Prenols and Ubiquinones in Single-strain and Mated Cultures of *Blakeslea trispora*

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SUMMARY

The prenols of *Blakeslea trispora* were found to be a mixture of C₆₀-C₉₀ prenols and α -dihydroprenols with C₇₀ as the main type; the principal ubiquinones were ubiquinone-9 and its (side-chain) dihydro-derivative. A *minus* strain contained higher levels of prenols, ubiquinones, ergosterol, and β -carotene than a *plus* strain, and the levels of all four types of isoprenoid were higher again in mated cultures. The overall increase in isoprenoid synthesis in mated cultures was sometimes tenfold but the different isoprenoid types were not all equally affected by the mating process; possible roles for the different types in sexual differentiation are considered.

INTRODUCTION

The accumulation of carotenoids in the differentiated sexual structures of many mucoraceous fungi (Burnett, 1965) and in submerged agitated cultures of opposite mating types of heterothallic species, such as *Blakeslea trispora*, is due to the action of the trisporic acids which oppositely sexed strains collaboratively produce. In particular, Thomas, Harris, Kirk & Goodwin (1967) showed that the enhanced synthesis of both β -carotene and ergosterol caused by additions of trisporic acids to single strains of *B. trispora* is blocked if protein synthesis has been prevented by prior addition of cycloheximide and, because this inducer-like action of the trisporic acids affects both carotenoid and steroid synthesis, they concluded that the induction primarily affected an enzyme or enzymes early in the isoprenoid biosynthetic pathway.

Since the trisporic acids are responsible for eliciting all the early stages of sexual differentiation in the Mucorales (van den Ende, 1968; Austin, Bu'Lock & Gooday, 1969) a wider understanding of their biochemical action is desirable, and the other branches of the isoprenoid biosynthetic pathway offer an obvious starting point. In this paper we describe the characterization of the functionally important ubiquinones (UQ-*n*, where *n* indicates the number of isoprene residues in the side-chain, e.g. UQ-9) and prenols (polyprenol alcohol, PP-*n*, where *n* indicates the number of isoprene residues, lettered α , ..., ψ , ω as in α -H₂PP-15, otherwise, 2,3-dihydropentadecaprenol). Their separation permits measurements of the four main types of isoprenoid in *Blakeslea trispora* (β -carotene, ergosterol, UQ and PP) and comparisons of their levels in single-strain, *plus* or *minus*, and mated (*plus* with *minus*) cultures.

METHODS

General. The strains of *Blakeslea trispora* used were NRRL2895 (*plus*) and 2896 (*minus*), and the culture procedures are fully described elsewhere (Bu'Lock & Winstanley, 1971). For the present work, shake cultures were grown on 10% commercial malt extract for

7 days after inoculation. Throughout, ether means diethyl ether, petrol means light petroleum b.p. 40 to 60 °C, solvent ratios are v/v and % is w/v.

Characterization of isoprenoids. Because the most exacting task was characterization of the PP fraction, which can occur both free and esterified, the well-established saponification work-up (Burgos, Hemming, Pennock & Morton, 1963; Stone, Butterworth & Hemming, 1967) was adopted for this aspect of the work even though this gives low recoveries of β -carotene, ergosterol and UQ. The non-saponifiable product was taken up in petrol, and the sterols mostly precipitated by refrigeration, before subjecting the mixture to chromatography on acid-washed alumina (grade III), first in 3:97 ether:petrol to elute carotenoids, etc., and then in 8:92 ether:petrol to elute a fraction containing PP and UQ. Direct further purification of the PP at this stage was impeded by the presence of ubiquinonols. This fraction was therefore acetylated with pyridine-acetic anhydride (overnight) and rechromatographed; 1:99 ether:petrol elution gave a clean PP-acetate fraction and 10:90 ether:petrol gave a UQ fraction. Each fraction was finally purified by thin-layer chromatography (t.l.c.) (Merck silica gel F 254 eluting with dichloromethane). The PP-acetate fraction was alternatively reconverted to free PP by saponification before final t.l.c.

