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ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES. IV.
MARASMIUS CONIGENUS*

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In a survey of Basidiomycetes for antibacterial activity, *Marasmius conigenus* (No. 6890) was reported to inhibit the growth of *Staphylococcus aureus* when tested by the strip or the "hole" method.¹ Dr. W. H. Wilkins kindly supplied us with a subculture of this organism with which we continued studies. Other species of *Marasmius* produce antibacterial substances.^{2, 3}

When grown on corn steep, thiamine peptone, or potato dextrose agar, and tested by the streak method,^{4, 5} *Marasmius conigenus* showed in our hands marked antibacterial activity against *Staphylococcus aureus* (H) slight activity against *Escherichia coli* and none for *Mycobacterium smegma*.

Considerable activity against *Staph. aureus* was observed with discs^{4, 5} cut from fungous cultures grown on the three media. The position of the disc in relation to the fungous colony determined the size and character of the inhibition zone. The disc cut from the oldest part of the colony produced the largest inhibition zone which was clear near the disc and surrounded by a zone of partial inhibition. Discs cut at a distance of 20 mm. from the edge of the fungous colony gave zones of partial inhibition. Isolations from these zones have not yielded strains of *Staph. aureus* resistant to the antibacterial material from this fungus.

Antibacterial Activity of Culture Liquids.—The fungus was grown at 25°C. in 2800-ml. Fernbach flasks containing beech shavings and a corn steep medium.⁵ In about four weeks, when the surface of the liquid was about one-half covered with mycelium, the activity of the culture liquid against *Staph. aureus* was 256 or 512 dilution units per milliliter. Reflooding such mats with fresh medium produced liquids of as high activity one week or more after reflooding. The culture liquid from a seven-

month old mat had an activity of 256 dilution units per milliliter 19 days after reflooding. Although year-old mats still produce culture liquids with an activity of 64 dilution units or more, the mats were usually not kept longer than five or six months as the increased thickness of mycelium made reflooding less practical. Mats were successfully reflooded six times.

The culture liquid in serial dilution evidenced little activity against *Escherichia coli* and the acid-fast bacteria as represented by *Mycobacterium phlei* and *M. smegma*.

The culture liquid lost its antibacterial activity at pH 8.5 and higher within an hour at 25°C. For the samples tested, there was no loss of activity at pH 3 on boiling for 10 minutes.

Isolation of a Crystalline Antibacterial Substance.—A white crystalline substance with antibacterial activity was isolated from the culture liquid of *Marasmius conigenus*. The procedure followed in the isolation of this compound may be illustrated by an example.

Ten liters of culture liquid with an activity of 64 dilution units per milliliter were acidified to pH 2 to 3 with hydrochloric acid and extracted twice, each time with one-tenth of its volume of chloroform. The chloroform was separated from the aqueous phase by passage through a de Laval separator. The chloroform was evaporated *in vacuo* to 25 ml. It contained 1373 mg. of dry matter. It was extracted twice, each time with 25 ml. of phosphate buffer at pH 5.8. The chloroform solution after extraction with buffer contained 1320 mg. of solids with an activity of 512 dilution units per milligram. The chloroform was removed by distillation *in vacuo*; the solids were dissolved in ether and extracted with 2% sodium bicarbonate solution. The bicarbonate removed 974 mg. of solids. The bicarbonate solution was acidified and extracted with ether. When the ether was evaporated slowly, white crystals mixed with a brown gum were formed. The gum was removed by washing carefully with ether. The crystals were purified by recrystallization from ether and additional crystals obtained from the gummy residue. The yield was 453 mg. of crystals which formed long white needles when crystallized from ether, dilute aqueous alcohol or acetone, water or ether upon the addition of hexane.

The maximum yield of crystals obtained was 100 mg. per liter of culture liquid. We have named this crystalline substance marasmic acid. Our observations indicate that culture liquids of 64 to 512 dilution units per milliliter all contained approximately the same amount of marasmic acid per liter (about 100 mg.).

