KALAFUNGIN, A NEW BROAD SPECTRUM ANTIBIOTIC

ISOLATION AND CHARACTERIZATION

M. E. BERGY

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan, U.S.A.

(Received for publication June 20, 1968)

Kalafungin is a new antibiotic extracted from the fermentation broth of *Streptomyces tanashiensis* strain Kala, and purified by chromatography on silica gel followed by crystallization. It is a weakly acidic, orange colored compound, pKa=10.0, with a molecular formula $C_{16}H_{12}O_6$ and a molecular weight of 300. It is dextrorotatory in chloroform, $[\alpha]_D^{25}+159$, and exhibits ultraviolet and visible absorption maxima at 212, 256, and 425 mμ in methanol.

Kalafungin melts at 163~166°C and is readily soluble in ethyl acetate, chloroform, and acetone but only slightly soluble in water.

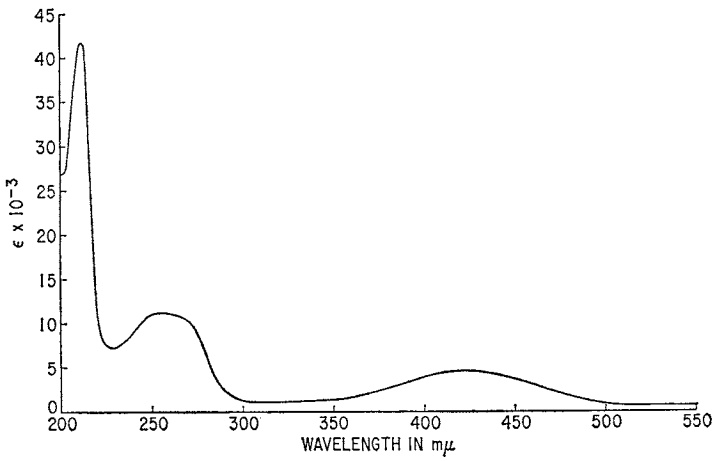
This paper describes the isolation and characterization of a new antibiotic, Kalafungin, which is produced in the fermentation broth of *Streptomyces tanashiensis* strain Kala. Its fermentation and biological properties are described by JOHNSON and DIETZ[1].

Isolation and Purification

A fermentation broth filtrate was extracted with petroleum hexane followed by methylene chloride. The latter extract was concentrated *in vacuo* with water to an aqueous solution which was freeze dried.

The impure kalafungin was purified by chromatography on silica gel buffered with KH_2PO_4 and Na_2HPO_4 with a solvent system composed of ethyl acetate and cyclohexane (1 : 3). On cooling, kalafungin crystallized from a concentrate of the combined active fractions.

Fig. 1. Ultraviolet absorption spectrum of kalafungin in methanol.



Characterization

Kalafungin is an orange-colored crystalline compound that is soluble in ethyl acetate, amyl acetate, chloroform, methylene chloride and methyl ethyl ketone, but only slightly soluble in water.

Fig. 2. Infrared absorption spectrum of kalafungin as a Nujol mull.