Visualization on thin-layer chromatograms was with iodine, phosphomolybdic acid (10% in ethanol), anisaldehyde (5% with 5% H_2SO_4 in ethanol: β -carotene, blue-green; ergosterol, red-brown; UQ, deep green; PP, deep green) or reduced methylene blue (reoxidized by UQ). For comparisons with authentic materials the most useful thin-layer system was the two-dimensional method of Dunphy *et al.* (1967), which resolved the various categories very adequately but did not tend to separate different homologues.

Mass spectra were determined on the AEI MS-12 instrument operating at 250 °C and 6 or 8 kV; n.m.r. spectra were obtained on the Varian HA-100 in deuterochloroform with tetramethylsilane as internal reference. Authentic UQ-9 and a dolichol (α - H_2 PP) were kindly provided by Dr F. W. Hemming.

Estimation of isoprenoids. Assays of β -carotene, ergosterol, and UQ were carried out on mycelium from single shake flasks, which was filtered off, pressed dry on paper, ground to a powder under liquid N_2 , and stirred for 30 min with 150 ml of acetone. The acetone slurry was filtered, the residue being used for dry-weight measurement, and the filtrate evaporated under reduced pressure to give an extract which was taken up in ether (100 ml) for spectrophotometric assay of β -carotene ($E_{1\text{cm}}^{1\%}$ 2500 at 452 nm) and ergosterol ($E_{1\text{cm}}^{1\%}$ 340 at 280 nm). The ether extract was then evaporated and the residue taken up in petrol for chromatography as above, eluting first with 3:97 and then with 8:92 ether:petrol. The latter fraction was evaporated and taken up in ethanol; the UQ content was determined from the change in extinction at 275 nm after addition of a little solid sodium borohydride to the cuvette (for UQ-9, $\Delta E_{1\text{cm}}^{1\%}$ (ox.-red.) 156 at 275 nm). Assays of up to five replicates at a time gave the variance indicated in Table 2.

Assays of PP were carried out by means of the saponification procedure with mycelium from at least five flasks. The 8:92 ether:petrol fraction from the non-saponifiable material was acetylated and subjected to t.l.c. on washed Merck F-254 silica gel analytical plates, applied as a 16 cm band and eluting with dichloromethane; the PP-acetate zone (R_F 0.50) was scraped off and eluted and the PP-acetate was estimated by direct weighing. Usually about 0.5 to 3.5 mg of PP-acetate was weighed, and the corresponding mycelial dry wt was obtained directly from replicate flasks; the PP-acetate obtained by this procedure on a somewhat larger scale was over 90% pure by n.m.r. observations and loss in recycling tests was under 20%. The main source of variability in the assays was apparently in the intensity of the mating response, and could most easily be judged by the β -carotene assays which

were very dependent upon the history and condition of the two inocula. The results in Table 2 are typical of those obtained when the response was high, but even with less good responses the qualitative picture was consistent throughout.

RESULTS

Identity of the ubiquinones. The UQ fraction behaved identically with authentic material in chromatography, colour reactions, and u.v. spectrometry. In the mass spectrum the strongest molecular ion group was at $m + e$ 794 and 796 (with attendant isotope peaks) corresponding to ubiquinones with 9 isoprene units, with a series of low-intensity fragmentation clusters ($M-69-n \times 68$) and a prominent fragment at $m + e$ 235 (Muraca *et al.* 1967). In the molecular ion group, $m + e$ 794 corresponds to UQ-9. The peak at $m + e$ 796 could be due to ubiquinol formed in the instrument or to a ubiquinone with a single reduced isoprene residue in the side-chain, UQ-9(H_2). A sample dissolved in ether was shaken vigorously with aqueous sodium dithionite under nitrogen, to convert quinones into quinols, and the ether layer evaporated under nitrogen and submitted to mass spectrometry. The molecular ion group now appeared at $m + e$ 796 and 798, showing that the original material was a mixture of UQ-9 and UQ-9(H_2).

