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*ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES IX. DROSOPHILA SUBATRATA. (BATSCH EX FR.) QUEL.**

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Drosophila subatrata[1] was found earlier in this laboratory to evidence substantial antibacterial activity when grown on agar media or in corn-steep liquid media.[2] In further investigation culture liquids of the fungus grown in Fernbach flasks in a corn-steep medium[3] at 25°C. developed an activity of 1000 dilution units or more against *Staphylococcus aureus*. After reflooding the fungus mat with fresh corn-steep medium high activity was obtained in 6 to 15 days.

Concentrates prepared from the culture liquid by extraction with methylisobutyl ketone or chloroform had activities against *Staph. aureus* of from 1000 to 6400 dilution units per mg. However, the stability of antibacterial activity of culture liquid and concentrates was variable. The data obtained on loss of activity when the concentrates were incubated with blood and on toxicity to mice by intravenous injection were inconsistent. Further investigation showed the presence in the culture liquid of at least four antibacterial substances which differed in stability, activity and other properties.

Isolation of Active Substances.—An activity of 1000 dilution units per ml. for *Staph. aureus* was obtained in the corn-steep medium in two weeks in shake culture. However, liquid from still cultures in the corn-steep medium was used for the isolation of the active substances as follows:

The culture liquid was acidified to between pH 3 and 4 and extracted with about one-tenth volume of chloroform. The chloroform was extracted with about one-tenth volume of 0.5 *M* sodium bicarbonate solution (*first* bicarbonate extract). About 37 per cent of the solids and 20 per cent of the activity against *Staph. aureus* were removed by the bicarbonate.

Two crystalline antibacterial substances (drosophilin A and B) were prepared from the chloroform after extraction with the bicarbonate by the

following procedure. The extracted chloroform was evaporated to about one-tenth volume in a flash-evaporator. The resulting dark brown concentrate was extracted with about an equal volume of 0.5 *M* sodium bicarbonate (*second* bicarbonate extract). This bicarbonate extraction removed about one-fourth of the antibacterial activity in the chloroform concentrate as measured against *Staph. aureus* and about 3 per cent of the solids. The second bicarbonate solution contained a highly active substance (later called drosophilin C).

Drosophilin A.—The chloroform concentrate after extraction with bicarbonate was further extracted with 0.5 *M* pH 10 carbonate buffer which removed a small amount of an active substance (drosophilin A) with a characteristic absorption spectrum (peak at 301 mμ). Crystalline drosophilin A was isolated from the carbonate buffer by acidifying and extracting with ether; pale yellow crystals formed when the ether evaporated. About 100 mg. of these crystals were obtained from 31 liters of culture liquid.

The crystals were purified by solution in benzene and treatment with decolorizing carbon. The white crystals obtained were recrystallized from hexane or from dilute aqueous alcohol; they melted at 118°C. (corr.) and remelted at the same temperature. The specific rotation was less than 0.5. Drosophilin A was soluble in hexane, benzene, acetone, alcohol, chloroform, ether and slightly soluble in water. It was not extracted from ether solution by concentrated HCl which indicated lack of basic properties. It was weakly acidic since it was not removed in appreciable amounts by extraction of the chloroform solution with bicarbonate but was removed by extraction with carbonate pH 10 buffer.

Drosophilin A was analyzed for C, H, methoxyl and Cl, and the oxygen found by difference; the molecular weight was determined cryoscopically in exaltone.[4]

	C	H	Cl	O	METHOXYL	MOL. WT.
Found	32.54	1.71	52.68	13.07	11.70	249
Computed for $C_{13}H_9O_4Cl_7$	32.70	1.90	51.99	13.41	13.00	477
Computed for $C_7H_4O_2Cl_4$	32.10	1.54	54.15	12.22	11.85	262

The empirical formula $C_{13}H_9O_4Cl_7$ fits the analysis better than $C_7H_4O_2Cl_4$ but not the molecular weight. Further work on drosophilin A is being undertaken.

Drosophilin A gave the same color reaction with alcoholic ferric chloride as pentachlorophenol. The absorption peak (301 mμ) was in the region of absorption by chlorinated phenols. It did not precipitate with silver nitrate; it reduced alkaline permanganate; it reduced alkaline silver

nitrate with the formation of silver chloride. It did not react with 2,4-dinitro-phenyl hydrazine and did not give reactions for α,β unsaturated ketones. Infra-red analysis[5] indicated the presence of hydrogen attached to unsaturated carbon, a hydroxyl group and a methyl or methylene group.

