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THE EFFECT OF CPTA ANALOGS AND OTHER NITROGENOUS COMPOUNDS ON THE BIOSYNTHESIS OF CAROTENOIDS IN *PHYCOMYCES BLAKESLEEANUS* MUTANTS*

MANZOOR ELAHI,† RICHARD W. GLASS, TUNG-CHING LEE, CLINTON O. CHICHESTER and KENNETH L. SIMPSON

Department of Food and Resource Chemistry, University of Rhode Island, Kingston RI 02881, U.S.A.

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Key Word Index—*Phycomyces blakesleeanus*; Mucorates carotenoid biosynthesis, CPTA and CPTA analogs; nitrogenous bases; ammonia derivatives.

Abstract—The inhibitory effect of a series of analogs of CPTA, 2-(4-chlorophenylthio)-triethylamine-HCl, and ammonia derivatives on carotenoid biosynthesis in *Phycomyces blakesleeanus* mutants was studied. The types of inhibition exhibited allowed no firm conclusions about the biosynthetic route to β-carotene from either β-zeacarotene or lycopene. However, the evidence suggests at present that both pathways are operative. It was found that a slight change in structure of inhibitor resulted in a different type of action. Conclusions based on a single inhibitor could be cited as "evidence" for a certain pathway.

INTRODUCTION

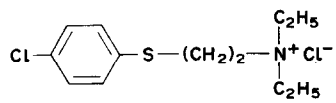
The effect of administering CPTA [2-(4-chlorophenylthio)-triethylamine hydrochloride [1–5], cycocel (2-chloroethyl-trimethyl ammonium chloride [5,6] nicotine [7–9] and other nitrogeneous heterocyclic compounds [10,11] on a wide spectrum of carotenogenic systems have been reported. Most of these compounds cause, in varying degrees, an accumulation of acyclic compounds such as lycopene and γ-carotene and a reduction in β-carotene and β-zeacarotene synthesis.

Previous communications from this laboratory [5,10] as well as from others [11] showed that minor structural changes in these compounds resulted in varying the degree of the effect and in some cases resulted in the compound becoming toxic. In one series of pyridine derivatives [10] the inhibition of β-carotene synthesis was related to compounds having a pK_a range of 6 ± 1.

In the present paper the biosynthesis of carotenoids by *Phycomyces blakesleeanus* mutants has been studied in the presence of analogs of CPTA and a series of derivatives of ammonia.

RESULTS

Table 1 lists the results of adding 1 mM of CPTA or its analogs to the growth medium on the biosynthesis of carotenoids in *P. blakesleeanus* strain C 115. Most of the CPTA analogs caused a slight increase in the total carotenes (μg/g dry weight) but caused little change in the total yield of mycelium. The addition of CPTA itself (**1**) caused an increased synthesis of phytoene, phytofluene and ζ-carotene, a large accumulation of lycopene and γ-carotene and a reduction of β-carotene and β-zeacarotene.



CPTA
(1)

The removal of the 4-chloro group (**2**), or the lengthening of the alkyl chain from 2 to 4 carbons (**3**) resulted in compounds less active than CPTA. The substitution of an *O* for the *S* of CPTA (**4**) resulted in the greatest stimulation of lycopene and γ-carotene and the greatest overall synthesis of carotenoids. The removal of the 4-chloro group from **4** (**5**) again resulted in a loss of activity. 2-Thiotriethylamine (**7**) was inactive whereas 2-hydroxytriethylamine (**8**) retained some activity over the

* Contribution No. 1533 of the Rhode Island Agricultural Experiment Station.

† Post-doctoral fellow, PCSIR Laboratories, Lahore 16 Pakistan.

