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EFFECTS OF AMINES ON THE CAROTENOGENESIS IN *BLAKESLEA TRISPORA**

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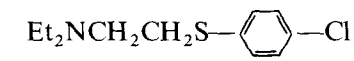
Key Word Index—*Blakeslea trispora*; carotenoid biosynthesis; diethylamines; triethylamine; α-Diethylaminopropiophenone; tributylamine; 2-(4-Chlorophenylthio)triethylamine (CPTA); lycopene; γ-carotene.

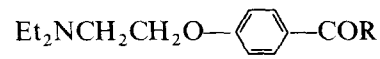
Abstract—Six amines profoundly affected carotenogenesis in *Blakeslea trispora*. When cultures were treated with the amines, namely 4-[β-(diethylamino)-ethoxy]-benzaldehyde, 4-[β-(diethylamino)-ethoxy]-acetophenone hydrochloride, 4-[β-(diethylamino)-ethoxy]-benzophenone hydrochloride, triethylamine hydrochloride, α-diethylaminopropiophenone hydrochloride and tributylamine hydrochloride, an increase in the lycopene accumulation was observed. The modes of action of these amines appear to be similar to that of 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA); however, they differ in relative effectiveness.

INTRODUCTION

PREVIOUS studies in this laboratory have shown that 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA) has a profound effect on the carotenogenesis in a wide variety of carotenogenic tissues including the fungus *Blakeslea trispora*.[1-4] In all cases, lycopene accumulated as the principal pigment with the concomitant increase in γ-carotene. In the present investigation, the effects of six amines, 4-[β-(diethylamino)-ethoxy]-benzaldehyde (**1**), 4-[β-(diethylamino)-ethoxy]-acetophenone hydrochloride (**2**), 4-[β-(diethylamino)-ethoxy]-benzophenone hydrochloride (**3**), triethylamine hydrochloride (**4**), α-diethylaminopropiophenone hydrochloride (**5**), and tributylamine hydrochloride (**6**), on the carotenoid biosynthesis in mated *B. trispora* are reported; also the mechanism of action in relation to CPTA is considered.



CPTA



(**1**) R = H
(**2**) R = Me
(**3**) R = C_6H_5

RESULTS AND DISCUSSION

Effects of 4-[β-(*diethylamino*)-*ethoxy*]-*benzaldehyde* (**1**) *and its analogs* (**2** *and* **3**)

The mycelia of mated *B. trispora* acquire a yellowish color because of the β-carotene

* Part VI in the series "Chemical Regulation of Carotenoid Biosynthesis". Part V *Phytochemistry* **12**, 2665 (1973).

[1] COGGINS, JR., C. W., HENNING G. L. and YOKOYAMA, H. (1970) *Science* **168**, 1589.
[2] YOKOYAMA, H., COGGINS, JR. C. W. and HENNING, G. L. (1971) *Phytochemistry* **10**, 1831.
[3] YOKOYAMA, H., COGGINS, JR., C. W., HENNING, G. L. and DEBENEDICT, C. (1972) *Phytochemistry* **11**, 1721.
[4] HSU, W. J., YOKOYAMA, H. and COGGINS, JR., C. W. (1972) *Phytochemistry* **11**, 2985.

accumulation.[4,5] The main carotenoids of the control mycelia were phytoene and β-carotene which constituted 80% of the total; lycopene and γ-carotene constituted only about 3% of the total (Tables 1 and 2). In the cultures treated with the compounds **1**–**3**, the carotenoid compositions of the mycelia were altered. Increase in lycopene accumulation was similar to that observed in cultures treated with CPTA.[4]

TABLE 1. EFFECT OF COMPOUND **1** ON THE CAROTENOGENESIS IN MATED *Blakeslea trispora*

	Compound **1**					
	Control		100 ppm		500 ppm	
*Treatment** Carotenoid	(μg/g dry wt)	(% of total)	(μg/g dry wt)	(% of total)	(μg/g dry wt)	(% of total)
Phytoene	417	63	640	60	520	57·7
Phytofluene	32	5	27	2·5	36	4
ζ-Carotene	16	2·5	26	2·5	17	2
Neurosporene	8	1·5	16	1·5	12	1·3
Lycopene	17	2·5	40	4	73	8
γ-Carotene	23	3	84	8	102	11
β-Carotene	134	20	233	21·5	132	15
β-Zeacarotene	16	2·5	20	2	9	1

* Compound **1** was added at the time of inoculation and the cultures were incubated for 1 week before harvesting.

