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CHEMICAL REGULATORS OF CAROTENOGENESIS BY *BLAKESLEA TRISPORA*

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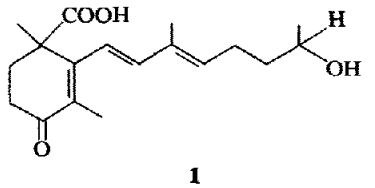
Key Word Index—*Blakeslea trispara*; Mucorales; fungi; carotenoids; regulation; trisporic acid; abscisic acid; ionones; vitamin A.

Abstract—The activation of the carotene biosynthetic pathway in *Blakeslea trispora* was found to occur by trisporic acid and many other compounds such as abscisic acid, β-ionone, α-ionone and vitamin A which share significant structural similarity with trisporic acid. The magnitude of stimulatory activities of these effectors was in the order trisporic acid > abscisic acid > β-ionone > α-ionone > vitamin A. Comparison of structures and stimulatory activities of all the effectors indicated that the short length of the side chain and the presence of a keto group in the ring structure of the trisporic acid molecule contributed significantly to the biological activity towards carotenogenesis.

INTRODUCTION

Trisporic acid TA(**1**), a well known sex hormone of the Mucorales, was first isolated from mated cultures of *Blakeslea trispora* [1], which produced much higher yields of carotene than unmated cultures [2, 3]. This rise in carotene production was attributed to the formation of TA. TA also regulates the sexual reproduction in heterothallic Mucorales [4] and conspicuously activates carotenogenesis in minus but not in the plus strain of *B. trispora* [2]. The stimulatory effect of trisporic acid in the minus strain is sensitive to cycloheximide, a protein synthesis inhibitor [5].

Earlier, it was reported that on mating two opposite mating types or on exogenous addition of TA to minus culture, there was an increase in mevalonate kinase activity indicating more synthesis of carotenogeneic enzymes in the presence of TA [6]. Later, we showed that the stimulatory activities of TA and β-ionone on the carotene pathway were competitive in nature suggesting the same site of action. It was proposed that TA might be depressing the enzyme(s) involved in the conversion of 5-phosphomevalonate to dimethylallyl-pyrophosphate [7]. In the present investigation, the effects of different compounds sharing a significant structural similarity with the TA molecule were studied on carotenoid production. By comparing their stimulatory effectiveness with that of TA, it might be possible to pinpoint the part(s) of the TA molecule which are essential for its stimulatory behaviour.



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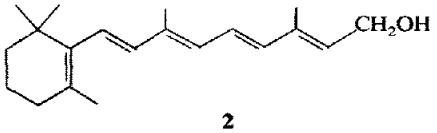

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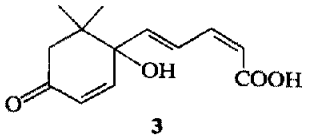

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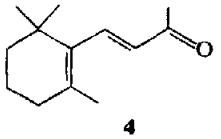

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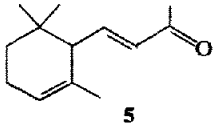
RESULTS AND DISCUSSION

Vitamin A (**2**), abscisic acid (ABA, **3**), β-ionone (**4**) and α-ionone (**5**) are strikingly similar to the structure of TA (**1**) and all have a common trimethyl cyclohexyl



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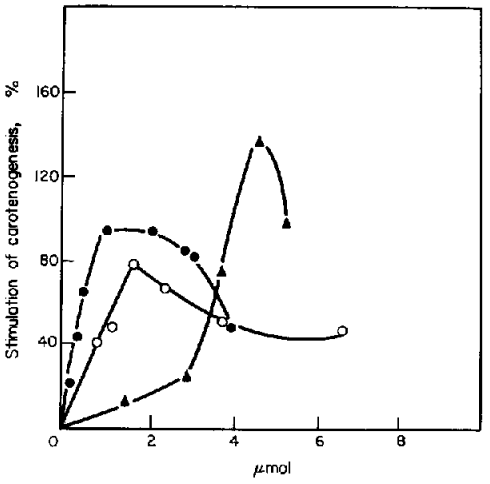

Fig. 1. Effect of abscisic acid (●—● μmol), vitamin A (○—○ μmol × 10^3) and α-ionone (▲—▲ μmol × 10^2) on carotene production by *B. trispora*.

ring, the differences being in the substitution and the position of unsaturation. All the compounds have side chains containing conjugated double bonds, vitamin A (**2**) has the longest side chain, α-ionone and β-ionone (**4**) have a shorter side chain. These effectors were added to 72-hr-old minus cultures and incubated further for 48 hr. The β-carotene content was then estimated for the harvested mycelia.