Table 1. Effect of CPTA and its analogs on carotenoid composition in *P. blakesleeanus* strain C 115

Carotenoid (μg/g dry wt basis)	CPTA analog added* 0	1	2	3	4	5	6	7	8	9
Phytoene	160	368	240	200	448	260	624	281	219	230
Phytofluene	35	68	43	42	96	55	132	32	44	70
ζ-Carotene	8	32	23	23	39	31	64	9	24	30
Neurosporene	10	16	13	13	19	16	23	9	13	10
Lycopene	4	425	78	57	638	132	20	10	105	22
γ-Carotene	2	124	51	37	141	59	12	9	52	15
β-Carotene	1265	698	1010	1165	577	977	400	1280	1152	1130
β-Zeacarotene	15		1	4		3		13	t	15
Total	1499	1739	1458	1541	1958	1560	1175	1630	1609	1522
Weight of mycelium (g/250 ml of medium)	2·11	2·07	2·10	2·09	2·04	2·11	2·08	2·13	2·16	2·14

* Added 24 hr after inoculation at a concentration of 1 mM, supplied by AmChem Products, Inc., Ambler, Pa., U.S.A.
† Aldrich Chem. Co., Milwaukee, Wisconsin.
0. Control; **1.** 2-(4-chlorophenylthio)-triethylamine HCl (CPTA); **2.** 2-phenylthio-triethylamine HCl; **3.** [2-(4-chlorophenylthio)-ethyl]-triethylamine HCl; **4.** 2-(4-chlorophenoxy)-triethylamine HCl; **5.** 2-phenoxytriethylamine HCl; **6.** 2-(4-chlorophenoxy)-triethylamine N-oxide; **7.** 2-mercaptotriethylamine HCl; **8.** 2-hydroxytriethylamine HCl; **9.** 4-[β(diethylamino)-ethoxy]-benzaldehyde†.

control. The formation of the *N*-oxide (**6**) resulted in the greatest inhibition of β-carotene; however, phytoene was accumulated rather than lycopene or γ-carotene.

In addition to the CPTA analogs available from commercial sources, a number of related compounds were synthesized in which the group substituents on nitrogen were varied. CPTA synthesized in our laboratory was identical to that supplied by AmChem and gave the similar results (Table 2). Where two thio ether groups were present (compound (**10**)) [2,2'bis(4-chlorophenylthio)-diethylamine hydrochloride] the compound was found to be toxic at a concentration of 1 mM. However, the trisubstituted compound (**11**) [2,2'2"tris(4-chlorophenylthio)-triethylamine hydrochloride] causes an increased accumulation of phytoene but otherwise resembles the control. Compound **12** (with only one ethyl group) caused an increased synthesis of lycopene, phytoene and γ-carotene. Compound (**13**) (with no ethyl groups) gave results essentially the same as the control.

Table 3 shows the effect of NH_4Cl, some of its derivatives, and urea in the biosynthesis of carotenoids in *P. blakesleeanus* strain C 115. Ammonium chloride appears to be used by the

Table 2. Effect of CPTA and its analogs on carotenoid composition in *P. blakesleeanus* strain C 115

Carotenoid (μg/g dry wt)	CPTA analog added* 0	1	10	11	12	13
Phytoene	172	470	—	680	261	170
Phytofluene	27	73	—	92	22	23
ζ-Carotene	7	39	—	16	13	11
Neurosporene	9	14	—	17	8	7
Lycopene	5	300	—	6	285	10
γ-Carotene	4	125	—	6	22	7
β-Carotene	1165	522	—	1175	1117	1133
β-Zeacarotene	11		—	7	6	13
Total	1400	1543	—	1999	1477	1374
Dry wt (g) mycelium/250 ml media	2·07	1·81	—	1·86	2·09	2·11

* Added 24 hr after inoculation at a concentration of 1 mM; CPTA and analogs synthesized in our laboratory.
0. Control; **1.** 2-(4-chlorophenylthio)-triethylamine HCl (CPTA); **10.** 2,2'-bis-(4-chlorophenylthio)-diethylamine HCl; **11.** 2,2',2"-tris-(4-chlorophenylthio)-triethylamine HCl; **12.** 2-(4-chlorophenylthio)-ethylamine-*N*-ethyl HCl; **13.** 2-(4-chlorophenylthio)-ethylamine HCl.