In cultures treated with compound **1** at 100 ppm, the levels of β-carotene, lycopene and neurosporene doubled; and the level of γ-carotene was increased almost 4-fold (Table 1). With a higher concentration (500 ppm) of **1**, the level of β-carotene returned to normal (132 μg/g dry wt of mycelia); however, γ-carotene further accumulated and became the second predominant pigment (102 μg/g dry wt of mycelia), while lycopene increased 4-fold (73 μg/g dry wt of mycelia) and became the third major pigment. In treated cultures the increase in the total carotenoids produced and the decrease in the percent of β-carotene indicate the multi-functional nature of compound **1**. It acts as a stimulator on the total carotenogenesis and simultaneously as an inhibitor on the cyclization reactions.

TABLE 2. EFFECTS OF COMPOUNDS **2**, **3**, **5**, AND **6** ON THE CAROTENOGENESIS IN MATED *Blakeslea trispora*

		2		**3**		**5**		**6**	
Treatment† Carotenoid	Control	100 ppm	500 ppm	100 ppm	500 ppm	100 ppm	500 ppm	100 ppm	1000 ppm
Phytoene	228*	154	38	51	56	432	159	40	133
Phytofluene	30	43	15	19	2	69	29	12	9
ζ-Carotene	17	10	4	Trace	5	20	11	11	12
Neurosporene	Trace	17	5	12	10	37	20	3	10
Lycopene	8	111	215	125	560	1312	2060	5	313
γ-Carotene	5	29	23	11	4	174	61	5	15
β-Carotene	173	154	47	15	4	270	67	33	18
β-Zeacarotene	15	29	10	Trace	Trace	35	5	Trace	Trace

* The numbers are expressed as μg/g dry wt of mycelia.

† Chemicals were added at the time of inoculation and the cultures were incubated for 1 week before harvesting.

In cultures treated with compound **2** at 100 ppm, the production of β-carotene was inhibited somewhat (154 μg/g dry wt of mycelia) (Table 2). However, the level of γ-carotene

[5] THOMAS D. M. and GOODWIN, T. W. (1967) *Phytochemistry* **6**, 355.

increased 6-fold (29 μg/g dry wt of mycelia); and the lycopene increased 14-fold (111 μg/g dry wt of mycelia) to become the second major pigment. In cultures treated with compound **2** (500 ppm), the β-carotene production was inhibited by 73% and lycopene became the major pigment (215 μg/g dry wt of mycelia).

Table 2 also shows that in cultures treated with 100 ppm of compound **3**, β-carotene production was inhibited by 90% and lycopene accumulated as the major pigment (125 μg/g dry wt of mycelia). At 500 ppm of compound **3**, the β-carotene level showed a further decrease and the level of lycopene increased by more than 4-fold (560 μg/g dry wt of mycelia) and constituted 96% of the total pigments.

The results indicated that the effects of compounds **1**–**3** on carotenogenesis are similar to that of CPTA. They stimulate lycopene synthesis and inhibit β-carotene production with differences in their relative effectiveness. The carotenoid patterns in CPTA treated *B. trispora* cultures depended on the concentrations of CPTA.[4] At 50 ppm of CPTA, γ-carotene accumulated as the major pigment (38% of the total) and β-carotene and lycopene each constituted 12% of the total; the β-carotene level increased slightly. At higher concentrations of CPTA (100–300 ppm), lycopene accumulated as the main pigment and γ-carotene was second. The carotenoid patterns shown in Table 1 and reported for various concentrations of CPTA[4] indicate that the effectiveness of the compound **1** on the induction of lycopene synthesis at the concentrations of 100 ppm and 500 ppm was less than CPTA at 50 ppm; compound **1** is only one-tenth as effective as CPTA. Figure 1 shows that **1**–**3** differ only in the R-groups. Results in Tables 1 and 2 show that the effectiveness increases as follows: **1** (R = H) < **2** (R = Me) < **3** (R = C_6H_5). The relative effectiveness of **3** is similar to that of CPTA.

