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Biological activity of trisporoids and trisporoid analogues in *Mucor mucedo* (−)

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Abstract

In the course of their sexual interactions, zygomycete fungi communicate via an elaborate series of carotene-derived compounds, namely trisporic acid and its biosynthetic progenitors. A novel building-block strategy allowed the systematic generation of structurally modified trisporoids along with putative early biosynthetic precursors for physiological tests. The impact of discrete structural elements was documented by the ability of individual compounds to induce sexually committed hyphae in *Mucor mucedo*. The activity screening contributed to establish general structure–function relationships for trisporoid action. Most crucial for activity were the dimension of the longer side chain, the polarity of functional groups at C(4) and C(13), and the number of conjugated double bonds in the side chain. The presence of an oxygen substituent at the cyclohexene ring is not essential for function. The overall biological activity apparently results from the combination of the various structural elements.

Keywords: *Mucor mucedo*; Zygomycetes; Sex hormone; Trisporic acid; Precursors; Chemosynthesis; Bioactivity

1. Introduction

The C_{18} β-carotene derivate trisporic acid and its precursors are pheromones involved in the regulation of mating reactions in Zygomycetes (Zygomycota) (Gooday, 1978; Schimek et al., 2003). As the same basic signal system is used throughout such a wide systematic range, interspecific reactions occur frequently and abundantly. Nevertheless, species-specificity of the mating reactions is maintained, ascertaining the formation of mature zygospores only in compatible mating pairs of the same species. The various trisporoid types produced during biosynthesis, i.e., trisporates, methyl trisporates, trisporols and trisporins, are defined by their substituents at C(1) and C(4) (Figs. 1 and 3).

Trisporic acids are the products of a complex, cooperative pathway involving both the (+) and the (−) mating type. In a simplified overview the early biosynthetic steps towards, for example, trisporic acid B take place in both mating partners, beginning with the cleavage of β-carotene until the production of 4-dihydrotrisporin (**2**). Compound **2** is the last common intermediate and is converted to trisporin (**4**) in the (−) mating type, and to 4-dihydromethyltrisporate (**3**) in the (+) mating type (Bu'Lock et al., 1976). The compounds act as pheromones towards putative partner strains (Fig. 1).

As each mating type is unable to convert its own intermediate into trisporic acid, production of the latter depends on exchange of the mating type-specific precursors between the complementary mycelia. The

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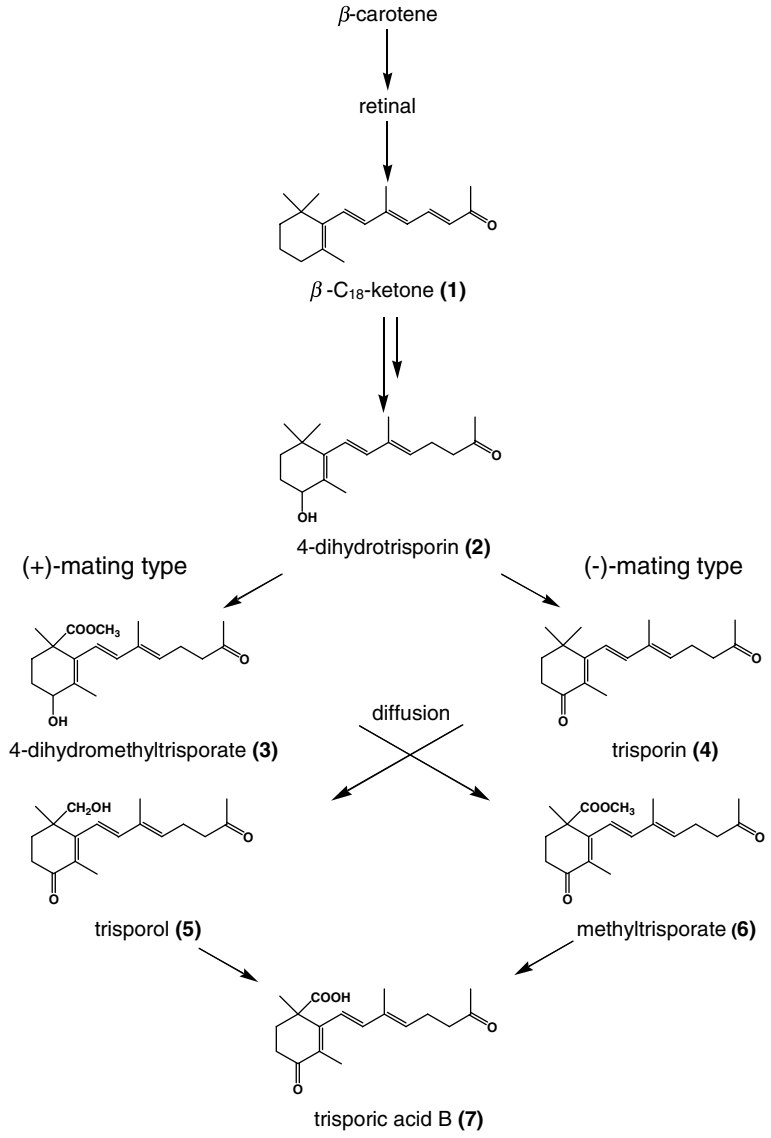


