Relationship Between Sexuality and Carotene Synthesis in *Blakeslea trispora*

H. van den Ende

Laboratorium voor Algemene Plantkunde, Plantenfysiologie en Farmacognosie, Universiteit van Amsterdam, Amsterdam, The Netherlands

Received for publication 15 July 1968

When stimulated by equivalent amounts of progametangia-inducing hormones, cultures of the minus mating type of *Blakeslea trispora* produce about the same quantities of carotenoids as mated cultures of the fungus, which suggests that the stimulation of carotene synthesis during the sexual activity of mated cultures is the result of hormonal action. These hormones were isolated and purified. From spectroscopic analysis of purified samples, it appears that the hormones are identical with trisporic acids B and C. When both mating types of *B. trispora* were cultivated in one vessel but were kept apart by membrane filters, the formation of sex hormones was not inhibited. Physical contact between the mating types is obviously not required for the induction of sexual activity. The sex hormones also formed in combined cultures of *B. trispora*-plus and *Zygorhynchus moelleri* (a homothallic species), but not in combined cultures of *B. trispora*-minus and *Z. moelleri*. This is evidence for the hypothesis that the hormones are produced by *B. trispora*-plus only.

The sexual activity of fungi belonging to the order Mucorales is often accompanied by an increased accumulation of carotenoids. *Blakeslea trispora* exhibits an enhanced carotene production when the plus and minus mating types are cultivated together (10). The suspensors and gametangia of *Mucor mucedo* are colored more intensely than the vegetative mycelium. In the bioassay of progametangia-inducing compounds with *M. mucedo* (9, 12), a change in color of the mycelium around the test spot can often be observed. Burgeff (6) showed that the mycelium of this fungus turns yellow, even though the mating types are kept apart by a collodion membrane. Barnett et al. (4), working with *Choanephora cucubitum*, reported the same phenomenon.

Several authors have expressed opinions about the relationship between sexuality and carotene production, but unequivocal evidence has never been put forward (15). Piemel (13) reports that the formation of "gamones" runs parallel with the accumulation of carotenoids in *B. trispora*, but he is unable to demonstrate the stimulation of carotene production in the separate mating types by his "chromatographically pure gamone" that was isolated from the medium of a mated culture. Thomas and Goodwin (17), however, show that carotene production in a culture of the minus mating type of *B. trispora* is increased by the addition of a crude extract of the medium of a mated culture. They suggest that trisporic acids, the structure of which has been elucidated by Caglioti et al. (7), are responsible for this increase.

The present report confirms that trisporic acids B and C induce the formation of carotenogenesis which accompanies the sexual activity in *B. trispora*. The isolation and improved purification of these compounds from the media of mated cultures of *B. trispora*, *M. mucedo*, and combined cultures of *B. trispora*-plus and *Zygorhynchus moelleri* are described. It is demonstrated that these trisporic acids should be considered as "sex hormones" according to Raper's definition (14), because they induce the formation of progametangia in *M. mucedo*.

Materials and Methods

**Cultures.** All cultures were obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Stock cultures of *M. mucedo* Bref. strain Saito (−) (CBS 109.16) and strain Burgeff (+) (CBS 144.24) were maintained on slants consisting of 20% beer wort (Amstel Brouwerij) and 1.5% agar, neutralized with KOH to pH 7 before sterilization. After inoculation, the cultures were kept at 20°C for 1 week, stored at 3°C, and transferred twice a year. Low temperature and infrequent transfer are necessary conditions for the preservation of sexual competence. Liquid culture
media consisted of 20% neutralized beer wort. Biological tests were performed in petri dishes with a medium consisting of 20 g of glucose, 10 g of KNO₃, 5 g of KH₂PO₄, 2.5 g of MgSO₄-7 H₂O, 1 g of yeast extract (Oxoid), 15 g of lonomar (Oxoid), and 1,000 ml of demineralized water.