Table 3. Effect of ammonium chloride and its derivatives and urea on the biosynthesis of carotenoids by *P. blakesleeanus* strain C 115

Carotenoids (μg/g dry basis)	Compound tested†													
	1	**14**	**14***	**15**	**15***	**16**	**16***	**17**	**17***	**18**	**19**	**20**	**21***	**22***
Phytoene	164	175	200	210	260	209	265	210	245	235	280	320	225	280
Phytofluene	31	33	40	37	39	38	61	34	41	38	39	50	45	—
ζ-Carotene	10	8	10	9	11	10	24	11	21	10	9	25	16	—
Neurosporene	9	9	11	11	9	10	9	10	11	10	11	14	10	—
Lycopene	4	5	4	13	28	32	94	22	44	14	42	120	6	—
γ-Carotene	3	3	2	11	23	20	65	17	36	11	26	42	5	—
β-Carotene	1280	1590	1996	1300	1325	1135	1130	1260	1230	1260	1160	1010	1465	1150
β-Zeacarotene	14	30	46	10	9	10	5	10	5	11	3	3	38	—
Total	1515	1853	2309	1591	1704	1464	1648	1564	1633	1589	1560	1484	1810	1430
Weight of mycellium (g/250 ml of medium)	2·04	2·29	2·38	2·06	2·12	2·07	1·95	2·07	2·03	2·07	1·95	2·04	2·86	0·25

* 5 mM; added 24 hr after inoculation.

† Concentration used 1 mM; where specified.

0. Control; **14.** ammonium chloride; **15.** methylamine HCl; **16.** dimethylamine HCl; **17.** trimethylamine HCl; **18.** ethylamine HCl; **19.** diethylamine HCl; **20.** triethylamine HCl; **21.** urea; **22.** hydroxylamine HCl.

mold as a nitrogen source; however, it also causes a stimulation of the synthesis of β-carotene and β-zeacarotene. Some molds [12] can use NH_4Cl as a nitrogen source and certain amino acids greatly stimulate carotenogenesis in *P. blakesleeanus* [13]. The effect of urea on *Phycomyces* was similar to that of NH_4Cl and in fact may act as a source of nitrogen.

The methyl (**15**), dimethyl (**16**), and trimethyl (**17**) amines caused a slight stimulation of lycopene and γ-carotene. The effect was the greatest with dimethyl amine. Triethylamine (**20**) was more active than diethylamine (**19**) which in turn was more active than ethylamine (**18**). The effect of triethylamine on the culture was very similar to that obtained with the use of 2-hydroxytriethylamine (Table 1). Hydroxylamine (**22**) was found to be toxic to the culture and only phytoene and β-carotene were isolated.

Table 4 shows that the phenyl derivatives of ammonia at a concentration of 0·25 mM have either no effect (aniline and triphenylamine) or are toxic (diphenylamine). Diphenylamine at a lower concentration (0·05 mM) results in the expected in-

Table 4. Effect of aniline, diphenylamine (DPA) and triphenylamine (TPA) on the biosynthesis of carotenoids by *P. blakesleeanus* strain C 115

Carotenoids (μg/g dry basis)	Control	Aniline* (0·25)†	DPA† (0·05)	TPA* (0·25)	CPTA† (1)	DPA and CPTA† (0·05 and 1)
Phytoene	178	200	1100	‡	940	1900
Phytofluene	36	39	143	38	105	291
ζ-Carotene	10	12	44	12	55	130
Neurosporene	11	12	29	9	19	46
Lycopene	4	5	—	4	410	320
γ-Carotene	2	2	—	3	192	93
β-Carotene	1295	1300	192	1180	1080	108
β-Zeacarotene	17	15	68	16	—	—
Total	1553	1585	1576	1252	2801	2888
Weight of mycelium (g/250 ml of medium)	2·29	2·21	2·14	2·20	1·90	1·94

* Chemicals added 24 hr after inoculation: DPA at 0·25 mM was toxic.