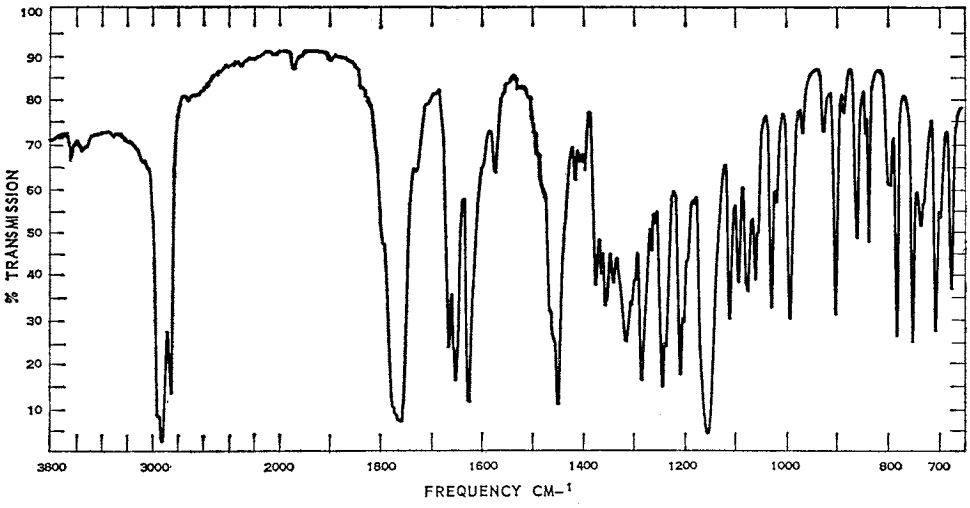
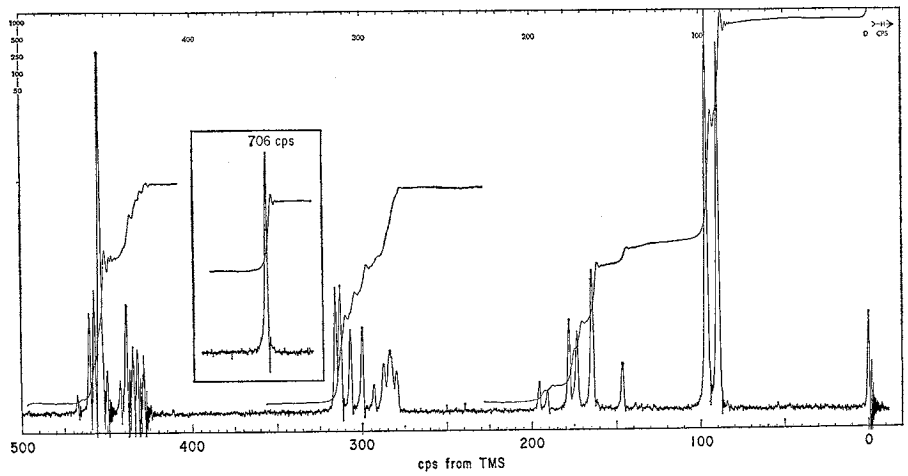


Fig. 3. Nuclear magnetic resonance spectrum of kalafungin in deuterated chloroform.



The equivalent weight determined by potentiometric titration is 288, and the molecular weight by mass spectrometry is 300. Based on the latter, elemental analysis indicates an empirical formula of $C_{16}H_{12}O_6$.

Kalafungin shows the following ultraviolet and visible absorption:

Methanol:	Max. at 212 mμ, ε=41,574
	Max. at 256 mμ, ε=11,016
	Infl. at 268 mμ, ε=10,557
	Max. at 425 mμ, ε= 4,452
0.01 N HCl in Methanol:	Max. at 212 mμ, ε=39,912
	Max. at 256 mμ, ε=10,998
	Infl. at 268 mμ, ε=10,443
	Max. at 425 mμ, ε= 4,458
Water:	Max. at 213 mμ, ε=37,326
	Infl. at 260 mμ, ε=11,223

Max. at 265 mμ, ε=11,265
Max. at 428 mμ, ε= 4,101

0.01 N KOH: Max. at 219 mμ, ε=33,138
Max. at 275 mμ, ε=10,791
Max. at 525 mμ, ε= 4,974

The ultraviolet absorption spectrum in methanol is shown in Fig. 1, the infrared absorption spectrum in Nujol mull in Fig. 2, and the nuclear magnetic resonance spectrum in deuterated chloroform in Fig. 3 (chemical shifts expressed from tetramethylsilane). Kalafungin melts at 163~166°C and has a specific rotation of $[\alpha]_D^{25}+159$ (1 %, $CHCl_3$).