During the work-up a ubichromenol fraction was encountered but there was no evidence that this was not an artefact.

Ubiquinones with side-chains shorter than that of the UQ-9, UQ-9(H_2) mixture will have molecular ions coinciding with the $+1$ isotope peaks of the fragmentation series of the larger molecules; detailed examination of the peak clusters with this principle in mind suggested that shorter UQ in our material were very minor components. More unambiguously, UQ-10, UQ-10(H_2) (M at $m + e$ 862, 864) was present in less than 1/50 of the amount of UQ-9, UQ-9(H_2).

Identity of the prenols. The PP fraction was authenticated by chromatography, colour reactions, and i.r., n.m.r., and mass spectra. It was a mixture in approximately 3:5 ratio, of true polyisoprenyl alcohols $H.[C_5H_8]_n.OH$ and corresponding dihydrocompounds, each type comprising a range of chain lengths. In the mass spectrum the molecular ions, the fragmentation peaks, and the associated isotope peaks, formed quite complex clusters. For example, for the (predominant) species with $n = 14$ the molecular ion cluster comprised peaks at $m + e$ 970, 971, 972, 973 in approximate ratio 0.6:0.4:1.0:0.7 (higher and lower neighbours < 0.2). The H_2PP behaved as expected if the proximal (HO-terminal, α) isoprene residue was saturated, and fragmented mainly by losses of C_5 units from the distal (ω) end giving a series ($M-69-n \times 68$); hence the $+1$ isotope peaks of this series coincided with the molecular ions of shorter H_2PP and the fragmentation peaks themselves coincided with the $+1$ isotope peaks of shorter chain PP. In contrast the true PP fragmented mainly by initial loss of H_2O giving a series ($M-18-n \times 68$); this difference was even more marked in the mass spectra of the acetates. With these observations as a guide (Stone *et al.* 1967; Gough & Hemming, 1970) and noting that the relative intensities of the peaks did not change significantly in spectra run serially (probe temperature 250 °C), the approximate chain-length composition of the PP- H_2PP mixture was worked out as in Table 1; the main component was H_2PP-14 .

The i.r. spectrum of the mixture agreed with expectation for a predominance of αH_2PP with mainly *cis*-double bonds (ν_{max} 886, 1060, 1090, 1130, 1240 cm^{-1}). More critically, both the prenol mixture and the derived acetates gave well-resolved n.m.r. spectra (at 100 Hz) which agreed with expectations (Feeney & Hemming, 1967) and in particular showed that

Table 1. Chain-length composition of the 3:5 PP- α H₂PP mixture from *Blakeslea trispora*

No. of isoprene units...	12	13	14	15	16	17	18
Approximate %	Trace	15	70	10	3	1	Trace

Table 2. Isoprenoid levels (mg/g) in *Blakeslea trispora*

Strain	Ergosterol*	β -Carotene*	UQ*	PP†
<i>plus</i>	3.5 $\pm$ 0.3	0.17 $\pm$ 0.03	0.09 $\pm$ 0.03	0.06
<i>minus</i>	9.2 $\pm$ 0.9	0.8 $\pm$ 0.1	0.26 $\pm$ 0.03	0.10
<i>plus</i> with <i>minus</i>	51.0 $\pm$ 8	11.0 $\pm$ 1.7‡	0.52 $\pm$ 0.11	0.35
Average increase	$\times$ 8	$\times$ 22	$\times$ 3	$\times$ 4

* Mean and s.d. of five samples each of 1 to 1.5 g dry weight.

† From saponification of 10 to 12 g dry weight.

‡ Also approx. 20 mg/g trisporic acids $\equiv$ 35 mg β -carotene.