Drosophilin B.—The chloroform concentrate after extraction with pH 10 buffer was extracted with 0.5 *M* sodium carbonate solution which removed a small residue of drosophilin A and colored substances. The chloroform was then extracted with 1 *N* KOH solution (which removed a small amount of colored substances), washed with water and acid and evaporated to dryness. The treatment with carbonate and hydroxide removed 17 per cent of the solids. The solids obtained by evaporation of the chloroform were dissolved in benzene and evaporated slowly. The crystals (drosophilin B) which formed were recrystallized from benzene and from dilute aqueous alcohol. The melting points, mixed melting points, analyses, molecular weight, infra-red and ultra-violet spectrum, solubilities, optical activity and the general chemical and biological properties showed drosophilin B to be identical with pleuromutilin.[6] The infra-red spectrum indicated the presence of hydroxyl, carbonyl and possibly an ester group.

TABLE 1.

POSITION OF ABSORPTION PEAKS IN mμ AND OPTICAL DENSITY FOR ALCOHOLIC SOLUTIONS OF DROSOPHILINS C AND D

C	207(0.72)	234(0.41)	269(0.31)	281(0.33)	
D	217(1.42)	259(0.33)	274(0.54)	290.5(0.76)	309(0.61)

Drosophilin C.—This substance which had the greatest antibacterial activity was obtained from the second bicarbonate extract. Since it was inactivated by drying in air it was necessary to avoid drying during isolation.

The acids in the bicarbonate extract were transferred to ether and the ether solution was shaken repeatedly with one-fourth volume of 0.1 *N* silver nitrate.[7] The yellow precipitate was removed each time and kept under water. The combined silver precipitates were washed with ether and water, ether added, the silver compound decomposed by addition of HCl and the organic acids transferred to ether. The substances in the ether were further purified by counter-current distribution between chloroform and 0.5 *M* phosphate buffer of pH 6.9. Most of the antibacterial activity and the solids were in the chloroform which had traveled through the system. This chloroform solution was distributed again in a four-funnel system using buffer of pH 7.5. The purest substance was found in the third funnel. A total of 4 mg. of drosophilin C was obtained from 31 liters of culture liquid. Probably at least as much was in the other fractions from the counter-current distribution system; the chemical properties indicate that drosophilin C should be found in other fractions and the

treatment with silver nitrate did not remove all of it from the ether solution.

The absorption spectrum was measured in alcoholic solution and the results reported (table 1) as optical density (in parentheses) for solutions with a concentration of 10 μg. per ml. in 1-cm. thick cuvettes.

Drosophilin D was obtained from the first bicarbonate extract. The extract was acidified and the acids extracted into chloroform. The chloro-

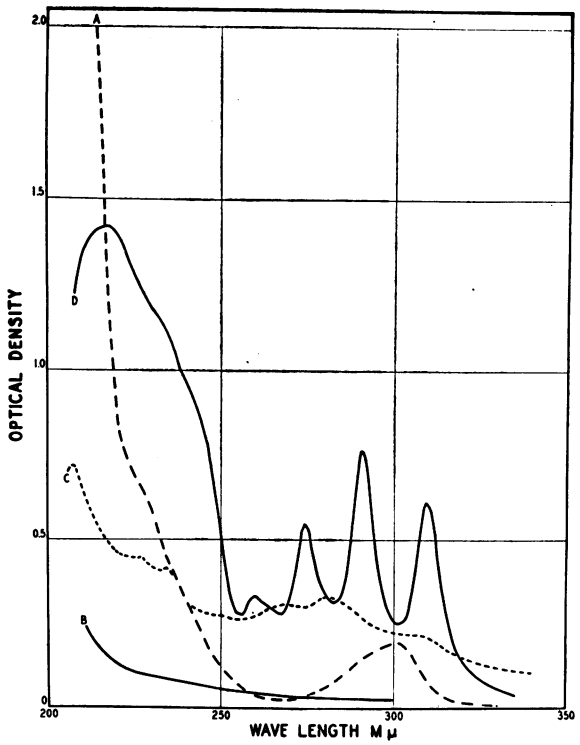

FIGURE 1

Absorption spectra of drosophilins A, C, and D measured in ethanol at a concentration of 10 μg. per ml. and B at a concentration of 100 μg. per ml.

