THE ISOLATION AND CHARACTERIZATION OF DERMATITIC COMPOUNDS PRODUCED BY MYROTHECIUM VERRUCARIA

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(Received for publication, August 10, 1954)

A number of reports on dermatitic or skin-irritating compounds produced by various molds have appeared in the recent literature. Several Russian workers, Vertinsky (1), Moseliani (2), Salikov (3), and Drobotko (4), have described the poisonous effects of the mold *Stachybotrys alternans* Borod. which produces a disease in horses known as stachybotryotoxicosis. The disease is caused by the consumption of hay on which the mold has been growing and is characterized by severe lesions of the nose, mouth, throat, and gastrointestinal tract. Ether extracts of cultures of the mold have dermatitic properties. Brian, Curtis, and Hemming (5) while handling large quantities of culture fluid during the isolation of glutinosin, an antifungal agent produced by *Metarrhizium glutinosum* S. Pope, experienced severe facial inflammation which was attributed to a volatile dermatitic compound produced by the organism. Lesions were also produced by dipping small filter paper disks in the culture fluid and strapping them in contact with the skin of the forearm for 48 hours. In later studies Brian, Hemming, and Jeffreys (6) found that other strains of the same fungus and several strains of *Myrothecium roridum* also produced dermatitic compounds. The present report deals with the production of several dermatitic compounds produced by *Myrothecium verrucaria* and the isolation and characterization of one of these compounds. The organism *M. verrucaria* (Alb. and Schw.) Ditm. ex Fr. used in these experiments has been shown by White and Downing (7) to be identical with the species *Metarrhizium glutinosum* S. Pope.

EXPERIMENTAL

Culture Medium—The medium used for the production of dermatitic compounds was a modification of Medium AS described by Brian, Curtis, and Hemming (5) and consisted of 50 gm. of glucose, 1.0 gm. of KH_2PO_4, 0.5 gm. of $MgSO_4 \cdot 7H_2O$, 1.0 gm. of $(NH_4)_2SO_4$, 1 mg. of $FeSO_4 \cdot 7H_2O$, 0.15 mg. of $CuSO_4 \cdot 5H_2O$, 1.0 mg. of $ZnSO_4 \cdot 7H_2O$, 0.1 mg. of $MnSO_4 \cdot 7H_2O$, and 0.1 mg. of K_2MoO_4 per liter of distilled water. The starting pH of all cultures was adjusted to 4.0.

Aeration and the addition of certain organic acids to the culture medium

were found to increase the yield of dermatitic compounds considerably (Table I). Of the organic acids tested the most effective were malic and tartaric. The best medium used for the production of the dermatitic compounds in quantities suitable for isolation, therefore, contained in addition to the ingredients mentioned in the preceding paragraph 1 per cent tartaric acid and 0.2 per cent malic acid.

Production of Dermatitic Compounds—For large scale production of the dermatitic compounds, 12 liter batches of medium were prepared and placed in 20 liter glass carboys. A typical isolation experiment was carried out in the following manner. Two 12 liter batches of medium were placed in individual glass carboys. The inoculum was prepared by suspending the spores from a surface culture of the mold (grown on Czapek-

TABLE I

Effect of Added Organic Acids on Sugar Utilization, pH, and Dermatitic Compound Production of Cultures of M. verrucaria

Acid added (1 per cent)	Glucose utilized	Final pH	Dermatitic compound
	per cent		*mg. per l.*
None	16	2.5	20
Tartaric*		3.8	8
Tartaric	76	4.3	94
Malic	70	5.0	58
Malonic	96	5.2	43
Oxalic	79	7.4	22

* This series was not agitated during incubation.

Dox agar in an 8 ounce glass bottle) in 5 ml. of autoclaved distilled water. 1 ml. of the spore suspension was placed in 100 ml. of medium in a 250 ml. Erlenmeyer flask. The flasks were placed in a rotating shaker which operated at 100 cycles per minute, and the culture was allowed to grow for 7 days at 25°. The medium in each carboy was inoculated with 100 ml. of this 7 day-old culture. The large cultures were also incubated at 25° and were aerated constantly during the incubation period by blowing filtered air into the cultures through sintered glass disks. Periodic analyses were made on portions of the medium during the growth period to follow the progress of dermatitic compound production.