B. trispora Thaxter strain NRRRL 2456 (+) (CBS 130.59) and strain NRRRL 2457 (−) (CBS 131.59) were maintained at 20 °C on slants consisting of 2 g of glucose, 300 ml of potato extract, and 15 g of agar per liter. The pH was adjusted to 8 with KOH before sterilization. Transfers were made once a month. Liquid media were composed of 20 g of glucose, 300 ml of potato extract, 100 ml of beer wort, and 600 ml of water (pH 8) before sterilization. Potato extract was prepared by homogenizing 100 g of peeled potatoes in 300 ml of water and filtering through cheesecloth. Liquid media (100 ml in 200-ml conical flasks) were inoculated with some mycelium which had been homogenized in its original culture medium in a Waring Blender for 30 sec at top speed. Liquid cultures were incubated at 20 °C in the dark on a shaking machine.

Zygothchythus moellori Vuill. (CBS 501.66) was treated in the same way as B. trispora.

Materials. Membrane filters were obtained from Sartorius-Membranfilter GmbH, 34, Götttingen, W. Germany. Silica gel for thin-layer chromatography (Merck 7731) and for column chromatography (Merck 7733) were obtained from Merck Sharp and Dohme, Rahway, N.J. Magnesium oxide (“Baker Analyzed” Reagent no. 1123) was obtained from J. G. Baker, Phillipsburg, N.J. All solvents were purified by distillation. Hexane for chromatography was purified by chromatography on silica gel followed by distillation.

Purification of sex hormones. A 10-liter amount of culture fluid was obtained from 7-day-old mated cultures of B. trispora. The fluid was filtered through cheese cloth and extracted with ethyl acetate. The extract was concentrated in vacuo and filtered. The resulting solution was extracted with 5% aqueous KHCO₃ which, after acidification, was extracted with diethyl ether. The extracted ethyl acetate fraction is called the neutral fraction. The final solution in diethyl ether is called the acidic fraction. It was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The concentrated solution was applied to about 15 layers of silica gel (20 X 20 cm), which were developed in the dark with a mixture of chloroform-diisopropyl ether-acetone-acetic acid (70: 15:10:5). The ultraviolet (UV)-absorbing spots with Rₚ = 0.37 (compound 2) and 0.51 (compound 3) were scratched off and were separately extracted with ethyl alcohol. The resulting preparations were used for experiments in which sexual activity and influence on carotene synthesis were studied. Samples for mass- and nuclear magnetic resonance (NMR)-spectrometry were further purified as follows. The ethyl alcohol was removed in vacuo; the residues were taken up in diethyl ether, cooled, and treated with a solution of diazomethane in diethyl ether at 0 °C for 10 min. The solvent and the excess diazomethane were removed in vacuo and the residues were taken up in a few milliliters of hexane. The solutions were extracted with 5% aqueous KHCO₃, washed with water, and dried over anhydrous sodium sulfate. After filtration and concentration, each solution was applied to a column of silica gel (9 X 3 cm). The columns were developed with 100 ml of hexane, 50 ml of 2% acetone in hexane, and 150 ml of 20% acetone in hexane, respectively. The last 100 ml of effluent contained the methyl ester of compound 2 or 3 (see Table 1). The solutions were stored in the dark at less than 0 °C.

Sexual activity was determined according to Plemel (12).

Carotenoids were extracted from lyophilized mycelium by homogenizing several times in hexane. After filtration, the optical density at 450 nm of the extract was used as a crude estimate of the concentration of carotenoids. Purification was effected by saponification and chromatography according to Anderson et al. (1). A column of 6 X 0.6 cm, filled with magnesium oxide-Celite (1:1) was used. The pigments were eluted with hexane containing 0 to 20% acetone. Lycopene was extracted with 10% ethyl alcohol in hexane after extraction of the column. The pigments were identified by their absorption spectra with the values given by Davies (8).

Mass- and NMR-spectrometry was performed at the Laboratorium voor Organische Scheikunde, Universiteit van Amsterdam. The NMR-spectra were obtained with a Varian model HA-100 instrument. The chemical shifts are expressed in parts per million (ppm, δ) relative to tetramethylsiline. The solvent was carbon tetrachloride. For mass spectrometry, an AEI MS-9 mass spectrometer was used.

RESULTS

Stimulation of carotene synthesis. By thin-layer chromatography on silica gel of an extract of the medium obtained from mated cultures of B. trispora, a number of UV-absorbing compounds were separated, which were not present in culture media of the separate mating types. In Table 1, the Rₚ values of these compounds are given, together with their sexual activity on M. mucido. The compounds numbered 2 and 3 induced the formation of progametangia on the mycelium of both plus and minus mating types of this fungus.

