Trisporic Acid Production by *Blakeslea trispora* and its Promotion by Barbiturate

By J. D. BU'LOCK AND D. J. WINSTANLEY

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

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SUMMARY

Defined procedures for the production of trisporic acids in laboratory-scale cultures of mixed *plus* and *minus* *Blakeslea trispora*, and specific assay procedures, are described. Increases in carotenoid and steroid synthesis in such cultures were observed when phenobarbitone was added to the medium; the effect was mainly due to an increase in trisporic acid yields, which in turn is ascribed to the induction of higher levels of mixed function oxygenase enzymes by the barbiturate.

INTRODUCTION

The fermentation production of trisporic acids (structures, Fig. 1), the diffusible sex-hormones and promoters of isoprenoid synthesis in mucoraceous fungi, is well known in outline, but few quantitative data are available. The patent literature (e.g. Upjohn Co., 1966; Societa Farmaceutici Italia, 1966) describes the production of 0.3 to 1.0 g./l. of trisporic acids in large-scale cultures of 'mated' *Blakeslea trispora* (i.e. *plus* and *minus* strains together) under rather special conditions in complex media with a variety of additives. In other accounts the yields of trisporic acids have been given semiquantitatively in terms of bioassays or other uncalibrated tests. Specific assays of defined substances seem particularly desirable in this field. Moreover important and inconvenient features of the fermentation are that two separate inocula are required, and that the yield and pattern of trisporic acids, etc. (and, independently, the yield and pattern of carotenoids), depend considerably upon pretreatments and the media used at each stage. Here we describe some procedures which we have found useful for trisporic acid production. Procedures for maximum carotene yield, either in mated cultures or in single strains with exogenous trisporic acids, are not considered here.

The biosynthesis of trisporic acids from β -carotene (Austin, Bu'Lock & Drake, 1970) requires a series of oxidative transformations which on general grounds we expected would involve mixed function oxygenase enzyme systems. In fungi, induction of such enzymes by added barbiturates has been demonstrated in *Claviceps* (Ambike, Baxter & Zahid, 1970) and inferred in *Trichoderma* (Bu'Lock & Tse Hing Yuen, 1971). We therefore examined the effect of phenobarbitone (5-ethyl-5-phenylbarbituric acid) on the *Blakeslea trispora* system.

METHODS

Organism. The strains of *Blakeslea trispora* used were NRRL 2895 (*plus*) and 2896 (*minus*); stock cultures were maintained at 25° on slopes of the following composition (g./l.): glucose, 2.0; asparagine monohydrate, 1.0; KH_2PO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2×10^{-4} ;

Trisporic acid A $R = \text{cis- or trans-CMe:CH} \cdot (\text{CH}_2)_3 \cdot \text{CH}_3$

Trisporic acid B $R = \text{cis- or trans-CMe:CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$

Trisporic acid C $R = \text{cis- or trans-CMe:CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_3$

Fig. 1. Structures of the trisporic acids; all are acidic trienones, but structure assigned to trisporic acid A is unconfirmed.

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2×10^{-4} ; thiamine hydrochloride, 1×10^{-3} ; agar, 16. Subcultures were made monthly with periodic renewal on potato dextrose agar (Oxoid).

Inoculum and fermentation. Suspensions of spores and mycelium scraped in sterile water from slopes of each strain were macerated together briefly, and then inoculated into one or two flasks of the following medium (g./l.): glucose, 40; asparagine monohydrate, 2.0; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; thiamine hydrochloride, 5×10^{-4} ; adjusted to pH 6.2 with N-NaOH . After 72 h. growth the cultures, frequently containing only a few very large pellets, were macerated in a sterile blender and dispensed as 5 ml. amounts into 500 ml. conical flasks containing 150 ml. of 5% commercial malt extract. All flask cultures were shaken on a gyrotatory shaker, 76 mm. throw, 180 rev./min., at 29°, mainly in darkness. The main cultures were kept for 5 to 6 days; somewhat lower yields of trisporic acids resulted if the *plus* and *minus* inocula were grown up separately but this procedure was preferable in certain types of experiment.

