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REVIEWS

Trisporoids and Carotenogenesis in *Blakeslea trispora*

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Abstract— The recent data on the pathways of carotenoid biosynthesis, which resulted in revision of the earlier concepts, are analyzed. Trisporoid diversity and their role in the sexual process of mucoraceous fungi, resulting in formation of sexual cells (zygospores) are discussed. Special attention is paid to the role of trisporic acids in carotenogenesis of combined culture of the (+) and (–) strains, in which zygospores are not formed.

Keywords: *Blakeslea trispora*, trisporoids, trisporic acids, carotenogenesis

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INTRODUCTION

Carotenoid cleavage results in formation of a wide spectrum of apocarotenoids, including retinal (C_{20}), an animal morphogen retinoic acid (C_{20}), a plant hormone abscisic acid (C_{15}), odoriferous substances (such as β-ionone (C_{13}) and safranal (C_{10})) [1]; trisporic acids (TSA), hormones of mucoraceous fungi (C_{18}–C_{19}), etc.

TSA and their precursors are produced during a sexual interaction and are signal molecules that initiate and control the sexual reactions between the (+) and (–) mycelia of heterothallic species, the (+) and (–) copulating branches of homothallic species, as well as the interaction between mycoparasites of mucoraceous fungi with the complementary strains of the host fungus [3–6].

An interest to trisporoids stems from the fact that sexual interaction between heterothallic strains stimulates the synthesis of carotenoids, which have a significant practical value [7–11]. Thus, biotechnologies of β-carotene and lycopene production [12–14], in which a joint submerged culture of the (+) and (–) strains of the fungus is used, were developed on the basis of *Blakeslea trispora*. The main attention in the review will be therefore paid to modern ideas about the biosynthesis of trisporoids and their role in carotenogenesis by submerged *B. trispora* cultures.

BIOSYNTHESIS OF TRISPOROIDS BY MUCORACEOUS FUNGI

As a result of intensive studies in the 1960–1990s (following the discovery of TSA as carotenogenesis stimulators in mucoraceous fungi), an idea on TSA biosynthesis pathways reflected on the presented scheme (Fig. 1) was formed. (1) TSA synthesis is a cooperative process involving the (+) and (–) strains; in the course of this process, precursors produced by one strain can be transformed into TSA only by a complementary strain. Thus, 4-dihydromethyltrisporates formed by the (+) strain are transformed into methyltrisporates only by the (–) strain, while trisporins generated by the (–) strain are transformed into trisporols only by the (+) strain. (2) First stages of TSA synthesis include a symmetric cleavage of β-carotene molecule with subsequent production of C_{20}-retinal, β-C_{18}-ketone, and 4-hydroxy-β-C_{18}-ketone. (3) 4-Dihydrotrisporin is the last common precursor, which is generated by both (+) and (–) strains [17–21].

However, this scheme incompletely elucidates the initial stages of trisporoid synthesis and does not explain a number of facts (for example, production of a small amount of TSA by the (+) strain not involving the (–) strain [22].

The studies conducted by a group of German scientists, who suggested a new approach to investigation of the pathways of trisporoid synthesis using tritium-labeled TSA precursors, became a new breakthrough in the discussed field [23]. Trisporoids (starting from D'orenone and ending with TSA) were added to submerged cultures of the *B. trispora* (+) and (–) strains in three variants, i.e., individual cultures of the (+) and (–) strains and their joint culture. Analysis of the structural changes 3, 6, 9, 12, and 24 h after introduction of the labeled compounds provided a possibility not only to identify the transformation products, but also to assess the rate of their production. The results obtained made it possible to conduct a revision of earlier ideas and to suggest a new scheme of TSA cooperative synthesis at sexual interaction of heterothallic strains of the fungus (Fig. 2), according to which (1) not retinal (C_{20}), but D'orenone (C_{18}) is the first product of β-carotene cleavage; (2) not 4-dihydromethyltrisporins, but trisporols are last common metabolites