(i) in the mixture it was the α -terminal isoprene residue that was predominantly saturated (for the acetate, CH₃-(sat) doublet at τ 9.07, 9.15; (sat)-CH₂(sat) multiplet at τ 8.75; AcO-CH₂ (sat) triplet at τ 6.30; AcO-CH₂ (allylic) doublet at τ 5.90; relative integrals in approximate agreement with the 3:5 mixture deduced from the mass spectra); (ii) the methyl signals at τ 8.34 and 8.41, due to methyl on *cis*- and *trans*-double bonds respectively (one of each kind on the ω -residue) had integrated intensities in the ratio 9.5:4.5; allowing for incomplete resolution of the smaller peak from the larger, for the presence of some α -unsaturated residues, and for the distribution of chain lengths (cf. Table 1) this corresponded to three internal *trans*-residues (theoretical for this case if fully resolved, 10:4; for two internal *trans*, 11:3).

Isoprenoid synthesis in single strains and mated cultures. Analytical data for *plus* and *minus* strains and for mated cultures of *Blakeslea trispora* are given in Table 2. All types of isoprenoid occurred at higher levels in our *minus* strain than in the *plus*, and all were further increased in the mated cultures. Proportionately the greatest increase was in the β -carotene but in terms of quantity the effect on ergosterol was largest. Because the growth rates of the *plus* and *minus* strains were not very different when they were grown separately, it is reasonable to assume that the two were present in very approximately equal proportions in the mated cultures, and hence to compare results for the latter with the averages for the two separate strains. With a good mating response such as Table 2 exemplifies, the overall increase in isoprenoid biosynthesis was about tenfold, but this was not equally reflected in the four separate categories.

DISCUSSION

The side-chains of ubiquinones are formed by the repeated *trans*-coupling of isoprene residues; in some ubiquinones one of the side-chain units is reduced. Table 3 summarizes data from Law, Threlfall & Whistance (1971) on the predominant UQ types in various fungi. The UQ-6 of *Saccharomyces cerevisiae* seems to be exceptional, but the predominance of UQ-9 or UQ-10, with or without side-chain reduction, does not seem to have any clear-cut taxonomic basis, at least on the present restricted range of data. The closest relative to *Blakeslea trispora* studied by these workers was *Phycomyces blakesleeanus*, in which UQ-9 predominates; the difference in *B. trispora* is the presence of an approximately equal amount of UQ-9(H₂).

Comparable data for fungal prenols are even more limited (Table 4). These differ from

Table 3. *Fungal ubiquinones (from Law et al. 1971)*

Dominant UQ	Genera
UQ-10	<i>Aspergillus</i>
UQ-10(H ₂)	<i>Alternaria, Aspergillus, Claviceps, Neurospora</i>
UQ-9	<i>Agaricus, Aspergillus, Penicillium, Phycomyces, Rhodotorula</i>
UQ-9(H ₂)	<i>Penicillium</i>
UQ-6	<i>Saccharomyces</i>

Table 4. *Fungal prenols*

Source	Total C ₅ units		Biosynthetically trans-C ₅ -units	Hydrogenated residues	Other modifications	References
	Predominant	Range				
<i>Saccharomyces cerevisiae</i>	?	13-18	3 ?	α	—	Dunphy <i>et al.</i> 1967
<i>Aspergillus fumigatus</i>	20-22	18-24	4	α/ω	—	Stone & Hemming, 1967
<i>A. niger</i>	20, 21	18-24	4	α/ω	ω is methylene-dihydro	Barr & Hemming, 1972a
<i>Blakeslea trispora</i>	14	12-18	4	None and α	—	Present work

the UQ side-chains in arising by *cis*-coupling steps from precursors with either three or four *trans*-coupled residues. The prenols of *Blakeslea trispora* have three internal *trans* residues, i.e. biogenetically four *trans* units. The true PP component of *B. trispora* represents a type hitherto found only in angiosperms (castaprenols) while the α -H₂PP type is more normal; however, the α -H₂PP of yeast is said to have only two internal *trans* residues, like the mammalian dolichols. The *Aspergillus* prenols have the same biogenetic stereochemistry as those of *B. trispora* but they are also reduced, and in one case alkylated, at the distal residues. Bacterial prenols are different again, with shorter chain lengths and only two internal *trans* residues; occasionally the α -residue is saturated but this is atypical (Scher, Lennarz & Sweeley, 1968; Durr & Habbal, 1972).