The minus strain of *B. trispora* was chosen as the test organism because it does not produce TA (**1**) but carotene synthesis is stimulated by TA. The presence of TA in the medium produced an enormous increase in the amount of β-carotene by the minus strain without affecting the growth rate of the mycelium. The increase in carotene levels was linear with increased TA addition [7]. The maximum increase of *ca* 340% was obtained at 5.8×10^{-4} mM TA. Above this concentration no further stimulation in carotene synthesis occurred. Thus, the highest activation of the carotene pathway by this hormone was *ca* 4.5 times more than the control cultures.

Abscisic acid (**3**), a plant growth hormone, stimulated carotene production by *ca* 100% in the single culture at concentrations of 9.5×10^{-4} mM (Fig. 1). There was a linear increase in carotene levels with increase in ABA concentrations up to 9.5×10^{-4} mM, above which a plateau was obtained. Addition of cycloheximide inhibited ABA-stimulated carotenogenesis

Table 1. Effect of abscisic acid on carotenogenesis by a minus culture of *B. trispora* in the presence of cycloheximide

Addition	Lipid-free mycelial wt (g)	β-Carotene (μg/g dry wt)
None	0.27	226
Abscisic acid and cycloheximide	0.14	88
Abscisic acid	0.28	306
Cycloheximide	0.15	73

Minus cultures (72 hr old) were supplemented with 0.3 μm ABA and cycloheximide (20 μg/ml) and harvested after 48 hr.

Table 2. Biological activities of trisporic acid and its structural analogues in *B. trispora*

Stimulator	K_{act}* (mM)	V_{max}† (%)	$\frac{V_{max}}{K_{act}}$
Trisporic acid	8.3×10^{-4}	500.00	6.0×10^5
Abscisic acid	5.0×10^{-3}	666.66	1.3×10^5
β-Ionone	3.0×10^{-2}	—	—
α-Ionone	3.7×10^{-2}	—	—
Vitamin A	10	666.66	66

*Activation constant.
†Maximum stimulation obtained.

as shown in Table 1. The data suggested that new protein synthesis was required for the ABA effect.

Vitamin A (**2**), another known carotenogenic stimulator, stimulated carotene production maximally (82% compared to the control at 0.18 mM in minus cultures). β-Ionone (**4**) [7] and α-ionone (**5**) addition to minus cultures resulted in 250 and 148% increases over the control at 0.57 and 0.48 mM, respectively (Fig. 1).

Response curves, i.e. concentration of stimulator vs % stimulation of carotene production plotted as double reciprocal plots, were linear for TA, ABA and vitamin A but sigmoidal for α-ionone and β-ionone [7]. It may be concluded from the data that compounds sharing structural similarity with TA also stimulate carotene production but to varying degrees. However, the affinity (activation) constants varied markedly. The concentrations of TA, ABA and vitamin A required for half maximal stimulation are presented in Table 2. Although the maximal stimulation obtained in the presence of these three activators was about the same, the concentrations required differed by many orders of magnitude and the effectiveness of each compound was in the order TA > ABA > vitamin A. Due to the sigmoidal nature of the response curves for α- and β-ionone, double reciprocal curves were not linear. It appears that these compounds may lead to interactions of a multiple order. In kinetic terms, binding of one molecule of β-ionone possibly affects the binding efficiency of the next molecule. β-Ionone was always added to the fermentations after a certain amount of growth was attained, since addition of β-ionone and α-ionone early in the growth phase resulted in the inhibition of growth. Thus, these terpenes affect growth also. The sigmoidal nature of the response curves with both these effectors may then be explained, partly by their dual effects. To find the concentrations of β-ionone and α-ionone required for half maximal stimulation, the log of effector concentration was plotted against V/V_{max}, i.e. stimulation observed/maximum stimulation obtained. The V_{max} value was taken from the response curve as the maximal per cent stimulation of carotenogenesis observed. Data in Table 2 show the K_{act} values for all carotenogenic stimulators along with TA indicating their effectiveness was in the order TA > ABA > β-ionone > α-ionone > vitamin A.

