[CONTRIBUTION FROM THE UPJOHN CO. AND NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Filipin, an Antifungal Antibiotic: Isolation and Properties

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A new antifungal antibiotic, filipin, has been obtained from a streptomycete isolated from Philippine soil. Its analysis best fits the empirical formula $C_{30}H_{50}O_{10}$ and it has been characterized as a conjugated polyene. The isolation and properties of this antibiotic are reported here.

Filipin, a potent antifungal agent, has been isolated from the mycelium and culture filtrates of a previously unreported actinomycete, *Streptomyces filipinensis*, found in a sample of Philippine soil.[2]

Crystalline filipin is a yellow, neutral compound whose analysis corresponds to an empirical formula of $C_{30}H_{50}O_{10}$, emp. wt. 571. The absence of nitrogen, sulfur and halogens, together with the high oxygen-to-carbon ratio makes filipin an extremely interesting metabolite. Filipin is non-aromatic and is unsaturated, taking up approx. 4 moles of hydrogen with either platinum oxide catalyst or 3% palladium on carbon catalyst based on a molecular weight of 571.

Many groups of polyene antifungals are reported in the literature. Vining,[3] Oroshnik,[4] and Umezawa,[5] have grouped most of these antifungals into families based on the position of their ultraviolet maxima. Filipin and fungichromin[6] appear to be the first reported members of a new family.

Filipin has an ultraviolet spectrum typical of a long chain polyene. In methanolic solution, crystalline filipin has three characteristic maxima, $E^{1\%}_{1\ cm.}$ 1330 at 355 mμ, 1360 at 338 mμ, and 910 at 322 mμ. A shoulder appears at 305 mμ (Fig. 1). This spectrum does not change in 0.1 *N* KOH or 0.1 *N* HCl. The failure of the ultraviolet bands to shift with *p*H is consistent with a structure having only C=C conjugation.

In common with other polyenes, filipin is susceptible to autoxidation and particularly so when exposed to light. Thus, solid filipin preparations lose activity in air in clear glass bottles, but not under nitrogen even when exposed to direct sunlight. Filipin is stable in the dark in air at refrigerator temperatures. It is relatively stable in very dilute solutions in ethanol (50 mcg. per ml.) but is unstable at 10 mg. per ml. under all conditions tested. Filipin deteriorates slowly in aqueous methanolic solutions at *p*H 2 and at *p*H 11 (28 and 7% loss of activity, respectively, in 3 days at 25°). Filipin is very soluble in dimethylformamide and pyridine. It is soluble in 95% ethanol, methanol, *n*-butanol, isopropyl alcohol, *t*-butyl alcohol, glacial acetic acid, ether, ethyl acetate and amyl acetate. It is nearly insoluble in water, chloroform, 50% ethanol, methylene chloride and Skellysolve B.

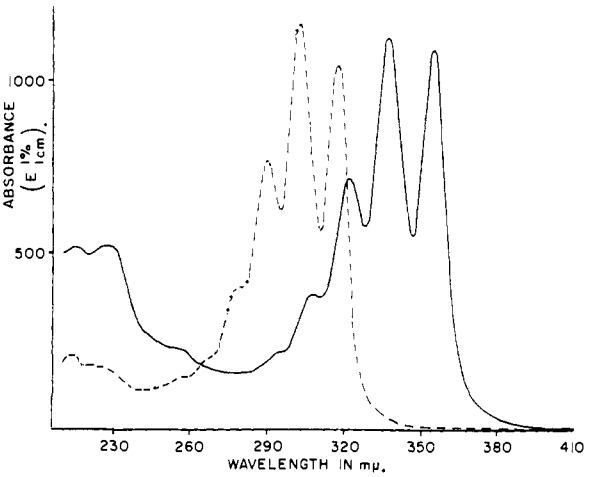

Fig. 1.—Solid line, filipin (10 mcg./ml.) in methanol; broken line, filipin degradation product (10 mcg./ml.) in methanol.

Fusion studies indicate that filipin exists in 2 solid modifications. It undergoes transition to a second form at 147°. This second form melts at 195–205° dec. Ultraviolet absorption spectra on the material converted to the higher form and allowed to cool indicate a partial degradation.

