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SPECIFICITY AND TRANSFORMATIONS OF THE TRISPORIC ACID SERIES OF FUNGAL SEX HORMONES

J. D. BU'LOCK, D. DRAKE* and D. J. WINSTANLEY†

Microbial Chemistry Laboratory, Department of Chemistry, The University, Manchester M13 9PL

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Abstract—Chromatographic and spectroscopic characterizations and comparative bioassay data are given for trisporic acid A, the separate 9-*cis*- and 9-*trans*-isomers of trisporic acids B and C, and trisporol C, all obtained from 'mated' (*plus* and *minus*) cultures of *Blakeslea trispora*. All five acids show comparable levels of hormone activity on both the mating types of *Mucor mucedo*, whereas natural trisporol C more specifically affects a *plus* strain and the laboratory-derived methyl esters are *minus*-specific. Similarly *plus* and *minus* strains of *B. trispora* convert trisporol C and the esters into trisporic acids at different rates, and they effect different transformations of administered methyl ^{14}C-trisporate C.

INTRODUCTION

HETEROTHALLIC fungi in the order Mucorales display a very simple form of sexuality and the processes involved are mediated by interdiffusing hormones, the situation being described in detail in a recent review.[1] The sexual designations *plus* and *minus* apply irrespective of species and are based upon the ability of two strains of opposite sex, growing in close proximity, mutually to elicit the first morphogenic step in the sex process, the production of zygophores (progametangia). Zygophores of opposite sex grow towards each other (zygotropism), but only if two zygophores are also of the same or of closely-related species are subsequent stages of the sexual cycle observed. Thus the sexual designation rests primarily on the process of zygophore induction. Morphological and simple biochemical differences between strains prove, in general, not to be linked to sexual type.

Zygophore induction is caused by the production of a series of hormones, the trisporic acids (structures I–III), which are characteristically produced (along with various cometabolites) only by mixed cultures of *plus* and *minus* strains. Because of contradictions in the literature[1] it is important to establish whether or not any of these hormones act selectively upon one or other mating type (i.e. whether *plus* and *minus* differ in their hormone response system). The mechanism by which the strains collaboratively produce the hormones is also of crucial importance: i.e. do *plus* and *minus* strains differ in their actual or potential ability to carry out individual reaction steps in the biosynthetic sequence?

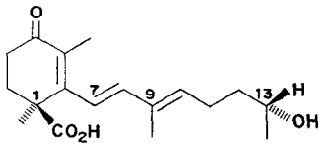
In this paper we give biological activity data for the main natural trisporic acids, having separated the principal stereoisomers. None of them is mating-type-specific. Some related substances are shown to have a degree of specificity for *plus* and *minus Mucor mucedo* (the preferred test organism). This apparent specificity is comparable to differences in the ability of *plus* and *minus* strains of *Blakeslea trispora* (the organism preferred for trisporic

* Present address: Department of Chemistry, Aarhus University, Denmark.

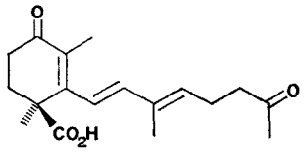
† Present address: Department of Nutrition and Food Sciences, Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.

[1] H. VAN DEN ENDE and D. STEGWEE, *Bot. Rev.* **37**, 22 (1971).

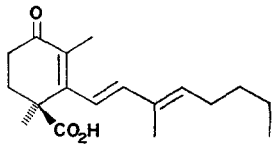
acid production[2]) to convert substances into trisporic acids and to effect related transformations. The separation and characterization of trisporic acid stereoisomers and some co-metabolites are described, together with preparations of ^{14}C-labelled substrates for the transformation studies.



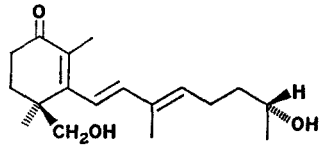
(I) Trisporic acid C (also 9-*cis*)



(II) Trisporic acid B (also 9-*cis*)



(III) Trisporic acid A (?)



