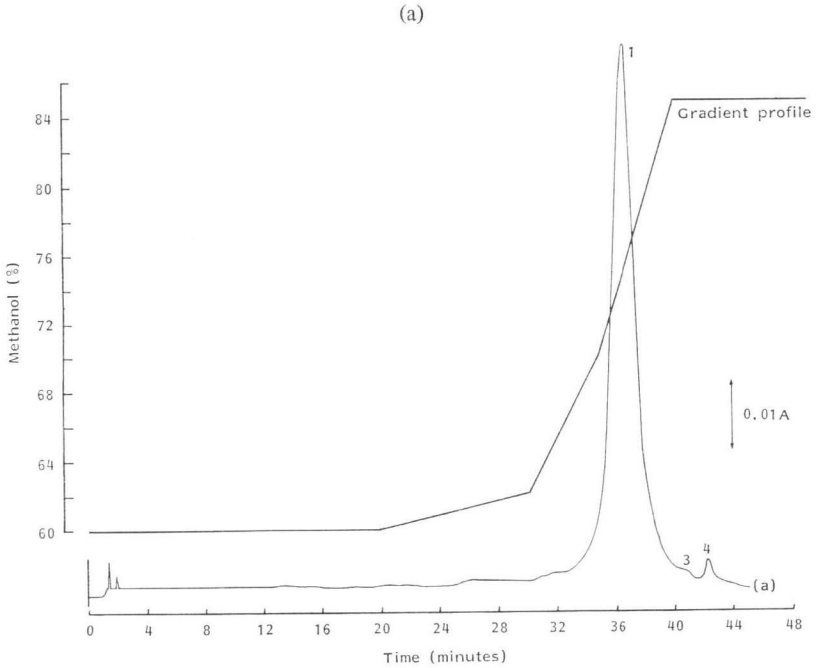
Although in isocratic conditions the MicroPak MCH-10 column equipping apparatus B was poorer in resolution capacity than the LiChrosorb RP-8 column equipping apparatus A, new components in both tetrafungin and nystatin, in addition to those described above, were detected by using a gradient elution. The selected gradient elution profile was the following: 60% (v/v) methanol was maintained

Fig. 2. HPLC chromatograms.

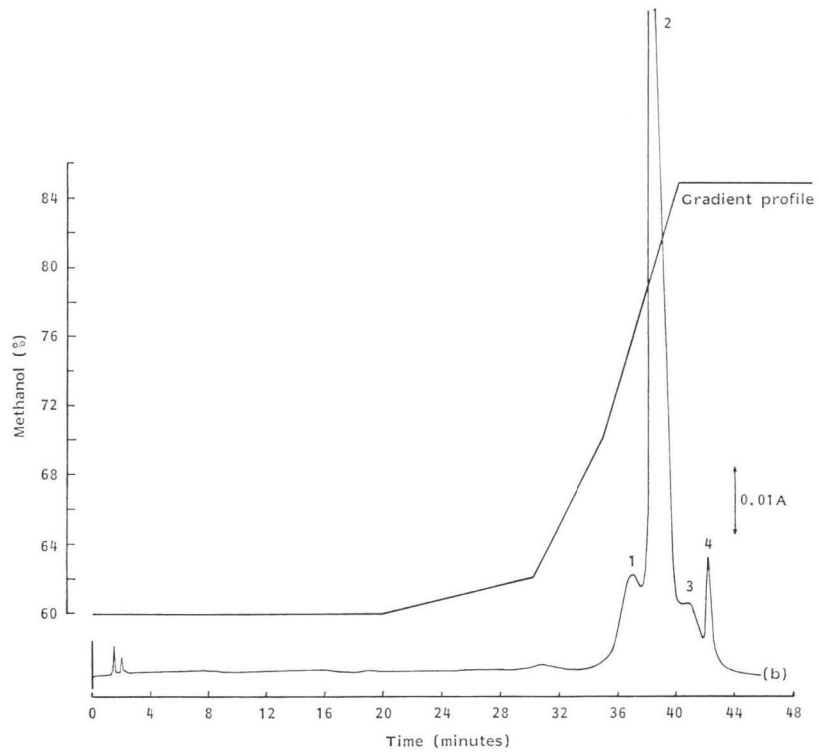
(a) Nystatin, sample 1, (b) pure tetrafungin, (c) mixture of nystatin sample 1 and pure tetrafungin (1: 1).

Column: MicroPak MCH-10, solvent: methanol - water, flow rate: 1.5 ml/minute, detector: UV 305 nm, 0.1 AUFS.

Retention times: peak 1, 37.4 minutes, peak 2, 39.45 minutes, peak 3, 41.2 minutes, peak 4, 42.5 minutes.



