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Cooperative Biosynthesis of Trisporoids by the (+) and (−) Mating Types of the Zygomycete *Blakeslea trispora*

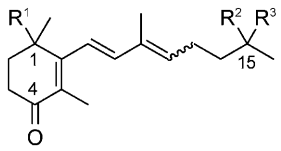
Doreen Schachtschabel,[a] Anja David,[a] Klaus-Dieter Menzel,[b] Christine Schimek,[c] Johannes Wöstemeyer,[c] and Wilhelm Boland*[a]

The fungal phylum zygomycota uses trisporic acids (TSAs), a family of apocarotenoids, during sexual reproduction to synchronize and control activity between the mycelial hyphae of opposite mating types. Separate as well as mixed cultures of Blakeslea trispora *were systematically supplemented with putative, deuterium-labeled precursors downstream of β-carotene en route to the bioactive TSAs. Analysis of the isolated metabolites allowed the reconstruction of the complete biosynthetic sequence between the first apocarotenoid, D'orenone (**1**), and the different series of TSAs B (**8**) and C (**13**). Both mating types produced a similar spectrum of early metabolites upstream of trisporols B (**7**) and C (**12**), while only the (+) type was able to further oxidize trisporols B (**7**) and C (**12**) to the corresponding methyltrisporoid B (**5**) and C (**11**), respectively. A novel 4-dihydrotrisporic acid B (**14**) that was not formed from the labeled precursors was isolated from mated strains; this compound might be derived from oxygenated β-carotene by a parallel pathway. The ester accumulated in the culture broth of the (+) strain and was only hydrolyzed by mycelia of the (−) strain; this corresponds to a synchronization of the biosynthetic activities of both mating types.*

Introduction

Fungi are heterotrophic organisms that are omnipresent in our environment. With few exceptions, fungi have filamentous bodies enclosed by cell walls, are nonmotile and reproduce both sexually and asexually by spores. The fungal division zygomycota—a group of more than 1000 species—is distinguished from all other eumycota by its ability to reproduce sexually by zygospores following gametangial fusion. If mycelia of complementary (+) and (−) mating types are present, the hyphal tips grow towards one another and develop into gametangia. Next, the walls between the two touching gametangia dissolve, and the two multinucleate compartments fuse. The (+) and (−) nuclei fuse to form a young zygospore with one or more diploid nuclei. The zygospores are typically thick-walled, highly resilient to environmental hardships and metabolically inert. When conditions improve, they can germinate, which is accompanied by meiosis to produce a germ sporangium or directly vegetative hyphae.[1]

The compounds coordinating and synchronizing sexual reproduction have been isolated and identified from members of the order mucorales, namely *Blakeslea trispora*,[2] *Phycomyces blakesleeanus*,[3] *Zygorhynchus moelleri*[4,5] and *Mucor mucedo*.[2,6] In all cases, the bioactive molecules belonged to the large family of trisporic acids (TSAs) representing a special group of apocarotenoids. As a part of their involvement in the recognition of mating partners, they induce the first steps of sexual differentiation, maintain the development of sexual structures and probably mediate the recognition between certain zygomycetes and mycoparasites, such as *Parasitella parasitica*.[7] As shown by Sutter et al.,[8] the production of the different series of bioactive TSAs (Scheme 1) requires an enzymatic contribution from both mating partners, since the TSAs and their early precursor β-carotene accumulate in the contact area of their hyphae.



Compound	Series B		Series C	
trisporin	R^1=CH_3;	R^2=R^3=O	R^1=CH_3;	R^2=H R^3=OH
trisporol	R^1=CH_2OH;	R^2=R^3=O	R^1=CH_2OH;	R^2=H R^3=OH
methyltrisporate	R^1=$COOCH_3$;	R^2=R^3=O	R^1=$COOCH_3$;	R^2=H R^3=OH
trisporic acid	R^1=COOH;	R^2=R^3=O	R^1=COOH;	R^2=H R^3=OH

Scheme 1. Functionalization patterns of trisporoids series B and C.

From pioneering analytical work of van den Ende,[9] Werkmann,[10] Austin[11] and Sutter[8] with the fungi *M. mucedo, P. blakesleeanus* and the trisporoid-overproducing species *B. trispora*,

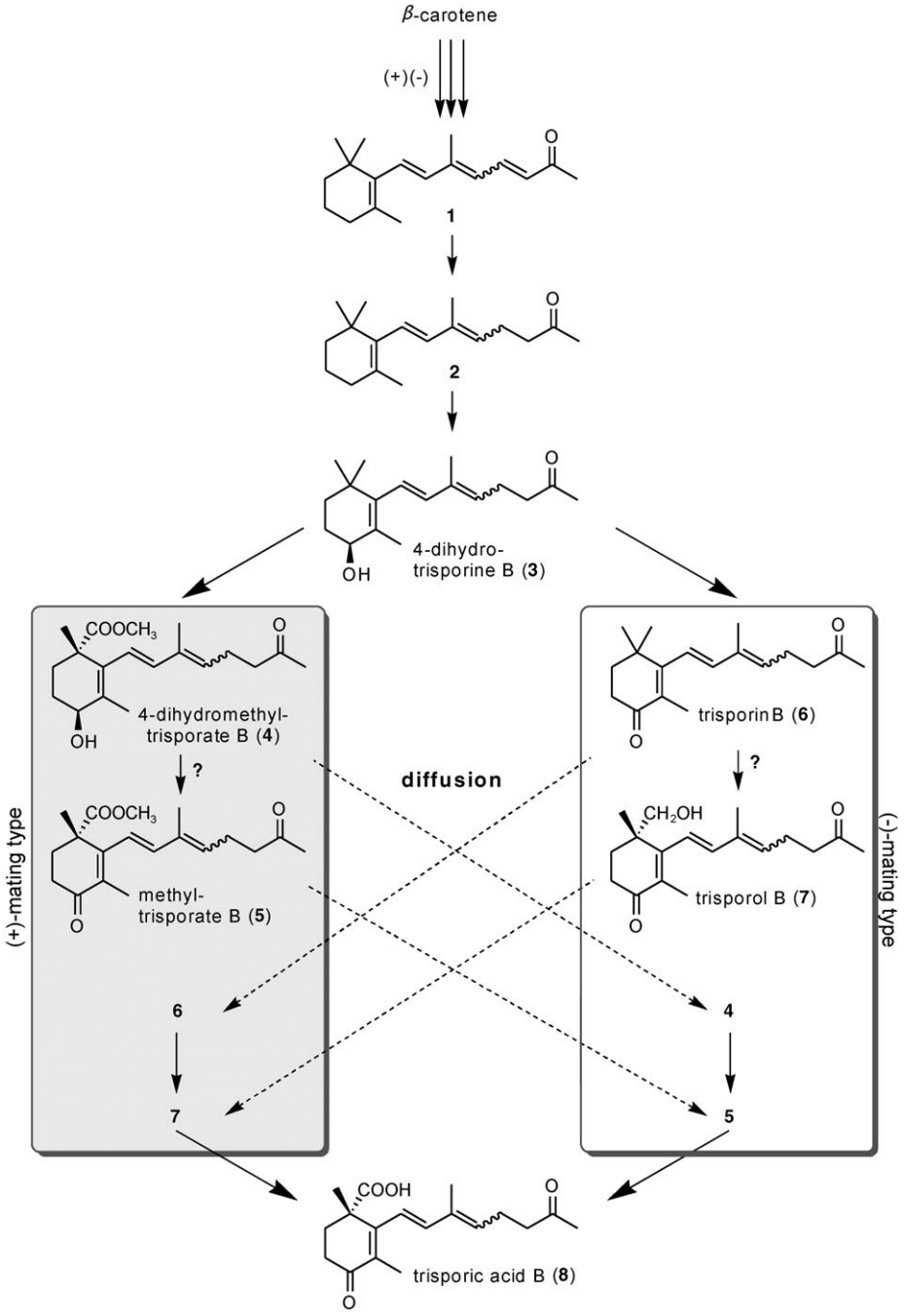
[a] *D. Schachtschabel, A. David, Prof. Dr. W. Boland*
Bioorganic Chemistry, Max Planck Institute for Chemical Ecology
Hans-Knöll-Strasse 8, 07745 Jena (Germany)
Fax: (+49) 3641-571202
E-mail: boland@ice.mpg.de