(IV) Trisporol C

In trisporic acid C (I) the *R*-configuration at C-13 has been established degradatively,[3,4] and the *S*-configuration at C-1 (and its biosynthetic origin) follows from circular dichroism and biosynthetic data.[5] Natural I comprises two geometric isomers, both 7-*trans*,[3,6] as does natural trisporic acid B (II). Trisporic acid A, for which we suggest structure III, is a very minor metabolite and its stereochemistry is conjectural. The 9-*cis*/*trans* pairs are conveniently separated as the methyl esters, from which the free acids can be regenerated for bioassays. In these trienones the chromophore is perturbed by the non-conjugated substituents at C-1 (for example, reduction of I to the alcohol IV causes a hypsochromic shift of 15 nm) so that assignments of the stereochemistry about Δ^9 from UV spectra[3] must be questioned. In synthetic racemic methyl 7-*trans*-9-*trans*-trisporate B, Edwards *et al.*[7] showed that the chemical shift of the proton at C-8 could be used to assign the stereochemistry about Δ^9 correctly, and here we correlate such assignments with the chromatographic behaviour and the biological activity.

RESULTS AND DISCUSSION

Methyl Trisporate A

This is the least polar of the three (pairs of) methyl esters found in working-up[2] mated *B. trispora* acids; it occurs in very small proportions (< 2%), but our encountering it as a transformation product of methyl trisporate C, as described below, led us to attempt a structural assignment. The trienone chromophore is perturbed as in I and II (λ_{max} 319 nm shifting by −24 nm on reduction with borohydride), but the mass spectrum, with $m^+/e =$ 304, requires the O-free side-chain shown in structure III. In agreement with this are the observed fragmentations of the molecular ion and the fact that the mass spectrum is unchanged after attempted acetylation. Hence the ester of III is the 13-deoxy derivative; we

[2] J. D. Bu'Lock and D. J. Winstanley, *J. Gen. Microbiol.* **70** (1972). (in press).
[3] T. Reschke, *Tetrahedron Letters* **39**, 3435, (1969).
[4] D. J. Austin, J. D. Bu'Lock and D. Drake, *Experientia* **26**, 348 (1970).
[5] J. D. Bu'Lock, D. J. Austin, G. Snatzke and L. Hruban, *J. Chem. Soc.* **D**, *Chem. Commun.* 225 (1970).
[6] O. K. Sebek and H. K. Jaeger, *Am. Chem. Soc. 148th Meeting*, Chicago (1964).
[7] J. A. Edwards, V. Schwarz, J. Fajkos, M L. Maddox and J. H. Fried, *J. Chem. Soc.* **D**, *Chem. Commun.* 292 (1971).

have not encountered Reschke's methyl anhydrotrisporate C, $m^+/e = 302$,[3] with which the trisporate A of the earlier literature (see Ref. 1) might alternatively be identified.

Trisporates B and C

A combination of column chromatography and TLC separates naturally-derived methyl trisporate B and methyl trisporate C each into a 'faster' (less polar) and a 'slower' (more polar) component[3,6] (R_f values, Table 1); CrO_3-pyridine oxidation of each component from methyl trisporate C gives the corresponding component of the methyl trisporate B

TABLE 1. SEPARATION AND BIOASSAYS OF ISOMERIC METHYL TRISPORATES

Product	R_f values* BEP	AM	C(8)proton δ	Bioassay *minus M. mucedo* zygophores/μg
Methyl trisporate C				
9 *cis*	0·33	0·31	6·40	380
9 *trans*	0·34	0·32	6·85	300
Methyl trisporate B				
9 *cis*	0·48	0·44	6·40	650
9 *trans*	0·50	0·46	6·85	380

* See Experimental.

mixture. For all four products the NMR spectra of the olefinic protons are essentially as previously discussed by Edwards *et al.*,[7] and in particular it is the less polar component in each pair which shows the C-8 proton (doublet) centred at $\delta = 6{\cdot}4$ and is the 9-*trans*-isomer, the more polar component being 9-*cis* with the C-8 proton signal centred at $\delta = 6{\cdot}85$ (Table 1). Bioassay results for the four esters against *M. mucedo* are also given in Table 1.

In freshly worked-up material from *B. trispora* the ratio of total trisporate C to total B is about 5, and of 9-*cis* to 9-*trans* about 3 in each case, but during handling and storage, since the free acids (and to a lesser extent the methyl esters) are unstable to light, peroxides, and extreme pH, and since the 9-*cis*-isomers are the less stable, the relative proportion of the 9-*trans*-isomers rises.