Analytical Methods—Analytical procedures used for the determination of these compounds were based on biological activity, ability to absorb ultraviolet light, and chemical properties. The biological assay was carried out by allowing 0.1 ml. of alcoholic solution to dry on a rabbit's skin from which the hair had been removed with a pair of small animal clippers. The greatest dilution which would just give a visible lesion in 48 hours was

taken as a measure of the quantity of dermatitic substance in the preparation. Later studies showed that the minimal quantity of purified active compound detected by the bioassay was 0.3 γ. A more convenient method was found for determining the concentration of the dermatitic substances which was simply to measure the ultraviolet light absorption of a diluted aliquot of the culture at 263 mμ. A third procedure consisted of determining the light absorption at 450 mμ of the reaction product formed when diazobenzenesulfonic acid was added to a solution of the active compounds. This method was adapted from a procedure by Snell and Snell (8) and was carried out by adding 1 ml. of the reagent to a properly diluted aliquot of the culture in 1 per cent sodium carbonate solution and measuring the absorption after 2 minutes. The optical methods, though not specific for the determination of the dermatitic compounds, were found to be sufficiently selective, when based on the bioassy, to be used as a rapid means for determining the concentration of the dermatitic compounds during the production and isolation.

Isolation of Biologically Active Compounds—At the end of 4 weeks when the concentration of the active compounds in the carboy cultures had reached a maximum, the cultures were filtered and the combined filtrates extracted three times with 2.4 liters of ethyl ether. Care was taken during this operation to protect the face against burns from volatile dermatitic substances by wearing a gas mask. All exposed skin surfaces were covered with a barrier cream. The product known as Chemiglov proved satisfactory. The ether extract was evaporated under a vacuum and the brown gummy residue extracted with 240 ml. of benzene. Benzene was found to be a more selective solvent for the dermatitic compounds than ethyl ether, but was not used in the initial extraction because of its tendency to form emulsions. The benzene was evaporated *in vacuo* and the residue dissolved in 5 ml. of butyl alcohol. This solution was prepared for a 100 transfer counter-current distribution in the Craig machine. The solvent system used in the Craig machine was composed of 4850 ml. of ligroin (b.p. 100–110°), 150 ml. of *n*-butanol, and 5000 ml. of 0.1 M acetate buffer, pH 4.0. When the components of this system are mixed, two layers of nearly equal volume are produced. A 100 glass tube Craig machine was used, each tube containing 40 ml. of each phase of the solvent system. Because of the relatively low solubility of the dermatitic compounds in the solvent system it was necessary to add the butanol concentrate to 395 ml. of the system (adjusted to allow for the extra butanol added) which in turn was divided into five equal portions and added to tubes 0 through 4 of the Craig machine. Both phases of the solvent system were added to the remainder of the tubes and the counter-current distribution was carried out in the usual manner at room temperature.

At the end of the Craig extraction the amount of dermatitic compound in each of the tubes was determined by the diazobenzenesulfonic acid method. The distribution of these compounds is presented in Fig. 1. The contents of tubes 35 to 50 were chosen for further purification and characterization studies, since the distribution of the substance reacting with the diazo reagent in these tubes closely resembled the theoretical distribution of a single substance. The distribution curves of the compounds reacting with the diazo reagent in tubes 0 to 20 and 70 to 99, however, were too wide to fit the calculated distribution curves of single substances;

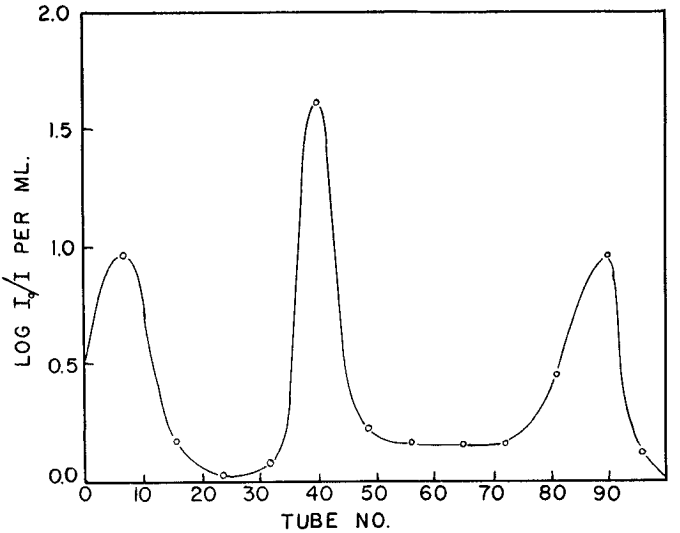

FIG. 1. Distribution of dermatitic compounds in the Craig machine. Absorption measurements were made at 450 mμ of the colored compound formed on addition of diazobenzenesulfonic acid.