Compounds 2 and 3 were also produced in smaller amounts by mated cultures of M. mucido and by combined cultures of B. trispora-plus and the homothallic species Z. moellori. However, sexually active substances were not produced by combined cultures of B. trispora-minus and Z. moellori.

The addition of compounds 2 or 3 to a culture of B. trispora-minus in a potato extract medium caused a remarkable increase in the production of carotenoids (Table 2). Compound 3, although occurring in much lower concentration than
TABLE 1. RF values of UV-absorbing compounds from mated cultures on thin-layer chromatograms on silica gel

<table>
<thead>
<tr>
<th>Compound</th>
<th>RF valuesa</th>
<th>Ultra-violet maxa</th>
<th>Biological activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mixture A</td>
<td>Mixture B</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.19</td>
<td>0.10</td>
<td>300</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>0.23</td>
<td>325</td>
</tr>
<tr>
<td>3</td>
<td>0.51</td>
<td>0.41</td>
<td>322</td>
</tr>
<tr>
<td>4</td>
<td>0.69</td>
<td>0.57</td>
<td>285</td>
</tr>
</tbody>
</table>

a Mixture A: chloroform-diisopropyl ether-acetone-acetic acid (70:15:10:5); mixture B: chloroform-acetic acid (95:5).
b UV-absorption maxima for solutions in ethyl alcohol (nm).

Biological activity was tested on M. mucido-plus and on M. mucido-minus. The results are for both mating types.

TABLE 2. Influence of addition of compounds 2 and 3 on the production of carotenoids by B. trispora-minus

<table>
<thead>
<tr>
<th>Additionsa</th>
<th>Dry weight (g)</th>
<th>OD450 per 100 ml of extract per gb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl alcohol (0.5 ml) ..........</td>
<td>1.521 ± 0.20</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Compound 2 ....</td>
<td>1.110 ± 0.22</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Compound 3 ....</td>
<td>1.166 ± 0.10</td>
<td>0.21 ± 0.04</td>
</tr>
</tbody>
</table>

a An 0.5-ml amount of a solution of compound 2 or 3 in ethyl alcohol (OD at 325 nm, 60) was added to 200 ml of 3-day-old cultures. The mycelium was analyzed after 24 hr of incubation on a shaking machine at 20 C in the dark.
b Amounts of carotenoids formed expressed by OD at 450 nm of 100 ml of hexane extract per g (dry weight). The values are averages of three determinations; maximal deviations of the average are indicated.

compound 2, appeared to be by far the most active in inducing the formation of progametangia in M. mucido as well as in enhancing the carotene production.

From the physiological point of view, it is important to add about the same quantities of stimulatory products as are present in mated cultures. Because the purification of these labile substances causes considerable losses, rather impure preparations were used to study the relationship between concentration and effect. Very low concentrations are sufficient to get maximal carotene production (Table 3).

The production of carotenoids in a minus culture, which had been stimulated by the addi-

TABLE 3. Influence of the addition of various amounts of the acidic fraction obtained from the medium of mated cultures on the production of carotenoids by B. trispora-minus

<table>
<thead>
<tr>
<th>Acidic fraction addeda</th>
<th>Dry weight (g)</th>
<th>OD450 per 100 ml of extract per gb</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.7336</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>0.7119</td>
<td>0.42</td>
</tr>
<tr>
<td>10</td>
<td>0.6734</td>
<td>0.51</td>
</tr>
<tr>
<td>20</td>
<td>0.7357</td>
<td>0.52</td>
</tr>
<tr>
<td>33</td>
<td>0.6629</td>
<td>0.51</td>
</tr>
<tr>
<td>100</td>
<td>0.7150</td>
<td>0.46</td>
</tr>
</tbody>
</table>

a Amounts added are expressed as percentages of the amount extracted from one 200-ml mated culture. To all cultures, including control, the same quantity of solvent (0.3 ml of ethyl alcohol) was added.
b Amounts of carotenoids are expressed by OD at 450 nm of 100 ml of hexane extract per g (dry weight). Cultural conditions as in Table 2.

