Zygophore-stimulating Precursors (Pheromones) of Trisporic Acids Active in (−)-Phycomyces blakesleeanus

ACID-CATALYZED ANHYDRO DERIVATIVES OF METHYL 4-DIHYDROTIRSPORATE-C AND 4-DIHYDROTIRSPORATE-C*

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The same pheromones apparently initiate sexual development in all mucoraceous fungi. We isolated methyl trisporates and methyl 4-dihydrotrisporates as pheromones from (+) cultures of Blakeslea trispora and used Sephadex LH-20 chromatography with ethyl acetate for the purification. The pheromones stimulated the development of zygophores (sex cells) in bioassays with (−) cultures of Phycomyces blakesleeanus and Mucor mucedo. Labeled methyl 4-dihydrotrisporate-C and methyl trisporate-C, prepared chemically from labeled trisporic acid-C, were incorporated into trisporic acids in (−), but not (+), cultures of P. blakesleeanus. Two other labeled compounds, Cpd85 and Cpd76, also were isolated from the incubation with methyl 4-dihydrortisporate-C. Cpd85 and Cpd76 apparently were not precursors of trisporic acids, zygopher-stimulating pheromones, or zygotropic pheromones, but were procedural artifacts. Methyl 4-dihydrotrisporate-C dehydrated to Cpd85 and 4-dihydrotrisporate-C dehydrated to Cpd76 in slightly acidic solutions. Cpd85 was formed when Cpd76 was treated with diazomethane. UV, IR, NMR, and mass spectra verified that Cpd85 was 1,3-dimethyl-2,3-dihydro-3-cyclohexene-1-carboxylic acid methyl ester. Rf and e values are given for each compound studied. We conclude that the distinguishing feature of pheromones made by (+) and (−) mating types is the degree of oxidation of the pro-S methyl group on carbon atom 1 and propose that the (+) mating type has a mechanism for inactivating trisporate precursors which would self-stimulate the formation of zygophores.

Phycomyces blakesleeanus is a single-celled, multinucleate organism which responds to a variety of external signals (1–3). Sexual development, for example, requires the cooperation of cultures of opposite mating types (3). Zygophores, sexually differentiated hyphae, develop on asexual hyphae prior to cell contact in crosses of (+) and (−) wild type cultures (4). Zygophores develop only on the carotene-deficient mutants in

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crosses of mutants and wild types (4). However, we were unable to detect pheromones at the 0.1 ng/ml level in the medium of 5-day-old (+) cultures of P. blakesleeanus, suggesting it does not make pheromones while undergoing hyphal elongation (nuclear division) or while committed to an alternate developmental pathway such as sporangioaphore formation.

Extracts isolated from (+) B. trispora grown in undefined medium stimulated both zygophore formation and the accumulation of TAs in (−), but not (+), cultures of P. blakesleeanus. Zygophore formation was stimulated by 0.6 A285 units of extract in (−) cultures, whereas 80 A285 units had no effect on (+) cultures. Twenty A329 units of purified acid fraction (TAs) were recovered from (−) cultures incubated overnight with 294 A285 units of extract. Thus, extracts isolated from 7-day-old cultures of (+) B. trispora have the same effect upon young cultures of (+) and (−) P. blakesleeanus as upon M. mucedo (7, 8) and B. trispora (7, 13). Are the same or different compounds active upon each culture?

Extracts from (+) B. trispora were resolved into 2 distinct components by Sephadex LH-20 chromatography with ethanol. The components exhibited a Vr/Vo of 0.77 and 1.04. Twenty A329 units of each component were tested for pheromone activity in bioassays with (−) cultures of P. blakesleeanus and M. mucedo. Only the first component, representing 90% of the A329 units in the initial extract, stimulated zygophore formation. It was active in both bioassays.