The branching of pathways of isoprenoid metabolism leading to the compounds studied here is schematized in Fig. 1. All the reactions are intensified in the mated cultures, in keeping with the views of Thomas *et al.* (1967) on the location of the inducer action of trisporic acids, but to different degrees (Table 2), which must reflect the capacity of later enzymes in the different branches of the sequence to handle an overall increase in substrate levels. The possible roles of each sequence branch in the mating process, and in particular in those parts of the process which can be induced directly in single strains by added trisporic acids, merit some consideration.

Exogenous steroids are essential for reproductive development in Oomycetes and endogenous sterols may be similarly implicated in a wider range of fungi (Hendrix, 1965; Nelson, Huisinigh & Webster, 1967; Elliott, 1969); in *Achlya* specific sterols have a hormone function (Barksdale, 1969). More generally, a steroid function in the lipids of membrane structures controlling permeability may be anticipated and could well have a role in the post-contact phases of the sexual process in Mucorales.

The carotenoid pathway is critical for sexual development in these fungi because β -carotene is itself the precursor from which the trisporic acids are formed when *plus* and *minus* strains collaborate (Austin, Bu'Lock & Drake, 1970). However, an actual accumulation of β -carotene in mated cultures is not required for such a role, and though such accumulations are conspicuous in many Mucorales, and particularly in *Blakeslea trispora*

Fig. 1. Schematized isoprenoid synthesis in *Blakeslea trispora*. t, *trans*-mechanism; c, *cis*-mechanism.

(Ciegler, 1965), other species do not accumulate carotenoids visibly (Burnett, 1965), though the converse, i.e. the strict absence of carotenoids in such species, has not been demonstrated and seems *a priori* unlikely. Carotenoids may have a direct function in the later stages of the mating process since they have been suggested as precursors of the zygospore coat-substance (G. W. Gooday, personal communication).

The general functions of UQ in respiration and of PP as carriers for the synthesis of extramembrane polymers (e.g. in *Aspergillus*; Barr & Hemming, 1972*b*) are well established, and it is noteworthy that the synthesis of both materials in *Blakeslea trispora* seems to be more tightly regulated, and less drastically affected by the mating effect, than the syntheses of β -carotene and ergosterol. However, zygophore development in Mucorales involves, as may readily be observed in plate cultures, the capacity for rather rapid outgrowths of a specialized hyphal type, with specific surface properties, which branch out from mycelia at points rather remote from the hyphal tips where wall synthesis is normally concentrated. In such circumstances an additional supply of UQ and PP could well be directly functional in zygophore outgrowth.

To these direct consequences of the trisporic-acid-mediated induction in the isoprenoid pathway we must add the indirect effects ensuing after a five- to tenfold increase in the consumption of acetyl-CoA, ATP and NADPH in this pathway, which will obviously be extensive. However, it remains for further investigation to establish whether the accumulated biochemical effects of a single-point inducer action are wholly sufficient to account for the range of phenomena elicited by trisporic acids in the mating process.