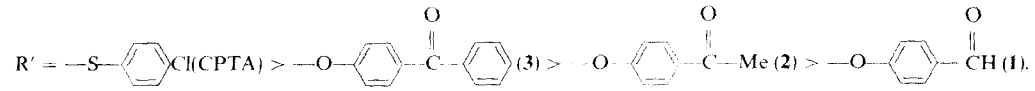
Benzophenone was shown to inhibit the carotenogenesis in *Mucor hiemalis* by inhibiting the dehydrogenation reactions thus causing the accumulation of phytoene.[6] Substitutions on one of the benzene nuclei, diminished the inhibitory effect of the benzophenone. 4-Hydroxybenzophenone was somewhat inhibitory; however, 2,4-dihydroxybenzophenone as well as the 2-carboxy- and 2-carboxy-4-methylbenzophenone were totally inactive in the carotenogenesis. In the present study, substitution at the 4-position with the β-diethylaminoethoxy group (compound **3**) not only diminished the inhibitory effect of benzophenone on the dehydrogenation of phytoene, but stimulated the dehydrogenation reactions, inhibited the cyclization reaction and consequently caused the accumulation of lycopene.

Effect of triethylamine hydrochloride **4**

Compounds **1**–**3**, like CPTA, inhibit the synthesis of β-carotene and stimulate the accumulation of lycopene. In order to investigate further the inhibitory effect of these compounds, triethylamine hydrochloride, the common group in the molecules of CPTA and compounds **1**–**3**, was applied to the *B. trispora* culture. Table 3 shows the carotenoid patterns of the cultures treated with a series of concentrations of triethylamine hydrochloride. At 35 ppm of compound **4**, no alteration of the carotenoid production and pattern was observed. However, as the concentration of compound **4** went up (70–350 ppm), the level of β-carotene decreased by 60%. The level of lycopene increased at the expense of β-carotene and all the intermediates leading to lycopene. The level of γ-carotene remained almost constant. These results indicate that probably the triethylamine portion of the molecules

[6] HERBER, R., MAUDINAS, B. et VILLOUTREIX, J. (1972) *Phytochemistry* **11**, 3461.

of CPTA, and of compounds **1**–**3** is responsible for the inhibitory effect on the β-carotene biosynthesis and the stimulatory effect on the lycopene biosynthesis. The relative effectiveness of these compounds apparently depends upon the β-substituting group (R′) on one of the ethyl-groups of the amine; the order of the effectiveness is



The same order of effectiveness was found in the citrus fruit systems.[7,8]

TABLE 3. EFFECT OF COMPOUND **4** ON THE CAROTENOGENESIS IN MATED *Blakeslea trispora*

Treatment† Carotenoid	Control	35 ppm	70 ppm	105 ppm	140 ppm	210 ppm	280 ppm	350 ppm
Phytoene	462*	464	436	212	181	238	202	345
Phytofluene	33	29	20	15	11	7	8	20
ζ-Carotene	13	Trace	8	12	3	8	6	6
Neurosporene	8	Trace	8	5	11	8	30	25
Lycopene	28	26	63	26	136	139	375	359
γ-Carotene	15	16	15	25	10	16	19	17
β-Carotene	199	174	102	85	60	48	97	59
β-Zeacarotene	60	46	20	7	4	2	6	3

* The numbers are expressed as μg/g dry wt of mycelia.
† Compound **4** was added at the time of inoculation and the cultures were incubated for 1 week before harvesting.

Effects of α-diethylaminopropiophenone hydrochloride (**5**) *and tributylamine hydrochloride* (**6**)

When cultures were treated with compound **5** at 100 ppm, total carotenoid production was enhanced by 5-fold; the levels of all the polyenes were increased (Table 2). Lycopene accumulated (1312 μg/g dry wt of mycelia) and became the main pigment. At a higher concentration of compound **5** (500 ppm), the levels of all the polyenes except lycopene decreased. The inhibitory effect of the compound **5** on the cyclization reactions was expressed and the massive accumulation of lycopene (2060 μg/g dry wt of mycelia) resulted.