Fig. 1. Postulated biosynthesis of trisporic acid B (van den Ende, 1976; Sutter et al., 1989; modified). Analogous pathways are expected to generate the trisporic acids A, C, D, and E.

precursors are taken up by the mating partner, and are converted in a sequence of mating type-specific enzymatic steps into trisporic acid (reviewed by Gooday, 1978, 1983).

Trisporoids exert a number of responses in susceptible mycelium. They induce directed growth and thus facilitate partner finding and contact between the putative partner hyphae. They also directly induce the formation of sexually committed hyphae, the zygophores, in *Mucor mucedo* and a number of other species. They further elicit a range of more general responses, among them up-regulation of β-carotene synthesis and, e.g., in *Phycomyces*, arrest of vegetative growth as preliminary step to sexual morphogenesis (Drinkard et al., 1982).

Previous studies established the presence of a mixture of derivates in each species (van den Ende, 1968; Sutter et al., 1974; Sutter and Whitaker, 1981a) as is exemplified for the different series of trisporic acids (**7**) shown in Fig. 2. This divergence may be the result of biosynthetic ramifications during speciation and the overall evolution of this fungal group. The compound pattern within each species is supposed to be the basis for species-specificity of the sexual response (Sutter et al., 1989). Complete reactions, culminating in the formation of mature zygospores, are restricted to crossings between compatible partners of the same species. On the other hand, the absolute amount of individual compounds of the blend may also influence the response of the mating partner. These conceptional uncertainties remain to be resolved. Also, the actual physiological functions of the biosynthetic intermediates (Fig. 1) are not yet known in detail; the same applies to the sequence

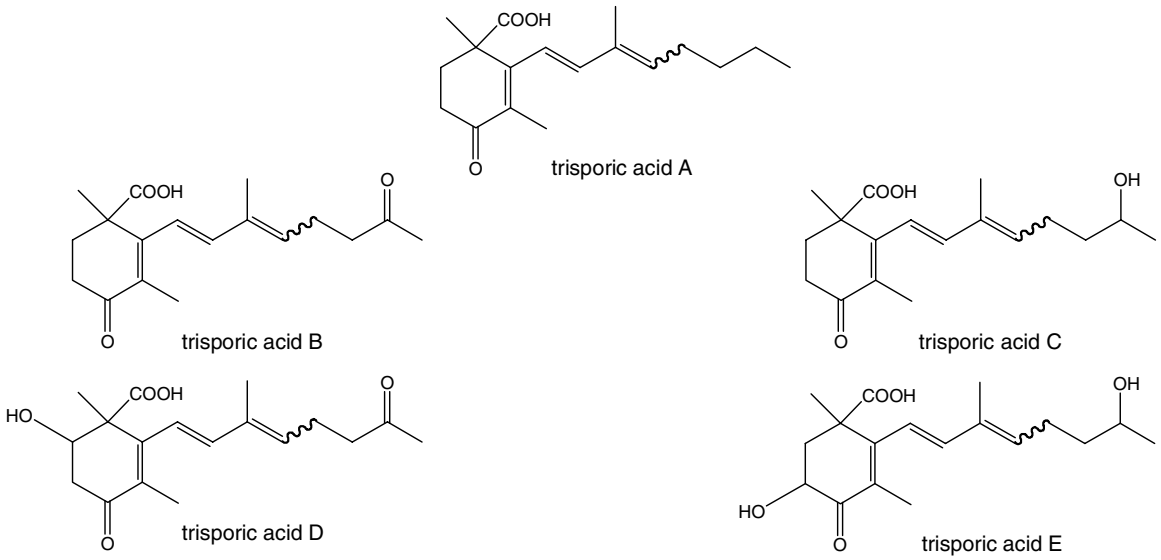


Fig. 2. The trisporic acids are shown as the end products of the pathways.