REFERENCES

- AUSTIN, D. J., BU'LOCK, J. D. & GOODAY, G. W. (1969). Trisporic acids: sexual hormones from *Mucor mucedo* and *Blakeslea trispora*. *Nature, London* **223**, 1178–1179.
- AUSTIN, D. J., BU'LOCK, J. D. & DRAKE, D. (1970). The biosynthesis of trisporic acids from β -carotene via retinal and trisporol. *Experientia* **26**, 348–349.
- BARKSDALE, A. W. (1969). Sexual hormones of *Achlya* and other fungi. *Science, New York* **166**, 831–837.
- BARR, R. M. & HEMMING, F. W. (1972*a*). Polyprenols of *Aspergillus niger*. *Biochemical Journal* **126**, 1193–1202.
- BARR, R. M. & HEMMING, F. W. (1972*b*). Polyprenol phosphate as an acceptor of mannose from guanosine diphosphate mannose in *Aspergillus niger*. *Biochemical Journal* **126**, 1203–1208.
- BU'LOCK, J. D. & WINSTANLEY, D. J. (1971). Trisporic acid production by *Blakeslea trispora* and its promotion by barbiturate. *Journal of General Microbiology* **69**, 391–394.
- BURGOS, J., HEMMING, F. W., PENNOCK, J. F. & MORTON, R. A. (1963). Dolichol: a naturally-occurring C₁₀₀ isoprenoid alcohol. *Biochemical Journal* **88**, 470–482.
- BURNETT, J. H. (1965). Functions of carotenoids other than in photosynthesis. In *Chemistry and Biochemistry of Plant Pigments*, pp. 381–403. Edited by T. W. Goodwin. London: Academic Press.
- CIEGLER, A. (1965). Microbial carotenogenesis. *Advances in Applied Microbiology* **7**, 1–34.
- DUNPHY, P. H., KERR, J. D., PENNOCK, J. F., WHITTLE, K. J. & FEENEY, J. (1967). The plurality of long-chain isoprenoid alcohols from natural sources. *Biochimica et biophysica acta* **136**, 136–147.
- DURR, I. F. & HABBAL, M. Z. (1972). The biosynthesis of C₅₅ polyprenols by a cell-free preparation of *Lactobacillus plantarum*. *Biochemical Journal* **127**, 345–359.
- ELLIOTT, C. G. (1969). Effects of inhibitors of sterol synthesis on growth of *Sordaria* and *Phytophthora*. *Journal of General Microbiology* **56**, 331–343.
- VAN DEN ENDE, H. (1968). Relationship between sexuality and carotene synthesis in *Blakeslea trispora*. *Journal of Bacteriology* **96**, 1298–1303.
- FEENEY, J. & HEMMING, F. W. (1967). Nuclear magnetic resonance spectrometry of naturally occurring polyprenols. *Analytical Biochemistry* **20**, 1–15.
- GOUGH, D. P. & HEMMING, F. W. (1970). The characterization and stereochemistry of biosynthesis of dolichols in rat liver. *Biochemical Journal* **118**, 163–166.
- HENDRIX, J. W. (1965). Influence of sterols on growth and reproduction of *Pythium* and *Phytophthora*. *Phytopathology* **55**, 790–797.
- LAW, A., THRELFALL, D. R. & WHISTANCE, G. R. (1971). Isoprenoid phenol and quinone precursors of ubiquinones and dihydroubiquinones in fungi. *Biochemical Journal* **123**, 331–339.
- MURACA, R. F., WHITTICK, J. S., DAVES, G. D., FRIIS, P. & FOLKERS, K. (1967). Mass spectra of ubiquinones and ubiquinolins. *Journal of the American Chemical Society* **89**, 1505–1508.
- NELSON, R. R., HUISINGH, D. & WEBSTER, R. K. (1967). Sexual differentiation in *Cochliobolus carbonum* as influenced by inhibition and repair of steroid biosynthesis. *Phytopathology* **57**, 1081–1085.
- SCHER, M., LENNARZ, W. J. & SWEeley, C. C. (1968). The biosynthesis of mannosyl-1-phosphoryl-polyisoprenol in *Micrococcus lysodeikticus*. *Proceedings of the National Academy of Sciences of the United States of America* **59**, 1313–1320.
- STONE, K. J., BUTTERWORTH, P. H. W. & HEMMING, F. W. (1967). Characterization of the hexahydro-polyprenols of *Aspergillus fumigatus*. *Biochemical Journal* **102**, 443–445.
- STONE, K. J. & HEMMING, F. W. (1967). The stereochemistry of hexahydroprenol, ubiquinone and ergosterol biosynthesis in the mycelium of *Aspergillus fumigatus*. *Biochemical Journal* **104**, 43–56.
- THOMAS, D. M., HARRIS, R. C., KIRK, J. T. O. & GOODWIN, T. W. (1967). The mode of action of trisporic acid. *Phytochemistry* **6**, 361–366.