Partially purified extract was resolved in 6 components designated A, B, C, D, E, and F by Sephadex LH-20 chromatography with ethyl acetate (Fig. 1A, solid line). These components exhibited Vr/Vo of 0.60, 0.76, 1.04, 1.17, 1.36, and 1.90. Twenty A329 units of each component were tested for pheromone activity in bioassays with (−) cultures of P. blakesleeanus and M. mucedo. Components A, B, and C stimulated zygophore formation in both bioassays whereas components D, E, and F were inactive in both bioassays. Components A, B, and C exhibited 4,800, 37,000, and 1,100 zygophore-stimulating units (total A329 units of component recovered from a liter of culture medium/smallest quantity of A329 units giving a distinctly positive response in the Mucor bioassay), respectively.

Components A, B, and C were resolved by thin layer chromatography into 2, 4, and 3 UV-absorbing spots, respectively. Only component spots A–1, B–2, and C–2 stimulated zygophore formation (in both bioassays). Some properties of these component spots are given in Table I. C–2 was identified as methyl 4-dihydroTA-C from the following observations. C–2 and authentic methyl 4-dihydroTA-C exhibited identical UV spectra, Vr/Vo, and zygophore-stimulating activities upon dilution in the Mucor bioassay. A mixture of C–2 and methyl 4-dihydroTA-C migrated as a single spot upon thin layer chromatography with solvent systems I, II, and III. B–2 was identified as 85% methyl 4-dihydroTA-B and 15% methyl TA-C. One A285 unit of B–2 migrated as a single discrete spot upon thin layer chromatography with solvent systems I, II, and III. B–2 migrated further than C–2 in all 3 solvent systems demonstrating that B–2 is less polar than C–2. The absorbance at 276, 283, and 296 nm indicated the presence of a methyl 4-dihydroTA-like compound and the absorbance at 328 nm indicated the presence of a methyl TA-like compound. Co-chromatography of B–2 and methyl TA-C using a Sephadex LH-20 column with ethyl acetate resulted in an elution profile with a constant A285/A329 ratio. The sodium borohydride reduction product of B–2 and authentic methyl 4-dihydroTA-C had identical UV spectra and Vr/Vo, in this figure only, represents the elution volume of a fraction divided by the total bed volume of the column.

![Fig. 1. Sephadex LH-20 chromatography](image)

**FIG. 1. Sephadex LH-20 chromatography.** Sephadex LH-20 chromatography with ethyl acetate of (A) neutral fractions isolated from the medium of (+) cultures of B. trispora grown in a potato extract-glucose medium (adjusted to pH 2 prior to extraction) and (B) from analyses of known compounds: 1, methyl trisporate-B; 2, methyl trisporate-C and methyl 4-dihydrotrisporate-B; 3, methyl 4-dihydrotrisporate-C; 4, trisporic acid-B; 5, trisporic acid-C. Absorbance measurements at λmax were made after fractions were diluted 50, 60, 69, and 100-fold for neutral fractions, methyl trisporates, methyl 4-dihydrotrisporate-C, and trisporic acids, respectively. A separate experiment revealed that methyl 4-dihydrotrisporate-B co-chromatographed with methyl trisporate-C. Vr/Vo in this figure only, represents the elution volume of a fraction divided by the total bed volume of the column.

**TABLE I**

Recovery of pheromones from the medium of (+) B. trispora

| Component | Thin layer chromatography | λmax | A285/A329 | A285 units recovered | % | Compounds in Component |
|-----------|--------------------------|-------|-----------|---------------------|----|-----------------------|
| A-1       | 0.61                     | III   | 283,328   | 3.5                 | 23.5 | 70% unknown           |
| B-2       | 0.39                     | III   | 276,283, 328, 328 | 6.4 | 75.5 | 79% methyl trisporate-B |
| C-2       | 0.52                     | II    | 276,283, 296 | 25.7 | 30% methyl 4-dihydrotrisporate-C |
compounds migrated as a single spot upon thin layer chromatography with the 3 solvent systems. A-1 was identified as 30\% methyl TA-B based upon its R\_f and UV spectra, V\_c/V, R\_f with solvent systems I and II, zygophore-stimulating activity in the *Mucor* bioassay (e.g. 0.38 pM if assuming lowest quantity giving a distinct positive response equals 108 pM), and the recovery of Cpd85 after treating the sodium borohydride reduction product of A-1 with dilute acid. We conclude that the same pheromones stimulate zygophore formation in

of cultures of both *M. mucedo* and *P. blakesleeanus*.