(b)


Methanol (%)
84
80
76
72
68
64
60
Gradient profile
0.01A
1
2
3
4
(b)
0
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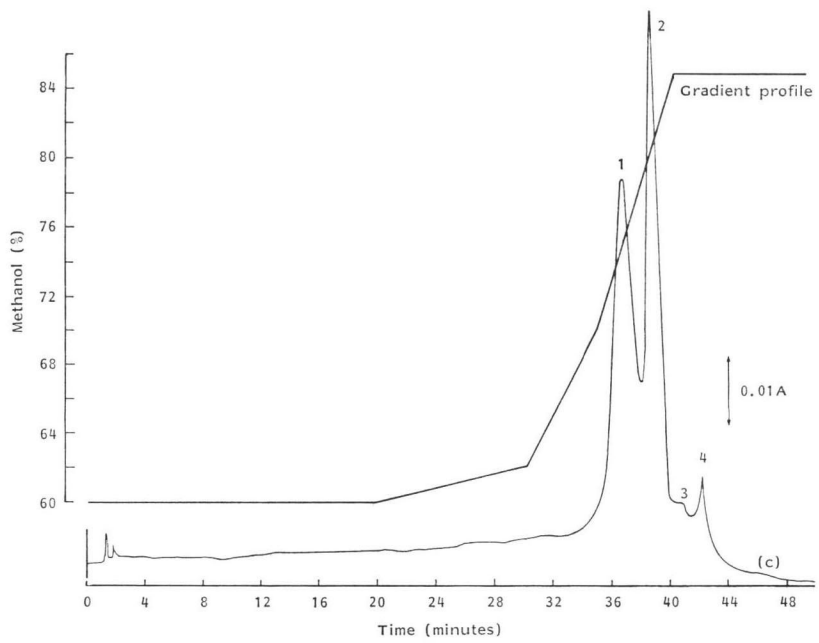
8
12
16
20
24
28
32
36
40
44
48
Time (minutes)

(c)


Methanol (%)
84
80
76
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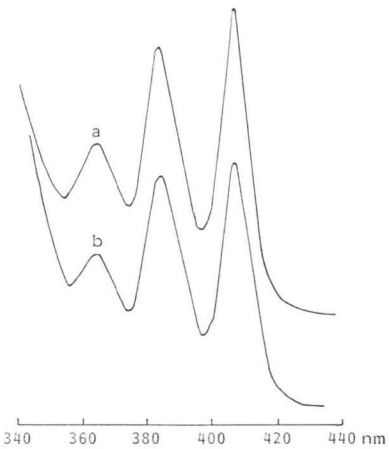
68
64
60
Gradient profile
0.01A
1
2
3
4
(c)
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (minutes)

for 20 minutes; linear increase in methanol concentration from 60 to 62% (v/v) in 10 minutes; a linear increase from 62 to 70% (v/v) methanol in 5 minutes; a new linear increase from 70 to 85% (v/v) methanol in 5 minutes, and then held for a further 15 minutes. Under these conditions, nystatin was resolved in a main component (peak 1) and a minor component (peak 4), with traces of a third component (peak 3). Tetrafungin was however resolved in four components: a main component (peak 2) and three minor components (peaks 1, 3 and 4). For both tetrafungin and nystatin, the retention time for peaks 1, 3 and 4 are identical, but quantitative differences are to be seen between both antibiotics. These quantitative differences with regard to peaks 3 and 4 may be due partly to differences in the purification procedures. However, quantitative differences with regard to peak 1 and the presence of peak 2 in the tetrafungin antibiotic are not due to this reason as has been demonstrated by HPLC, since peaks 1 and 2 were resolved in an identical way for both purified and non-purified samples of this antibiotic (Fig. 1).

Fig. 3. UV spectra in methanol.
(a) Nystatin sample 1: nm ($E^{1\%}_{1cm}$) 364 (12.48), 383 (22.1), 406 (26), (b) pure tetrafungin: nm ($E^{1\%}_{1cm}$) 364 (5.72), 383 (9.49), 406 (10.14).



By varying the wave-length from 305 to 382 nm in the UV detector, it has been demonstrated that the peaks appearing in HPLC chromatograms are all tetraenes and no heptaene has been detected in this way. However, both tetrafungin- and nystatin-complex antibiotics contain a heptaene component as has been demonstrated by UV spectrophotometry (Fig. 3). The presence of a heptaene in nystatin has been shown by previous workers[5].

From this study, it may be deduced that tetrafungin and nystatin are closely related complex antibiotics: Both antibiotics are produced by strains belonging to *S. albulus*; both antibiotics contain a minor heptaenic component detectable by spectroscopic UV analysis; in both antibiotics component 4 is present, and perhaps also component 3; main component of nystatin (presumably nystatin A1 or a mixture of A1 and A2 and/or A3) is present in tetrafungin as a minor component.

However nystatin and tetrafungin are different, since besides quantitative differences in their common components, these antibiotics differ qualitatively in, at least, one main component.

Similarly, polifungin and nystatin complexes were differentiated from each other by the presence of a minor component in polifungin which is absent in nystatin, though no differences were found in their main components[14]. HPLC studies involving closely related heptaene macrolide antibiotics[8~6] also indicated that many of them have numerous common components but differ in their relative amounts and/or in the presence of additional components.

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