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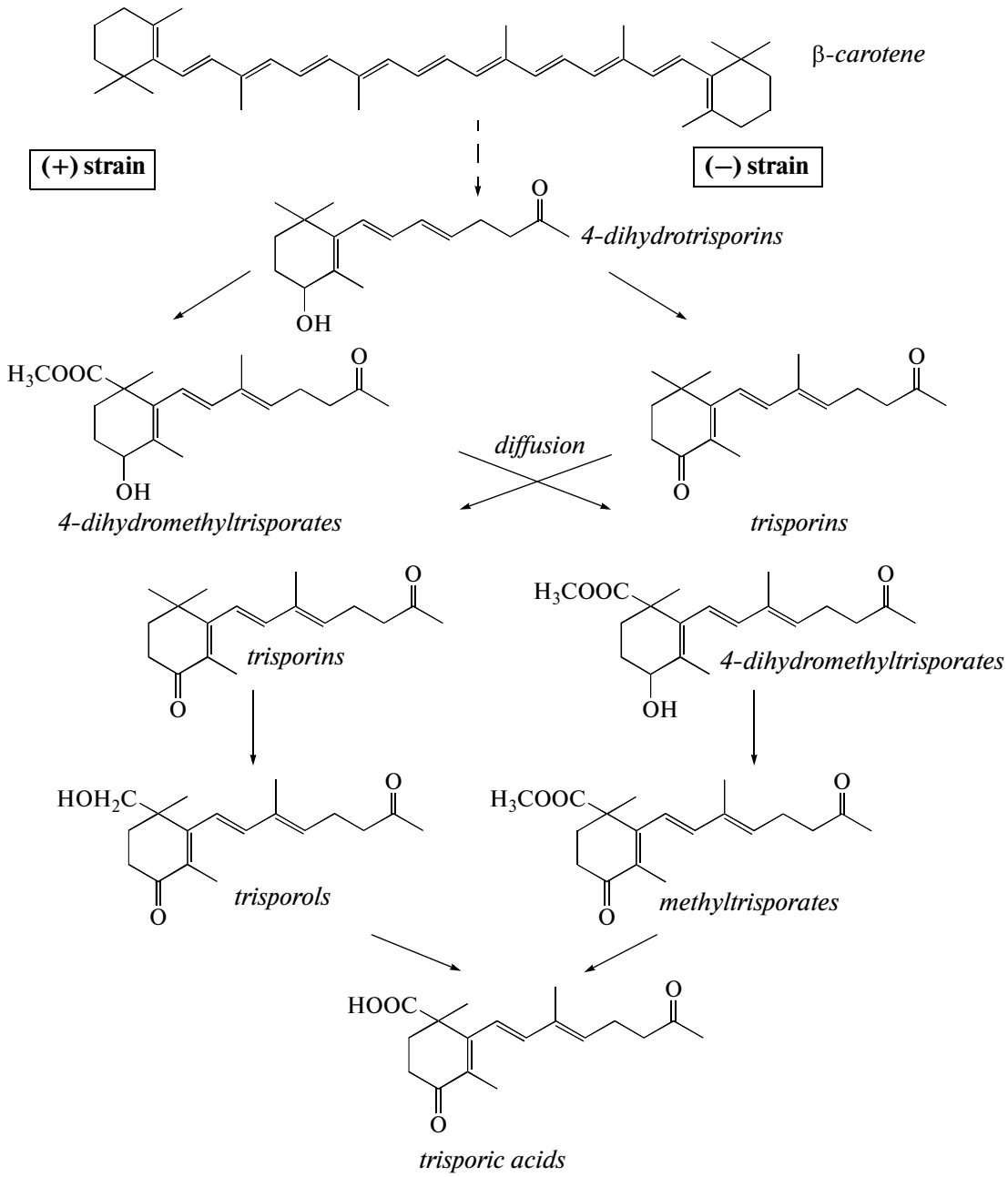


Fig. 1. Scheme of trisporic acid synthesis [15–21].

in the (+) and (–) strains; (3) production of a small amount of TSA by the (+) strain without involvement of the (–) strain was proved; (4) trisporols are oxidized to methyltrisporates only by the (+) strain, while saponification of methyltrisporates to TSA is carried out by both strains, albeit with a different rate; (5) 4-Dihydromethyltrisporates that do not contain a label were found in a joint culture; (6) the reaction of methyltrisporate transformation is irreversible; (7) the study of kinetics of trisporin transformation to trisporols and methyltrisporate transformation to TSA demonstrated that the (–) strain had a considerably higher activity than the (+) strain.

Further study of the products of β-carotene cleavage confirmed these data and demonstrated that cleavage of β-carotene molecule occurs asymmetrically to form C_{18}, C_{15}, and C_7 compounds and, consequently, C_{20} retinal is not an intermediate product in TSA biosynthesis. It was established that C_{15} compounds were formed during the cleavage of β-carotene molecule, rather than as a result of shortening of the side chain of C_{18} trisporoids; consequently, they could not be termed apotrisporoids [24–27]. The role of new