A partially purified extract isolated from cultures grown on "defined medium was resolved into 7 components designated A, B, B*, C, D, E, and F by Sephadex LH-20 chromatography with ethyl acetate (Fig. 1A, dotted line). The R\_f and UV spectra of the component spots in B, C, D, E, and F were qualitatively identical with those obtained from the former extract. For example, B-2 represented 87\% of B and exhibited an A\_\text{350}/A\_\text{254} of 12.2. About 71 A\_\text{350} units of B-2 (3.4 pmol of methyl 4-dihydroTA-B and 0.3 pmol of methyl TA-C) were recovered from 1,000 A\_\text{350} units of extract. The new component, B\*, with a V\_c/V of 0.92 exhibited 1,100 zygophore-stimulating units compared with 35,000 zygophore-stimulating units in B. The zygophore stimulating activity in component B\* was present in component spot B\*-1 which had an R\_f similar to C-2 upon thin layer chromatography with solvent system III. B\*-1 exhibited a complex UV spectrum suggesting it was a mixture of compounds. The identity of the compound responsible for the zygophore-stimulating activity in B\*-1 was not determined. Component A contained only A-1 (no A-2). In summary, these extracts contained about the same amount of pheromone as extracts isolated from cultures grown on undefined medium.

**Biological Characterization of Zygophore-stimulating Pheromones**—The relative zygophore-stimulating activities of the pheromones methyl 4-dihydroTA-B, methyl 4-dihydroTA-C, methyl TA-B, and methyl TA-C of and of their metabolites TA-B and TA-C upon cultures of *P. blakesleeanus* and *M. mucedo* are presented in Table II. The activity of each compound is expressed as the lowest quantity of that compound required to elicit a distinctly positive response in that species relative to the compound which was active in the lowest absolute picomole amount. Two-fold differences in activity are within experimental error and not significant. In bioassays with both species, methyl 4-dihydroTA-Bs were active only on (−) cultures, methyl TA-B were about 100 times more active on (−) than (+) cultures, and TA-Cs were equally active on both (+) and (−) cultures. The B form (oxygen at C-13) of each class of compounds was more active than the C form (hydroxyl at C-13). This difference was at least 4-fold in *P. blakesleeanus* and 10-fold in *M. mucedo*.

There was one major difference between the bioassays with *P. blakesleeanus* and *M. mucedo*. The pheromone methyl 4-dihydroTA-B was the most active compound in bioassays with *P. blakesleeanus*.

It was 3 times more active than methyl TA-B and 90 times more active than TA-B. In contrast, TA-B was the most active compound in bioassays with *M. mucedo*, being 4 times more active than the pheromones methyl 4-dihydroTA-B and methyl TA-B.

