COMPARATIVE ANALYSIS OF HEXAENE ANTIBIOTICS

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A study of nine hexaene antibiotics resulted in their assignment to three subgroups on the basis of their bioactivities. Separation of individual components of the nine antibiotic complexes was accomplished by thin-layer chromatography. Similarities and differences among members of the subgroups were established by thin-layer chromatography, spectrophotometry and high performance liquid chromatography. Two antibiotics (endomycin and hexafungin) were found to be similar.

The polyene antibiotics constitute a large group of antifungal antibiotics produced by *Streptomyces* and other closely related genera. They are divided into groups based on the number of conjugated double bonds in their structures. The location of the UV-visible absorption peaks indicates the number of double bonds, which allows for the classification of the compounds as trienes, tetraenes, pentaenes, hexaenes or heptaenes based on their having three, four, five, six or seven double bonds in series, respectively[1,2].

The presence of a methyl group on the allylic carbon to the double bonds of a number of pentaenes results in a bathochromic shift of about 6 nm in their absorption peaks. A ketone at this position causes the typical UV-visible spectrum to be changed into a single peak[3].

The hexaenes constitute the smallest and most poorly characterized group of the polyenes. Only some 12~15 true hexaenes have been reported. Some of the hexaenes are very active against fungi[4]. The polyene mode of action has been studied by many investigators[5~9]. The polyenes are thought to interact with membrane sterols causing the formation of pores which allow leakage of some vital cell constituents.

Thin-layer chromatography (TLC) techniques have been commonly used for the separation of components of polyene antibiotics as well as for qualitative determination of these components[10~12]. MARTIN and MCDANIEL[13] prepared the active and non-active fractions of candihexin by preparative TLC and determined the characteristics of the different components.

High performance liquid chromatography (HPLC) techniques have also been employed for the characterization and quantitative analyses of polyene antibiotics. MECHLINSKI and SCHAFFNER[14,15] reported various aspects of HPLC techniques for polyenes. They were able to prove that some aromatic heptaenes which were originally reported to be different were essentially the same[15].

The objective of this study was to investigate the similarities and differences among hexaene antibiotics. Active fractions of the antibiotic complexes were isolated using preparative TLC plates and comparisons were made on the basis of TLC, HPLC and bioassay studies.

The fradicin group of antibiotics which consists of fradicin[16], mycelin[17], mycelin IMO[18], sapromycetin[19] and kotomycin[20] was excluded from this study since this group exhibits a distinctly different

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UV spectrum. The "tentative" empirical formula reported for fradicin is quite different from the formulae reported for polyene macrolides[21,22].

Some of the reported hexaene antibiotics could not be included in this study because neither the producing cultures nor the products were available. These were hexamycin[23], hexin[24], flavacid[25] and grecomycin[26]. In the case of cryptocidin[27] the culture was available but it no longer produces the antibiotic. Dermostatin, which was not available for this study, is included in the summary since it is a conjugated hexaene, and thus is distinctly different from all those included in this investigation.

Materials and Methods

Hexaene Antibiotic Complexes

The hexaene antibiotics which were studied are listed in Table 1. Hexafungin was obtained from Dr. THRUM (Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, GDR). The others were produced in the pilot plant of the Waksman Institute of Microbiology where the producing cultures of candihexin, hexaene-80, hydrohexin, L-72 and streptohexin were all isolated.

Table 1. A list of the hexaenes studied.

Antibiotic	$E^{1\%}_{1cm}$	Wavelength of $E^{1\%}_{1cm}$ estimation (nm)	References
L-72	493	363	*
Candihexin	318	359	28
Tetrahexin	574	351	29
Streptohexin	380	354	30
Hydrohexin	376	354	31
Hexaene-80	639	357	**
Endomycin	68	356	32
Hexafungin	826	357	33
Mediocidin	590	356	34

* J. B. TUNAC (unpublished, 1974)
** J. A. POPE, Jr. (unpublished, 1975)

The absorption characteristics ($E^{1\%}_{1cm}$) of each crude product used are listed to give an indication of their purities. Much of the endomycin which was present in fermentation broths was lost during product recovery, and thus the final product was of relatively low purity.