form was shaken with one-fourth volume of pH 6 buffer which removed most of the colored substances but only traces of antibacterial activity. The chloroform was then extracted repeatedly with silver nitrate with removal of the precipitated silver compound before repeating the treatment with fresh silver nitrate. About three-fourths of the activity of the bicarbonate solution was lost at this step and most of that remaining was in the chloroform. The chloroform solution was treated to a counter-current

distribution using pH 7.2 buffer. A substance with an ultra-violet absorption spectrum[8] characteristic of polyacetylene substances was found in largest amount in the middle funnels. The distribution was repeated and material from the middle funnel of a five-funnel system was taken as representative of the active substance. This compound, drosophilin D, was soluble in organic solvents, somewhat soluble in water, was precipitated from aqueous alcoholic solution by silver nitrate as was drosophilin C. Drosophilin D titrated in 20 per cent ethanol as a monobasic acid with a pK of 4.2 and an equivalent weight of 405.

The absorption spectrum (table 1 and Fig. 1) was determined in the same way as for drosophilin C. The frequency differences of the position of the absorption peaks of drosophilin D are 224.2, 63.4, 62.2 and 61.8*f*. The latter three differences are characteristic of polyacetylenes.[8] Drosophilin D is the sixth polyacetylene (identified in this laboratory) which has antibacterial activity and is produced by species of Basidiomycetes.[9]

TABLE 2

MINIMUM INHIBITORY CONCENTRATION IN μg. PER ML.

BACTERIA	DROSOPHILIN A	B	C	D
Bacillus mycoides	64	125	16	125
Bacillus subtilis	32	16	0.12	2
Escherichia coli	250	250	16	250
Klebsiella pneumoniae	64	0.5	32	500
Mycobacterium smegmatis (smegma)	64	8	16	64
Pseudomonas aeruginosa	250	500	32	250
Staphylococcus aureus	4	0.5	0.03	4

Antibacterial Activity.—The activity of these four compounds against seven bacteria was measured by the methods used in this laboratory.[10] The substances were active mainly against Gram-positive bacteria.

Some culture filtrates had an antibacterial activity consistent with the assumption that most of the activity was due to drosophilin C.

The activity of the drosophilins was tested against 60 bacteriophages.[11] Drosophilin A was active against only staphylophage 8, drosophilin B (pleuromutilin) against streptophage 6 and 7; the other two drosophilins were inactive. Observations of the antibacterial activity were made incidental to the determination of antiphage activity. Drosophilin A was active against the strains of staphylococci and *B. subtilis* but inactive against streptococci and Gram-negative bacteria. Drosophilin B was active against most of the strains of bacteria. Drosophilin C showed some activity against all Gram-positive bacteria. Drosophilin D was active against all of the bacteria with activity greatest against Gram-positive bacteria.

Effect of Blood.—When the four drosophilins were incubated for four

hours with 5 per cent human blood in beef extract test medium, A and B were not inactivated, C was $^7/_8$ inactivated and D lost one-half of its activity. The inactivation of some culture filtrates by blood suggested that most of the activity was caused by drosophilin C.

Animal Toxicity and In Vivo Tests.[12]—Drosophilin B (pleuromutilin) was administered in single doses immediately after infecting mice with *Diplococcus pneumoniae* (ATCC 6302). The mice receiving the largest dose (1 mg.) did not live longer than the untreated controls. Drosophilin B had an *in vitro* activity of 3 μg. per ml. against *D. pneumoniae*. Drosophilin B was not toxic to the mice when given at 1 mg. to 20 g. mice by either the intravenous or the intraperitoneal routes. The lack of animal toxicity and the ineffectiveness of drosophilin B was another indication of its identity with pleuromutilin.

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[1] The culture of the organism was obtained from Dr. José Emilio Santos Pinto Lopes, Instituto Botanico, Lisbon, Portugal, to whom the authors express their thanks. The fungus is also known as *Agaricus subatratus* Batsch ex Fr. and *Psathyrella subatrata* (Batsch ex Fr.) Gell.

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[4] The quantitative analyses and molecular weight determinations were made by the Huffman Microanalytical Laboratories, Denver, Colo.

[5] Infra-red spectra and interpretations were made by Dr. H. L. Dinsmore, Commercial Solvents Corporation.

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[12] The authors are indebted to H. Payne and R. Baldwin, Commercial Solvents Corporation for these determinations.