The labeled compounds isolated from (+) and (−) cultures of *P. blakesleeanus* incubated with the labeled pheromones methyl 4-dihydroTA-C and methyl TA-C are presented in Table III. TA-C, formed only by (−) cultures, was the major metabolite of both pheromones. It represented 45\% and 64\% of the label originally added to cultures as methyl 4-dihydroTA-C and methyl TA-C, respectively. (In a separate experiment (data not shown), 360 \mu g of TA-B and 140 \mu g of TA-C were recovered from (−) cultures incubated with 1.1 mg of unlabeled methyl 4-dihydroTA-B. Two unexpected compounds, Cpd76 and Cpd85, were found in (−) cultures incubated with labeled methyl 4-dihydroTA-C, Cpd76 was present in both the acid and neutral fractions. Cpd85 was found only in the neutral fraction. Unlabeled compounds absorbing UV radiation were not detected among the labeled compounds isolated from the cultures.
Identification and Characterization of Cpd76 and Cpd85—Ninety per cent of the 4-dihydroTA-C in acidified 6\% KH₂PO₄, dehydrated to Cpd76 upon extraction with CHCl₃, and evaporation to dryness (Fig. 2). Over 21 μmol of Cpd85 were formed when 24 μmol of methyl 4-dihydroTA-C in 5 ml of CHCl₃ were exposed to 2.5 mg of p-toluene sulfonic acid for 30 min at 22°C. (Less than 0.1 μmol of Cpd85, if any, was formed when 24 μmol of methyl TA-C were treated identically.) This Cpd85 was identical (as judged by UV spectral data and co-chromatography with solvent systems III, V, and VI) to the: 1) Cpd85 isolated in the tracer experiments, 2) product formed upon treating Cpd76 with diazomethane, and 3) major compound (90\%) of component spot A-2 isolated from (+) culture medium acidified prior to extraction with CHCl₃.

UV spectra of Cpd85 and Cpd76 exhibited λₘₐₓ 276, 286, 298 nm, and molar absorptivities 1.7 times greater than TA-C. IR spectra of Cpd85 and Cpd76 exhibited absorptions at 1715 cm⁻¹ and 1703 cm⁻¹, respectively, but none in the 3450 cm⁻¹ region, indicating that neither compound has a hydroxyl group. The NMR spectrum of Cpd85 is shown in Fig. 3. The parent peak indicates a Mᵣ = 304. The parent peak plus one is 20.3\% as abundant as the parent peak, which is consistent with a molecular formula of C₃₀H₅₄O₄ for Cpd85. A 60 MHz NMR spectrum of Cpd85 indicated one OCH₃ group (3.68 ppm) for every 28 protons. A single scan of the same sample with a 250 MHz NMR spectrometer indicated the presence of 4 diastereoisomers as judged from the OCH₃ peaks: 3.687 ppm (52.4\%), 3.672 ppm (40.5\%), 3.644 ppm (3.3\%), and 3.636 ppm (3.8\%). (Cpd85 had been prepared from an unresolved mixture of TA-C (~80\%) and TA-B.) The NMR spectra for the 2 diastereoisomers of Cpd85 prepared from 13R-TA-C are given in Table V. We conclude (based upon chromatographic, synthetic, and spectral data) that Cpd85 is 1,3-dimethyl-2-[3-(tetrahydro-5-methyl-2-furanyl)-2-butenylidene]-3-cyclohexene-1-carboxylic acid methyl ester. We have assumed in Fig. 2 that Cpd85 has the same stereochemistry at carbon atoms 1 and 13 as natural TA-C (14, 15).

Cpd85 and Cpd76 did not have any detectable effects upon (+) and (−) cultures. For example, a micromole of either compound did not stimulate the formation of zygophores in either P. blakesleeanus or M. mucedo. A micromole of compound did not attract any zygophores of M. mucedo when placed upon a 4-mm² filter paper between (+) and (−) cultures which were 0.7 mm apart, even though zygophores of opposite mating types attracted each other. In tracer experiments, 80\% of the label was recovered after (+) and (−) cultures and uninoculated medium were incubated with 14,400 dpm of Cpd76 for 14 h. All of the label recovered was in Cpd76. Only 30\% of the label was recovered after (+) and (−) cultures were incubated with 44,400 dpm of Cpd85, whereas 60\% was recovered from uninoculated medium. All of the label recovered was recovered.