Chemicals

Malt extract, yeast extract, agar, nutrient agar, and Sabouraud dextrose agar were obtained from Difco Laboratories (Detroit, Michigan) and Cerelose from Corn Products (Englewood Cliffs, New Jersey). Other materials were commercial grade chemicals.

Spectrophotometry

Absorbance spectra were obtained by using a Beckman UV 5270 UV Spectrophotometer. Since the $E^{1\%}_{1cm}$ of most pure polyene macrolide antibiotics is about 1,000, determinations of purity of antibiotic samples were based on this assumed value. Concentrations of antibiotics in solutions were calculated according to the following equation:

$$C(\mu g/g) = OD \times \text{Dilution factor} \times 10$$

where OD is the optical density of the second longest wavelength peak in the range of 350 and 365 nm. Samples were scanned between 420 and 250 nm to cover any heptaene or tetraene in addition to hexaene peaks. Samples of the components of the hexaene antibiotics were prepared from the scrapings of the fractions on the TLC plates. These fractions were dissolved in MeOH and filtered through Whatman #1 filter paper and also Millipore Microfilters (Millex-SR, 0.5 μm).

Thin-layer Chromatography

Samples for TLC were prepared by dissolving 10 mg of the antibiotic preparations in 10 ml of MeOH. Ten μl of the samples were spotted on silica gel plates for spectrodensitometry and for bioautography (E. Merck, Darmstadt, FRG, F-254, 0.25 mm). For preparative TLC, 2 ml of each sample were introduced homogeneously on a line on the preparative silica gel plates (E. Merck, Darmstadt, FRG, F-254, 0.5 mm). The following solvent systems were used for the development of the preparative and analytical TLC plates:

System A: $CHCl_3$ - MeOH - NH_4OH, 4: 10: 1
System B: $CHCl_3$ - MeOH - H_2O, 6: 10: 1

The solvent mixtures were prepared fresh for each run. Thin-layer plates were developed in rectangular glass tanks of dimensions 30×10×25 cm with a glass lid. The tanks were lined with Whatman #1 filter paper sheets which were immersed in the solvent system about an hour before development was started. Development was carried out at room temperature.

Spectrodensitometry

Antibiotics developed on 0.25 mm thick silica gel plates were scanned on a Schoeffel Instrument Corporation SD3000 Spectrodensitometer at 380 nm.

Bioassays

Bioautography: TLC plates which had been developed with solvent system B were dried under a nitrogen gas stream. Molten agar at 50°C which was seeded with *Saccharomyces cerevisiae* ATCC 9763 was poured onto the plates. The plates were then incubated at 27°C for 24 hours and observed for zones of inhibition.

Qualitative Tests for Bioactivity: Qualitative tests for bioactivity of the components which were separated on preparative TLC plates were done by scraping various amounts of the silica gel onto agar plates seeded with *S. cerevisiae* ATCC 9763. The plates were then incubated at 27°C for 24 hours and observed for inhibition of growth.

Minimum Inhibitory Concentrations (MIC): The *in vitro* activities of the antibiotic complexes were determined using the agar dilution procedure of LECHEVALIER *et al.*[35]. A stock solution of each antibiotic was prepared in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml, calculated on the basis of pure material having $E^{1\%}_{1cm}=1{,}000$. Dilutions of this solution were made by adding distilled water and mixing with an agar medium to obtain final concentrations of 100, 60, 30, 10, 6, 3, 1, 0.6, 0.3, 0.1, 0.06, 0.03, and 0.01 μg/ml of pure material. Bioassays were performed using nutrient agar for bacteria and Sabouraud dextrose agar for fungi. The bacteria were incubated at 37°C and yeasts and filamentous fungi at 27°C. MIC's were estimated at 24 hours for bacteria and yeasts and at 48 hours for filamentous fungi.