A frequently-encountered artefact derived from methyl trisporate C is a more polar ester giving a conspicuous pink colour with ceric sulphate. The mass spectrum shows m^+/e 336 [after Ac_2O-pyridine treatment, 378 (monoacetate)] and in the NMR spectrum the absorbance seen in methyl trisporate C at 5·5δ (olefinic H on C-10) is absent, while the signal at 1·83δ (CH_3 on C-9) is shifted to 1·28δ. The shift of the C-13 (methyl) resonance on acetylation is the same as for methyl trisporate C. We conclude that the additional oxygen indicated by the molecular weight and increased polarity is attached at C-9/C-10 and formulate the product as a 9,10-epoxide of methyl trisporate C. It is not found in freshly worked-up material.

Trisporol C

We reported this ketodiol (IV) as a minor metabolite found in the neutral fraction from mated *B. trispora* cultures[4] but it is more readily obtained by chemical reduction of methyl

trisporate C, and some fuller characterizations are given in the Experimental. Identical material has also been obtained from *minus B. trispora* fed with methyl ^{14}C-trisporate C (see below).

Zygophore Induction

Bioassays of hormone activity against *minus M. mucedo* (see Experimental) for the 9-*cis*- and 9-*trans* isomers of methyl trisporates B and C are compared in Table 1; methyl 9-*cis*-trisporate B is the most active but all four are comparable (within ×2). However, the esters are much less active on *plus M. mucedo*. Since the natural hormone mixture is most conveniently manipulated after methylation, this has lent continued credibility to an older view that the system is mating-type-specific, despite accumulating contrary evidence which has recently been summarized.[1] To investigate this point, we converted separate samples of the four B and C esters and a sample of methyl trisporate A back to the corresponding acids, bioassay results for which are given in Table 2. Clearly both mating types of

Table 2. Comparative bioassays on *plus* and *minus M. mucedo*

Compound	Zygophores/μg *plus*	*minus*
9-*cis*-Trisporic acid C	150	550
9-*trans*-Trisporic acid C	150	420
9-*cis*-Trisporic acid B	260	910
9-*trans*-Trisporic acid B	160	450
Me trisporate A	50	100
Trisporic acid A	50	120
Trisporol-C	20	⩽1†
β-C_{18}-ketone*	7	⩽1†

Inactive compounds include: retinyl acetate, retinol, retinal, retinoic acid, β-ionone, β-cyclocitrylideneacetic acid, and abscisic acid.

* 3-Methyl-1-(2,6,6-trimethylcyclohexenyl)-octa-1,3,5-trien-7-one.

† No zygophores with 10 mg/assay.

M. mucedo respond similarly to all of the trisporic acids which make up the natural hormone system, i.e. the *response* to the hormones is identical in both sexes.

That this does not preclude strain differences in the response to substances which may be convertible into trisporic acids is shown by other bioassay data. The much lower activity of the methyl esters upon *plus M. mucedo* is a case in point; of more interest in relation to the natural system is the selective activity of trisporol C upon the *plus* strain (Table 2). Similarly selective activity, at a level which is much lower but still genuine, is shown by the 'β-C_{18}-ketone' (Table 2) which conceivably is an intermediate in the biosynthesis[4] of trisporic acids from β-carotene. A variety of other substances (Table 2) proved to be quite inactive.

Transformations

As a parallel to the different responses shown by *plus* and *minus M. mucedo* to derivatives and precursors of trisporic acids, we studied the metabolism of methyl ^{14}C-trisporate C

(prepared by biosynthesis from 2-^{14}C-acetate) by *plus* and *minus* cultures of *B. trispora*. Figure 1 summarizes the results and Table 3 lists individual products. These included methyl trisporates A and B, trisporic acids, trisporol C, and some new compounds, which were assigned the structures shown on the basis of the tabulated data, i.e. their polarity on TLC, the extent of perturbation of the trienone chromophore and the shift on reduction with borohydride (to trienols), and the molecular weight, framentation pattern, and number of acetylable —OH groups as shown by mass spectroscopy. Some of the products were only