Fig. 2. Chemical modifications of trisporic acid-C. Chemical modifications of trisporic acid (I) and of its derivatives: methyl trisporate-C (II), methyl 4-dihydrotrisporate-C (III), 4-dihydrotrisporate-C (IV), Cpd76 (V), and Cpd85 (VI). The reactions are: 1, esterification with CH₂N₂; 2, reduction with NaBH₄; and 3, dehydration catalyzed by traces of acid. The wiggly line representing a bond from a chiral carbon indicates that both R and S diastereoisomers were made.

Table V

| Signal (J) | δ major | δ minor | Assignment |
|-----------|---------|---------|------------|
| d(6)      | 1.26    | 1.33    | 13CH₃      |
| s         | 1.75    | 1.79    | 1CH₃       |
| s         | 1.92    | 1.92    | 5CH₃       |
| s         | 3.67    | 3.67    | 1-OCH₃     |
| m         | 4.12-4.22 | 3.97-4.07 | 13H         |
| t(7)      | 4.42    | 4.24    | 10H         |
| broad s   | 5.74    | 5.74    | 4H          |
| d(12)     | 6.08    | 6.13    | 7H         |
| d(12)     | 6.29    | 6.29    | 8H         |
from uninoculated medium was Cpd85. Over 98% of the label recovered from cultures was in the neutral fraction, primarily as Cpd85, but traces of 2 and 4 unidentified compounds were found in (+) and (-) cultures, respectively. The purified acid fraction contained the remaining label. In summary, Cpd85 and Cpd76 apparently are not zygophore-stimulating pheromones, zygotropic pheromones, or precursors of trisporic acids.

**DISCUSSION**

**Zygophore-stimulating Pheromones in (+) Culture Medium**—Four pheromones were isolated from the medium of (+) cultures of *B. trispora*: methyl TA-B, methyl TA-C, methyl 4-dihydroTA-B, and methyl 4-dihydroTA-C (Table I). This is the first report of the isolation of methyl TAs from (+) cultures. The amount of pheromones isolated from a given (+) culture is a function of both the composition of the medium used for growing the culture (3) and the extraction procedure. Over 90% of the TA-stimulating components (pheromones) was recovered when (+) culture medium was extracted directly, whereas only 44% was recovered when the culture medium was adjusted to pH 2 prior to extraction (16). If the appropriate corrections are made for losses of pheromone upon injection of the column and to pooling the fractions, the difference in pheromone levels between neutral fraction extract and culture medium were due solely to the destruction of methyl 4-dihydroTA-C by acid with the corresponding formation of an anhydro derivative when exposed to acid (Table III). They proposed a structural formula for the anhydro ester based upon its polarity and m/e 304. UV, IR, NMR, and mass spectral data of Cpd85 verify that it has the structure which Bu′Lock proposed for the anhydro ester (see Fig. 2). We were unable to find evidence that Cpd85 or Cpd76 possessed either zygophore-stimulating or zygotropic pheromone activity, or that they were metabolized significantly by separate mating type cultures of *P. blakesleeanus*. The artificial origin of Cpd85 and Cpd76 is supported by the finding that, in the absence of cultures, traces of acid catalyzed their formation from methyl 4-dihydroTA-C and 4-dihydroTA-C, respectively. The reactions appear irreversible. We propose that any metabolite of TAs with hydroxyl groups at carbon atoms 4 and 13, such as 4-dihydrotrisporin-C, will form the corresponding anhydro derivative when exposed to acid.

The identification of Cpd76 as an anhydro derivative of 4-dihydroTA-C indicates that the esterase in (-) cultures is not absolutely specific for methyl TAs but can catalyze the hydrolysis of methyl 4-dihydroTAs too. The presence of large amounts of Cpd76 in the medium of (-) cultures incubated with methyl 4-dihydroTA-C implies that the esterases are ineffective in oxidizing the hydroxyl group on carbon atom 4 of 4-dihydroTA-C or that 4-dihydroTA-C dehydrates to Cpd76 in the presence of cultures. In either case, methyl TAs would be the predominant biosynthetic intermediates of TAs made from methyl 4-dihydroTAs.