Experimental

Isolation from Fermentation Broth

The whole broth (5,000 liters) was filtered at pH 7.1 with 5 % (w/v) diatomaceous earth filter aid. Impurities were first extracted from the clear broth with petroleum hexane (600 liters discarded), and the kalafungin was then extracted with methylene chloride (3,000 liters). The latter extracts were mixed with water and concentrated *in vacuo* to an aqueous solution which was freeze-dried. The dried impure kalafungin weighed 535 g and contained 160 mcg of kalafungin per mg.

Silica Gel Chromatography and Crystallization

The kalafungin present in impure preparations was purified by silica gel chromatography and crystallization. Five kg of silica gel #7734 (E. Merck AG, Darmstadt, Germany) was mixed with 4.0 liters of buffer solution which contained 136 g of KH_2PO_4 and 142 g of Na_2HPO_4 (pH 6.7). The moist gel mixture was dried initially at <100°C followed by activation at 120~130°C for approximately 2 hours. After cooling, the dry buffered silica gel was mixed with cyclohexane and poured into a 10 cm diameter glass column and packed to a constant height of 113 cm with flowing cyclohexane.

Crude kalafungin (565 g) was dissolved in 1.0 liter of acetone and mixed with 1.0 kg of buffered and activated silica gel #7734 which had been prepared as above. The acetone was removed by evaporation at room temperature, and the dried mixture was distributed evenly into the layer of cyclohexane remaining on top of the column bed. The level of cyclohexane was drained to within 6~8 cm of the column load, and a solvent mixture composed of ethyl acetate and cyclohexane (1 : 9) was layered onto the layer of cyclohexane. The flow was started and the column was washed with 20 liters of this solvent followed by development with a mixture of ethyl acetate and cyclohexane (1 : 3). Fractions (4 liters) were collected after the addition of the developing solvent and were designated as liters of effluent. The kalafungin was detected in the fractions by saturating 12.7 mm assay discs with liquid from each fraction and placing the dried discs on a agar tray which has been seeded with *S. pastorianus*. Fractions 43~95 contained the kalafungin and they were refrigerated overnight whereupon kalafungin crystallized from fractions 47~58. The crystals were removed by filtration, washed with cyclohexane, and dried *in vacuo* to a constant weight (21.7 g). U. V.: (MeOH) Max. 258 mμ, ε=10,200.

The mother liquor from fractions 47~58 and the fractions 43~46 and 59~86 were combined and the solution was concentrated to a volume of 750 ml. Two liters of ethyl acetate was added and the solution was clarified by filtration. Three liters of cyclohexane was added and the solution was concentrated *in vacuo* to volume of 2.0 liters and stored overnight at 5°C. The crystalline kalafungin was removed by filtration, washed with cyclohexane and dried *in vacuo* to a constant weight (55.5 g). U. V.: (MeOH) Max. 258 mμ, ε=11,100.

Analysis : $C_{16}H_{12}O_6$ Calculated : C 63.99, H 4.03, O 31.97
Found : C 63.80, H 4.07, O 31.81

Thin-Layer Chromatography

Kalafungin was analyzed by thin-layer chromatography on plates prepared with silica gel HF_{254} (E. Merck, A. G.-Darmstadt, Germany) suspended in a solution of buffer salts consisting of equal volumes of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 (pH 6.7). The plates were activated at 120~130°C for 2 hours prior to use. Development was with ethyl acetate and cyclohexane (1 : 1). Visualization by ultraviolet light (254 mμ) and potassium permanganate-sodium metaperiodate spray[2] revealed only the presence of kalafungin (Rf, 0.4).

Acknowledgements

The author wishes to thank members of The Upjohn Company who contributed to this work ; in particular W. D. MAXON and F. L. CUNNINGHAM and their associates for large scale preparations and W. A. STRUCK and his associates for microanalyses.

References

1) JOHNSON, L. E. & A. DIETZ : Kalafungin, a new broad spectrum antibiotic. Taxonomy, fermentation and biological properties. In preparation, 1968.
2) LEMIEUX, R. U. & H. F. BAUER : Spray reagent for the detection of carbohydrates. Anal. Chem. 26 : 920~921, 1954.