† Chemicals added at the time of inoculation. Concentration mM in brackets.

‡ Phytoene was observed but could not be separated from triphenylamine due to a similar absorption maximum and R_f values.

Table 5. Effect of ammonium chloride, CPTA and β-ionone on the biosynthesis of carotenoids in *P. blakesleeanus* strain C115

Carotenoids (μg/g dry basis)	(A) Control	NH_4Cl (5)	CPTA (1)	CPTA + NH_4Cl (1 + 5)	(B) NH_4Cl (5)	CPTA (1)	CPTA + NH_4Cl (1 + 5)	β-Ionone*	β-Ionone* + CPTA
Phytoene	180	190	860	547	195	334	305	530	644
Phytofluene	38	36	115	75	38	82	66	75	125
ζ-Carotene	10	8	60	40	8	40	31	23	72
Neurosporene	9	8	18	20	12	15	14	12	18
Lycopene	5	4	400	152	5	380	128	3	410
γ-Carotene	t	4	202	99	3	178	82	2	142
β-Carotene	1180	1524	1026	1592	1541	727	1175	1250	682
β-Zeacarotene	13	27	—	—	24	—	—	30	—
Total	1435	1801	2681	2525	1826	1756	1801	1925	2083
Weight of mycelium (g/250 ml of medium)	1·95	2·18	1·85	2·05	2·21	1·90	1·95	1·97	1·80

(A) Compounds added at time of inoculation: concentration in mM in brackets.
(B) Compounds added 24 hr after inoculation.
* β-Ionone was added 24 hr after inoculation according to the method of Reyes *et al.* [17].

hibition of the dehydrogenation reactions and stimulation of phytoene synthesis [14]. The dehydrogenation of β-zeacarotene was also blocked by DPA as a significant build-up of this compound was observed. CPTA when added at the time of inoculation has been observed to cause a large increase in the total carotenes as well as cause an inhibition of the cyclase reactions in *Blakeslea trispora* [4] and *P. blakesleeanus* [5]. When DPA and CPTA were added together a very large increase in the level of phytoene and an almost equal decrease in the accumulation of β-carotene was observed. Effectively, the addition of DPA to a CPTA culture did not change the total pigment synthesis but caused the more saturated polyenes to accumulate. This is in contrast to the results obtained by Hsu *et al.* [4] with *B. trispora* where the addition of DPA and CPTA caused a large increase in the synthesis of carotenoids over that obtained by the use of each chemical by itself. However, the effect suggests (as in *B. trispora* [4]) that CPTA competes with DPA for the dehydrogenases, thus allowing a greater percentage of polyenes biosynthesis after phytoene to accumulate. *P. blakesleeanus* would appear to be more sensitive to DPA than *B. trispora* as half of the DPA concentration produced a much greater inhibition of the dehydrogenation reactions.

Table 5 shows that the effect of NH_4Cl in stimulating the synthesis of β-carotene and β-zeacarotene was the same irrespective of the time of the addition. It had been shown previously [5] that CPTA is most effective when added at the time of inoculation (see Table 5). When NH_4Cl (5 mM) and CPTA (1 mM–280 ppm) were added together the combined effect was observed to be generally in between the extremes obtained with the use of each chemical singly. The time of the addition made a difference in the absolute amounts of the pigments that were isolated but generally a competitive effect was observed between the two bases.

The effect of β-ionone vapours on *Phycomyces* was shown by Reyes *et al.* [15] to be one of stimulating the synthesis of phytoene, phytofluene and β-carotene. When the mold was treated with both

Table 6. Effect of ammonium chloride on the biosynthesis of carotenoids by *P. blakesleeanus* strain C9

Carotenoids (μg/g dry basis)	Control	NH_4Cl (5 mM)
Phytoene	266	325
Phytofluene	78	85
ζ-Carotene	22	35
Neurosporene	16	24
Lycopene	851	845
γ-Carotene	25	29
β-Carotene	4	6
β-Zeacarotene	—	—
Total	1262	1349
Weight of mycelium (g/250 ml of medium)	1·98	2·19

β-ionone and CPTA greater amounts of phytoene, phytofluene, ζ-carotene, neurosporene and lycopene and somewhat less β-carotene was observed than when each was used singly. Where CPTA was used either with NH_4Cl or β-ionone, β-zeacarotene was not observed.