We conclude that under physiological conditions both methyl 4-dihydroTAs and methyl TAs are mating type-specific zygotropic stimulating compounds in *P. blakesleeanus* because TAs, which are more active in bioassays with (+) cultures than these compounds, are inactive in crosses of (+) and (-) TAs. We do not know which compounds initiate and maintain (regulate) the formation of zygophores. Are they the pheromones themselves or their metabolites, intrazygalfal TAs? The regulatory molecules could be inactivated either by being converted to TAs or by being released into the medium. The prevalent opinion that TAs are the regulatory compounds is based upon circumstantial evidence. First, TAs were the initial compounds to be identified as zygophore-stimulating agents in *M. mucedo* (20). This was 3 years before it was realized that TAs might be synthesized by way of mating type-specific precursors (21) and 7 years before mutant studies verified the existence of mating type-specific pheromones (4). Second, TAs were about 4 times more active than methyl 4-dihydroTAs in stimulating zygophore formation in (-) *M. mucedo* based upon a dilution bioassay (10). However, 1) the relative stability of the two compounds under bioassay conditions is unknown, and 2) other workers have reported that TAs and methyl 4-dihydroTAs have equal zygophore-stimulating activities in (-) *M. mucedo* based upon the amount of compound needed for a half-maximal response (22). Third, (-) mutants of *M. mucedo* such as N301 are defective in their response to TAs under certain conditions, and to methyl 4-dihydroTAs under all conditions tested (22). Although these mutants demonstrate that TAs enter the cell, they do not indicate which compounds are the regulatory molecules. *M. mucedo* could...
pheromones are isolated. They are (+) pheromones, mating type-specific precursors of TAs; trisporins and trisporols are (−) pheromones. The distinguishing feature of (+) and (−) pheromones is the degree of modification of the pro-S methyl group on carbon atom 1 of 4-dihydrotrisporin, a putative intermediate made by both mating types. The methyl group is either unaltered (e.g., trisporins) or oxidized to an alcohol (e.g., trisporols) in (−) pheromones and oxidized to a carboxyl group which is esterified in (+) pheromones. The finding that (+) cultures do oxidize the hydroxyl group on carbon atom 4, possibly by the same enzyme protein which catalyzes this reaction in (−) cultures (23), implies that (+) cultures possess a mechanism, unrecognized to date, for inactivating TA precursors which would self-stimulate the formation of zygophores.

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![Diagram](image-url)
Materials and Methods

Tricyclic acids and their derivatives are sensitive to near UV radiation (24, 25) oxygen (26), and mineral acids. All work involving these compounds was done under red lights. Glassware was washed with slightly alkaline detergent. Merck compounds were extracted with 1:3 methanol near the end of the procedure for their purification to neutralize traces of any acid that might be present. All compounds were stored in ethanol at a convenient room temperature until needed. A nitrogen atmosphere was used at 25°C.

The quantity of TAs, extracts, and extracts was determined spectrophotometrically and was expressed as mp/mg unless otherwise stated. The absorbance reading of the sample at 254/190 (A254/A280) times the dilution factor times the total ml of undiluted sample. Quantities of compounds also were expressed in mg and mol. Melanogenic activities for samples in ethanol are given in Table IV. Ethanol always refers to 95% ethanol.

Culture and Culture Medium

The white type strain used in this work are Phoma clavata (Berk.) Sacc., and P. blakeanum (Jones et al.) H. Thom. In the dark at 23°C (± 2°C), cultures were grown on Potato Dextrose agar slants containing glucose (2%), potato (1%), and malt extract (0.5%) and were transferred monthly. Cultures containing melanin on the plug and inoculated with TAs separately are referred to as (+) cultures, (+) cultures, TAs extract, and (+) cultures. Cultures containing melanin both plug and inoculated with TAs (++) cultures are referred to as (+++) cultures.

