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The Isolation and Properties of Antimycin A[1]

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In a previous investigation[3] antibiotic preparations effective against fungi were obtained from cultures of an unidentified species of *Streptomyces*. Because the properties of these preparations appeared unique, a name, antimycin, was proposed for the active material. Continued investigation has now resulted in the isolation of an active crystalline substance, which is described in this report. The name "antimycin A" is proposed for this crystalline substance, since there is evidence of additional antibiotic factors in the crude preparations.

The isolation was accomplished by means of solvent fractionation procedures, the active material being taken successively into alcohol, ether, and petroleum ether. Residues from the latter solvent were readily induced to crystallize.

Antimycin A is an optically active, nitrogenous phenol with the probable molecular formula $C_{28}H_{40}O_9N_2$. Its properties appear to differentiate it from other previously known antibiotics. It is an extremely potent fungicide, producing inhibitory effects against *Nigrospora sphaerica*, for example, at dilutions as high as 1:800,000,000.

Experimental

Culture Methods.—Aseptic technique was used throughout. Inoculum for large-scale fermentations was produced from a dried soil culture of the *Streptomyces* species by transferring into 30 ml. of a soybean medium containing 40 g. of soybean oil meal, 20 g. of glucose, and 1.5 g. of powdered calcium carbonate per liter. This culture was shaken at 26° on a reciprocating shaker with six inch strokes at 66 cycles per minute for forty-eight hours, and then distributed among four 2-l. Erlenmeyer flasks each containing 500 ml. of the same medium. These cultures were shaken for forty-eight hours as before, and were then pooled and used to inoculate 70 l. of the soybean medium in an iron fermentation tank.

Fermentation was allowed to continue at 24–28° for 80–96 hours with continuous stirring and with aeration at the rate of 50–100 liters of air per minute. The tank was kept under air pressure of 10–15 p. s. i. For foam control, 100 ml. of a sterile, finely dispersed mixture of "D. C. antifoam A"[4] with 10 parts of soybean oil was added to the tank before sterilizing the medium, at the time of inoculation, and at twenty-four-hour intervals thereafter until the fermentation was finished. Foam control was also aided by the automatic addition of 25–50 ml. of sterile soybean oil each time foam contacted an electrode placed in the top of the tank. The *p*H at inoculation was 7.0, and at the end of the fermentation period was 7.6–7.8.

Assay Method.—The previous assay method[3] was modified by substituting peptone agar (5 g. of Bacto peptone and 17 g. of agar) for the nutrient agar, and by using a specified number of spores (*ca.* 100,000) per plate. The standard solution was made up in concentrations of 0.5, 1, 2, 4 and 8 units[3] per ml. Samples to be assayed were dissolved in alcohol and diluted to give four solutions each one-half as concentrated as the preceding, and estimated to fall in the range 0.5–8 units per ml. Alcohol alone caused no inhibition.

In carrying out an assay, each petri plate received one disc for each concentration of the sample or standard, that is, either 4 or 5 discs per plate, and all plates were set up in triplicate. After 40–48 hours incubation at 28° the inhibition zones were measured with a mm. scale, and the diameters averaged for each dosage level. Curves were plotted with the log of the concentration (relative concentrations in the case of unknowns) and the diameter of the inhibition zones as coördinates. Straight line functions nearly always resulted, and most of the sample curves were parallel to the standard. From the curve of each sample, the concentration that gave the same size inhibition zone as the standard containing one unit per ml. was determined. This concentration of the unknown was then regarded as containing 1 unit of antimycin activity per ml.

Since the test organism grew rather slowly, no appreciable error resulted from the one to two-hour time interval required for placing the discs on the agar plates. The reproducibility of the method is indicated by the average values and standard deviations found when each of three preparations was assayed seven different times. The values were 148 ± 8.8, 64.7 ± 5.7 and 10.6 ± 1.2 units per ml.

Isolation of Antimycin A.—A 60 l., ninety-six-hour culture containing 24×10^6 units was adjusted to *p*H 9.0, mixed with 3600 g. of Celite 503 and passed through a filter press. The cloudy filtrate was brought to *p*H 2.5 with hydrochloric acid, mixed with 1300 g. of Celite and again filtered. After this filter cake had been washed by pumping 10 l. of acidulated water, *p*H 2.5, through the press, it was extracted with five 6-l. portions of 95% alcohol. For this purpose each portion of alcohol was recycled through the press for fifteen minutes.

The alcohol extract, which contained 15×10^6 units of antimycin activity, was concentrated *in vacuo* at 30–40° to about 2 l. The aqueous suspension so obtained was thoroughly extracted by dividing the material into small portions and shaking out each one seven times with equal volumes of ether. Stable emulsions formed which were broken by centrifuging. Evaporation of the ether extract left 32 g. of a dark colored, oily paste containing 10.4×10^6 units. This was dissolved in about 200 ml. of absolute ether, a small amount of inactive, insoluble solid filtered off, the ether removed, and the residue stirred up with about 100 ml. of petroleum ether.[5] This solvent dissolved 6.25 g. of a nearly inactive, oily liquid, and left the rest of the material as an insoluble, dark-colored solid.