of biosynthetic step(s) in the different species. Especially, the sequence of the oxidative functionalization of the early intermediates and the physiological importance of the resulting products remain to be established. This lack of knowledge prompted us to start a systematic and comprehensive study of structure–function relationships in trisporoid action and biosynthesis. As the existing methods do not allow the isolation and purification of natural trisporoids at a preparative scale, the approach depends on the synthesis of potential metabolites. Owing to their interesting biological activities, the trisporoids have been repeatedly targets for synthesis. Methyl trisporates have been synthesized by six groups so far (Edwards et al., 1971; Isoe et al., 1971; Secrist et al., 1977; Trost and Ornstein, 1983; Takabe and White, 1983; White et al., 1985; Takahashi et al., 1988). The free trisporic acids have been produced by different strategies by White et al. (1985), and by Reeder and Meyers (1999). Several of the proposed early precursors were synthesized by the groups around Bu'Lock et al. (1973, 1976) and Sutter (1986), and trisporol was synthesized also by White and coworkers (Prisbylla et al., 1979). However, most previous routes are lengthy and suffer from low overall yields. Since the current project focuses only on the early intermediates of trisporoid biosynthesis, the major obstacle of previous syntheses, namely the presence of the carboxylate group in the molecule, could be ignored. The most important structural features to be analyzed are exemplified in Fig. 3.

As shown, the analysis of structure–functional relationships of the early trisporoids addresses in particular the functional and structural principles linked to the oxidative functionalization at C(4) and C(13). This covers the presence of keto- or alcohol functions at both carbon atoms as well as combinations of ketones and alcohols. In the case of alcohols both centres become chiral. Moreover, the overall chain length, the stereochemistry, the number of double bonds, and the relative distance between the double bond and the position of the oxygen functions are of principal interest. Here we report on the biological activity of compounds obtained along a novel and highly flexible synthesis towards early trisporoids. First data on their physiological effects (growth arrest and zygophore induction) of the compounds are presented.

2. Results and discussion

2.1. Synthetic concept

The novel concept towards trisporoid-type compounds addresses only compounds that lack the carboxyl- or ester group of the late trisporates. To minimize the synthetic effort towards the different test compounds (Figs. 3 and 4), we developed a building block strategy which allows the generation of all of the different trisporoids from a single intermediate.

The strategy is outlined in Fig. 4. Commercial β-ionone (**8**) is first converted into the central (*E*,*Z*)-vinyl bromide **9**. The bromide can be easily functionalized at C(4) by oxidation with $CrO_3 \cdot$ pyridine (Sarett et al., 1952) to ketone (**10**), or to an acetate by allylic oxidation with $Pd(CF_3COO)_2$ (McMurry and Kocovsky, 1984). The latter can be readily converted to **10**. The second strategic element of the novel synthesis is based on the facile alkylation of the vinylic bromides **9** and **10** with organozinc reagents in the presence of Pd(0)-catalysts (Knochel et al., 1998). The organozincates can be readily obtained from functionalized halides without the

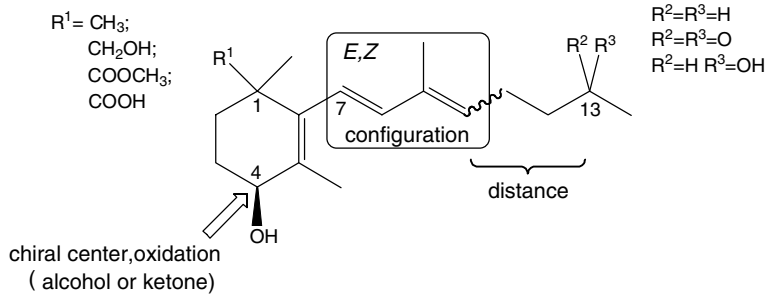


Fig. 3. Relevant structural elements of trisporoids for biological activity.