Experimental cultures were grown on medium containing 24% glucose, amino acids, thiamine-HCl, and salts described in (1). The amount and kind of amino acids used in the medium varied as follows: Medium I, 0.16% monosodium L-glutamate and 0.5% L-serine; Medium II, 0.14% monosodium L-glutamate, 0.5% L-tyrosine, and 0.5% L-serine; and Medium III, 0.12% monosodium L-glutamate and 0.5% L-asparagine. Medium I and II were prepared with 1:3 methanol. An ethanol medium was prepared by adding 0.5% ethanol to Medium I. Medium containing potato extract and glucose-thiamine (16) was used initially with cultures described for TAs. Cultures containing melanin both plug and inoculated with TAs (+++) cultures are referred to as (+++) cultures.

Extraction of medium was carried out using a 125 ml Erlenmeyer flask containing 20 ml of medium. Cultures were removed from the medium, washed with 30 ml of slightly alkaline detergent solution, and the remaining medium was filtered on glass wool. The filtrate and the washing solution then were added to the flask with 20 ml of ethanol (95%). The mixture was transferred to a separatory funnel and was extracted three times with 20 ml of ethyl acetate. The combined organic layers were washed with 20 ml of a 5% aqueous NaCl solution, and the resulting organic layer was concentrated to approximately 1 ml. The resulting concentrated extract was chromatographed on a Sephadex LH-20 column. Each type of chromatography was done under red lights. Aliquots of concentrated extract were eluted with ethyl acetate and eluted with 1 ml of ethyl acetate. Each type of chromatography was done under red lights. The concentration of TAs, derivatives, and extracts were determined as described in (17).

Supplement to "PHYCOMYCES STIMULATING PRECURSORS (PHEROMONES) OF TRISOPORE ACIDS ACTIVE IN PHYCOMYCES BLAKEANUS: ACID-CATALYZED WINHYDRO-DERIVATIVES OF METHYL 4-DIHYDROTRISOPORATE-C AND 4-DIHYDROTRISOPORATE-C"

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Culture and Culture Medium

The white type strain used in this work are Phoma clavata (Berk.) Sacc., and P. blakeanum (Jones et al.) H. Thom. In the dark at 23°C (± 2°C), cultures were grown on Potato Dextrose agar slants containing glucose (2%), potato (1%), and malt extract (0.5%) and were transferred monthly. Cultures containing melanin on the plug and inoculated with TAs separately are referred to as (+) cultures, (+) cultures, TAs extract, and (+) cultures. Cultures containing melanin both plug and inoculated with TAs (++) cultures are referred to as (+++) cultures.

Experimental cultures were grown on medium containing 24% glucose, amino acids, thiamine-HCl, and salts described in (1). The amount and kind of amino acids used in the medium varied as follows: Medium I, 0.16% monosodium L-glutamate and 0.5% L-serine; Medium II, 0.14% monosodium L-glutamate, 0.5% L-tyrosine, and 0.5% L-serine; and Medium III, 0.12% monosodium L-glutamate and 0.5% L-asparagine. Medium I and II were prepared with 1:3 methanol. An ethanol medium was prepared by adding 0.5% ethanol to Medium I. Medium containing potato extract and glucose-thiamine (16) was used initially with cultures described for TAs. Cultures containing melanin both plug and inoculated with TAs (+++) cultures are referred to as (+++) cultures.

Extraction of medium was carried out using a 125 ml Erlenmeyer flask containing 20 ml of medium. Cultures were removed from the medium, washed with 30 ml of slightly alkaline detergent solution, and the remaining medium was filtered on glass wool. The filtrate and the washing solution then were added to the flask with 20 ml of ethanol (95%). The mixture was transferred to a separatory funnel and was extracted three times with 20 ml of ethyl acetate. The combined organic layers were washed with 20 ml of a 5% aqueous NaCl solution, and the resulting organic layer was concentrated to approximately 1 ml. The resulting concentrated extract was chromatographed on a Sephadex LH-20 column. Each type of chromatography was done under red lights. Aliquots of concentrated extract were eluted with ethyl acetate and eluted with 1 ml of ethyl acetate. Each type of chromatography was done under red lights. The concentration of TAs, derivatives, and extracts were determined as described in (17).