TABLE 4. Influence of the addition of the neutral fraction and the acidic fraction of the extract from mated cultures on the production of carotenoids by B. trispora in 100-ml cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>Additiona</th>
<th>Dry weight (g)</th>
<th>OD450 per 100 ml of extract per gb</th>
</tr>
</thead>
<tbody>
<tr>
<td>minus</td>
<td>Neutral fraction</td>
<td>0.345 ± 0.008</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>minus</td>
<td>Acidic fraction</td>
<td>0.340 ± 0.010</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>minus</td>
<td>Acidic fraction</td>
<td>0.361 ± 0.011</td>
<td>0.73 ± 0.22</td>
</tr>
<tr>
<td>plus</td>
<td>Neutral fraction</td>
<td>0.423 ± 0.014</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>plus</td>
<td>Acidic fraction</td>
<td>0.403 ± 0.013</td>
<td>0.95 ± 0.06</td>
</tr>
<tr>
<td>mated</td>
<td>Acidic fraction</td>
<td>0.422 ± 0.014</td>
<td>1.02 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.386 ± 0.024</td>
<td>0.95 ± 0.11</td>
</tr>
</tbody>
</table>

a Quantities added provide a concentration of active substances equal to the natural concentration in mated cultures.
b Details as in Table 2. The values are averages of three determinations; maximal deviations of the average are indicated.
The same stimulation of carotene synthesis of the minus mating type was observed when the plus and the minus mating types were grown in a liquid medium on opposite sides of a membrane filter (pore diameter 10 nm) which was clamped between ground edges of two horizontal, cylindrical glass containers. Even when the barrier consisted of two membrane filters separated by 25 ml of permanently sterile medium, neither the formation of progametangia-inducing hormones nor the stimulation of carotene synthesis was prevented; the processes were only retarded. The only way to explain this result is to assume that the formation of compounds 2 and 3 was induced by soluble substances in the medium, produced by one or both mating types and able to penetrate membrane filters with pores of 10 nm diameter.

Structure of compound 2. The UV-spectra of this compound in ethyl alcohol (maximal absorption at 230 and 325 nm) and its methyl ester in hexane (maximal absorption at 320 nm, with shoulders at 305 and 335 nm) correspond to the description of the spectrum of trisporic acid C (7). By high resolution mass spectrometry of the methyl ester of compound 2, a molecular weight of 320.19670 was found, in accordance with the formula C_{19}H_{28}O_{4} (calculated: 320.19875). The NMR-spectrum of the methyl ester of compound 2 is in agreement with the structure (Fig. 1 and 3) proposed by Caglioti et al. (7). On the basis of this evidence, compound 2 is considered to be identical with trisporic acid C.

Structure of compound 3. The UV-spectra of this compound and its methyl ester are practically identical with the spectra of compound 2 and the methyl ester of compound 2. The molecular weight of the methyl ester, as obtained by mass spectrometry, is 318. The NMR-spectrum of the methyl ester is different from the spectrum of the ester of compound 2: instead of a doublet at $\delta = 1.14$, a singlet at $\delta = 2.06$ ppm is present (Fig. 2). Therefore, it is probable that this substance has the structure of compound 2, in which a carbonyl group instead of a hydroxyl group is present (Fig. 3). Caglioti et al. (7) described a similar substance, lacking the hydroxyl group, which they called trisporic acid B. We propose to attempt identification of compound 3 with trisporic acid B.

### Table 5. Influence of compounds 2 and 3 on the production of various carotenoids in B. trispora-minus

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phytofluene</th>
<th>β-Carotene</th>
<th>Zeaxarotene</th>
<th>y-Carotene</th>
<th>Neurosporene</th>
<th>Lycopene</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>15</td>
<td>2</td>
<td>13</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Compound 2</td>
<td>9</td>
<td>44</td>
<td>8</td>
<td>20</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Compound 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Amounts are expressed as micrograms per gram (dry weight) of mycelium. Experimental details as in Table 2.

* The maxima in the absorption spectrum of this pigment are 400, 423, and 450 nm. It might consist of a mixture of α- and β-zeaxarotene (11).