need for protecting groups (Huo, 2003). This minimizes the number of transformations on the generally acid- or base sensitive trisporoid backbone. For example, trisporin (**4**) is synthesized in a single operation by a Pd(0)-catalyzed alkylation of **10** with an organozinc reagent directly obtained from 4-iodo-butan-2-one. The same strategy can be followed to generate a derivative with a secondary alcohol **14** or to produce aliphatic sides chains of variable chain length, e.g., **11** and **12**. Heck-type alkylation of **9** with buten-2-one (Naora et al., 1988) leads to **1** and biosynthetic precursors of trisporin (**4**). Aromatic derivatives representing analogues with restricted conformational freedom or fluorescent probes can be obtained along a similar protocol. The approach also allows the preparation of deuterium labelled compounds via $^{1}H/^{2}H$-exchange on the level of β-ionone. Full experimental details for the novel route will be published elsewhere.

2.2. Biological activity tests

The biological activities of the test compounds have been monitored in *M. mucedo* (−) as the most sensitively reacting species (Sutter and Whitaker, 1981a). Also with respect to the previously postulated intermediate preferences (Fig. 1), the (−) strain of *M. mucedo* is particularly well suited. Despite its preference for complementary precursors, *M. mucedo* (−) also responds, albeit less pronounced, to (−)-mating type-specific compounds from other species (Sutter and Whitaker, 1981a; Schimek

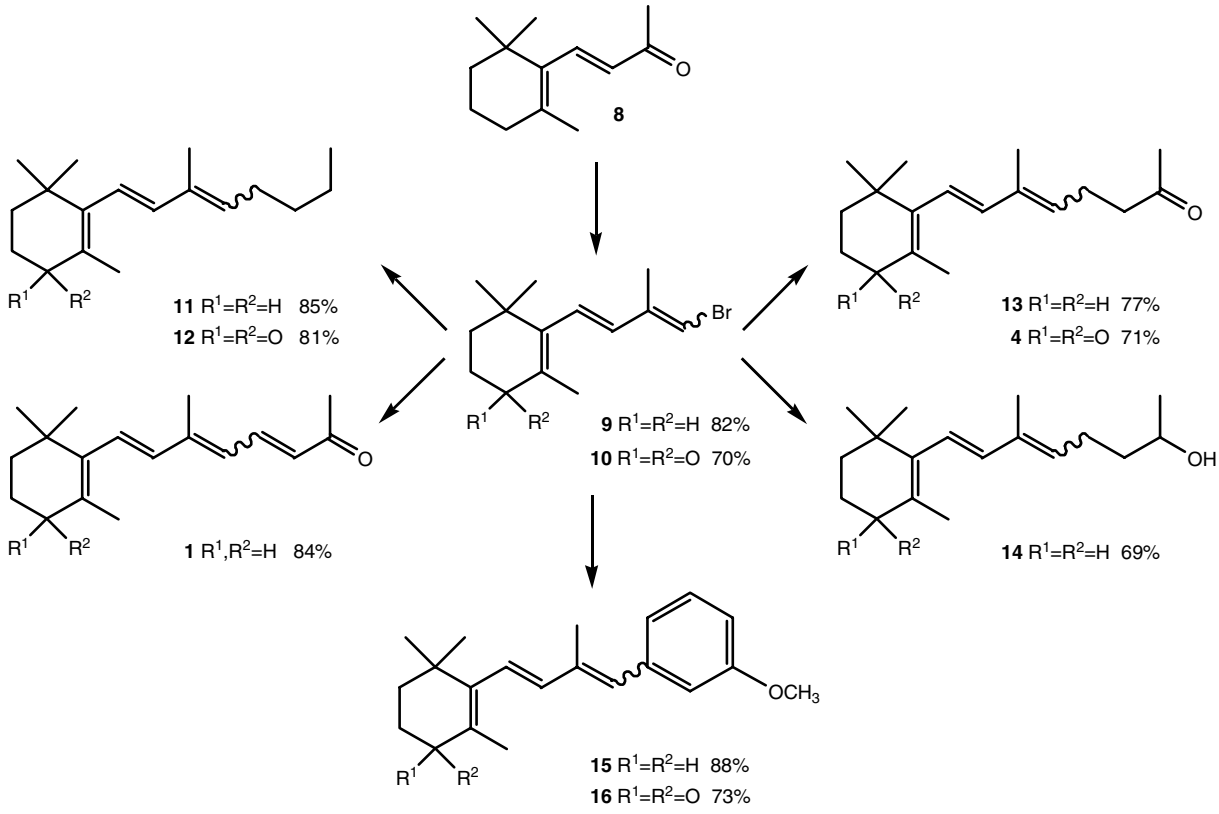
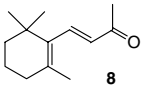
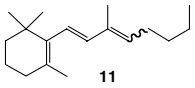
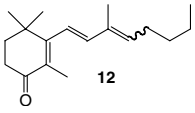
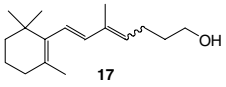
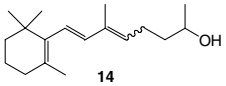
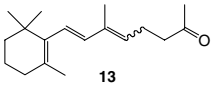
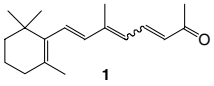
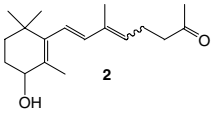
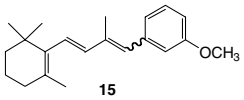
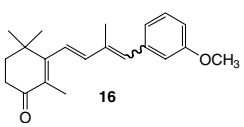