The biological activity of TA was much higher than abscisic acid. ABA (**3**) has a shorter side chain with a —COOH group and the position of the keto group and the unsaturation are different from those in TA

(**1**). β-Ionone (**4**) which comes next to ABA (**3**) as an efficient stimulator has a trimethyl cyclohexyl ring with unsaturation in the β-position without a keto group and a small side chain. This could mean that the smaller length of the side chain and the presence of a keto group in the ring structure are essential for biological activity. Stimulations mediated by TA [5], β-ionone [8, 9], ABA (Table 1) and vitamin A [8] were cycloheximide-sensitive indicating that new protein synthesis was required. Trisporic acid and β-ionone mediated stimulations were competitive in nature suggesting the same site of action in the carotene biosynthetic pathway [7]. Precursors of TA, ABA and vitamin A were shown to be carotenoids. β-Carotene served as a precursor of TA and vitamin A [10, 11]. Many reports have appeared in recent times indicating that ABA is formed by a photo-oxidation of epoxy carotenoids [12, 13].

ABA is a recently discovered growth regulating hormone which promotes senescence and abscission of leaves and induces dormancy in buds and seeds [14]. It has been detected in many carotenogenic systems such as tomatoes, rose petals, avocado etc. and it is generally noticed that amounts of carotene change depending upon the growth stage of that particular system. A possible involvement of ABA in triggering off high carotene synthesis in these systems is implicated. Similarly, it was found that various citrus oils enhanced carotene production in *B. trispora* [15]. Vitamin A was shown to be involved in sexual reproduction and the structural similarity of it to another sex hormone, TA, may be one of the important features. Thus all the information available regarding TA and its structural analogues suggests that they might be stimulating carotenogenesis apart from other functions in the organisms where they are found.

EXPERIMENTAL

Mold strains. The plus (NRRL 2895) and minus (NRRL 2896) strains of *Blakeslea trispora* were obtained from the U.S. Department of Agriculture, Peoria, Illinois. The cultures were maintained on slants containing synthetic Mucor medium solidified with 1.8% agar [16]. Slants were kept at 20° for 4–5 days and stored at 0–4°. Transfers were made once a month.

Media for cultivation. (A) Synthetic Mucor medium (SMM) described in ref. [17] was used for most of the expts. It contained glucose (40.0 g), asparagine, (2.0 g), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$, (0.25 g) and thiamine–HCl (10.0 mg) in 1 l. of glass-distilled H_2O. The pH of the medium was adjusted to 6.2. 250 ml Erlenmeyer flasks containing 100 ml of the medium were autoclaved at 10 lbs for 15 min. (B) Potato Glucose Thiamine (PGT) medium described in ref. [18] was used for trisporic acid production. Potato extract (12.5 g), glucose (20.0 g) and thiamine–HCl (0.002 g) were dissolved in 1 l. glass-distilled H_2O. Portions of 100 ml media were distributed in 250 ml Erlenmeyer flasks, and autoclaved at 10 lbs for 15 min.

Inoculum preparation and cultivation. Mycelia from 4–5-day-old agar slants were inoculated into 100 ml SMM containing 1.0% malt extract and were incubated for 2 days at 28 ± 2° on a gyrotory shaker (185 rpm). Under these conditions, the mycelia formed one solid mass. Inoculum for the fermentation was prepared by aseptically transferring the thoroughly washed mycelial mass to a Sorvall omnimixer and macerating it to make a homogeneous suspension. Equal vols. of the homogenate were used in all flasks for a given set of fermentations. Single and mated cultures were incubated for 5 days at 28° on a shaker for the time specified. For analytical and preparative work on trisporic acid, the cultures were incubated in the dark.

Growth determination. After the extraction of carotenoids, lipid-free mycelia were dried at 50° in an oven and weighed.

Extraction and estimation of β-carotene. The cultures were filtered through muslin in a Buchner funnel, washed thoroughly with H_2O and pressed dry. The mycelium was cut into small pieces and ground in Me_2CO—Et_2O (1:1). The mixture was filtered and the lipid-free residue reextracted until the filtrate was colourless. The solvent extract was washed with H_2O and dried (Na_2SO_4). Total carotene content was estimated as β-carotene by using an $E^{1\%}_{1cm}$ value of 2500 at 450 nm as described by ref. [19].

Extraction and estimation of trisporic acids. Trisporic acid was extracted from the culture medium of mated cultures by the method described by ref. [18]. The hormone was dissolved in 0.1 M Tris–sulphate buffer, pH 7.5. The trisporic acid content was calcd using an $E^{1\%}_{1cm}$ value of 700 at 325 nm. All operations were performed under diffused light at 0–4°.

Addition of different effectors. The compounds soluble in H_2O were sterilized by passing through a membrane filter (0.45 μm) before addition to the cultures. Cultures (72 hr old) were supplemented with the specified compound and incubated for a further 48 hr.

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