Chemical Properties of Marasmic Acid.—Marasmic acid was very soluble in acetone, soluble in ethanol, ether, chloroform and water (1.1 mg./ml. at 25°) but insoluble in hexane.

The melting point in an open capillary tube was 165°C. (uncorr.). There was a temperature range through which part of the substance decomposed and the rest of the crystals dissolved in the melt. Determined in a capillary sealed off *in vacuo*, the melting point was sharp at 174–175°C. corr. without decomposition; it remelted at the same temperature even after the second or third melting.

The crystals titrated in 30% alcohol as a monobasic acid with a pK of 5.8 and an equivalent weight of 276. Qualitative tests did not indicate the presence of nitrogen, sulfur or halogens.

The quantitative analysis⁶ was:

	C	H	MOL. WT.
Found	68.21	7.17	
Computed for C ₁₆ H ₂₀ O ₄	69.6	7.25	276

The optical activity of marasmic acid was $[\alpha]_D^{25} = +176$ (1.4% in acetone).

Marasmic acid in alcohol had absorption peaks in the ultra-violet region at from 240 to 242 m μ with a molecular extinction coefficient $\epsilon = 10,400$ and at 314–318 m μ with $\epsilon = 45.6$. In phosphate buffers of increasing pH, the position of the main peak was shifted slightly toward the red and the extinction decreased. In pH 1.45 and 3.52 phosphate buffers, the peak was found at 245 m μ and $\epsilon = 11,400$; in pH 5.40 buffer at 246 and $\epsilon = 10,400$; in pH 7.0 buffer at 248 m μ and $\epsilon = 8900$; in pH 8.8 at 248 m μ with $\epsilon = 8400$, and in pH 11.0 buffer at 260 m μ with $\epsilon = 3900$. Marasmic acid in the three most acid buffers had isobestic points at 230 and 255 m μ . There were no isobestic points at pH 7 and higher, indicating that a change in the light absorbing groups took place.

The positions of the peaks and the absorption coefficients is the same as that of several α,β -unsaturated ketones⁷ in which the double bond is conjugate to the carbonyl group.

Marasmic acid reduced Fehling's reagent slowly in the cold and rapidly when heated. Ammoniacal silver nitrate was not reduced at room temperature but was reduced when boiled. When marasmic acid and hydroxylamine hydrochloride were mixed, there was no change in pH suggesting that the two substances did not react. Bromine in carbon tetrachloride did not add. Marasmic acid reduced cold potassium permanganate in sodium carbonate solution but not in cold acid solution. The acid did not give a color reaction with alcoholic ferric chloride, thus indicating that it probably is not a phenolic compound or an α -hydroxy acid. Marasmic acid reacted after a few seconds with alkaline nitroprusside to give a reddish color which became a very pale gray green when the solution was acidified. It formed an orange colored 2,4-dinitrophenylhydrazone with a corrected melting point of 136° to 138°C.

The reactions given above indicate that marasmic acid is unsaturated,

contains a carbonyl group, and probably a carboxyl group, and changes at high pH so that increased reducing power results.

Marasmic acid added one molecule of cyanide for each equivalent of acid when the reaction (Kiliani) was conducted in 0.1 *N* acetic acid solution.⁸ The 2,4-dinitrophenylhydrazones of marasmic acid and mesityl oxide had similar absorption bands in the near ultra-violet with the peak at 375 $m\mu$ when measured in alcohol. When the optical densities of the two dinitrophenylhydrazones were compared at 375 $m\mu$ on a molecular basis, the one from marasmic acid absorbed 1.12 times as much as the one from mesityl oxide, indicating that marasmic acid probably has one carbonyl group per equivalent weight.

A sample of marasmic acid was dissolved in four equivalents of 0.1 *N* sodium hydroxide, incubated at 37°C. for 2 hrs., and then back titrated with hydrochloric acid. The alkaline solution had a yellow color which disappeared as the solution became acid. There was no evidence for a second hydrogen, thus indicating the absence of a lactone ring.