hence further resolution of these mixtures would be necessary before characterization studies could be carried out on the pure compounds. High dermatitic activity was found only in tubes 35 to 50 and 70 to 99.

The dermatitic compounds present in other cultures of *M. verrucaria* when subjected to the fractionation procedure described above were found to produce Craig distribution patterns which varied to some extent from the one given in Fig. 1, but in all cases two or more compounds were detected. The partition coefficients of each of these compounds, however, seemed to fall close to one of the following values: 0.1, 0.9, 4.0, and 11.0.

In the typical experiment described above, the contents of tubes 35 to 50 were combined, the solvents were removed by vacuum distillation, and the remaining material was extracted three times with 200 ml. of ether.

The ether extracts were combined and the solvent was removed by evaporation *in vacuo*. The residue, an oily liquid, weighed 280 mg. When this residue was lyophilized from 20 ml. of benzene, a white microcrystalline residue was produced.

Characterization of Crystalline Compound—The crystals melted at 38°. An alcoholic solution of the compound had a specific rotation of $[\alpha]_D^{25}$ +94°. The compound consisted solely of carbon, hydrogen, and oxygen. No nitrogen, sulfur, phosphorus, or halogen was found on analysis, and no ash was produced on combustion. The molecular weight as determined by freezing point depression of a benzene solution was 270 ± 15. The mo-

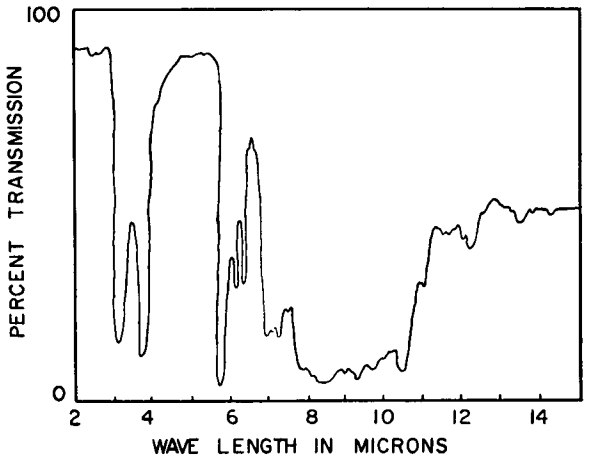

FIG. 2. Infra-red spectrum of the dermatitic compound isolated from tubes 35 through 50 of the Craig machine.

lecular formula $C_{15}H_{22}O_4$ fits best the molecular weight determination and the analyses.

$C_{15}H_{22}O_4$ (266.3). Calculated, C 67.6, H 8.3; found, C 68.1, H 8.4

Analysis for methoxy groups revealed that none was present. Methylation of the compound was accomplished in the following manner. 5 ml. of a 2 per cent ether solution of diazomethane were added to 8.9 mg. of the compound in 1 ml. of ethyl alcohol. The solution was stored at 5° for 24 hours after which the solvents were evaporated and the sample was reweighed. A gain in weight of 1.24 mg. was noted which is equivalent to the addition of about three methyl groups.

The dermatitic compound was very soluble in chloroform, benzene, ethanol, methanol, butanol, and ether, but considerably less soluble in water, ligroin, and carbon tetrachloride. The compound was weakly acidic, since

it could be extracted into aqueous solutions at pH 11 but not at pH 8. Decomposition as observed by loss of dermatitic activity and discoloration took place rapidly above pH 11. The compound reacted readily with diazobenzenesulfonic acid to give a yellow color. It also produced a bright red color in the Liebermann nitrous acid reaction (9) but failed to form a colored complex with ferric chloride in methanol in concentrations as high as 1 mg. per ml. Millon's test ((9) p. 134) and the titanous chloride test of Weygand and Csendes (10) were negative.