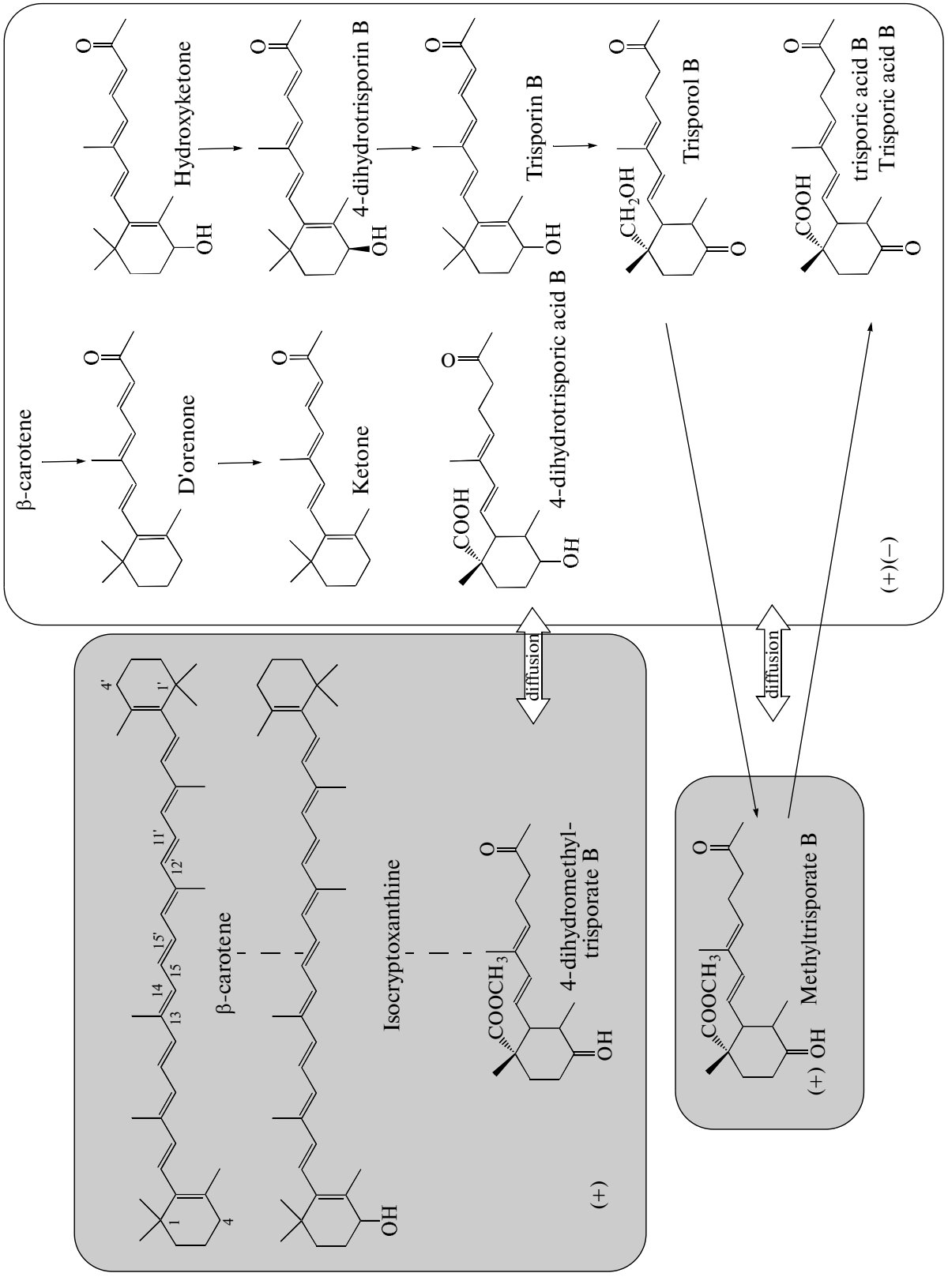


Fig. 2. Scheme of trisporic acid synthesis [23].

apocarotenoids, C_{15} (cyclofarnesoids) and C_7 compounds, remains unstudied.

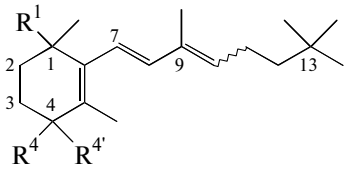
The origin of 4-dihydromethyltrisporates is a main riddle in the new scheme, since the data on their role in TSA synthesis are contradictory. Previously, it was considered that the (–) strain produced trisporins from 4-dihydrotrisporin, while the (+) strain produced 4-dihydromethyltrisporates possessing a high biological activity [28]. However, other studies demonstrated that 4-dihydromethyltrisporates did not incorporate the labels upon addition of tritium-labeled precursors to submerged cultures of the (+) and (–) strains [23]. Moreover, the rate of 4-dihydromethyltrisporate transformation to TSA was low. These facts indicate that 4-dihydromethyltrisporates are possibly generated not from β-carotene, but from its oxidized derivative (isocryptoxanthin) by a parallel pathway, and are almost not included in TSA synthesis [29]. The results of studies of nonenzymatic oxidative cleavage of the β-carotene molecule [30], including data on a decrease in the activity of labile β-carotene oxygenases under the influence of reactive oxygen species (ROS) [31], support this assumption. Thus, the role of 4-dihydromethyltrisporates in the synthesis of trisporic acids remains unclear. The reaction of their transformation into 4-dihydrosporic acid was described; however, its functions in the cell are unknown.

Although the products of β-carotene (and/or isocryptoxanthin) cleavage were not isolated, the following sequence of reactions is proposed based on data on inclusion of tritium-labeled chemically synthesized analogues of trisporoids in the TSA biosynthesis. It is possible that D'orenone (β-C_{18}-ketone) is one of the first products of β-carotene cleavage; it can subsequently (1) be modified into another ketone as a result of reduction of the C_{11}–C_{12} double bond or (2) be transformed into 4-hydroxy-β-C_{18}-ketone by incorporation of oxygen atom (in the form of a hydroxyl group) into the C_4 position in the ring under the action of a monooxygenase [17, 23]. As a result, both products are transformed into 4-dihydrotrisporin (Fig. 2).

Oxidation of the C_4 hydroxyl group of 4-dihydrotrisporin to a ketogroup to form trisporin is the next stage of TSA synthesis. Oxidation of trisporin C_1 methyl group results in formation of trisporol (the last common precursor in the (+) and (–) strains); however, the oxidases that are involved in this process have not been studied.

Oxidation of trisporol C_1 hydroxyl group to a carboxyl group results in formation of methyltrisporate. This reaction is typical of the (+) strain and does not occur in the (–) strain. The enzymes that catalyze this reaction have not been studied.

Methyltrisporate saponification to form TSA under the effect of an esterase occurs in both strains, but the rate of this reaction in the (–) strain is considerably higher [23]. TSA B predominates at initial stages of TSA synthesis; then TSA C starts accumulating as a result of its transformation [33]. The enzyme that performs this transformation was also not studied.