[b] *K.-D. Menzel*
Leibniz Institute for Natural Product Research and Infection Biology
Hans Knöll Institute, Beutenbergstrassee 11a, 07745 Jena (Germany)

[c] *C. Schimek, Prof. Dr. J. Wöstemeyer*
Institute for Microbiology, Friedrich-Schiller-Universität Jena
Neugasse 24, 07743 Jena, (Germany)

a putative chemical dialogue between sexual partners was envisaged, which involves an exchange of early trisporoids as precursors for the bioactive TSAs.[12,13]

According to Scheme 2, both mating types are able to convert β-carotene to 4-dihydrotrisporin B (**3**) but lack the ability to generate significant amounts of the downstream metabolites required for the production of hormones, such as TSA B (**8**). Therefore, for further transformations to occur, mating partners were supposed to exchange early metabolites, such as trisporin B (**6**) and 4-dihydromethyltrisporate B (**4**), with their complementary mating partners. According to this concept, TSA B (**8**) is generated in the (−) type by the saponification of methyltrisporate B (**5**) or in the (+) mating type by the oxidation of trisporol (**7**).[14] The details of the biosynthesis scheme were composed from experiments with radiolabeled precursors,[14] with compounds isolated from the mixed growing cultures and the logic of enzymatic transformations connecting the metabolites in a functional biosynthesis pathway. Moreover, experimental results from different zygomycetes were unified in the general pathway outlined in Scheme 2.



Scheme 2. Postulated cooperative biosynthetic pathway for TSA B (**8**). The production of **8** required the exchange of intermediates between the mycelia of the different mating types.

Although the sequence of Scheme 2 apparently matches currently known aspects of sexual recognition and differentiation in zygomycetes, many details of the elegant and convincing scheme remain speculative. Early intermediates, such as the carotene cleavage product **1**, recently termed D'orenone,[15] have never been isolated or characterized. Most importantly, although the sequence within the pathway is biochemically plausible, it is, as yet, unconfirmed by precise labeling experiments, which would unequivocally establish the sequence of reactions.

Herein, we present a new approach with which to evaluate and validate the previously postulated biosynthesis pathway for *B. trispora*, employing systematic metabolic profiling based on the administration of isotopically labeled precursors[16] to cover and probe the whole pathway (Scheme 2). High-resolution gas chromatography combined with mass spectroscopy (GC-MS) was used to characterize and identify the correct stereochemistry of the resulting metabolites. We provide evidence that both mating types are able to produce all early intermediates en route to trisporol B (**7**) albeit with different degrees of efficiency. The further oxidation to methyltrisporate B (**5**) was achieved exclusively by the (+) strain, while the saponification of the ester into the bioactive TSA B (**8**) was faster in the (−) strain. Combining pulse-chase experiments with labeled com-

pounds and isolated metabolites from the large-scale fermentation of the individual strains, we revise previous schemes of the chemical dialogue between the mating types of *B. trispora*.

Results

Administration of labeled precursors and metabolite analysis

TSAs stimulate zygophore (sexually determined hyphae) development in *M. mucedo* and in many other mucoralean fungi, including the fungus *B. trispora*, which overproduces β-carotene and trisporoids.[2,11,17–19] Separate (+) and (−) cultures of *B. trispora* synthesize appreciable amounts of TSA B (**8**) and C (**13**) after biosynthetic precursors from a neutral fraction of the culture medium stimulate the complementary mating type.[20] Without stimulation, the (+) strain is able to produce small amounts of TSAs, but the (−) strain is not. To establish the complete biosynthetic pathway from β-carotene to the individual TSAs and to address the biosynthetic capacity of the individual strains, we systematically administered deuterium-labeled early precursors from Scheme 2—namely, the ketone cleavage products of β-carotene ([D_3]**1**, [D_3]**2** and [D_3]**9**; Figure 3)—and analyzed their metabolites. The later transformations en route to TSAs were studied by administering labeled 4-dihydrotrisporin B ([D_3]**3**), trisporin B ([D_3]**6**), trisporic C ([D_3]**10**), trisporol B ([D_3]**7**), trisporol C ([D_3]**12**), and unlabeled methyltrisporate B (**5**), methyltrisporate C (**11**), TSA B (**8**) and TSA C (**13**). All compounds were individually added to shaken, liquid (+), (−) and mixed cultures of *B. trispora* (21 °C, 120 rpm) in the dark, and their metabolites were extracted after 3, 6, 9, 12, 24 and 48 h following the standard protocol shown in Scheme 3. After the mycelia were removed, an aliquot of the culture broth was adjusted to pH 8, and the neutral components were removed with $CHCl_3$/propan-2-ol (20:1). After the aqueous medium was acidified to pH 2, the TSAs were extracted with the same binary solvent. For analytical purposes, free acids were converted to methyl esters (with diazomethane), and additional hydroxy groups were converted to trimethylsilyl ethers upon treatment with *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA) when necessary.