The infrared absorption spectrum of filipin in Nujol suspension (Fig. 2) shows bands at 3580 and 3360 cm.$^{-1}$, characteristic of OH groups. A broad general absorption extends to about 1715 cm.$^{-1}$, indicating a conjugated or non-conjugated lactone, or a non-conjugated ketone. A band at 1177 cm.$^{-1}$ is characteristic of the C–O of an ester or lactone. The bands at 1137, 1085, 1040 and 1005 cm.$^{-1}$ probably indicate R–OH or R–OR groups. The band at 840 cm.$^{-1}$ is indicative of isoprenoid groupings. A 5% solution of filipin in dimethylformamide has characteristic bands at 1303, 1160, 1005, 958 and 846 cm.$^{-1}$.

Filipin has a specific rotation of $[\alpha]^{22}D$ −148.3 (*c* 0.89 in methanol); it gives a positive Molisch test and negative ninhydrin, Benedict, anthrone, ferric chloride and 2,4-dinitrophenylhydrazine reactions.

Because a crystalline 2,4-dinitrophenylhydrazone does not form, the carbonyl absorption is probably not due to a ketone, but to an ester or lactone. Filipin contains no O–CH_3 groups but approx. 2CCH_3 groups per $C_{30}H_{50}O_{10}$.

(1) Communications should be addressed to: Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

(2) A. Ammann, D. Gottlieb and H. E. Carter, *Plant Disease Reporter*, **39**, 219 (1955).

(3) L. C. Vining, W. A. Taber and H. A. Lechevalier, *Congr. intern. Botanique*, 106 (1954).

(4) W. Oroshnik, L. C. Vining, A. D. Mebane and W. A. Taber, *Science*, **121**, 147 (1955).

(5) Y. Okami, R. Utahara, S. Nakamura and H. Umezawa, *J. Antibiotics (Japan)*, Ser. A, **7**, 98 (1954).

(6) A. A. Tytell, F. J. McCarthy, W. P. Fisher, W. A. Bolhofer and J. Charney, "Antibiotics Annual, 1954–1955," Medical Encyclopedia, Inc., New York, N. Y., 1955, p. 716. We are indebted to Dr. A. A. Tytell, Sharp & Dohme Division, Merck & Co., for a sample of authentic fungichromin.

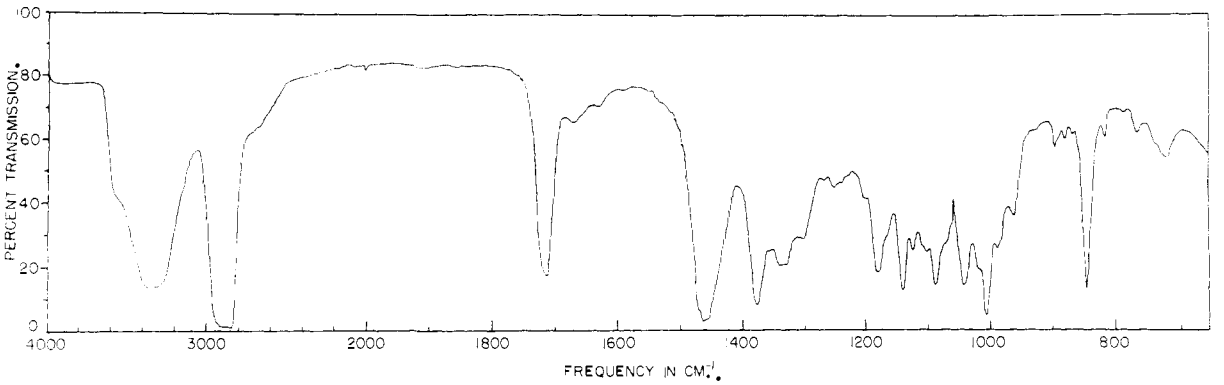

Fig. 2.—Filipin in Nujol mull.

Filipin may be differentiated from fungichromin which it resembles very closely. Fungichromin demonstrates only one allotropic modification, has distinct differences in its infrared absorption spectrum and is separable from filipin on papergrams developed with acidic or basic wet *n*-butanol. In both systems filipin has an R_f of 0.85, fungichromin, 0.70.

Filipin degrades in two different ways. Under certain conditions of heat and light, the biological activity and ultraviolet absorption maxima disappear simultaneously. The second and more striking degradation occurs when concentrated methanolic or ethanolic solutions are allowed to stand at 4°. A transition takes place, affording a white, crystalline substance, whose analysis indicates $C_{30}H_{50}O_{11}$. Neither the solid material nor more dilute solutions (10 or 50 mcg./ml.) show this shift but degrade in the customary fashion. The mother liquor contains only traces of filipin.