Trisporin	$R^1 = CH_3$	$R^4 = R^{4'} = O$
Trisporol	$R^1 = CH_2OH$	$R^4 = R^{4'} = O$
Methyltrisporate	$R^1 = COOCH_3$	$R^4 = R^{4'} = O$
trisporic acid	$R^1 = COOH$	$R^4 = R^{4'} = O$
4-Dihydromethyl-trisporate	$R^1 = COOCH_3$	$R^4 = H$; $R^{4'} = OH$
4-Dihydrotrisporic acid	$R^1 = COOH$	$R^4 = H$; $R^{4'} = OH$

Trisporoid derivatives	Carbon atom		
	2	3	13
A			
B			=O
C			–OH
D	–OH		=O
E		–OH	–OH

Fig. 3. Trisporoid structure.

It should be noted that the (+) strain is able to generate a small amount of TSA in a separate culture, but considerable stimulation of trisporoid synthesis occurs only in a joint culture. The (+) and (–) strains are responsible for different stages of this process: the reaction of trisporol transformation into methyltrisporate occurs only in the culture of the (+) strain; on the other hand, the activity of a number of enzymes (4-dihydrotrisporin dehydrogenase, 4-dihydromethyltrisporate dehydrogenase, and esterase) is considerably higher in the (–) strain.

DIVERSITY OF TRISPOROIDS

Structurally, trisporoids are biologically active oxidized isoprenoid compounds containing 18 or 19 carbon atoms and having a skeleton out of fourteen carbon atoms [5, 33].

Substituents at C_1 and C_4 determine which group of trisporoids the compound belongs to (TSA, trisporins, trisporols, 4-dihydromethyltrisporates, methyltrisporates, and 4-dihydrotrisporic acids) (Fig. 3). Functional groups at C_2, C_3, and C_{13} determine the type of derivative (A, B, C, D, E) [5]. TSA A, B, C, D, and E [19, 34–36], trisporins and trisporols B and C [15, 34],

4-dihydromethyltrisporates B and C [17, 35, 37], methyltrisporates B, C, and E [38, 39], and 4-dihydrotrisporic acid [23] were isolated. Quantitative ratios of trisporoids vary. Thus, for example, C, B, and A forms account for 55–87%, 12–39%, and up to 16%, respectively, of the *B. trispora* TSA [40]. Taking into account a large diversity of the forms of trisporoids, their composition may be considered specific for each fungus. There is as yet no answer to the question of why such diversity of trisporoid forms is required. The question of whether TSA precursors possess some regulatory function or are only metabolites of TSA synthesis pathway is discussed in the literature [5]. The data on the inhibition of the growth of root fibrils in different plant species by D'orenone may indicate the existence of such functions [41].

Biological activity of trisporoids is usually assessed according to the amount of sexual hyphae (zygosporophores), the formation of which is induced by these compounds in separate surface cultures of the (+) and (–) test strains [2]. It is possible to obtain the most clear sexual response when using the *Mucor mucedo* (+) and (–) strains as test organisms, since this fungus forms zygosporophores on aerial hyphae, unlike other studied members of the *Mucorales* (*Phycomyces blakesleeanus* and *B. trispora*); as a result, it is easy to record them.

Biological activity of trisporoids is different. Thus, TSA B and C induce the production of zygosporophores in both test strains, methyltrisporates and 4-dihydromethyltrisporates act only on the (–) strain, while trisporins B and C act only on the *M. mucedo* (+) strain [33–37]. The biological activity of trisporoids increases in the following series: A-derivatives < C-derivatives < B-derivatives [5, 40]. The presence of polar functional groups at C_4 and C_{13}, R conformation of the C_4 chiral center, *cis*-conformation of C_9, and a saturated C_{11}–C_{12} bond are the structural features that cause the biological activity of trisporoids [5, 33].

Thus, a wide spectrum of trisporoids with diverse biological activity is generated at sexual interaction of heterothallic strains of mucoraceous fungi. The fungus *B. trispora* is the most studied in this respect; as compared to other mucoraceous fungi, it generates the largest amount of trisporoids, which makes it an attractive model object for the study of their synthesis and physiological role.

ENZYMES OF TRISPORIC ACID SYNTHESIS

In spite of the success in the study of pathways of TSA synthesis, molecular foundations of sexual interaction of the (+) and (–) strains are very poorly studied. Only three enzymes (β-carotene oxygenase, 4-dihydrotrisporin dehydrogenase, and 4-dihydromethyltrisporate dehydrogenase) have been isolated, and regulation of the relevant gene expression was partially studied.

The β-carotene oxygenases TSP3 and TSP4 (encoded by *tsp3* and *tsp4*) are biocatalysts of the process of β-carotene cleavage. The structure, activity, and specificity of these enzymes, as well as the products of reactions that they catalyze, were not studied; however, information about the expression of the encoding genes is available. It was demonstrated that the *tsp4* transcription depends only on the stage of the culture development, while the *tsp3* expression occurs in response to sexual stimulation in a mixed culture or to TSA addition to the cultures of individual strains. The response was five times higher in the (+) than in the (–) strain. The functions of TSP4 and TSP3 oxygeneses in *B. trispora* are different. It is known that the (+) strain is individually able to synthesize small amounts of TSA; therefore, it has both active oxygenases, while TSP3 plays a more important role in the (–) strain, providing additional inflow of precursors for TSA synthesis. It is assumed that the role of TSP4 consists in providing of a minimal TSA level, required for the activation of expression of the genes of their own biosynthesis [42] and genes for the synthesis of their precursor (β-carotene) [44, 45], at initial stages of sexual interaction between the (+) and (–) strains.