Although the composition of the extracts proved to be quite complex, we successfully analyzed the individual metabolites including their stereoisomers by GC-MS (Figure 1). The presence of a deuterium label in the methyl group at C(3′) of the side-chain (> 98% D_3) facilitated the identification of the metabolites (Scheme 1). This position proved to be completely inert, and we obtained all identified metabolites without significantly affecting the original isotope level.

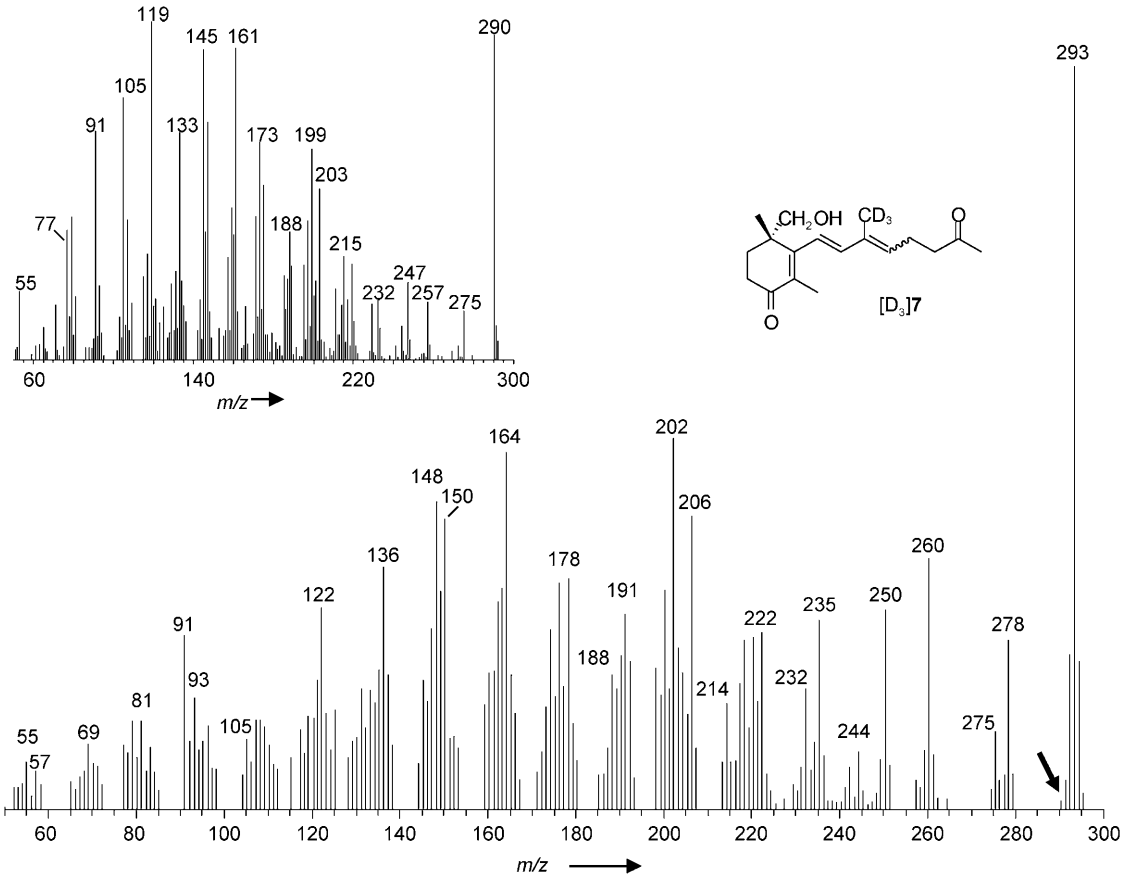
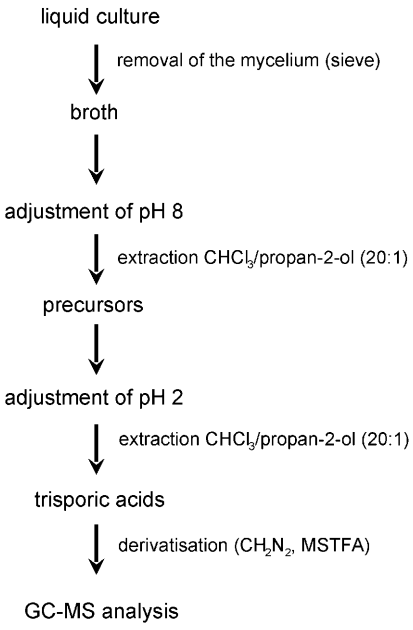


Figure 1. Mass spectrum of trisporol B ([D_3]**7**) obtained from the sequential transformation of [D_3]**1** by (+) or (−) mating type cells of *B. trispora*. The insert shows the upper mass range from the mass spectrum of natural trisporol B.



Scheme 3. Extraction and derivatization protocol for early and late trisporoids from the culture broth of growing mycelia of (+) and (−) mating types of *B. trispora*.

Administration of early cleavage products of β-carotene

Administration of the putative first β-carotene cleavage product, [D_3]**1**, resulted in the culture broth of both mating types of *B. trispora* to produce a complex pattern of metabolites that changed greatly over time. After 3 h, we observed the transformation of [D_3]**1** into labeled 4-dihydrotrisporin B ([D_3]**3**), trisporin B ([D_3]**6**) and trisporin C ([D_3]**10**). After 6 h, the oxidation of the pro-*R* methyl group at the cyclohexene ring of the intermediates generated labeled trisporol B ([D_3]**7**) and trisporol C ([D_3]**12**). All compounds were unequivocally identified by their retention times and mass spectra by using authentic standards.[16] The ratio of the originally administered *E*/*Z* isomers was channeled relatively unchanged through a series of transformations. Moreover, we performed independent administration experiments with the identified metabolites to exclude artifacts caused by detoxification reactions not related to the biosynthetic pathway en route to TSAs. Furthermore, the same ratio of products was observed in fermenter experiments (30 L); this indicates that the biosynthetic capacity of the strains was not exhausted by the amount of added precursors.