High Performance Liquid Chromatography (HPLC)

A Varian 500 Liquid Chromatograph equipped with a SP4100 computing integrater was employed for this study. A prepacked Lichrosorb RP-18 column (10 μm, 250 mm×4.6 mm (ID)) was obtained from E. Merck, Darmstadt, FRG. The UV detector was an LDC Phosphor converted to a 350 nm UV monitor featuring an 8 μl cell and a double beam optical system. The solvent system employed consisted of a mixture of acetonitrile and 0.06 mol ammonium citrate (45: 55) at a final pH of 6.1. Only HPLC grade solvents and certified ACS grade salts were used in the preparation of the HPLC solvent systems. Prepared solvents were kept at 4°C. The chromatograph was operated at pressures of 70.3~84.4 kg/cm^2 supplied by a pump and at a flow rate of 1 ml/minute. The attenuation of the UV detector was 0.08 unit full scale.

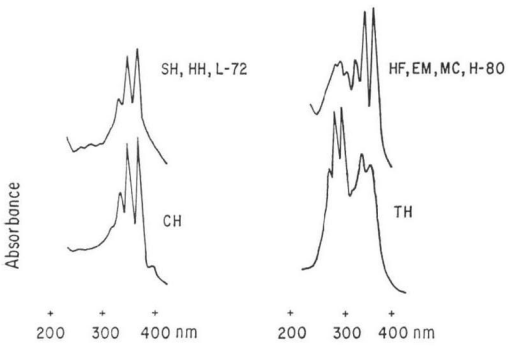
Antibiotic stock solutions were prepared by dissolving 10 mg of each antibiotic in 10 ml of MeOH. These solutions were filtered using Millipore microfilters (Millex-SR, 0.5 μm) to remove insoluble particles. Injections were made using a 10 μl sampling syringe (Precision, Baton Rouge, LA). Samples of components of the antibiotics which are described earlier were also run to observe and compare individually.

Results

Spectrophotometry

The UV-visible spectra of the nine hexaenes used in this study are shown in Fig. 1. Mediocidin, endomycin, hexaene-80, hexafungin, and tetrahexin each have a significant tetraene spectrum in addition to a hexaene spectrum. The hexaene spectrum is predominant in all except tetrahexin which has much higher absorption in the tetraene region. Candihexin, L-72, hydrohexin and streptohexin showed no tetraene absorption peaks. Candihexin has a very small peak at 405 nm which suggests the presence of a minor heptaene component. Dermostatin, a conjugated hexaene, has a single absorption peak[38].

Fig. 1. UV-visible spectra of the hexaenes studied. (Abbreviatons: see Table 2).



The peaks of L-72 are 5~7 nm higher than the peaks of normal hexaenes.

Biologically active fractions of the antibiotic complexes isolated on preparative TLC plates demonstrated spectra similar to the original complexes. It was not possible to isolate separate tetraene and hexaene components from the complexes.

Minimum Inhibitory Concentrations

The results of MIC determinations are presented in Table 2. As a group the hexaenes have activity against Gram-positive bacteria as well as against fungi. No other group of polyenes has antibacterial activity, although there are a few individual exceptions (*e.g.* lienomycin, a pentaene)[37].

Table 2. MIC's of the hexaenes against various microorganisms (calculated μg/ml on equal OD).