NADP-dependent 4-dihydrotrisporin dehydrogenase TSP2 (the *tsp2* gene) catalyzes the oxidation of 4-dihydrotrisporin hydroxyl group at C_4 to the ketogroup to form trisporin [46]. The enzyme is the most active in the (–) strain, and its activity does not depend on sexual stimulation. It is assumed that in addition to its involvement in trisporoid synthesis, 4-dihydrotrisporin dehydrogenase probably performs a sensory function in the process of sexual partner recognition at initial stages of the sexual process [29].

Data are available about the incorporation of 4-dihydromethyltrisporate in TSA synthesis with the involvement of NADP-dependent 4-dihydromethyltrisporate dehydrogenase TSP1 (the *tsp1* gene), which transforms it into methyltrisporate and is the most active in the (–) strain under conditions of sexual stimulation [47, 48]. The *tsp1* gene was isolated and characterized in the (+) and (–) strains of *Mucor mucedo, Absidia glauca, Parasitella parasitica*, and *B. trispora*. Later, as a result of genomic analysis, it was also found in 30 species of zygomycetes, including *Phycomyces blakesleeanus* and *Thamnidium elegans* [5]. It was established that transcription of this gene was carried out constitutively, and regulation of its expression probably occurred at later stages. However, it should be emphasized that the rate of 4-dihydromethyltrisporate oxidation to TSA is extremely low as compared to the rate of TSA formation from trisporol [23], and its function is unknown.

Both enzymes (4-dihydromethyltrisporate dehydrogenase and 4-dihydrotrisporin dehydrogenase) are low-specific enzymes [29] that have similar enzymatic activity (dehydrogenase) and cofactors (NADP), but

differ in their primary structure and spatial conformation (including the active center structure). While 4-dihydromethyltrisporate dehydrogenase is an aldo-keto reductase that contains a TIM-barrel, 4-dihydrotrisporin dehydrogenase is a short-chain dehydrogenase with a Rossman fold. In spite of the differences in the structures of these dehydrogenases, active centers of both enzymes contain lysine and tyrosine [49].

Localization of the enzymes of the TSA synthesis pathway and signal transduction pathway have not been studied. Only one study exists, in which the preferential localization of 4-dihydromethyltrisporate dehydrogenase in the *M. mucedo* zygosporophores was demonstrated [50]. Theoretically, it is possible to assume that only the cleavage of lipophilic β-carotene is intracellular. The synthesis of trisporoids can further occur both inside and outside the cell, up to the stage of trisporol (the last common precursor for the (+) and (–) strains). Subsequent stages are associated with exchange of the intermediate products between the (+) and (–) strains and should probably occur inside the cell. A number of facts indicate this. First, the transformation of tritium-labeled TSA precursors (starting from D'orenone and ending with methyltrisporates) was demonstrated in the culture liquid of submerged *B. trispora* cultures [23]. This suggests that the enzymes that catalyze these stages can be exogenous or be localized in the cellular membrane. Second, it was demonstrated that when adding exogenous TSA, the protein content in the cellular wall of mucoraceous fungi *B. trispora* [51] and *Cunninghamella japonica* [52] increased by 1.5-fold, which was probably (at least partially) due to the presence of the TSA synthesis enzymes. Thus, available information suggests that lipophilic β-carotene cleavage occurs in the cell, while subsequent transformations of C_{18} compounds are extracellular.

It should be noted that in spite of intensive studies, many ambiguities remain concerning the trisporoid biosynthesis. Early stages of TSA synthesis are insufficiently studied. Our knowledge about the enzymes of TSA biosynthesis is fragmentary; only three enzymes were isolated and partially characterized [42, 43, 46, 47]. Localization of the enzymes was not studied, which makes it impossible to answer the question about the correlation between the extracellular concentration of trisporoids and intracellular processes.

ROLE OF TRISPOROID IN SEXUAL REPRODUCTION OF MUCORACEOUS FUNGI

In 1978, Gooday [2] proposed a hypothesis about TSA universality as sexual hormones of fungi of the order *Mucorales*, although TSA synthesis was by then partially studied only in three members of this order (*B. trispora, P. blakesleeanus*, and *M. mucedo*). To date, this hypothesis was additionally confirmed. Thus, the existence of a sexual communication system on the basis of trisporoids in fungi of the genus *Mortierella* was shown in 2003 [3].

In addition to interaction between the (+) and (–) strains of the same species, existence of a common system of hormonal regulation in mucoraceous fungi also provides (a) passage of sexual reactions between the complementary (+) and (–) strains of different species (and even families) and (b) interaction between the host organism and a parasitic organism that is sexually complementary to it [3, 4, 6]. Sexual reactions are manifested in cessation of vegetative mycelium growth and in zygosporophore and gametangium formation; however, no mature zygosporophores are produced [41, 53].