This white degradation product has no antifungal activity, but is still an unsaturated polyene as evidenced by its ultraviolet absorption spectrum and hydrogenation studies. The degradation product shows the following maxima in the ultraviolet: $E^{1\%}_{1\ cm.}$ 1050 at 318 mμ, 1170 at 303 mμ, 770 at 290 mμ, and a shoulder at 281 mμ (Fig. 1). The quantitative transition of filipin to this new compound is non-reversible and seems to be autocatalytic. The infrared spectrum of this material is nearly identical with that of filipin. The carbonyl and C–O positions and intensities are not much changed. The degradation product in Nujol suspension shows bands at 3590, 3370 and 3170 cm.$^{-1}$ characteristic of OH groups. A broad, general absorption extends to about 1722 cm.$^{-1}$ and is characteristic of carbonyl. The spectrum shows characteristic bands at 1162, 1137, 1095, 1045 and 1005 cm.$^{-1}$. Additional bands at 849 and 845 indicate the presence of the isoprenoid grouping. These data are consistent with the hypothesis that a carbon–carbon double bond has been removed from conjugation with the polyene chromophore.

Chemical tests performed on the degradation product have given the same results as noted for filipin. However, filipin dissolves in concentrated H_2SO_4 with the formation of a deep blue-purple color, whereas the degradation product dissolves with a wine-red color. Catalytic hydrogenation of the degradation product with platinum results in an uptake of only 3.3 moles of hydrogen; with 3% palladium on carbon, 2.9 moles, based on a molecular weight of 571.

Experimental

Assay.—Filipin is bioassayed by the agar diffusion method using filter paper discs against *Saccharomyces pastorianus* ATCC 2360 or *Penicillium oxalicum*. When correlation was established between the 338 and 355 mμ maxima in the ultraviolet and the antifungal activity, routine use was made of this relationship as an assay and excellent results were obtained even from dilutions of culture broths. Crystalline filipin serves as the standard in both assays.

Fermentation.—Filipin was produced on a small scale in shake flasks. One hundred ml. of a selected medium in a 500-ml. erlenmeyer flask was inoculated with a 3-day-old shake culture of *S. filipinesis* NRRL 2437, and incubated for 4 days at 28° on a rotary shaker.

Isolation of Filipin from the Mycelium.—On a somewhat larger scale, a 6,000-l. aliquot of a typical culture broth was mixed with 500 lb. of diatomaceous earth[7] and filtered. The filter cake was extracted three times with a total of 646 l. of *n*-butanol. The resulting two-layer extract containing the antibiotic activity was centrifuged in a DeLaval separator to remove the aqueous layer which was discarded. The clarified butanol was concentrated *in vacuo* to 90 l. and this solution was added to 180 l. of petroleum ether.[8] The resulting precipitate was filtered, washed with 38 l. of petroleum ether, and dried *in vacuo* to afford 3.3 kg. of amorphous filipin, 76% pure.

Isolation of Filipin from the Culture Filtrate.—A typical culture broth, 250 l. was mixed with 20 lb. of diatomaceous earth and filtered. The culture filtrate and the cake wash were saturated with ethyl acetate and then extracted with 60 l. of ethyl acetate. The spent beer was inactive and was discarded. The ethyl acetate extract was concentrated *in vacuo* and the residue was extracted with 1.5 l. of petroleum ether. The resulting mixture was filtered, washed with 50 ml. of petroleum ether and the amorphous material was dried *in vacuo*. The yield was 35 g. of amorphous material, 70–80% pure.

Crystallization of Filipin.—Twenty grams of the amorphous antibiotic was slurried with two 370-ml. portions of chloroform, the filipin forming a suspension of fine, feathery needles. The crystalline filipin was filtered from the yellow mother liquor, washed with 10 ml. of chloroform, two 10-ml. portions of petroleum ether, and dried *in vacuo*. The yield was 17 g. of crystals (m.p. 195–205°).

A sample was dried for 48 hours at 37° *in vacuo*.

Anal. Calcd. for $C_{30}H_{50}O_{10}$: C, 62.96; H, 8.83; sapon. equiv., 570.7. Found: C, 62.95; H, 8.88; sapon. equiv., 574.

Hydrogenation.—Three grams of filipin was dissolved in 200 ml. of 25% dimethylformamide in methanol. The solution was hydrogenated at 30 lb. hydrogen pressure with 1 g. of 10% palladium on charcoal as catalyst for 2 hours.

(7) Dicalite 4200, trade-mark, Great Lakes Carbon Co., Chicago, Illinois.

(8) Skellysolve B, trade-mark, Skelly Oil Co., Kansas City, Missouri.