Organisms*	MC**	EM	H-80	HF	TH	CH	L-72	HH	SH
Bacillus subtilis (ATCC 9763)	1	0.3	1	0.3	0.1	30	10	6	40
Bacillus megaterium (IMRU 10)	0.6~1	0.3	0.6	0.3	0.3	30	—	6	40
Staphylococcus aureus (ATCC 6537P)	1	0.6	10	0.6	10	30	100	10	>100
Micrococcus luteus (IMRU 14)	3	0.6	3	—	0.6	30	10	100	>100
Escherichia coli B (PP 01)	>100	>100	>100	—	>100	>100	>100	>100	>100
Saccharomyces cerevisiae (ATCC 9763)	0.01	0.06	0.1	0.1	0.06	3	10	30	>100
Candida albicans (IMRU 204)	0.01	0.3	0.06	0.1	0.3~0.6	3	10	30	>100
Candida utilis (NRRL Y-900)	0.3	0.3	0.06	0.6	0.3	3	10	30	100
Candida lipolytica (PP 47)	0.1	0.06	0.3	0.1	0.6	>10	—	—	>100
Aspergillus foetidus (ATCC 10254)	0.3	3	0.06	0.3	0.06	10	>100	10	>100
Aspergillus niger (PP 29)	0.3	3	0.06	>0.3	0.06	10	>100	30	>100
Aspergillus sulfureus (PP 17)	0.01	0.3	0.03	0.3	0.06	10	>100	100	>100
Penicillium chrysogenum (ATCC 12690)	0.01	0.03	0.03	0.1	0.06	6	>100	100	>100
Penicillium terrestre (PP 84)	0.03	0.03	0.03	0.3	0.1	6	10	10	80
Trichoderma viride (PP 24)	0.06	—	0.06	—	0.1	10	>100	—	—

* Sources of organisms. ATCC: American Type Culture Collection, IMRU: Waksman Institute of Microbiology, Rutgers University, PP: Pilot Plant of Waksman Institute of Microbiology, Rutgers University, NRRL: Northern Regional Research Laboratories.

** MC: Mediocidin, EM: endomycin, H-80: hexaene-80, HF: hexafungin, TH: tetrahexin, CH: candihexin, L-72: L-72, HH: hydrohexin, SH: streptohexin.

The nine hexaenes can be placed in three subgroups based on their levels of activity. Mediocidin, endomycin, hexaene-80, hexafungin and tetrahexin are very active, comparable to the most active heptaenes (*e.g.* candicidin and trichomycin)[4] against fungi. Most members of this group are several fold more active against fungi than against Gram-positive bacteria. Tetrahexin has been shown to cause leakage of potassium but not phosphate ions from *B. subtilis*[12]. Candihexin has only moderate activity, somewhat more against fungi than against Gram-positive bacteria. Hydrohexin, streptohexin and L-72 have very low activity against both fungi and Gram-positive bacteria.

TLC, Spectrodensitometry and Bioactivity Tests

The individual components of the antibiotics were isolated on preparative TLC plates (0.5 mm thickness) using solvent system A. These samples were used later for HPLC analysis. The location of each component was determined by scanning at 380 nm in a spectrodensitometer. A small amount of silica gel for each component was scraped off and placed on a plate seeded with *S. cerevisiae* as a test of activity. The spectrodensitometric scans are given in Fig. 2. The minor components of tetrahexin showed activity as did the major components of hydrohexin and streptohexin.

In order to verify the activities thus found, bioautograms were run with analytical plates (0.25 mm) using solvent system B which contained no ammonia. The spectrodensitometric scans are given in Fig. 3. The Rf values of the active components of the complexes studied here are reported in Table 3.

Although the procedures for these two studies of hexaene fractions were different (*i.e.* thickness of TLC plates, solvent systems, and bioassay method), the results were quite similar. A shift of the location of the active component can be noticed in the case of tetrahexin. Minor variations in the locations of the similar peaks of the other antibiotics can also be observed.

Fig. 2. Spectrodensitometry of the hexaenes in solvent system A at 380 nm.

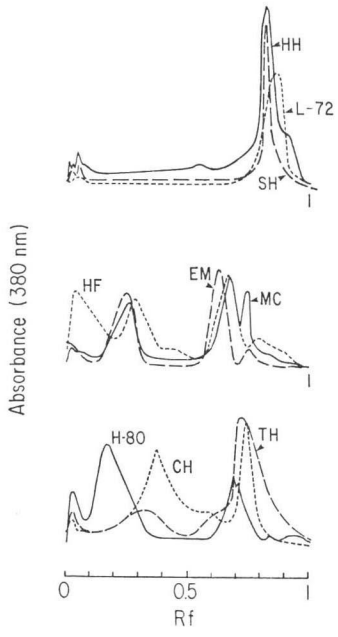


Fig. 3. Spectrodensitometry of the hexaenes in solvent system B at 380 nm.