Trisporoids play a role of signal molecules during the transition from vegetative development to sexual reproduction. Meeting of the (+) and (–) mycelia is the first stage in this process. Their mutual recognition is provided by means of the signal transfer between mycelia of the opposite sign. Sex-specific precursors (or precursor) of trisporoids (pheromones) that are synthesized by both strains (or by one of them) act as signals. The mechanism of pheromone action is unknown. Synthesis of special surface proteins (agglutinins) is one of the studied mechanisms of cellular communication in yeasts at the stage of recognition of sexually complementary cells [54]. These proteins favor the "adhesion" of sexually complementary cells and thus provide their rapid contact and efficient interaction. The synthesis of agglutinins is increased under the effect of pheromones. It is possible that action of fungal pheromones can be similar to the action of yeast pheromones.

At the next stage, the exchange of TSA precursor molecules occurs between the (+) and (–) strains, resulting in enhanced synthesis of the fungal sexual hormones, TSA. As a result of TSA action according to the positive feedback principle, (1) derepression of key genes encoding the enzymes of their precursor (β-carotene) synthesis, such as mevalonate kinase [44], phytoene synthase (*carB*), and lycopene cyclase (*carRA*) [45], occurs, and (2) the activity of TSA synthesis enzymes (such as β-carotene oxygenase (*tsp3*) [42] and 4-dihydromethyltrisporate dehydrogenase (*tsp1*) [43]) is increased.

The question of which of the trisporoids are true hormones (sex-specific TSA precursors or TSA themselves) is discussed for a long time in the literature. The assumption that TSA are an inactive metabolite pool from which true hormones, trisporols, and methyltrisporates are synthesized [3, 7] is discussed. However, recent studies using labeled compounds demonstrated that the reaction of methyltrisporate saponification to TSA is irreversible [23], which contradicts the suggested hypothesis.

There is experimental evidence that trisporoids induce sexual cytodifferentiation: (1) development of sexual structures, zygosporophores, and their directed growth towards each other (zygotropism), finally

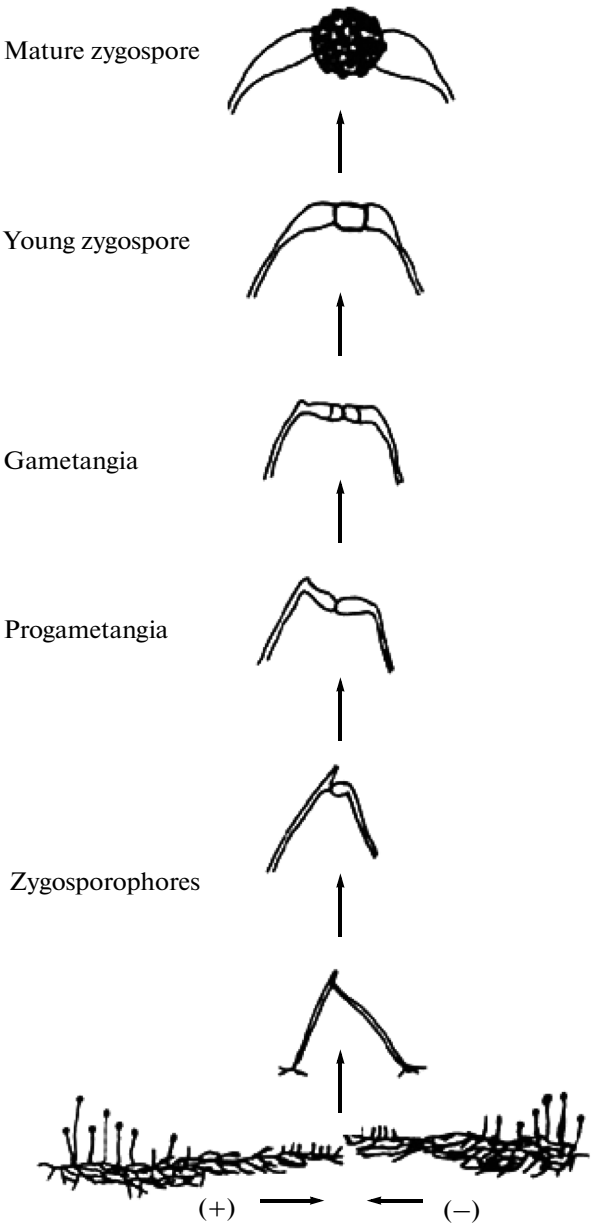


Fig. 4. Sexual reproduction of *Mucor mucedo* [29].

resulting in the contact; (2) isolation and fusion of gametangia; (3) formation of zygotes; (4) synthesis of a thick sculptured envelope around the zygote and its transformation into a mature zygosporophore (Fig. 4).

TSA precursors, trisporols, and methyltrisporates may be theoretically involved in the partner recognition and initial sexual reactions. Moreover, volatility of trisporoids and the biological properties of fungi should be taken into consideration. Thus, trisporine and methyltrisporates are volatile compounds, while trisporols and TSA are nonvolatile. Volatile precursors can play a role in induction of zygosporophore generation, for example, in *M. mucedo*, in which zygosporophores are formed on aerial hyphae. Meanwhile, diffusion of nonvolatile compounds in a solid medium can be of higher importance for the fungi in which zygosporophores are generated on substrate hyphae, such as *B. trispora* and *P. blakesleeanus*) [5]. Data on formation of large amount of trisporins and trisporols (but not methyltrisporates) in the *B. trispora* submerged culture supports this suggestion [40, 55]. It is possible that regulation of trisporoid synthesis is sufficient for blocking the sexual reactions in separate cultures of the (+) and (–) strains of mucoraceous fungi (and, on the contrary, for their induction in mixed cultures). The genes that determine sex (*sexP* in the (+) strain and *sexM* in the (–) strain) are apparently involved at later stages of sexual differentiation [56–58]. It is known that SexM and SexP are transcription factors; however, their functions and target genes are unknown. The transcription level of both genes considerably increases in joint cultures as compared with separate cultures. It was demonstrated that an increase in *sexM* (but not *sexP*) transcription was associated with the effect of trisporoids; however, it is unclear whether trisporoids are the only transcription regulators or if other factors (such as physical contact between the (+) and (–) strains) may affect it.