The putative first metabolites from the reduction and oxidative functionalization of [D_3]**1**, namely [D_3]**2** and [D_3]**9**, were observed owing to the rapid turnover of labeled 4-dihydrotrisporins, such as [D_3]**3**. The fact that the addition of the putative intermediates [D_3]**2** and [D_3]**9** resulted in a similar pattern of

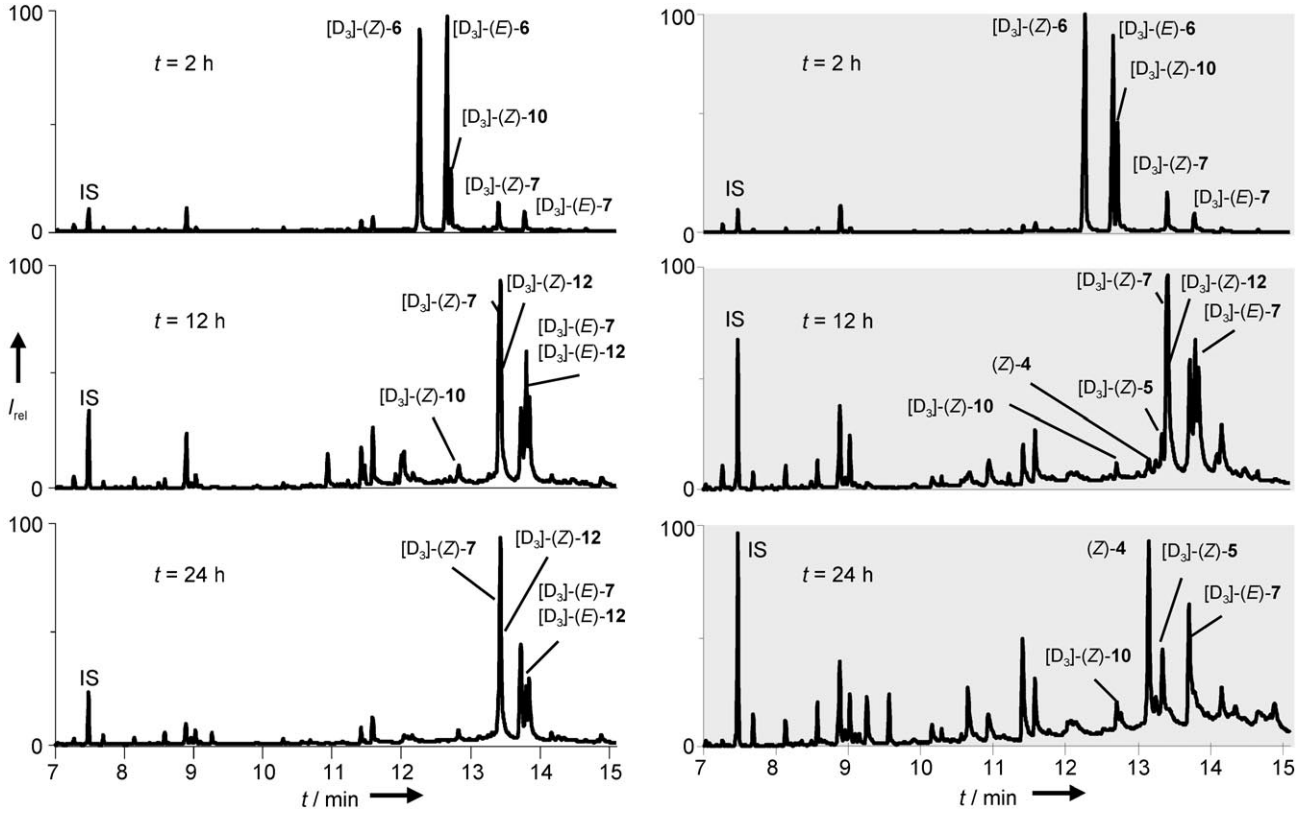


Figure 2. Time course of the transformation of (*E*/*Z*)-trisporin ([D_3]**6**) in (+) and (−) mating type cells of *B. trispora*. Compounds were extracted at pH 8 and do not contain the TSAs. Left: time course of the formation of (*E*/*Z*)-trisporol B ([D_3]**7**) and C ([D_3]**12**) in (−) mating type cells of *B. trispora*. Right (gray background): time course of the formation of 4-dihydromethyltrisporate ((*Z*)-**4**) and methyltrisporate B ([D_3]**5**) and trisporol C ([D_3]**7**) in (+) mating type cells of *B. trispora*.

Table 1. Metabolite profile after administration of D'orenone ([D_3]**1**), ([D_3]**2**), ([D_3]**9**), and dihydrotrisporin B ([D_3]**3**) in the culture broth of the (+) and (−) mating types after 12 h.

Administered precursor mating type	[D_3]**1**		[D_3]**2**		[D_3]**9**		[D_3]**3**	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Metabolite								
D'orenone ([D_3]**1**)	/		–[a]		–[a]		–[a]	
ketone ([D_3]**2**)	–[a]		/		–[a]		–[a]	
hydroxyketone ([D_3]**9**)	–[a]		–[a]		/		–[a]	
4-dihydrotrisporin B ([D_3]**3**)	X	X	X	X	X	X	/	
trisporin B ([D_3]**6**)	X	X	X	X	X	X	X	X
trisporin C ([D_3]**10**)	X	X	X	X	X	X	X	X
trisporol B ([D_3]**7**)	X	X	X	X	X	X	X	X
trisporol C ([D_3]**12**)	X	X	X	X	X	X	X	X
methyltrisporate B ([D_3]**5**)	X	–	X	–	X	–	X	–
methyltrisporate C ([D_3]**11**)	X	–	X	–	X	–	X	–
4-dihydromethyltrisporate B ([D_3]**4**)	X	–	X	–	X	–[b]	X	–[b]
trisporic acid B ([D_3]**8**)	X	–	X	–	X	–	X	–
trisporic acid C ([D_3]**13**)	X	–	X	–	X	–	X	–