Ammonium chloride promoted the synthesis of β-carotene and β-zeacarotene in the high β-carotene mutant C 115. The addition of NH_4Cl at 5 mM had no effect on lycopene or β-carotene synthesis in the high lycopenelow β-carotene mutant C 9 (Table 6).

DISCUSSION

It is possible to survey a large number of organic and inorganic compounds and compare their biological activity in terms of cell growth and production of a finite number of biological by-products. Differences in the levels of inhibition of various compounds may range from permeability problems associated at some level of cell structure to inhibition of a given enzyme. However certain conclusions and generalizations can be made regarding structural/activity relationships of various chemicals based on the observed carotenoid biosynthesis in an organism.

Within the CPTA analog series (Tables 1 and 2) a few generalizations are possible: (1) In each case the oxy-ether is more active than the thiol ether. (2) The removal of the 4-chloro group or substitution with another phenyl ring deactivating group results in a loss of activity. (3) The removal of the phenyl group results in almost a complete loss of activity. (4) The formation of an N-oxide completely changes the nature of the inhibition. (5) Substitution of the ethyl groups with H eliminates almost any effect. (6) A di-2(4-chlorophenylthio)-ethyl group is toxic and a tri-2(4-chlorophenylthio)-ethyl group results in an additional accumulation of phytoene in an otherwise normal culture.

Within a broader sense the compounds that we have assayed would appear to fall into several categories: (1) Those that inhibit β-carotene and form lycopene in its place. Invariably β-zeacarotene drops out and γ-carotene accumulates. Most, but not all compounds tested with an unshared pair of electrons on a nitrogen atom had some activity in this manner; although their activity was not limited to the cyclization reaction. (2) Those compounds that stimulate β-carotene synthesis usually also stimulate the formation of β-zeacarotene. β-Ionone, NH_4Cl and urea were reported in the present paper to have this action and were shown to moderate the action of CPTA on the carotene forming system. Other types of compounds with this activity often are of a terpenoid nature. (3) Compounds that inhibit the stepwise dehydrogenation of phytoene to the more unsaturated polyenes. Diphenylamine has been the classic example of this type of inhibition. Many of the compounds that inhibit cyclization or stimulate β-carotene formation also stimulate phytoene and its products and thus some dual effects can be seen. Monophenyl amine (aniline) and triphenyl amine do not have the same effect even at higher concentrations. The simple conversion of the amine [2-(4-chlorophenyl)-triethylamine] to the N-oxide [2-(4-chlorophenoxy)-triethylamine N-oxide] changed a CPTA analog from a type 1 to a type 3 compound. (4) A fourth and rather curious type of inhibition has been mentioned before [10] and is shown by hydroxylamine hydrochloride. These compounds inhibit the formation of intermediates between phytoene and β-carotene. These have been discussed previously [10].

Finally, the data of this paper and the previous ones [5,10] allow no firm conclusion about the biosynthetic route to β-carotene. If one selects an inhibition of type (1) or type (3) above it would be possible to obtain evidence for one or the other. When all the evidence is taken together one would have to conclude at this point that both pathways are operative.

EXPERIMENTAL

P. blakesleeanus strains C 115 and C 9 were kindly supplied by Dr. M. Delbrück of the California Institute of Technology, Pasadena, California, U.S.A. CPTA and its analogs were kindly supplied by AmChem Products, Inc., Ambler, Pennsylvania, U.S.A.: β-ionone and 4-[β-(diethylamine)-ethoxy]-benzaldehyde were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Methylamine and dimethylamine were obtained from Fisher Scientific Co., Medford, MA. All other chemicals were obtained from Eastman Kodak Co., Rochester, N.Y. Solvents were distilled before use. A Cary 14 was used for the spectrophotometric measurements.