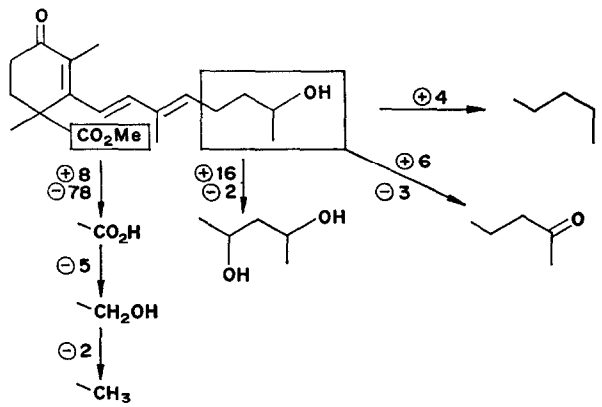


FIG. 1. TRANSFORMATIONS OF Me ^{14}C-TRISPORATE C.

The figures are the % (of activity fed) recovered in transformation products of each structural type after 48 hr incubation with *plus* (+) or *minus* (−) *B. trispora*; results at 24 and at 72 hr were qualitatively similar.

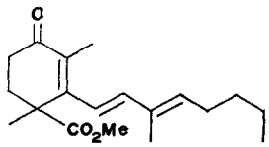
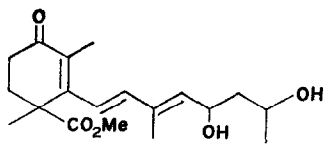
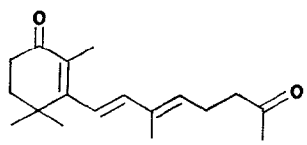
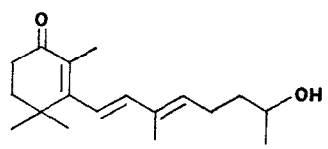
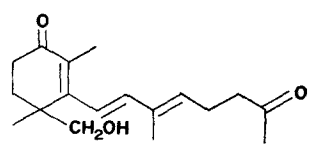
obtained in very small amounts (<0·1 mg, see Fig. 1) but within the limits this imposed all were found to have the same specific activity as the methyl trisporate C fed; there was no significant dilution by endogenous materials.

With *plus B. trispora* there was very little hydrolysis of the methyl ester; 65% of unchanged methyl trisporate C was recovered and the main transformations of the remainder—deoxygenation to trisporate A, oxidation to trisporate B, and allylic hydroxylation—occurred in the side-chain. In contrast, *minus B. trispora* effected these side-chain transformations to a much lesser extent, the main reaction being ester hydrolysis; less than 18% of unchanged ester was recovered. A somewhat surprising reaction, apparently occurring subsequent to hydrolysis, was reduction of the acid to —CH_2OH (e.g. in trisporol C) and —CH_3 (see Table 3).

Similar but less searching experiments were carried out with ^{14}C-labelled methyl trisporate B and trisporol C; only major products were recovered. As shown in Table 4 the pattern of results is similar; the *minus* mycelium was more effective in hydrolysis and reduction (B → C) steps and the *plus* strain more effective in oxidations.

The extent to which these differences in transforming ability between our *plus* and *minus* strains of *B. trispora* are in broad agreement with the different response to hormone derivatives and precursors shown by our *plus* and *minus* strains of *M. mucedo* suggests that some of these phenomena may be genuinely linked to the sex-determinant, even though in both cases we are dealing with pairs of distinct cultures which are admittedly not isogenic in other respects. Such differences are also implied by the results of various experiments on

Table 3. Transformation products from methyl trisporate C incubated with single strains of *B. trispora**

Compounds isolated	R_f(BEP)	λ_{max}	λ_{max} (BH_4)	m^+/e	m^+/e after Ac_2O-pyridine
Product from incubation with *plus* mycelium					
	0·63	319	285	304	Unchanged
Me trisporate B	0·50	318	285	318	Unchanged
	0·13	315	282	336	420
Product from incubation with *minus* mycelium					
	0·51	290	260	274	Unchanged
	0·35	285	260	276	314
	0·23	289	250	290	332
Trisporol C	0·10	298	250	292	376

* For unchanged methyl trisporate C and for trisporic acids B and C (isolated as methyl esters) see Fig. 1.

† All gave a yellow colour with Ce^{IV}; that from trisporol C turns purple with phosphomolybdate.

the limited formation of trisporic acids which occurs following transfers of media from one type of strain to cultures of the opposite type.[1] However, they are not in themselves sufficient to explain the process of intensive hormone synthesis which occurs when cultures of complementary types are maintained in continuous diffusion-contact.