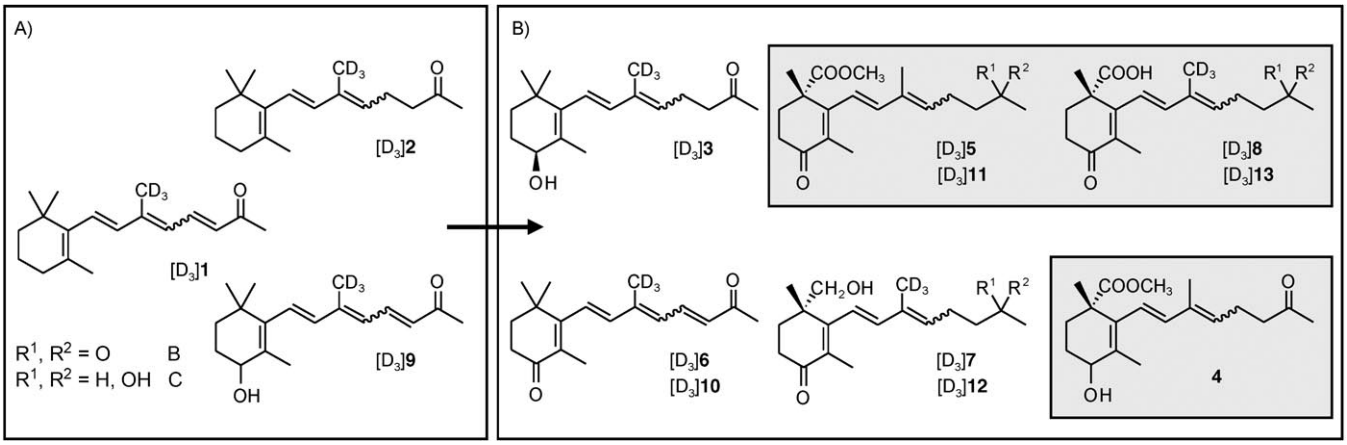
[a] Compound not observed due to rapid turnover; [b] < 6% deuterium label; X: compounds produced during 24 h after administration of precursors; –: compound not produced; /: precursor incubated substance.

metabolites as observed for [D_3]**1** (Figure 3) confirmed their involvement and turnover. Alternative cleavage products of β-carotene, namely retinol, retinal and retinoic acid, were not metabolized by the growing cultures and could be recovered unchanged (Figure 3 and Table 1).[11,14]

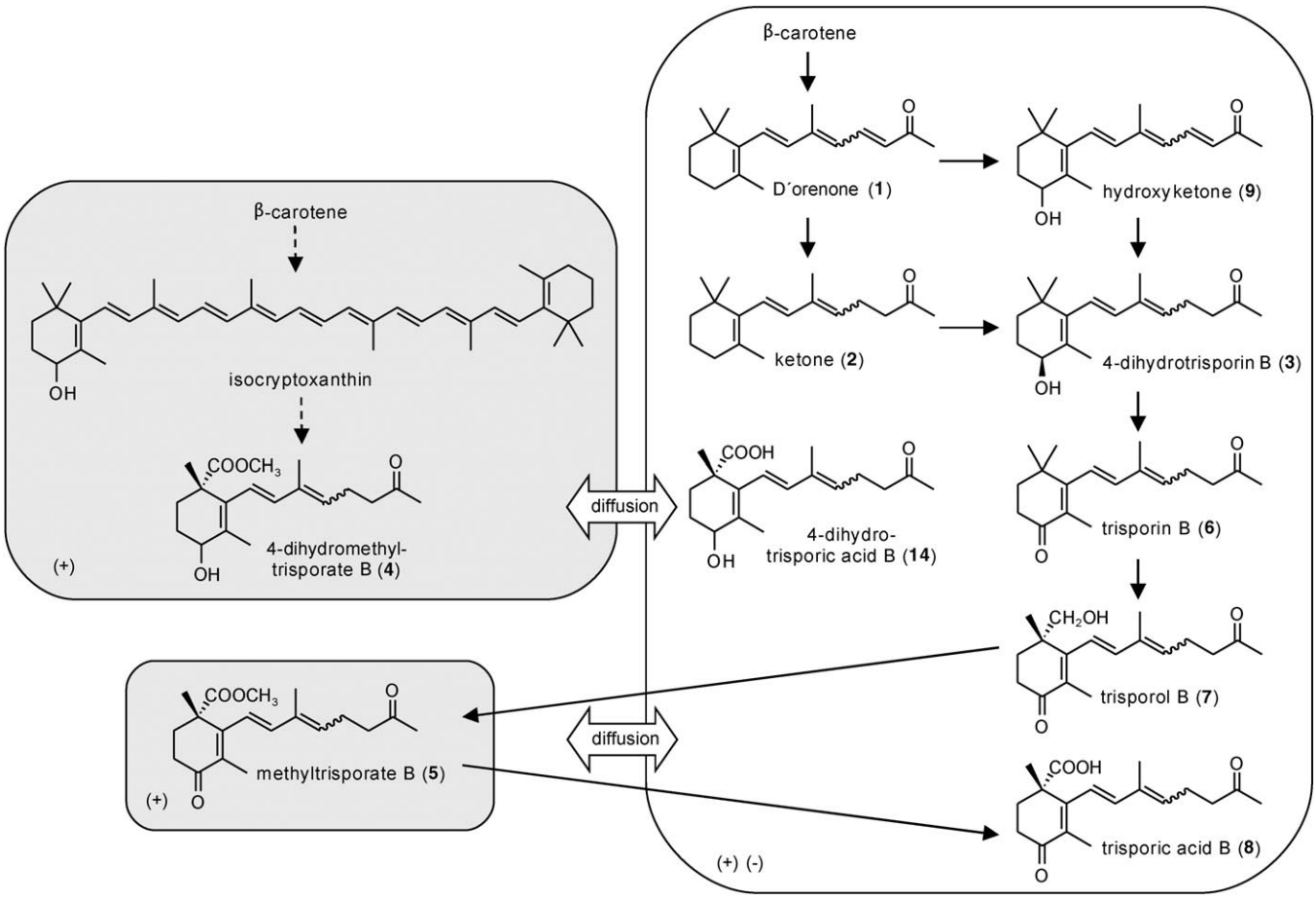
The cultures of both mating types produced the same spectrum of early trisporoids, in which trisporols B ([D_3]**7**) and C ([D_3]**12**) represented the last common intermediates. Figure 1 shows a mass spectrum of the downstream metabolite trisporol B ([D_3]**7**) obtained by administering [D_3]**3** to growing hyphae of the (+) or (−) mating type of *B. trispora*.