The hypothesis about TSA universality as the *Mucorales* sexual hormones is widely distributed. However, it is known that mature zygospores are not formed in all pairs of the (+) and (–) heterothallic strains of a single species and or in all homothallic strains. Data on zygote formation in eight (+) and four (–) wild type *B. trispora* strains may be provided as an illustration (Table 1) [40]. It can be seen from the table that all studied strains were able to form zygospores with at least one sexual partner. However, it remains unclear why one strains forms zygospores with all sexual partners, while other strains only with some partners. These data contradict the hypothesis of TSA universality as a sexual hormone in all mucoraceous fungi and suggest the presence of additional mechanisms for control of sexual interaction.

The reasons for the inability of pairs to form mature zygospores are currently unknown. Since zygospore formation is a complex multistage process (Fig. 3), dysfunctions at any of its stages associated with (1) TSA synthesis and (2) synthesis of β-carotene synthesis—which is a precursor for both TSA and sporopollenin, an important component of the cell wall in mature zygospores—as well as (3) regulation of zygospore formation, may theoretically result in the inability of pairs of the (+) and (–) strains to form mature zygospores [59, 60].

It is assumed that lack of TSA and/or weak hormonal response to the action of hormones is a reason for the inability to produce zygospores in a homothallic *Zygorhynchus moelleri* strain, since exogenous addition of TSA and their precursors (4-dihydromethyltrisporates and trisporins) results in restored sexual reproduction [50].

In the case of *B. trispora*, it was found that the level of β-carotene production decreased considerably in the pairs of (+) and (–) strains of the wild type (which are unable to form zygospores) as compared with zygote-forming pairs. It was also shown that the pairs of wild type strains which do not produce zygospores accumulated less trisporoids (including TSA) than zygote-forming pairs, while the qualitative composition of trisporoids did not differ considerably [40]. Interesting data were obtained for the pairs of β-carotene overproducing strains T (+) and T (–), in which T (–) is a mutant strain [61]. This pair generates larger amount of both β-carotene and TSA; however, it does not produce zygospores, which means that zygore formation in it is blocked at a later stage than TSA synthesis (namely, at the stage of the development of a young zygospore) [40]. At the same time, biochemical mechanisms of induction of zygosporophore formation are completely unstudied, also indicating our insufficient knowledge of the role of TSA role in sexual interaction between mucoraceous fungi.

Table 1. Zygote formation in different *B. trispora* strain pairs [40]

Strains BKMF	Width of zygote formation zone, mm				
	811 (–)	921 (–)	826 (–)	987 (–)	T(–)*
1201 (+)	–	3	1	4	–
666 (+)	is.	4	1	2	–
904 (+)	2	4	2	6	–
812 (+)	3	3	–	7	–
989 (+)	5	8	2	7	–
701 (+)	6	3	is.	6	is.
902 (+)	2	2	is.	7	–
T (+)	1	4	–	7	is.

Designations. "is.", isolated immature zygospores; "–", absence of zygospores.
*, mutant strain.

ROLE OF TRISPORIC ACIDS IN CAROTENOID SYNTHESIS

If β-carotene synthesis is a necessary condition for TSA formation, TSA in turn are one of a number of factors that affect carotenogenesis [62–68]. Two approaches for carotenogenesis stimulation, namely, the use of a mixed culture of the *B. trispora* (+) and (–) strains [12–14] and the use of an individual *B. trispora* (–) strain culture with carotenogenesis stimulated by TSA [69], have been described in biotechnologies of carotenoid production. The first approach is based on sexual interaction between the (+) and (–) strains of the fungus in submerged culture, where no zygospores are produced. In spite of this, a considerable amount of trisporoids is extracted from the culture liquid, and stimulation of carotenogenesis is observed. This fact itself indicates that TSA production does not necessarily correlate with induction of zygospore formation, and the absence of zygospores can not be considered an indicator of the absence of TSA synthesis. However, the study of relationships between the intensity of zygote formation and carotenogenesis using a representative sample of wild *B. trispora* strains made it possible to conclude that the pairs of (+) and (–) strains with intensive zygote formation in the surface culture produce more β-carotene and trisporoids in submerged culture than the pairs with low zygote formation [40]. This conclusion does not apply to mutant strains, since the mutant *B. trispora* T (–) strain, which does not form zygospores with any of wild (+) strains, produced large amounts of both carotenoids and trisporoids in submerged culture with wild (+) strains [40].