Mold growth conditions. Equal vol. of spore suspension of the respective strains of *P. blakesleeanus*, in sterile H_2O, were added to eq. vol. (250 ml) of standard sterile medium [1] contained in a 1 liter flask. The mold was allowed to grow in a controlled environment incubator as described previously [5]. The stock solutions of chemicals in H_2O (sterilized by filtration) were added in the concn of 1 and 5 mM of medium either 24 hr after or at the time of inoculation. The mold was allowed to grow

for 4 days in each case before harvesting. Aniline, diphenylamine and triphenylamine were dissolved in 95% EtOH and added to the medium. The final conc. of EtOH in the medium was 0·6%. Control cultures were also grown in the presence of the same amount of EtOH.

Extraction and chromatographic separation of carotenoids were made according to the methods described earlier [16].

Synthesis of CPTA analogs (*Table 2*). All chemicals were obtained from Aldrich Chimical Co., Milwaukee, WI., in highest purity grade available. *2-(4-chlorophenylthio) triethylamine HCl* (*CPTA*). Prepared from *p*-chlorothiophenol and 2-diethylaminoethyl chloride–HCl [17]. Recrystallized from iso-ProH. HCl salt, m.p. 125–126° (lit. [1] 123–124·5°). IR and UV spectrum were identical to an authentic sample of CPTA (m.p. 125–126°) obtained from AmChem Products, Inc., Ambler, Pa. *2,2'-bis(4-chlorophenylthio) diethylamine HCl.* Prepared by reacting diethanolamine with $SOCl_2$ according to the procedure of Ward [18] to yield dichloroethylamine–HCl, m.p. 214–216° (lit. [18,19] 216–217°). Di-CPTA was prepared by reacting dichloroethylamine HCl with *p*-chlorothiophenol in an analogous manner as CPTA [17]. Recrystallized from EtOH–Et_2O, m.p. 120–122°. *2,2',2''-tris(4-chlorophenylthio)-triethylamine HCl.* Prepared by reacting triethanolamine–HCl with $SOCl_2$ according to the procedure of McCoombie and Purdie [20] to yield trichloroethylamine–HCl, m.p. 129–130° (lit. [18,20] 130–131°, 133·5°). Tri-CPTA was prepared by reacting trichloroethylamine–HCl with *p*-chlorothiophenol in an analogous manner as CPTA [17]. Recrystallized from EtOH–Et_2O, m.p. 157–159°. *2-(4-chlorophenylthio)-ethylamine–N-ethyl–HCl.* Prepared by reacting 2-(ethylamino)-ethanol with $SOCl_2$ according to the procedure of Parkkari *et al.* [21] to yield 2-(ethylamino)-ethyl chloride–HCl, m.p. 220–221·5° (lit. [21,22] 218–219°, 223°). 2-(4-chlorophenylthio) ethylamine–N–ethyl–HCl was prepared by reacting *p*-chlorothiophenol in an analogous manner as CPTA [17]. Recrystallized from EtOH–Et_2O, m.p. 135–136°. *2-(4-chlorophenylthio) ethylamine–HCl.* Prepared by reacting 2-chloroethylamine–HCl with *p*-chlorothiophenol according to the procedure of Kulka [23] to yield 2-(4-chlorophenylthio)-ethylamine. The free amine was extracted into Et_2O and dry HCl gas was bubbled through the solution to form the HCl salt. Recrystallized from EtOH, sinc. 148–150°, m.p. 230–232° (lit. [24] sinc. 147–152°, m.p. 231–233°). IR spectrum was identical to an authentic sample of the free amine obtained from the Alfred Bader Library of Rare Chemicals, Aldrich Chemical Co., Milwaukee, Wisc.

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