Table 2 also shows that when compound **6** (100 ppm) was applied to the culture, the carotenoid synthesis of the culture seemed to be inhibited by 78%; however, β-carotene is still the major pigment. The growth of this culture appeared to be normal in comparing the 533 mg dry wt of mycelial mass/100 ml media for this culture to the 508 mg dry wt of mycelial mass/100 ml media for the control culture. The low total carotenoid produced in this culture might be attributed to the cultural variation in onset time for carotenogenesis. In the cultures treated with 1000 ppm of compound **6**, the β-carotene production was inhibited by 90%, lycopene accumulated (313 μg/g dry wt of mycelia) and became the major pigment.

The above results indicate that neither the β-substitution of phenoxy-derivatives on one of the ethyl groups of triethylamine nor the ethyl groups of the amine part is exclusively necessary for the regulatory activity of the amine compounds on the carotenogenesis. Amines with other alkyl groups might have the same effect, but might differ in relative effectiveness. In the case of compound **6**, a much higher concentration (1000 ppm) was required

[7] POLING, S. M. HSU, W. J. and YOKOYAMA, H. (1973) *Phytochemistry* **12**, 2665.
[8] HSU, W. J. and YOKOYAMA, H. In preparation.

for the accumulation of lycopene and the inhibition of β-carotene synthesis. This could be due to the bulkiness of the butyl groups on the nitrogen atom.

Compounds **1–3** also induced lycopene production in grapefruit and navel orange systems.[7,8] However, compounds **4–6** although effective in the *B. trispora* system, were not effective in citrus fruit systems.[8] This phenomenon may indicate the problem of penetration of the compounds into the flavedo. The β-substitution of certain groups on one of the ethyl groups of the triethylamine molecule is probably necessary to enable the compounds to enter the flavedo tissue of the fruits and to induce lycopene synthesis. The phenomenon may also indicate that the two systems may differ in the functional groups involved at the active sites of carotenogenic enzymes, which will accommodate compounds with different structures. Despite this, the six amines tested in this study all possess the same inhibitory affect on the β-carotene synthesis as CPTA and cause the lycopene to accumulate.

EXPERIMENTAL

Culture. The (+) strain (NRRL 2456) and (−) strain (NRRL 2457) of *Blakeslea trispora* were obtained from Dr. Alex Ciegler of the Northern Regional Laboratory, USDA. The organisms were cultured in glucose-potato extract media in Erlenmeyer flasks on a gyrotary shaker (120 rpm) at 30°.

Extraction of lipid and preparation of unsaponifiable matter. The washed cultures were homogenized and the disrupted mycelia were extracted with acetone, followed, in some cases, by $CHCl_3$ extraction in order to extract all the lycopene from the tissue. The lipid was saponified and the unsaponifiable material extracted by standard procedures.[9]

Separation and identification of pigments. The unsaponifiable matter, dissolved in petrol. (30–60°) (PE), was chromatographed on MgO: Hyflo-Supercel (1:1, W/W), and the various fractions were eluted with PE containing an increasing amount of acetone. The lycopene zone was eluted with acetone, then with EtOH and $CHCl_3$. The pigments were identified by their UV and visible spectra and adsorption behaviors relative to known compounds.

Quantitative determination. The method used has been described by Davies.[9]

Chemicals. **1**, **5**, and free amine forms of **4** and **6** are available from Aldrich Chemical Company, Inc. **2** and **3** were chemically synthesized by published methods.[10] With diethylaminoethyl chloride as the common starting reactant, *p*-hydroxyacetophenone and *p*-hydroxybenzophenone were used as the other reactant for **2** and **3** respectively. The compounds synthesized were checked on preparative silica gel G TLC plates using EtOH (96%): 25% aq. NH_3 (4:1) as the solvent system and ninhydrin as the spray reagent; the compounds were shown to be chromatographically pure with R_f 0·66 for **2**, and 0·69 for **3**, **4** and **6** were prepared by bubbling HCl gas into ethereal solutions of triethylamine and tributylamine respectively.

Acknowledgements—This work was supported in part by the California Citrus Advisory Board.

[9] DAVIES, B. H. (1965) *Chemistry and Biochemistry of Plant Pigments* (GOODWIN, T. W., ed.), Academic Press, New York.

[10] SCHUETZ, R. D. and BALDWIN, R. A. (1958) *J. Am. Chem. Soc.* **80**, 162.