The metabolite was obtained undiluted by a background of unlabeled compounds as evidenced by the large molecular ion at *m/z* 293 and the very low abundance of the one at *m/z* 290 (Figure 1; arrow: unlabeled **7**). The high abundance of the 293 signal for [D_3]**7** also proved that the deuterium label was completely maintained during subsequent transformations. The next step, namely the oxidation of the pro-(*R*)-CH_2OH group of [D_3]**7** to methyltrisporate B ((D_3)**5**), was only observed in the (+) cultures; the mycelia of the (−) strain accumulated trisporol ([D_3]**7**; Scheme 4). Parallel to the oxidative functionalization of the cyclohexene moiety, the keto group within the longer C8 side-chain was reduced to a secondary alcohol yielding the corresponding trisporins and trisporols of the C series (Table 1). The configuration of the resulting chiral center was not determined. Trisporols of both the B ([D_3]**7**) and C ([D_3]**12**) series were oxidized only by the cells of the (+) strain to the corresponding methyltrisporate B ([D_3]**5**) and methyltrisporate C ([D_3]**11**), respectively. The saponification of the esters to TSA B ([D_3]**8**) and C ([D_3]**13**), respectively, was achieved by both mating types, but the cells of the (−) mating type proved to be more efficient (vide infra).

In addition to the esters [D_3]**5** and [D_3]**11**, 4-dihydromethyltrisporate B (**4**) was also found in the culture broth of the (+) mating type (Schemes 2 and 5). All three esters accumulated transiently between 9 to 12 h after the precursors were administered, but only [D_3]**5** and [D_3]**11** were rapidly saponified to the final TSA B ([D_3]**8**) and C ([D_3]**13**), respectively. Ester **4**—previously considered to be a central precursor to TSA B (**8**; Scheme 2)—apparently had another biosynthetic origin and metabolic fate than [D_3]**5** and [D_3]**11**. First, unlike [D_3]**5** and [D_3]**11**, **4** was not significantly labeled (approximately 6% D_3). The same low degree of labeling was obtained when, for example, the more advanced intermediate [D_3]**9** was fed to the



Scheme 4. Survey of administered precursors and their metabolites from the culture broth of (+) and (−) mating types of *B. trispora*. A) Administered precursors; B) isolated metabolites from (−) mating type cultures (white background) and (+) mating type cultures (gray background). Compounds were identified by their retention time and mass spectra with authentic references.[16,29]



Scheme 5. Revised cooperative biosynthetic pathway for the biosynthesis of TSA B (**8**) by individual and mated cultures of *B. trispora*. The pathway illustrates the production of the B series of trisporoids. The reduction of the keto group in the C8 side-chain to a secondary alcohol generates the C series, which is present in the culture broth of both mating types. In mated cultures, there is a free exchange of all early and late intermediates, which are secreted into the medium.

growing culture of the (+) strain. Second, although ester **4** accumulated transiently, its exclusive saponification by the mycelia of the (−) mating type preferentially led to a novel and rather unstable 4-dihydrotrisporic acid B (**14**) without any notable oxidation of the ring hydroxy group (Scheme 5). In fact, the oxidation of **4** to TSA B (**8**) was very slow and insignificant compared to the main route to **14** (Figure 2 and Scheme 5).

Kinetics of trisporol and TSA production

Figure 2 illustrates the time-dependent conversion of synthetic trisporin B ([D_3]**6**) to trisporin C ([D_3]**10**) and trisporols B ([D_3]**7**) and C ([D_3]**12**) for both mating types. Cells of both mating types metabolized the *E* and *Z* isomers of the administered [D_3]**6** in the first 12 h; the corresponding *E* and *Z* isomers of trisporols [D_3]**7** (neutral extract, pH 8) were also metabolized with similar efficiency. Minor amounts of (*E*)- and (*Z*)-trisporol B ([D_3]**7**) were produced within 2 h by both mating types (Figure 2). The *E*/*Z* ratio of [D_3]**6** isomers was largely retained in the end products (vide supra). In cultures of both mating types, after 12 h the intermediate trisporin C ([D_3]**10**) was largely consumed and converted to the corresponding trisporols B

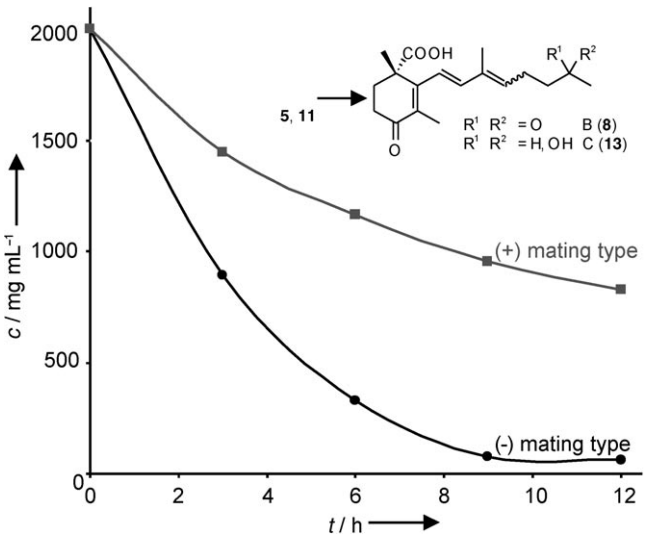


Figure 3. Time course of the saponification of methyltrisporate B (**5**) and C (**11**) by mycelia of the (+) and (−) mating type of *B. trispora*.

([D_3]**7**) and C ([D_3]**12**). After 24 h, cells of the (−) mating type had converted all intermediates to an *E/Z* mixture of trisporols B ([D_3]**7**) and C ([D_3]**12**) with the *E* isomers prevailing. After the same time, the *Z* isomer of unlabeled 4-dihydromethyltrisporate (**4**) dominated in cultures of the (+) mating type; minor amounts of trisporin C ([D_3]**10**) and methyltrisporate B ([D_3]**5**) were also present.

The kinetics of the ester hydrolysis to TSAs B (**8**) and C (**13**) was independently studied by administering methyltrisporate B (**5**) and methyltrisporate C (**11**) to separate cultures of similar biomass. The progress of the reaction was followed by GC-MS analysis of aliquots without pretreatment with diazomethane. In both cases, the externally added pure methyltrisporates B (**5**) and C (**11**) were not only hydrolyzed but partially reduced or oxidized; this generated a mixture of the two series prior to hydrolysis to TSAs B (**8**) and C (**13**), respectively. Within 12 h, the mycelia of the (−) type hydrolyzed methyltrisporate B (**5**) almost completely to the acid (**8** and **13**), whereas the cells of the (+) type achieved only about 50% conversion. The hydrolysis proved to be irreversible since adding free TSA B (**8**) or C (**13**) did not result in the formation of methyltrisporates (**5** or **11**, respectively).