Fig. 4. Unified synthetic route to early intermediates of trisporic acids.

et al., 2003). Altogether this is the most reliable strain to monitor a broad response. The typical responses, observed after exposure of growing cultures of (−) *M. mucedo* are surveyed in Table 1.

The induction of zygophores can be considered as a reliable proof for sexual stimulation and can be quantified, if certain limitations are taken into consideration. That special type of aerial hypha is easily discernible from the sporangiophores, the vegetative reproductive structures. The zygophores are small, not exceeding about 500 μm in height and 15 μm in width in the (−) mating type (data not shown). In contrast, young sporangiophores have a diameter of at least 50 μm, and later their tips swell characteristically. Differentiation can be induced at the nanogram level of trisporoids (Sutter and Whitaker, 1981a), however at very low concentrations zygophore production is no longer quantifiable and occurs as an all-or-nothing reaction. At high concentrations, quantification is hampered by the high density of zygophores that can be no longer reliably counted.

Table 1
Physiological effects of synthetic trisporoid analogues in *Mucor mucedo* (−)

compound	zygophore induction	growth arrest
mixture of natural trisporoids extracted from mated cultures of *Blakeslea trispora*	yes	no
 **8**	no	yes
 **11**	yes	no
 **12**	yes	no
 **17**	no	no
 **14**	no	no
 **13**	yes	no
 **1**	yes	yes
 **2**	no	no
 **15**	no	yes
 **16**	n.d.[a]	no

[a] not determined

2.3. Biological activity of individual compounds

As safeguard in case of weak responses, rather high amounts (10–100 μg) of the compounds were applied. The strongest reaction, determined as the ability to induce zygophores, was detected for compound **13** with 50 zygophores at the most. Zygophore induction was also observed for compounds **1**, **11** and **12**. Their effects were constantly weaker, yielding on average 4–20 zygophores for each of the three compounds.

Compound **12** induced typical zygophores only at high concentration (ca. 100 μg). At lower concentrations (25 μg) formation of atypical, branched aerial hyphae (designated as antlers) was observed. The antlers have about the same dimensions as the non-branched zygophores and have been observed with natural trisporoids, too (Schimek, unpublished observations). Antlers are probably the result of weakly positive reactions, where the stimulus is not strong enough to override all other morphogenetic signals.