The catalyst was filtered and the resulting solution was concentrated and freeze-dried. Three grams of a light yellow, amorphous powder was obtained. It contained less than 0.2% residual filipin and was inactive against *S. pastorianus*. The 2,4-dinitrophenylhydrazine and ferric chloride tests were negative.

Anal. Calcd. for $C_{30}H_{58}O_{10}$: C, 62.25; H, 10.11. Found: C, 62.88; H, 10.26.

Preparation of the Degradation Product.—Five grams of crystalline filipin was suspended in 350 ml. of 95% ethanol, stirred, and filtered. The filtrate was concentrated in a nitrogen stream to 220 ml. and the clear solution was placed in the deepfreeze overnight. The feathery, white crystals were filtered, washed with ice cold ethanol, ether, and air-dried. Successive crops were obtained totaling 705 mg. (m.p. 195–205°; specific rotation, 0).

Anal. Calcd. for $C_{30}H_{50}O_{11}$: C, 61.41; H, 8.59. Found: C, 61.28; H, 8.75.

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Alkaloid Studies. VIII.[1] The Structures of the Diterpenoid Alkaloids Laurifoline and Cuauchichicine

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The isolation and structure elucidation of two new diterpenoid alkaloids, laurifoline and cuauchichicine from *Garrya laurifolia* Hartw. is described. Laurifoline (VI) is 19-epiveatchine and its ready isomerization with acid to cuauchichicine (V) and with hot alcohol to isolaurifoline (VII) is reported.

Garrya laurifolia Hartw., a tree, commonly known as "cuauchichic," is widely distributed throughout Mexico[4] and extracts of the bark are used in indigenous medical practice as an antidiarrhetic agent.[4] Our interest in this plant was stimulated by an earlier report[5] in which it was indicated that it contained one or more unidentified alkaloids and by Oneto's publication[6] on the isolation of two new alkaloids, veatchine and garryine, from the related *Garrya veatchii* Kellogg as well as from five other *Garrya* species.

Our initial isolation experiments, patterned after those of Oneto[6] and proceeding *via* the hydrochlorides, suggested that the alkaloid composition of *G. laurifolia* was quite similar to that of the other *Garrya* species. Shortly thereafter, the first of a series of outstanding papers appeared by Wiesner and collaborators[7a] culminating in the structure elucidation[7c,d] of veatchine (I) and garryine (II).[8] The Canadian investigators,[7c] recognizing the close similarity between the *Garrya* alkaloids and the atisines,[9] also proposed a skeletal structure for the latter class of alkaloids and supporting experimental evidence has recently been provided by Pelletier and Jacobs.[10]

The facile and nearly quantitative separation of the isomeric alkaloids veatchine (I) and garryine (II) by countercurrent distribution[7a] prompted us to apply a similar procedure to the crude alkaloids of *Garrya laurifolia*, which had been extracted with ethanol and subsequently separated with dilute *hydrochloric acid* (*vide infra*). From such a separation scheme, there was isolated a crystalline alkaloid, $C_{22}H_{33}NO_2$, which proved to be isomeric but not identical with veatchine and garryine. We have named this alkaloid "cuauchichicine" after the indigenous name ("cuauchichic") of the plant and our original structure studies were carried out with this substance.[11]

The most important difference between cuauchichicine and the other *Garrya* alkaloids (I, II) is demonstrated by the infrared spectrum (Fig. 1) which shows the complete absence of NH or OH absorption but a strong carbonyl band at 5.78 μ, which can be attributed to a five-membered ring ketone. The carbonyl group is moderately reactive, as shown by preparation of an oxime (of isocuauchichicine X); semicarbazone or 2,4-dini-

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(2) Eli Lilly Predoctorate Research Fellow, 1953–1955.

(3) Postdoctorate Research Fellow.

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(8) We are proposing a numbering system which follows as closely as possible that of abietane (W. Klyne, *J. Chem. Soc.*, 3072 (1953)) in order to emphasize the structural similarity to the diterpenes.

(9) *Cf.* E. S. Stern, in R. H. F. Manske and H. L. Holmes, 'The Alkaloids," Vol. IV, Academic Press, Inc., New York, N. Y., 1954, chapter 37.

(10) S. W. Pelletier and W. A. Jacobs, THIS JOURNAL, **76**, 4496 (1954).

(11) For preliminary communication see C. Djerassi, C. R. Smith, S. K. Figdor, J. Herran and J. Romo, *ibid.*, **76**, 5889 (1954). The constants (m.p. and $[\alpha]D$) for cuauchichicine reported in that communication have to be changed slightly since that material was probably contaminated with isocuauchichicine, which is described in the present paper.