Discussion

The fungal phylum zygomycota uses the degradation products of β-carotene, namely TSAs (Schemes 1 and 2) and many of its isoforms and precursors, as a communication system. The compounds, which are involved in the recognition of mating partners, induce the first steps of sexual differentiation and possibly maintain the development of sexual structures. Their origin in β-carotene has been demonstrated with inhibitors of β-carotene biosynthesis[2] and β-carotene-deficient mutants[21,22] and by administering radiolabeled β-carotene[8] to growing cultures. *B. trispora*, *M. mucedo* and *P. blakesleeanus* in particular, have been studied with respect to the diverse substances they contain, which are active during the sexual reproduction cycle. Pioneering work by Austin, Sutter, Gooday and Bu'lock led to the proposal of a cooperative biosynthesis of trisporoids (Scheme 2) by both mating partners, which was reinvestigated in this contribution by the administration of specifically labeled precursors to individual cultures of the (+) and (−) strains, as well as to mixed cultures. Owing to the inert position of the deuterium atoms in a methyl group of the side-chain, the metabolites could be unequivocally identified by mass spectrometry. Moreover, the administration and recovery of the metabolites resulting from the transformation of both early and late trisporoids by (+) and (−) mycelia gave reliable information on the biosynthetic capacity of the two mating types and allowed the sequence of transformations to be reconstructed.

The procedure is illustrated by the administration of the postulated early product of β-carotene cleavage, namely the conjugated ketone D'orenone (**1**), to mycelia of both mating types. Mass spectrometric analysis of the isolated metabolites revealed that both strains were able to produce the full spectrum of early trisporoids between D'orenone (**1**) and trisporol (**7**). However, cells of the (−) mating type generated larger amounts in less time. Interestingly, 4-dihydrotrisporine B (**3**) was produced from ketones **9** and **2**, since both intermediates were converted to **3** when added to the growing cultures. However, the administration of **9** resulted in higher levels of **7** and **12** (Table 1), and consequently, of TSA B (**8**) and TSA C (**13**), respectively; this suggests that the allylic oxidation at C-4 of the cyclohexene ring might precede the reduction of the conjugated double bond. Most importantly, cells of the (+) mating type were able to generate small amounts of the end-product TSA B (**8**) without any assistance by the (−) type.[20] Consistent with previous findings (Scheme 2), cultures of the (−) mating type were not able to proceed beyond trisporol B (**7**). Further oxidation required the contribution of the complementary cell type to convert the hydroxymethyl group of (**7**) to the methyl ester of methyltrisporate B (**5**). The biosynthetic flexibility and capacity of both strains were further illustrated by their ability to generate simultaneously trisporoids of the B and C series from the same early intermediates **2** and **3** (Table 1). Since all intermediates are secreted into the medium, the hyphal cells of a mixed culture can contribute at almost all early levels to the production of the final TSA. The oxidation of trisporol B (**7**) to methyltrisporate B (**5**), however, is limited to cells of the (+) strain, which then synchronize the biosynthesis of TSAs in mixed cultures. The final saponification of the methyltrisporates **5** and **11** to the free TSAs **8** and **13**, respectively, can be achieved by both strains, but again, metabolic activity is much higher in the (−) strain (Figure 3). Accordingly, the complementary partner serves as a source of early intermediates and stimulants, which enhances the efficiency of the cooperative biosynthesis dramatically since much higher amounts of the bioactive TSAs are obtained from mixed cultures than from a single (+) mating type.[20]

In contrast to previous findings,[20,23] in this study, trisporol B (**7**) was formed by both the (+) and (−) cells of *B. trispora*. Accordingly, the overall transformation requires at least three different enzymes. The initial oxidation of the methyl group to the primary alcohol is achieved by an enzyme common to both cells. The subsequent oxidation of the alcohol **7** to the carboxylic acid **8** and its esterification only occur in cells of the (+) type. Whether this transformation requires one or two distinct enzymes, including a methyl transferase, remains to be established. Overall, the oxidative functionalization of the exocyclic methyl group to a carboxyl group resembles the action of certain multifunctional P450-type enzymes of the steroid metabolism required for dealkylation processes.[24] The final saponification of methyltrisporates to the free TSAs can be achieved by both strains, but, as in the case of the biosynthesis of the early intermediates, this occurs faster in the (−) strain (Figure 3, Scheme 5).

A striking result was the observation that 4-dihydromethyltrisporate B (**4**), which was isolated from growing cultures of the (+) strain, was not labeled (about 6% D_3) after the administration of [D_3]**1** and other early precursors. Since TSA B ([D_3]**8**) was found to be highly labeled (>95% D_3) when obtained from the transformation of [D_3]**1**, the isolated 4-dihydromethyltrisporate B (**4**) seems to have another origin. Following an experimentally unproven postulate of Gessler et al.,[25] we

assume that the (+) strain might be able to use oxygenated carotenes, such as isocryptoxanthine, as substrate for the biosynthesis of the accumulating 4-dihydromethyltrisporate B (**4**). The oxidative degradation and transformation of this alternative and oxygenated carotene precursor could account for the lack of deuterium in **4**. For further transformation into TSA B (**8**) this compound would require the oxidation of the ring hydroxyl group and the saponification of the resulting methyl ester. Interestingly, **4** did not follow this line, but instead accumulated in the fermentation broth of the (+) type mycelia and required the hydrolytic capacity of the (−) type for the saponification to the corresponding unstable 4-hydroxytrisporic acid (**14**). The biological function of **14** is not yet known.

Since the biosynthesis of **14** requires cooperative activity from both mating types, this novel pathway also indicates the synchronization of the production of the bioactive compounds that control the progress of sexual reproduction. The further oxidation of **14** to TSA B (**8**) was not observed (Figure 2). Additional experiments with labeled isocryptoxanthine are necessary to explore this pathway. It also remains to be clarified whether or not the previously described 4-dihydromethyltrisporate dehydrogenase from *M. mucedo*, which is claimed to be responsible for the oxidation of **4** to methyltrisporate (**5**) in the (−) strain,[25] can alternatively oxidize 4-dihydrotrisporine B (**3**) to trisporin B (**6**). Since 4-dihydromethyltrisporate (**4**) is not oxidized to TSA B (**8**) in *B. trispora* in significant amounts, the oxidation of **3** to **6** seems to be the more likely reaction for this enzyme.

In summary, the administration of labeled early trisporoids to growing cultures of the (+) and (−) strains of *B. trispora* showed that metabolic activity in both mating types is highly flexible. Our experiments clearly demonstrate that both are able to use the previously postulated[26] early cleavage product of β-carotene, recently called D'orenone (**1**),[15] to produce trisporins **6** and **10** and trisporols **7** and **12**, albeit with different efficiencies. Besides β-carotene, oxygenated derivatives of β-carotene like isocryptoxanthine (Scheme 5), might also contribute to the metabolic diversity of these compounds.