For natural metabolites upstream of 4-dihydrotrisporin (**2**) only very few data on physiological activities are available. As yet, these compounds have not been isolated from natural sources. With the synthetic racemate of 4-dihydrotrisporin B (**2**) (custom synthesized by ARC Laboratories, Minden, The Netherlands) we could demonstrate that 4-dihydrotrisporin (**2**) does not elicit zygophore induction in *M. mucedo* (−) or (+) strains, although the compound already exhibits all necessary structural features. Since biological activity was postulated only for compounds downstream of 4-dihydrotrisporin (**2**), its putative biosynthetic progenitor **1**, the β-C_{18}-ketone, should also be inactive in *M. mucedo* (−) (Bu'Lock et al., 1976). However, synthetic compound **1** was found to be active in our assays. Compound **13**, which differs from **1** only by reduction of the α,β-conjugated double bond in the carbonyl compound, was the most active analogue of all. This confirms the data presented by Sutter et al. (1996) who observed zygophore production in carotene deficient mated mutant strains of *Phycomyces blakesleeanus* when stimulated with synthetic compound **13**. Compound **13** differs from inactive 4-dihydrotrisporin (**2**) by lacking the polar hydroxyl group at C(4). The other two active analogues, namely **11** and **12**, correspond to the series

of trisporate A. Published data on the trisporic acid A series are inconsistent; trisporic acid A is described as zygophore inducing by Bu'Lock et al. (1972), and as inactive by White et al. (1985). In the latter study, trisporol A, on the other hand, was found active towards *M. mucedo* (+). As the trisporins are the biosynthetic precursors of trisporols (Gooday, 1983), which are functional pheromones in some species, the activity of **12** does not surprise, and supports the statement of physiological effects of intermediates of the trisporic acid A series.

The lack of zygophore induction for **14** and **17** was unexpected, since the compounds possess an oxygen function at C(13), thus resembling intermediates of the trisporic acid C series. On the other hand, a terminal hydroxyl group is also present in the inactive and chain-shortened apotrisporol (van den Ende, 1968; Sutter and Whitaker, 1981b), representing a degradation product of trisporol. Formally, analogue **14** can be considered as an intermediate within the C or E-series of trisporic acids which are generally physiologically active. From the E-series, only methyl trisporate E has been analyzed so far and was found to exhibit biological activities similar to the corresponding methyl trisporate C (Miller and Sutter, 1984). Trisporoids C have been described as less active (Bu'Lock et al., 1972) by a factor of 10–20 (Sutter and Whitaker, 1981a) when compared to those of the B series.

Steric, electronic and solubility factors may account for the inactivity of the two aromatic compounds **15** and **16**. Inactive are also compounds with a short side chain, such as β-ionone (**8**) and a diketone, derived from **8** by oxidation at C(4). This is consistent with the inactivity of apotrisporins and apotrisporols, which are considered as products of an inactivation process (Sutter and Whitaker, 1981a; Sutter and Zawodny, 1984; Sutter, 1986). In summary, from the data in Table 1 several functional correlations can be made:

(1) The length of the side chain appears to be critical for activity. Chain-shortened compounds such as β-ionone (**8**), abscisic acid, or apotrisporins, with a C_{10}- or C_{11}-backbone, are inactive throughout. A side chain with the correct length immediately leads to active compounds, even if the carbonyl group at C(13) is missing (cf. compounds **11** and **12**). Earlier attempts with C_{15}-backbone retinoids, retinol, retinal, and retinoic acid demonstrated the lack of biological activity for such compounds (Bu'Lock et al., 1972).
(2) The polarity of functional groups at the longer side chain is critical. While a hydrocarbon segment or keto group at C(13) lead to the active compounds **11** and **13**, a hydroxy group at C(13) **14** or an ester moiety in the same position result in inactive products.
(3) An oxygen substituent at C(4) is not essential. Analogues **11** and **13** are active without having an oxygen atom at C(4). Compounds with a non-functionalized cyclohexene ring may represent early intermediates from asymmetric cleavage of β-carotene. Formally, these compounds could be considered as new trisporoid F derivates.
(4) With some reservation, the saturation of the $C_{11–12}$ bond with the resulting enhanced rotational freedom may be important to adopt a conformation, which is essential to fit to the macromolecular targets (receptors).
(5) None of the above structural features alone seems to be correlated with a specific response pattern. Instead, the biological activity is conveyed by the combination of the various elements. For example, in the physiologically active C series the negative effects of the hydroxyl group may be overcome by the presence of other substituents at the ring carbon atoms.