It was empirically established that the amount of β-carotene in submerged *B. trispora* cultures depended on the ratio of the (+) and (–) strains in the inoculum [70, 71]. Moreover, the trisporoid composition in submerged cultures varied considerably depending on the ratio of (+) and (–) strains in the inoculum (1 : 7, 1 : 1, and 7 : 1) (Table 2) [40]. The largest amount of trisporoids and TSA were produced at equal strain ratio and at (+) strain predominance, respectively. The prevalence of the (–) strain resulted in a small decrease in the amount of trisporoids and TSA, while carotene formation was maximal. It is possible that two factors, TSA and a considerably larger caroten-producing capacity of the (–) strain as compared with the (+) strain, affected carotenogenesis in this case. The absence of a direct correlation between the level of β-carotene and TSA indicates that not only generation of TSA but also the maximal amount in inoculum of the carotene-producing (–) strain—and therefore its preferential development—are important for active carotenogenesis. While the synthesis of β-carotene increased considerably with sexual stimulation ("sexual carotenogenesis"), with carotene level increasing 1.5–2 times at joint cultivation of the (+) and (–) strain, the content of β-carotene increased 16–30 times upon TSA addition to a separate culture of the (–) strain [69, 70]. This effect was explained by the stimulating action of TSA on the expression of the genes of β-carotene biosynthesis [72, 44, 73, 74]. Apart from sexual stimulation, chemical stimulators are also used in biotechnologies of β-carotene production for intensification of carotenogenesis. It is possible to see what happens in this case with the synthesis of trisporoids from the studies of the action of two carotenogenesis stimulators, including β-ionone and 6-methyl-2-aminopyridine (MAP) [55, 74, 75]. MAP is used as a lycopene cyclase inhibitor, which blocks lycopene cyclization into β-carotene, so that lycopene is accumulated in the fungal cells [75, 76]. The use of β-ionone is associated with its stimulating action dur-

Table 2. Influence of the ratio of the (+) and (–) strains in the inoculum on trisporoid composition of a joint *B. trispora* culture [40]

Ratio of the (+) and (–) strains	Biomass, g/L	β-carotene, % of dry biomass	Trisporoids									
			% of the sum								sum	
			NF			AF					mg/g dry biomass	g/L
			TN and TL	X_{290}	m-TSA	TSA A	TSB B	TSC C	ΣTSA	X_{260}		
7 : 1	24.9	0.073	13.3	–	1.3	13.6	10.6	2.8	54.0	10.8	77.2	1.92
1 : 1	22.1	0.135	31.1	13.0	3.4	5.6	9.1	31.5	46.2	9.0	148.2	3.28
1 : 7	34.3	0.244	25.3	16.2	5.6	–	3.2	26.0	29.2	10.6	60.6	2.08

Designations. NF, neutral fraction; AF, acid fraction; TN and TL, trisporins and trisporols; TSA-A, B, C, trisporic acids; X_{290} and X_{260}, unidentified compounds with maximum absorption at λ = 290 and λ= 260, respectively; "–", not found.

Table 3. Trisporoid composition in a joint culture of T (+) and T (–) *B. trispora* strains under conditions of carotenogenesis stimulation by MAP and β-ionone [55]

Variant	Biomass, g/L	Trisporoids								
		% of the sum							sum	
		NF		AF					mg/g dry biomass	g/L
		TN and TL	X_{280}	TSAB	TSAC	TSA	ΣTSA	X_{250}		
Control	43.1	24.2	3.0	10.3	19.5	29.8	10.2	–	29.8	1.28
β-ionone	46.1	9.7	5.1	–	10.3	10.3	11.4	33.4	117.0	5.39
MAP	50.3	19.9	8.8	–	–	–	8.5	44.5	19.3	0.97
MAP + β-ionone	44.4	7.8	3.6	–	–	–	3.3	41.2	43.2	1.92

Designations. TN and TL, trisporins and trisporols; TSA-B, C, trisporic acids B, C; X_{280}, X_{260}, and X_{250}, unidentified compounds with maximum absorption λ = 280, λ = 260, and λ = 250, respectively.

ing the early stages of carotenoid synthesis at the level of mevalonate [77]. This stimulator, which has structural similarity with TSA, is their competitor in the effect on carotenogenesis. Its use against the background of carotenogenesis inhibition by MAP results in increased lycopene synthesis. TSA synthesis inhibition was observed in all variants of chemical regulation of the production of carotenoids: partial inhibition of TSA generation when using β-ionone or complete inhibition in all variants of MAP application (Table 3). Only a small amount of their neutral precursors (trisporins and trisporols) was found in the culture liquid, indicating an influence of effectors not only on carotenogenesis, but also on TSA synthesis (and on the contrary, not stimulating, but inhibiting this process). However, the fact that β-ionone was introduced in the analyzed studies not at the beginning of growth of a mixed culture of the (+) and (–) strains, but at the early stationary stage (when the complex of carotenogenous enzymes was already developed as a result of sexual interaction, particularly TSA action), should be taken into consideration. These data make it possible to assume that the role of TSA in stimulation of carotenogenesis in submerged cultures consists in the development of a complex of carotenogenous enzymes, while β-ionone influences these enzymes by increasing their activity, resulting in a further increase in the level of β-carotene.

In contrast, the use of barbiturate as a stimulator resulted in stimulation of both carotenogenesis and TSA synthesis, which was associated with induction of β-carotene oxygenases by barbiturate [78].

Thus, a complex interconnection between carotenogenesis and TSA synthesis exists in submerged cultures, where no zygospore production is observed; several factors, including the prevalence of the carotenogenous (–) strain in a joint culture, intensity of TSA formation, and regulation of a cascade of processes of β-carotene production and transformation by specific stimulators, influence the carotenogenesis.

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