Treating (+) or (−) mycelia with individual early and late trisporoids at given concentrations revealed that many of them are bioactive and that they are able to stimulate differential reactions in *B. trispora*,[27,28] or other members of the zygomycetes, such as *M. mucedo*.[29] The biosynthesis and oxidative degradation of β-carotene, in particular, can be differentially stimulated by various early and late trisporoids in *B. trispora* and *M. mucedo* (M. Richter et al., unpublished results). To evaluate the metabolic profile of other zygomycetes, the current approach can be easily modified or adapted. Since D'orenone (**1**), the first cleavage product of β-carotene, strongly inhibits the growth of root hairs of various plant species,[15] the bioactivity of individual trisporoids needs to be evaluated even beyond the zygomycota. This is further supported by the recent discovery of **1** in cyanobacteria and cell cultures of rice (Salim Al-Babili, University of Freiburg, Germany, 2008, personal communication). New and unexpected functions in the interaction of zygomycetes with other organisms (the mycorrhization of plant roots, parasitic interactions with other fungi, colonization of insect guts, colonization of mycelia by bacterial endobionts and establishment of the *Geosiphon* fungus–cyanobacteria interaction) might be discovered, since several structurally related apocarotenoids (e.g., retinoic acid and abscisic acid) control fundamental processes in living organisms.[30,31]

Experimental Section

Strains and culture conditions: A compatible mating pair of *B. trispora* strains (FSU 331 (+) and FSU 332 (−)) were grown on solid glucose for 30 days in standard Petri dishes (diameter 9 cm) with aeration vents and containing complete medium.[32] Three pieces of mycelium (5 mm × 5 mm) were inoculated and incubated for 4 days for the (−) type and 3 days for the (+) type at 21 °C in liquid SUP medium and shaken at 120 rpm. The different inoculation times accounted for the different growth rates and generated similar biomasses, as determined by dry weight, at the onset of the administration experiments. The mycelium was filtered and transferred into a medium containing maltose instead of glucose.[33]

Trisporoid standards: D'orenone (**1**),[15,16] 4-dihydrotrisporin B (**3**), trisporin B (**6**) and trisporin C (**10**) were synthesized as defined E/*Z* isomers, according to Schachtschabel et al.[16] Methyltrisporate B (**5**) and methyltrisporate C (**11**) were obtained from TSA B (**8**) and TSA C (**13**), respectively, by esterification with ethereal diazomethane. TSA B (**8**) and TSA C (**13**) were produced by a large-scale coculture of both mating types of *B. trispora* in a 30 L fermenter. The free acids were extracted from the acidified medium (pH 2) and purified as described.[16] The ester, 4-dihydromethyltrisporate B (**4**), was purified from an alkaline extract (pH 8) of the (+) strain. Trisporol B (**7**) and trisporol C (**12**) were generated by the biotransformation of trisporin B (**6**), as previously described.[16] Ketone **2** was generated from 2-(4-brom-3-methylbuta-1,3-dienyl)-1,3,3-trimethylcyclohex-2-ene by Pd^0-mediated alkylation with the zincate of 4-iodobutan-2-one. Hydroxyketone **9** was synthesized via a Heck-type alkenylation of 3-((1*E*)-4-bromo-3-methylbuta-1,3-dienyl)-2,4,4-trimethylcyclohex-2-enol with but-3-en-2-one.[16,34]

Mass spectrometric analysis of trisporoids: Samples were analyzed by using a Trace GC (Thermo Finnigan, San Jose, CA, USA) connected to a Trace MS detector (Thermo Finnigan, San Jose, CA, USA). An EC-5 column (15 m × 0.25 mm i.d., 0.25 μm film thickness; Alltech, Deerfield, IL, USA) was used to separate the reaction products. Helium, at a constant rate of 40 cm sec^{-1}, served as the carrier gas, and samples (1 μL) were introduced in the splitless mode by an autoinjector. The GC injector, transfer line and ion source were set at 220, 280 and 280 °C, respectively. Spectra were taken in the total-ion-current mode (TIC) at 70 eV. The separation of the compounds was achieved with a temperature program from 50–280 °C at 15 °C min^{-1}. The final temperature of 280 °C was maintained for 2 min prior to cooling. Trisporoids were identified by comparing their mass spectra and retention times to those of authentic standards.[16]

Feeding experiments: Deuterium-labeled precursors (5.0 mg) in ethanol (100 μL) were added to sterile cultures of mated or individual (+) and (−) mating strains of *B. trispora* grown in liquid cultures on a maltose medium (100 mL in 250 mL Erlenmeyer flasks). The cultures were shaken in the dark at 21 °C (120 rpm) for 3, 6, 9, 12 and 24 h. Incubations were performed with D'orenone ([D_3]**1**), 4-dihydrotrisporin ([D_3]**3**), trisporin B ([D_3]**6**), trisporin C ([D_3]**10**), methyltrisporate B (**5**), methyltrisporate C (**11**), 4-dihydromethyltrisporate B (**4**), 4-dihydrotrisporic acid, TSA B (**8**), TSA C (**13**), the two ke-

tones [D_3]**2** and [D_3]**9** and the apocarotenoids retinol, retinal and retinoic acid.

Extraction and derivatization of metabolites: All operations were carried out in the dark (with red light) to avoid the isomerization of double bonds. The culture broth was filtered and adjusted to pH 8 with aqueous NaOH (1 M), and β-ionone (25 μg) was added as an internal standard (IS). Neutral constituents were extracted with $CHCl_3$/propan-2-ol (20:1, *v/v*). The aqueous phase was then acidified (pH 2, 1 M HCl), perhydrogenated retinoic acid (25 μg) was added as an IS, and the aqueous phase was extracted with $CHCl_3$/propan-2-ol (20:1, *v/v*). Both extracts were dried (Na_2SO_4), and the solvent was removed under reduced pressure. The residue of the alkaline extract was redissolved in CH_2Cl_2 (1.0 mL) before being treated for 5 min with ethereal diazomethane (0.5 mL). Excess diazomethane and solvent were removed by a stream of argon. Additional treatment with MSTFA (50 μL) for 90 min at 55 °C converted hydroxylated derivatives into silylethers. Following the removal of the reagent under a gentle stream of argon gas, the sample was dissolved in CH_2Cl_2 (1.0 mL).

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