Extraction and assay of trisporic acids. The culture filtrate (e.g. 100 ml.) was acidified to pH 1.0 and extracted with 2×100 ml. peroxide-free diethyl ether; the extracts were combined and washed with water (100 ml.), and the acids then extracted into 2×50 ml. of 5% aq. NaHCO_3 . The yield could be estimated spectrophotometrically at this stage (λ_{max} 335 nm.) but we preferred to acidify the bicarbonate extract, and extract with 2×100 ml. diethyl ether. The combined ether extracts were washed with water and treated with excess diazomethane; after 20 min. the excess diazomethane was distilled off under reduced pressure leaving a solution of methyl esters of the trisporic acids which was relatively stable in the dark. The trisporic ester content was estimated spectrophotometrically (λ_{max} 318 nm., $E_{1\text{cm}}^{1\%}$ 710) but the presence of related substances with different chromophores was always checked by running the complete ultraviolet absorption spectrum. The composition of the

Table 1. *Methyl esters of major trisporic acids*

Compound	Total trienone acid esters (%)	R_F^*	Visualization*	
			(1)	(2)
Methyl trisporate A	1.0	0.61	Yellow	No change
Methyl trisporate B	15	0.50	Yellow	No change
Methyl trisporate C	82	0.34	Orange	Magenta

* Thin-layer chromatography on SiO_2 gel in benzene + ethyl acetate + pentane (7 + 5 + 1, by vol.), sprayed first with ceric ammonium nitrate solution (1) and subsequently with phosphomolybdic acid solution (2); R_F values are for the predominant stereoisomer.

ester mixture was established by thin-layer chromatography on Merck F254 silica-gel plates in benzene + ethyl acetate + pentane (7 + 5 + 1, by vol.), visualized with 2% (w/v) ceric ammonium nitrate in 10% sulphuric acid, oversprayed with 5% (w/v) phosphomolybdic acid in ethanol. R_f values and colour reactions are tabulated in Table 1, together with typical yields for the major trisporic acids.

Other assays. Mycelium from one flask was pressed dry, ground to a powder under liquid N_2 , and put into 150 ml. of acetone for 1 h. in the dark. The acetone filtrate (plus washings) from the slurry was evaporated under reduced pressure and the residue taken up in ether. After checking the total absorption spectrum, β -carotene was estimated at 452 nm. [$E_{1\%}^{1\text{cm}}$ 2500] and ergosterol at 280 nm. [$E_{1\%}^{1\text{cm}}$ 340]; in normal runs other carotenoids and non-absorbing steroids were quantitatively insignificant.

Effect of barbiturate. To each culture-flask was added sufficient filter-sterilized phenobarbitone in N-NaOH to give a final concentration of 1 mg./ml., and 2 N- H_2SO_4 added to restore the normal pH of the medium (pH 4.5).

RESULTS

Neither mating type of *Blakeslea trispora* produced trisporic acids when grown alone; typical yields from the mated cultures are detailed in Table 2. The pattern of trisporic acid production was similar to that observed by van den Ende, Weichmann, Reyngood & Hendriks (1970) in that with 'pre-mated' inocula production was most rapid as growth slowed down.

The effects of barbiturate addition are summarized in Table 2, similar results being obtained throughout the fermentation. In the mated cultures the yield of trisporic acids was approximately doubled and the accumulation of carotene was trebled, while there was a smaller increase in ergosterol; the effect on growth was not significant. To ascertain whether the effect on carotene accumulation was a direct effect or a secondary consequence of the increase in trisporic acid production, the effect of barbiturate on the single strains (which do not convert carotene into trisporic acids) was followed; under the conditions used, the *minus* strain normally produced nearly as much β -carotene as the mated cultures while the *plus* strain produced much less. The addition of barbiturate had little effect on the *plus* strain and only a small, though definite, effect on the *minus* (Table 2).

Table 2. *Effect of phenobarbitone on yield of Blakeslea trispora and its production of metabolites (72 h. data)*

Cultures used	Fungus dry wt (g./l.)	β -Carotene (mg./l.)	Ergosterol (mg./l.)	Trisporic acids (mg./l.)
Controls				
(<i>plus</i>)	3.6	1.1	17.2	Nil
(<i>minus</i>)	4.7	6.0	52.9	Nil
(<i>plus</i>) and (<i>minus</i>)	3.7	6.4	65.9	115.9
+ Phenobarbitone (1 mg./ml.)				
(<i>plus</i>)	3.6	1.1	16.0	Nil
(<i>minus</i>)	4.5	8.5	56.9	Nil
(<i>plus</i>) and (<i>minus</i>)	3.1	18.6	80.9	227.8

DISCUSSION

The yields of trisporic acids obtained by our procedures, up to 150 mg./l., compare reasonably with the higher figures claimed for large-scale fermentations; doubtless they could be improved. The effect of barbiturate on trisporic acid levels is striking and entirely consistent with our view of the mechanism by which the trisporic acids are produced, given the evidence that it induces higher levels of mixed function oxygenases. Its effect on carotenoid accumulation seems mainly to reflect the enhanced production of trisporic acids, but the small effect noted in the *minus* strain growing alone could be a more direct one, since β -carotene is itself formed by a series of O_2 -requiring dehydrogenation steps. A probable parallel to the effect of barbiturate on the mated cultures is the action of a variety of 'promoters' claimed to increase carotenogenesis in this system, at least some of which are substances structurally resembling phenobarbitone (Ninet, Renaut & Tissier, 1969).

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