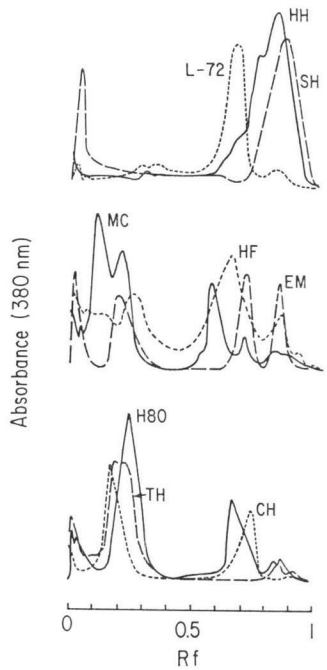


Table 3. Rf values and retention times of active components of hexaene antibiotic complexes.

Anti-biotic	Rf (TLC)		Retention time (HPLC) (minutes)	
	A*	B*	Complex	Individual components
L-72	0.85	0.7	6.9	—
CH	0.37	0.2	7.9	—
TH	0.3	0.25	11.4	—
	0.7	—	—	—
SH	0.8	0.9	5	—
HH	0.8	0.85	5.8	—
H-80	0.2	0.25	5.4	—
	0.7	0.65	3.7	—
EM	0.25	0.2	9.3	9.6
	0.65	0.7	5.1	5.2
HF	0.3	0.25	8.7	9.6
	0.65	0.65	4.9	5.2
MC	0.25	0.1	9.5	9.6
	—	0.2	8	—
	0.65	0.6	4.7	—
	0.75	0.7	—	—
	0.85	0.85	—	—

* Solvent system.

Fig. 5. HPLC of the similar active components of endomycin, hexafungin and mediocidin and their mixture.

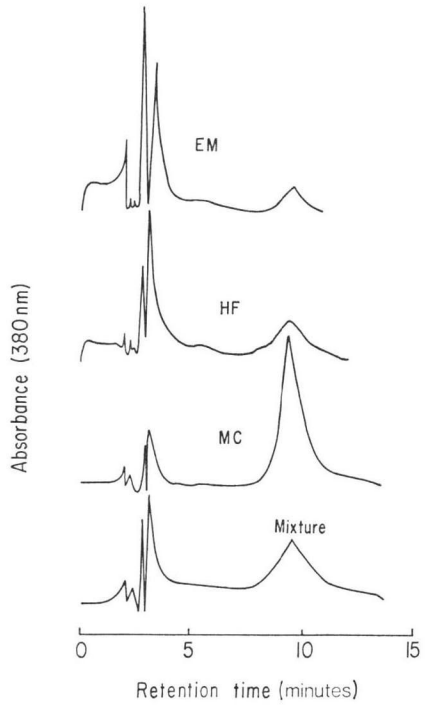


Fig. 4. HPLC of the hexaene complexes. (The shaded areas indicate bioactivity)

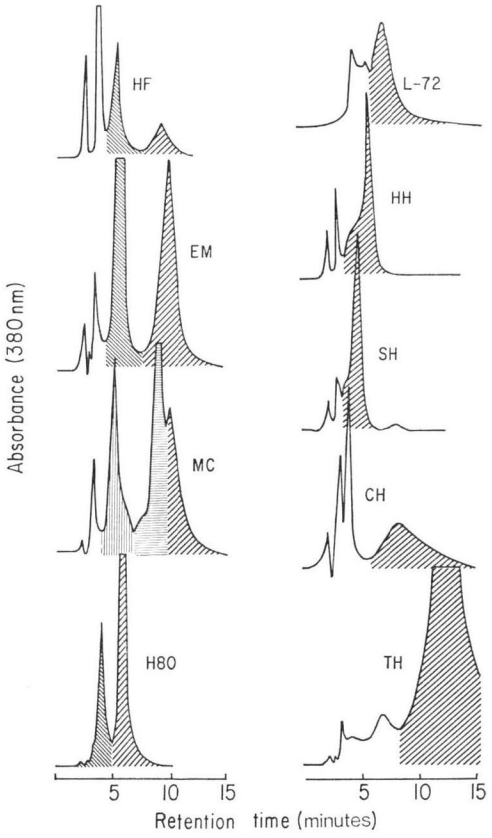
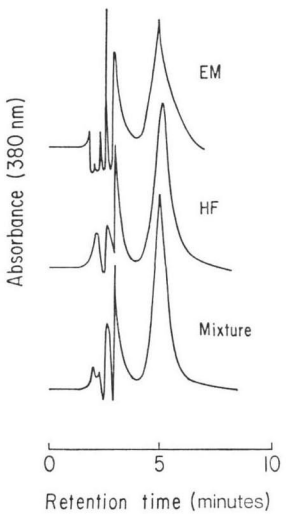