Analysis of acid and alcohol fractions: tracer experiments. Culture medium was obtained from strain of P. blakeanum by passage through a filter of agarose. A number of cultures in a 250 ml Erlenmeyer flask containing 50 ml of medium and 10 ml of ethanol (95%) were incubated to the point where the culture was concentrated to 5 ml. A 1 ml aliquot of the culture was removed, and the remaining sample was stored at -20°C. When a sample was used, a 0.5 ml aliquot was removed from the culture, and the remaining sample was stored at -20°C. A 0.5 ml aliquot of the culture was removed, and the remaining sample was stored at -20°C. The concentration of TAs, derivatives, and extracts were determined as described in (17).

Pheromones and Artifacts in Phycomyces

Supplement to "PHYCOMYCES STIMULATING PRECURSORS (PHEROMONES) OF TRISOPORE ACIDS ACTIVE IN PHYCOMYCES BLAKEANUS: ACID-CATALYZED WINHYDRO-DERIVATIVES OF METHYL 4-DIHYDROTRISOPORATE-C AND 4-DIHYDROTRISOPORATE-C"

RICHARD P. BOTTGER AND JOANNE P. WHITAKER

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Duplicate 0.1 ml portions of each neutral and purified acid fraction were counted; duplicate 0.05 ml portions of each fraction were diluted for absorbance measurements (Table I). All absorbance determinations were made with light yellow solutions at pH 7.4. No significant differences were noted in the absorbance of each fraction containing 0.005 ml units were filtered quantitatively and then concentrated to 0.0 ml units/m1 by bubbling with nitrogen gas. Duplicate 0.0 ml portions of each fraction were determined by thin-layer chromatography of 0.02 ml units using solvent systems 1 and 2 separately. Subsequently, 0.06 ml units were chromatographed on 3 strips with one solvent system for qualitative analyses. Each compound was dissolved in 0.5 ml of ethanol and then filtered through Whatman No. 4 filter paper to remove particles of silica gel. Duplicate 0.1 ml portions were analyzed for radioactivity and triplicate 0.05 ml portions were diluted (or for absorbance measurements) and dried with nitrogen gas. The thin-layer chromatographic determinations were calculated from the same 0.02 ml units for each. In the calculations, all radioactivity measurements were made in a Beckman 1205 liquid scintillation counter. As the sample in ethanol mixed with 3 ml of Bray's [27] solution without 1% (w/v) 2-propanol was incubated for 30 minutes at 0°C, then filtered and the filter was washed with 5 ml of ethanol. The radioactivity of the filter was then measured with a liquid scintillation counter. To prepare the antigen, the antiserum was exposed to the chloroform layer. In a solution, was quantitatively transferred to a separatory funnel with the aid of a CMC. One-third the volume of the chloroform fraction was added. The reaction was broken by shaking and the chloroform fraction was removed by filtration; 0.2 ml of chloroform and 0.2 ml of ethyl alcohol. The volume of each fraction was not less than 40 times the volume of the chloroform fraction. The volume was less than 10 times the volume of the chloroform fraction. The volume was less than 20 times the volume of the chloroform fraction. The volume was less than 50 times the volume of the chloroform fraction. The volume was less than 100 times the volume of the chloroform fraction. The volume was less than 200 times the volume of the chloroform fraction. The volume was less than 500 times the volume of the chloroform fraction. The volume was less than 1000 times the volume of the chloroform fraction. The volume was less than 2000 times the volume of the chloroform fraction. The volume was less than 5000 times the volume of the chloroform fraction. The volume was less than 10000 times the volume of the chloroform fraction. 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