EXPERIMENTAL

General. The *B. trispora* strains used are NRRL-2895 (*plus*) and NRRL-2896 (*minus*), and the fermentation, work-up, and chemical assay procedures are fully described elsewhere.[2] Reagents are analytical grade; Et_2O, peroxide-free. For scintillation counting, compounds soluble in EtOH, PhH, or PhMe were counted

TABLE 4. METABOLISM OF Me TRISPORATE B AND TRISPOROL C BY *plus* AND *minus B. trispora*

Fed	Reaction	Per cent recovered* from incubation with *plus* mycelium	Per cent recovered* from incubation with *minus* mycelium
Me trisporate B	$-CO_2Me \rightarrow -CO_2H$	3	90
	$>C=O \rightarrow >CHOH$	0	70
trisporol C	$-CH_2OH \rightarrow -CO_2H$	18	10
	$>CHOH \rightarrow >C=O$	2	1

* After 48 hr; results after 24 hr similar.

in PPO–POPOP–xylene; solutions in $CHCl_3$, Me_2CO, or H_2O in naphthalene–PPO–POPOP–MeOH–glycol; zones scraped from TLC plates in the latter scintillant with 25% of NE-221 gel. At least 10^5 counts were recorded and efficiencies checked both automatically and by spiking with ^{14}C-hexadecane.

TLC systems. Standard systems employed Merck F-254 silica gel plates, eluted with either BEP (7:5:1, PhH–EtOAc–C_5H_{12}) or AM (1:9, Me_2CO–CH_2Cl_2) (R_f values in Tables 1, 3, etc.), visualizing with a spray of Ce ammonium nitrate (2% in 10% H_2SO_4) followed by 5% phosphomolybdic acid in EtOH.

Isomeric trisporic acids and esters. The mixture of methyl esters [prepared[2] from the acid fraction from the medium of mated (*plus* + *minus*) *B. trispora* cultures] was chromatographed with a slow gradient of 0–10% Me_2CO in CH_2Cl_2 on neutral silica gel, monitoring by TLC. Methyl trisporate A is eluted with 1% Me_2CO and was purified by TLC without separating stereoisomers. Methyl trisporate B is eluted with *ca.* 5% Me_2CO in two overlapping bands, further purified by TLC to give as the less polar component methyl 1-*S*-7-*trans*-9-*trans*-trisporate B, λ_{max} (Et_2O) 317 nm (log ϵ 3·33) and as the more polar component the 7-*trans*-9-*cis*-isomer, λ_{max} (Et_2O) 319 nm (log ϵ 3·35). Methyl trisporate C is similarly eluted at *ca.* 10% Me_2CO and resolved to give the less polar methyl 1-*S*-13-*R*-7-*trans*-9-*trans*-trisporate C, λ_{max} (Et_2O) 317 nm (log ϵ 3·34), and the more polar 7-*trans*-9-*cis*-isomer, λ_{max} (Et_2O) 320 nm (log ϵ 3·36). The R_f, relevant NMR, and bioassay (*minus M. mucedo*) data are given in Table 1.

For bioassays of the free acids (Table 2) the ester samples, in EtOH, were hydrolysed with a slight excess of aq. NaOH (overnight, 20°) and recovered with Et_2O after acidification; concentrations were checked spectrophotometrically. R_f data for the free acids in 95:5 $CHCl_3$–AcOH are: trisporic A, 0·51; 9-*trans*-trisporic B, 0·44; 9-*cis*-trisporic B, 0·42; 9-*trans*-trisporic C, 0·22; 9-*cis*-trisporic C, 0·20.

About 40 mg of methyl 9-*cis*-trisporate C and about 8 mg of the 9-*trans*-isomer were separately oxidized with a slight excess of the CrO_3-pyridine complex (15–30 min in CH_2Cl_2 at 20°, monitored by TLC); the products, recovered chromatographically in *ca.* 55% yield, were chromatographically and spectroscopically identical with 9-*cis* and 9-*trans*-isomers, respectively, of methyl trisporate B.