Fig. 6. HPLC of the second similar components of endomycin and hexafungin and their mixture.



High Performance Liquid Chromatography

HPLC of the antibiotic complexes and their fractions was performed. The scans of the complexes are given in Fig. 4. One component of endomycin, hexafungin, and mediocidin were similar on the HPLC scan as they were by TLC (Fig. 4). They were also similar when individual components, as well as a mixture of the three, were run by HPLC (Fig. 5). Similarly, another component of endomycin and hexafungin behaved the same in HPLC scans when run separately or as a mixture (Fig. 6). When other components which were suspected of being the same were run together more than one peak appeared, indicating that they were not identical. The retention time of each active component is reported in Table 3.

Discussion

MARTIN and MCDANIEL[28] reported that there are two active and two inactive components in the candihexin complex. In this study only one active and one inactive component were isolated. This difference is probably due to the use of different solvent ratios in these studies. The objective of this investigation was not to find the solvent system which separated the most fractions of each antibiotic complex but to compare them using a common solvent system for all. PATEL[10] reported that there were five major fractions recovered from the mediocidin complex by using preparative HPLC and TLC systems. Our studies also revealed the presence of five active components.

On the basis of this study, ten hexaenes were assigned to three subgroups (see Table 4). Dermostatin, although not available for this study, is included in our summary since it is known to be distinctly different from the other nine. The members of group I, which showed the highest bioactivity, contained more than one active component and each showed a tetraene spectrum in addition to a hexaene spectrum.

An active component was found to be common in hexafungin, endomycin and mediocidin. In addition, the second active components of hexafungin and endomycin were found to be similar. It is possible to conclude that there is no significant difference between endomycin and hexafungin. Although some of the other antibiotics showed similarities when only TLC's were available, it was possible to find differences using HPLC techniques (see Table 3). Tetrahexin is distinctly different from the other members of group I in absorption spectrum and HPLC pattern. The two members of group II

Table 4. Classification of hexaene antibiotics.

- I. High activity against fungi and moderate activity against Gram-positive bacteria — both hexaene and tetraene absorption spectra
  - A. Hexaene peaks considerably higher than tetraene peaks
    1. Mediocidin. 2. Endomycin. 3. Hexafungin. 4. H-80.
  - B. Tetraene peaks considerably higher than hexaene peaks
    1. Tetrahexin (tetraesin)
- II. Moderate activity against fungi and Gram-positive bacteria — no tetraene peaks
  - A. Absorption peaks at 379, 357, 340 and 323 (sh) nm (small at 405 nm)
    1. Candihexin
  - B. Single major peak at 384~386 nm (carbonyl hexaene)
    1. Dermostatin
- III Low activity against fungi and Gram-positive bacteria — no tetraene peaks
  - A. Soluble in H_2O (17 mg/ml)
    1. Hydrohexin
  - B. Not soluble in H_2O
    1. Absorption peak at 380 nm.
       a. Streptohexin
    2. Absorption peak at 385 nm.
       a. L-72

are different in that candihexin is a normal hexaene and dermostatin, a conjugated hexaene. The three members of group III are distinctly different; L-72 has a peak at 385 nm, which is 5~7 nm higher than normal hexaenes. Hydrohexin gives a true solution in water at low concentrations, and streptohexin is insoluble in water.

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