Marasmic acid is not a methyl ketone or a β,γ -unsaturated lactone as judged by the slowness of the nitroprusside reaction.⁹

One equivalent of marasmic acid reacted with one molecular weight of cysteine as judged by the disappearance of the nitroprusside test for sulfhydryl. The reaction occurred within 2 hrs. at 37°C. and pH 6.

Marasmic acid was converted in alkaline solution in a short time into a product or products with chemical properties different from those of marasmic acid. The solution prepared by allowing marasmic acid to stand in about 0.1 *N* alkali for an hour and neutralizing, reacted immediately with nitroprusside to give a red color which faded to amber color within a minute or two. The 2,4-dinitrophenylhydrazone prepared from the solution did not crystallize from alcohol, chloroform, ether or benzene. The ultra-violet absorption spectrum of the alkaline-treated marasmic acid showed general absorption without a pronounced peak.

The reaction of marasmic acid with Fehling's solution, alkaline permanganate and nitroprusside, may depend upon conversion by alkali into the more reactive substance or substances referred to above.

On standing for a longer time in alkaline solution, the reactivity with nitroprusside was lost, indicating further change.

The behavior of marasmic acid suggests that it may be an α,β -unsaturated ketone which shifts in alkaline solution so that the double bond is not conjugated with the carbonyl double bond.

Antibacterial Activity of Marasmic Acid.—The antibacterial and anti-luminescent activities of the neutralized crystalline material were measured by the methods in use in this laboratory.¹⁰ The results are given as the minimum concentration in micrograms per milliliter for inhibition of growth or luminescence.

ANTIBACTERIAL ACTIVITY

<i>Bacillus mycoides</i>	1
<i>Bacillus subtilis</i>1
<i>Escherichia coli</i>	64
<i>Klebsiella pneumoniae</i>	128 (64 partial)
<i>Mycobacterium smegma</i>	32
<i>Photobacterium fischeri</i>	0.016
<i>Pseudomonas aeruginosa</i>	250 (128 partial)
<i>Staphylococcus aureus</i>	2

ANTILUMINESCENT ACTIVITY

10 minutes.....	.8
1 hr.....	.4
2 hrs.....	.2
3 hrs.....	.1
24 hrs.....	0.25

Effect of Blood.—Marasmic acid was incubated with blood and the residual antibacterial activity determined for *Staph. aureus*. Human blood was diluted ten times in beef extract medium containing 0.7% sodium chloride. One milliliter of neutralized solution of marasmic acid was added to 1 ml. of the diluted blood, incubated for 3 hrs. at 37°C. centrifuged, and the antibacterial activity of the supernatant liquid determined by serial dilution. A control was diluted in beef extract medium without blood and incubated simultaneously. The activity of penicillin G, streptomycin and chloromycetin did not decrease on incubation with blood; the activity of marasmic acid was decreased. The greater the proportion of blood to marasmic acid, the greater was the decrease in activity.

Antifungal Activity.—The antifungal activity¹¹ of marasmic acid was measured by serial dilution in a peptone medium¹² at pH 6 using spore suspensions of the fungi. *Trichophyton* was incubated at 30°C.; the others at 25°C. The tests were read after from 42 to 48 hrs. incubation. The activity reported is the minimum inhibitory concentration in micrograms per milliliter; *p* = partial inhibition.

ORGANISM	ACTIVITY
<i>Aspergillus niger</i>	32 (16 <i>p</i>)
<i>Chaetomium globosum</i> (USDA 1042.4).....	64
<i>Gliomastix convoluta</i> (PQMD4c).....	250 (8–128 <i>p</i>)
<i>Memnoniella echinata</i> (PQMD1c).....	64
<i>Myrothecium verrucaria</i> (USDA 1334.2).....	64
<i>Penicillium notatum</i> (832).....	32 (16 <i>p</i>)
<i>Phycomyces Blakesleeanus</i> (+ strain).....	16 (8 <i>p</i>)
<i>Saccharomyces cerevisiae</i> (188).....	32
<i>Sterphylium consortiale</i> (PQMD41b).....	128 (64 <i>p</i>)
<i>Trichophyton mentagrophytes</i>	4

Animal Toxicity.—Toxicity for mice was determined by injecting 0.5 ml. of neutralized marasmic acid made up in 0.7% sodium chloride solution into a tail vein of Carworth Farms CF1 male white mice. Each treatment group contained five mice. Four of the mice that received 32 mg./kg. in a single dose died within 3 days. All of the mice that received 16 mg./kg. were alive 30 days later. The LD50 lies somewhere between 16 and 32 mg./kg.