Bioassays. The *M. mucedo* strains were provided by Dr. G. Gooday: Z-46 (*plus*) and Z-43 (*minus*). The basic procedure of Plempel[8] as carried out by Gooday[9] was followed; test material at a range of concentrations was put into wells ahead of the advancing front of a plate culture and after 24 hr the total number of zygophores in a 2-mm transect from the well towards the colony centre were counted. The counts were then plotted against the amount of substance tested. Such plots normally show an extensive linear section, from the slope of which an 'activity', in zygophores/μg, is calculated. This method obviates a correction for the pseudozygophores sometimes seen in blank tests and reduces operator error. Activities $< 10^{-3}$, i.e. no zygophores seen with 1 mg of test material, are taken as zero.

^{14}C-labelled methyl trisporates. To each of 50 flasks each containing 150 ml of (*plus* + *minus*) *B. trispora* cultures, at approximately 30 hr from inoculation when trisporate production is accelerating, was added 4 ml of filter-sterilized NaOAc-2-^{14}C (2·73 mg, 1 mC, in 250 ml H_2O). After a further 30 hr incubation and the usual work-up[2] the crude ester fraction (440 mg spectroscopic assay, 2×10^8 dpm, 13% ^{14}C incorporation) was chromatographed to give methyl trisporate B, 29 mg, $2·7 \times 10^8$ dpm/mmol, and methyl trisporate C, 260 mg, $2·0 \times 10^8$ dpm/mmol. These were purified by TLC before use; part of the methyl trisporate C was converted into ^{14}C-trisporol-C (see below).

Trisporol-C. Ether extracts of the neutral fraction from mated *B. trispora* cultures subjected to TLC in BEP afford three main components, trisporone[10] (R_f 0·07), anhydrotrisporone[10] (R_f 0·47), and trisporol-C, m^+/e 292 ($C_{18}H_{28}O_3$)[after treatment with Ac_2O-pyridine, m^+/e 376 (diacetate)], λ_{max} (EtOH) 235 and

[8] M. PLEMPEL, *Planta* **59**, 492 (1963).

[9] G. W. GOODAY, *New Phytol.* **67**, 815 (1968).

[10] G. CAINELLI, B. CAMERINO, P. GRASSELI, R. MONDELLI, S. MORROCCHI, A. PRIETO, A. QUILICO and A. SELVA, *Chim. e Ind.* (*Milano*) **49**, 748 (1967).

302 nm (log ϵ 4·04), ν_{max} 3400, 1650 cm^{-1}, R_f (BEP) 0·13, yellow with Ce^{IV} changing to magenta with phosphomolybdate. Chromatographically and spectroscopically identical material was prepared from methyl trisporate C by, (a) treatment with ethane-1,2-diol in refluxing dry benzene with a trace of TsOH to prepare the ketal, purified by chromatography, (b) reduction of the ketal with $LiBH_4$ in refluxing dry tetrahydrofuran, (c) decomposition of the product in acetone with dilute HCl, (d) final chromatographic purification. Prepared from the methyl ^{14}C-trisporate (above) the product had $2·0 \times 10^8$ dpm/mmol.

Transformations by single strains. To each of 3 flasks of *plus B. trispora* and 3 of *minus*, grown for 24 hr on 5% malt extract medium, was added methyl trisporate C (2 mg, $1·33 \times 10^6$ dpm) (in H_2O with 0·5% Tween-80). At 24, 48, and 72 hr one flask of each strain was removed and the Et_2O extract from the medium divided into neutral and acidic fractions which were resolved by TLC, the latter after methylation (CH_2N_2). The labelled products were rechromatographed as necessary, and counted by direct counting of silica gel scraped from the plates; other aliquots were eluted for UV spectroscopy (and hence quantitative estimation) and mass spectrometry; within experimental error, all had the same specific activity as the trisporate C fed. Outline R_f and spectroscopic data, Table 3. Recoveries from the 48 hr experiment are expressed as % transformations in Fig. 1 assuming that in the *minus* strain reduction of the oxycarbonyl group occurs after ester hydrolysis; the results at 24 and 72 hr were qualitatively similar. Transformations of methyl trisporate B and trisporol C were studied similarly but only the major reaction products were observed—methyl trisporates B and C, the corresponding acids (after methylation) and unchanged trisporol-C.

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Key Word Index—Blakeslea trispora; Mucorales; Fungi; sex hormones; trisporic acids; interconversions.