![Fig. 1. NMR-spectrum of compound 2.](image-url)
that all of progametangia are easily distinguished because its progametangia are
produced by mated cultures of several members of the Order Mucorales, e.g.,
M. mucedo, B. trispora, Phycomyces blakesleeanus, and also by mixed cultures of
heterothallic species of this Order (13). Moreover, illegitimate copulations can be
observed between many species of the Mucorales (5, 6). Thus, a great similarity in
sexual development within this Order is demonstrated. Therefore, it seems justified
to assume that all substances which evoke progametangia on M. mucedo play a similar
role in the sexuality of the fungus by which they are produced. The described
compounds are thus considered as "sex hormones" (14).

There are two possible ways in which stimulation of carotene synthesis and
sexual activity may be coupled: (i) the formation of compounds 2 and 3 and the stimulation of carotene synthesis are both simultaneously induced by one common
mechanism; (ii) one of the first expressions of sexual activity is the induction of the synthesis of compounds 2 and 3, which induce the formation of progametangia as well as stimulate the carotene synthesis.

The quantities of carotenoids in minus mating type cultures, which have been stimulated by the addition of sex hormones, and in mated cultures are almost the same. From this fact it is concluded that the second possibility (ii) is the way in which stimulation takes place in mated cultures.

The physiological role of compounds 1 and 4 is uncertain. Compound 1, which is present in the neutral fraction, has little or no influence on the production of carotenoids (Table 4). Compound 4 occurs in very small and varying amounts. It has not been possible to purify this substance sufficiently to permit its characterization.

It is interesting that only the minus mating type of B. trispora is stimulated; this has also been observed by other workers (16, 17). It is an example of a physiological difference between the plus and the minus mating type of B. trispora. However, one cannot generalize, because the cultural conditions appear to have a strong influence on the physiological behavior of the fungus, particularly with respect to carotene accumulation. This may explain, in part, the considerable variability which is observed in the values for carotene content in Tables 2, 3, and 4. For the strains used, we have unfortunately found no satisfactory synthetic medium in which sexual activity as well as substantial carotene synthesis takes place.

It is noteworthy that the plus mating type of B. trispora produces about the same quantities of carotenoids as a mated culture. The possibility of contamination of this strain by the other mating type was checked several times by the transfer of one-spore cultures. However, Anderson et al. (2), reporting the same strain numbers, did not observe any significant difference in the carotene production between the plus and the
minus mating types when grown on a corn-based medium. This has been verified with our cultures. Evidently potato extract has a stimulating influence on this strain.

Very little is known about the primary induction of sexual differentiation. Physical contact between the mycelia of the two mating types is obviously not necessary for the induction of sexual activity and stimulation of carotene production, as was demonstrated in the experiment in which the mycelia were kept apart by one or two membrane filters. This result strongly supports the hypothesis put forward by Plempel (12) and Banbury (3), that the synthesis of “gamones” is induced by diffusible “progamones” which are regularly formed by the separate mating types. Since the dispersion of the mycelia of both mating types before combination results in a higher production of factors that stimulate carotenogenesis, Sutter and Rafelson (16) suggested that physical contact is necessary for the induction of these factors. According to their view, no soluble metabolites are involved in the induction process, which contradicts the result of the membrane experiment described above. Sutter and Rafelson’s result could, however, be explained by assuming that dispersed mycelia are more susceptible to the soluble substances that are responsible for the induction of sexual activity.

The results of the experiments with Z. moelleri may be explained by supposing that the sexual hormones are produced only by the plus mating type of B. trispora. The same supposition was made by Sutter and Rafelson (16), who observed a proportionality between the amount of plus inoculum and the production of stimulating factors in mated cultures. However, more research is needed to support this assumption.

From spectroscopic data, it appears that compounds 2 and 3 are identical with trisporic acids C and B, respectively. The NMR-spectra reveal that even the most highly purified samples contain several isomers. It has been observed (9) that isomerization is caused by illumination, which results in a transformation of the UV- and NMR-spectra and in a decrease of biological activity.

This isomerization is reversible: biological activity is fully restored by a short illumination in the presence of catalytic amounts of iodine. The nature of this isomerization, however, is not fully understood.

ACKNOWLEDGMENTS

I thank D. Stegwee and G. Marsman-Goertz for helpful advice and criticism, and P. S. Tims for excellent technical assistance.

The assistance of Th. J. de Boer (Laboratorium voor Organische Scheikunde, Universiteit van Amsterdam) for facilities with respect to NMR- and mass spectrometry is gratefully acknowledged.

LITERATURE CITED