The high toxicity and the inactivation by blood militate against the possible therapeutic usefulness of marasmic acid.

Additional Antibacterial Substances.—The existence of additional antibacterial substances in the culture liquid of *Marasmius conigenus* was suggested by some of our observations.

The chloroform extracts of some lots of culture fluid were more active on a milligram basis than marasmic acid. For example, a spectrophotometric analysis of a chloroform extract indicated a maximum of 78% marasmic acid. The activity of this extract against *Staph. aureus* was 6 times that of an equal weight of crystalline marasmic acid. Five other strains of Staphylococci were from 4 to 32 times as sensitive to the material in the extract as to an equal concentration of marasmic acid. However, the activity for other bacteria tested was no different from that of marasmic acid. It appeared, therefore, that the culture liquid contained a factor to which Staphylococci were especially sensitive.

The antistaphylococcus factor was found in only four of 13 lots of culture liquid examined. Two of the lots of culture liquid assayed 64, one 256, and one 512 dilution units per milliliter against *Staph. aureus*. The concentrates containing the antistaphylococcus factor were all prepared from solutions in which the fungus had been grown from the inoculum; the factor was not observed in solutions from flasks that had been reflooded. The antistaphylococcus factor was destroyed by heating a dry fraction in an oven for 3 hrs. at 100°C. Marasmic acid was unaffected by this treatment. The antistaphylococcus factor was not affected by air drying overnight nor by bringing it to a boil in a solution of pH 2 to 3. Incubation with blood reduced the activity of the antistaphylococcus factor.

A further series of observations suggested that *Marasmius conigenus* may produce a third antibacterial substance. Chloroform removed all or nearly all the antibacterial activity from the various lots of acidified culture liquid as indicated by tests on the aqueous phase after extraction. However, the proportion of original activity of the culture liquid present in the chloroform extract varied with different lots from 3 to 100%. We did not determine the cause for this variability. It seems improbable that loss or destruction of either marasmic acid or the antistaphylococcus factor accounts for the variability in recovery of antibacterial activity in

the chloroform extracts, and, therefore, a third less stable substance may have been present.

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¹¹ We are indebted to Dr. W. L. White for the cultures with the USDA or PQMD numbers, to Dr. R. F. Light, Fleischmann Laboratories, for the culture of *S. cerevisiae*.

¹² This medium contained per liter 1.5 g. KH_2PO_4 , 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 g. dextrose, 2 g. neopeptone, 1500 m μ moles of thiamine, and a mixture of minor mineral elements as used in this laboratory.

CHROMOSOME SEGREGATION IN MAIZE TRANSLOCATIONS IN RELATION TO CROSSING OVER IN INTERSTITIAL SEGMENTS*

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In maize, the second meiotic division in the microsporocytes results in a quartet of four microspores in which it is possible to distinguish the two division planes. In normal material, the prominent feature of these spores, as revealed by acetocarmine smears, is the single nucleolus. In translocations involving chromosome 6 which carries a nucleolar organizer region, non-disjunction may result in spores with two nucleolar organizers (potentially two nucleoli), or with no organizer (nucleolar material remains scattered or diffuse) in the same quartet. By this method, McClintock⁴ established the fact that the chromosomes which crossover in an interstitial segment (between the centromere and the translocation break) pass to opposite poles.

A further relation between chromosome segregation in such maize translocations and the frequency of crossing over in an interstitial segment has been reported in abstracts.^{1, 2} This is a report of additional studies of