The solid was placed in the thimble of a Soxhlet extraction apparatus, and exhaustively extracted with petroleum ether. Light-colored solid material accumulating in the extraction flask was collected at one or two day intervals. After six days, 14.2 g. containing 5.45×10^6 units was obtained. This was dissolved in the minimum amount of boiling ether, petroleum ether added to incipient turbidity, and the solution allowed to stand,

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds from the Schenley Research Institute.

(2) The authors are indebted to W. H. Peterson and M. J. Johnson for the use of fermentation equipment and for helpful advice, to George J. Stessel and Paul W. Barnhill for assistance in carrying out assays, and to Eugene Herrling for help in preparing illustrations.

(3) Leben and Keitt, *Phytopath.*, **38**, 899 (1948).

(4) A silicone product manufactured by the Dow Chemical Co., Midland, Mich.

(5) The petroleum ether used throughout was Skellysolve A, b. p. 30-40°.

first at room temperature and then at 4°, for several days. The crystals were collected with suction, washed with petroleum ether, and dried to yield 3.63 g. of a light tan product, m. p. 126–128.5°, containing 4.79×10^6 units, *i. e.*, 20% of the original activity.

Properties of Antimycin A.—The product obtained as above, when recrystallized repeatedly from methanol, aqueous methanol, isopropyl ether or isopropyl ether–petroleum ether mixtures, came to constant m. p. 139–140°, $[\alpha]^{25}D$ +64.8° (c = 10 in chloroform). For analysis the material was dried to constant weight over phosphorus pentoxide at 60° and 0.2 mm.

Anal. Calcd. for $C_{28}H_{40}O_9N_2$: C, 61.30; H, 7.35; N, 5.11; mol. wt., 548.6. Found: C, 61.11, 61.09; H, 7.26, 7.47; N, 5.24, 5.24; alkoxyl, nil; mol. wt. in camphor (Rast), 506, 493.

The acid equivalent was determined by titrating a solution of antimycin A in 95% alcohol with alcoholic sodium hydroxide, thymolphthalein being used as the indicator. Calcd. for $C_{28}H_{40}O_9N_2$ as a monobasic acid: neut. equiv., 548.6. Found: neut. equiv., 556, 560.

The substance is colorless, but shows two bands in the ultraviolet (Fig. 1).

Antimycin A gives strong positive Millon, ferric chloride, and Gibbs phenol tests,[6] but negative Molisch, ninhydrin, Ehrlich, fuchsin aldehyde and 2,4-dinitrophenylhydrazine tests, and no color with cold, concentrated sulfuric acid. It is freely soluble in ether, alcohol, acetone and chloroform but very slightly soluble in petroleum ether, benzene, and carbon tetrachloride. It is insoluble in water and in 5% aqueous solutions of hydrochloric acid, sodium carbonate and sodium bicarbonate. In aqueous sodium hydroxide the crystals disintegrate and form a milky suspension which clears on warming. Active antimycin A cannot be recovered from such solutions.

Alcoholic solutions of antimycin A appear to be stable indefinitely under ordinary laboratory conditions. No particular sensitivity to light or to atmospheric oxygen has been noted. The substance has no basic properties, but is weakly acidic, probably because of the presence of a phenol function in the molecule. Various crystalline preparations have shown an activity of about 900–1300 units per mg. by the assay test described above.

Discussion

The antibiotic potency of the large scale cultures used for isolation of antimycin A has usually been in the range of 200–400 units per ml. The crystalline material isolated accounts for some 15–20% of this original total activity.

There are some indications that factors other than antimycin A are present in the crude ethanol extract. Crude extracts produce an inhibition zone in the usual assay which is entirely free from the test organism, whereas with antimycin A, although the zones are often equally distinct and are always sufficiently clear to be measured easily, enough of the test organisms ordinarily survive in the inhibited areas to produce a visible cloudiness. Furthermore, certain crude preparations have been found to be more potent, weight for weight, than the crystalline material, when tested by the agar streak method[3] against *Colletotrichum circinans* and *Stemphylium sarcinaeforme*. Efforts to isolate further active materials are, therefore, being made.

(6) The crude material previously tested[3] was evidently too low in antimycin A to respond to phenol color tests.

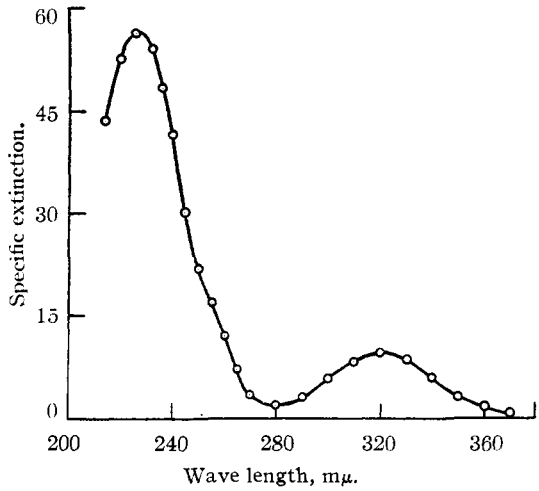

Fig. 1.—Ultraviolet absorption spectrum of antimycin A in alcohol.

Summary

A new, highly potent, fungicidal antibiotic, antimycin A, has been isolated from cultures of a *Streptomyces* species. The substance is an optically active, nitrogenous phenol with the probable molecular formula $C_{28}H_{40}O_9N_2$.

MADISON 6, WIS. RECEIVED DECEMBER 27, 1948