An aqueous solution (pH 6.5) of the compound absorbed in the ultraviolet with a single maximum at 263 mμ (ϵ = 7740). A dried film of the compound was prepared on a silver chloride plate for infra-red absorption studies which were carried out with a Perkin-Elmer spectrophotometer model 12C. Major absorption peaks occurred at the following wavelengths: 3.0, 3.5, 5.85, 10.4, and 12.3 μ (Fig. 2).

Similar Compounds Present in M. verrucaria Cultures—Another compound isolated from a different batch of culture filtrate had a partition coefficient of 0.12 in the liquid counter-current distribution system. Upon fractionation the compound was distributed in the Craig machine relatively free of other overlapping substances. It was isolated as a yellow oil, and the yield per liter of culture was 1.4 mg. The compound had very little dermatitic activity and an alcoholic solution had no optical rotation. Like the first compound it contained only carbon, hydrogen, and oxygen, but the carbon content, 58.1 per cent, was appreciably lower than that of the more active compound. The molecular weight was determined as 253 $\pm$ 10. The ultraviolet and infra-red spectra of the two compounds were very similar.

At least two more compounds were detected in *M. verrucaria* culture filtrates which have distribution coefficients in the two phase solvent system near 4 and 10. These compounds have not been isolated, although mixtures of crystals have been obtained. Preliminary studies have shown that these compounds have higher molecular weights and greater optical rotation than the compounds previously described. They also possess high dermatitic activity.

DISCUSSION

According to the analytical data presented for the first crystalline dermatitic compound described, a likely explanation for the weakly acidic properties is the presence of a phenolic structure. The hydroxyl group is strongly indicated by the infra-red absorption at 3.0 μ. NH structures which also show a maximum in this region can be eliminated because of the absence of nitrogen in the compound. The maximum at 3.5 μ can most probably be attributed to CH structures, while the peak at 5.85 μ is most

often associated with the C=O double bond. In view of the weakly acidic properties of the compound it seems more likely that this structure can be attributed to an aldehyde, ketone, or ester group than to a carboxyl group. Ester groups are probably somewhat less likely to be present than aldehyde or ketone groups, since the ester maximum in this region usually falls closer to 5.75 than 5.85 μ. In general the infra-red spectrum, including the maxima at 10.4 and 12.3 μ, is quite characteristic of aromatic or alkene structures. The aromatic nature of the compound is confirmed by the positive Liebermann reaction and the production of a yellow color with diazotized sulfanilic acid. If the compound is phenolic in nature the absence of color production in the presence of ferric chloride indicates polysubstitution on the aromatic ring. The positive diazotized sulfanilic acid and Liebermann reactions indicate an unoccupied para position, while the negative Millon's test is characteristic of di-*o*- and di-*m*-substituted phenols. The negative titanous chloride test reveals the lack of *o*-quinone structures. In spite of the evidence for an aromatic ring, the ratio of hydrogen to carbon atoms in the molecular formula $C_{15}H_{22}O_4$ is too great to permit a completely aromatic structure to be written for the compound; thus a partially aliphatic structure must be considered.

The skin-irritating compounds of poison ivy, which possess aromatic rings, hydroxyl groups, aliphatic side chains, as well as dermatitic activity, also represent an interesting series. These and other natural products of similar chemical and biological properties have been reviewed by Wasserman and Dawson (11).

SUMMARY

At least three compounds possessing high dermatitic activity have been produced by culturing the organism *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr. One of these compounds has been isolated in crystalline form and its molecular formula determined as $C_{15}H_{22}O_4$. Another compound produced by the mold and isolated as a yellowish oil had low biological activity but was chemically and spectrophotometrically similar to the active compounds.

The authors are indebted to Dr. Edwin V. Hill and to Dr. James D. Mold for suggestions on the problem, to Dr. P. W. Brian of Butterwick Laboratories, Welwyn, Herts, England, for supplying the organism, to Dr. Benjamin Warshowsky of Camp Detrick and Mr. Erna J. H. Rhodes of the Army Chemical Center for analyses, and to Dr. George S. Melville and Mr. Edward J. Weneck of Camp Detrick for infra-red spectrophotometric determinations.

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