Another phenomenon often encountered as reaction to chemical stimuli is a localized growth arrest. No mycelium grows directly up to and beyond the filter disc, which is then left in a more or less circular bay of uncovered substrate. Growth arrest is a prerequisite to sexual reactions in *P. blakesleeanus* (Drinkard et al., 1982), but is generally not observed in *M. mucedo*. This behavioural difference reflects the difference in zygophore action in the two species. In *M. mucedo*, the aerial zygophores with their limited length can only get into contact with a partner zygophore, if the distance between them does not exceed ca. 1 mm. As zygophores are not formed from very young mycelium directly at the front of the growing colony, the partner mycelia are forced to intermingle to allow for sexual reactions. In *Phycomyces*, zygophores are the much shorter, lobed, and bloated hyphal branches formed at the substrate surface from the foremost hyphae of the growing colony. As they cannot move freely in the air, their range of action is very limited, and contact with a partner hypha relies on the possibility of a direct encounter. It cannot be excluded, however, that in *Mucor*, vegetative growth might be reduced, too, in the course of switching commitments. Another explanation for growth inhibition is, of course, a systemic toxic effect exerted by the compound applied. Such toxic reactions can be triggered by β-ionone (**8**), as was confirmed in this study for *Mucor* (Table 1) and also for *Phycomyces* (data not shown). Growth arrest was furthermore observed as reaction to compounds **1** and **15.** In *Phycomyces*, growth inhibition was triggered also by **11**. Carotene induction as a marker for sexual stimulation was occasionally observed, but is not included in the table as no basis for evaluation exists. A quantifiable assay for β-carotene production is currently developed. The physiological reactions

monitored in this study are not absolutely quantifiable at the present stage. Nevertheless, the data obtained provide a reasonable basis for unravelling of common principles. With a synthetic concept allowing systematic variation of individual structural features, a number of very early trisporoid analogues have been generated and their analysis helped to unravel basic correlations determining the bioactivity of trisporoids, especially of the C and E series (Fig. 2). Systematic evaluation of the bioactivity of the trisporoids from the different series with other members of the group of zygomycetes will show, whether or not and to which extent discrete structural features have been developed to individualize a species- and mating-type specific interaction. The large number of currently known trisporoids may be indicative for the development of a complex metabolic network serving as a pool from which different bioactive compounds may have been recruited during evolution to generate functional and highly selective communication systems for sexual reproduction of the zygomycete fungi.

3. Experimental

The biological activity of the synthesis products was tested using strains of *M. mucedo* FSU 621 (+) and FSU 620 (−) (Mucoraceae, Mucorales, Zygomycetes) and *P. blakesleeanus* FSU 2486 (+) and FSU 2487 (−) (Phycomyceteaceae, Mucorales). Cultures were grown on 9 cm Petri dishes on an enriched medium (SUP) as described by Wöstemeyer (1985). Vegetative spore stocks were kept at −20 °C in a 10% glycerol solution. Biological activity was tested on solid "induction" medium containing 20 g maltose, 10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4 \times 7H_2O$, 1 g yeast extract and 12 g agar per 1 l H_2O dest. (van den Ende, 1968; modified). *P. blakesleeanus* was grown on solid S-IV medium as described by Sutter (1975). The biological activity of the trisporoids and their analogues was estimated by their ability to induce zygophore formation in *M. mucedo* (+) and (−) strains and to provoke sexual responses in *P. blakesleeanus* as described by Schimek et al. (2003). 10–100 μg of the various compounds were dissolved in 15 μl of ethanol and applied directly in front of the growing mycelium on a 5-mm disc of Whatman No. 1 filter. After 16 h of incubation in the dark at 21 °C, the zygophores were counted with a dissecting microscope in an area of 6 mm^2 adjacent to the application spot. Test compounds were added in the range of 10–100 μg and their activity was compared to parallel runs with an extract from *Blakeslea trispora* containing natural trisporic acid and precursors. All compounds were tested in several independent assays, each using four plates per compound and concentration. As the absolute numbers of zygophores varied considerably between measurements, they are not included in the table. In the control reactions, more than 200 zygophores were counted in all plates.

Absorbance spectra were recorded on a V-560 UV/VIS Spectrophotometer (Jasco, Tokyo, Japan). Approximative measurements of trisporoid concentrations were calculated using the specific extinction coefficients for trisporic acid ($E^{1\%\ cm}_{325\ nm} = 572$) and 4-dihydromethyl trisporate ($E^{1\%\ cm}_{282\ nm} = 547$) as derived from the work of Nieuwenhuis and van den Ende (1975).

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