Inhibition of Carotene Biosynthesis in Cell Extracts of *Phycomyces blakesleeanus*

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Cell extracts of the C115 (β-carotene-accumulating) strain of *Phycomyces blakesleeanus* were incubated with either [2-^{14}C]MVA or [1-^{14}C]IPP and a range of possible inhibitors of carotenogenesis, including bleaching herbicides, biphenyl compounds and geranylacetone. Several of these compounds were potent inhibitors of β-carotene formation and caused the accumulation of phytoene. No other carotenes were found to accumulate, *in vitro*. The structures of these inhibitors, compared to that of phytoene, suggest that they affect the enzymic activity of "phytoene dehydrogenase", possibly by competitive inhibition.

Introduction

A wide range of compounds are known to inhibit the biosynthesis of carotenoids. These include bleaching herbicides [1], algal excretion products [2], diphenylamine and related compounds [3, 4] and a large number of onium derivatives [5]. In most cases, especially in plant tissues, the desaturation of carotenes is affected, resulting in the accumulation of phytoene and/or β-carotene [6]. Most studies on the mode of action of inhibitors of carotenoid formation have used whole cells or tissues, mainly because of the lack of effective carotenogenic enzyme systems from photosynthetic tissues. Although this problem has been somewhat alleviated with the development of an active preparation of thylakoids from *Aphanocapsa* [7, 8], cell extracts of the fungus *Phycomyces blakesleeanus* represent one of the best characterized cell-free systems yet available for *in vitro* studies on carotene biosynthesis. In this publication we describe the effects of a range of compounds, including bleaching herbicides, on carotenogenesis by this model system.

Abbreviations: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; J852, 4-isobutoxy-2-isopropylamino-6-methyl-pyrimidine.

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Materials and Methods

The C115 *car S42 mad 107* (−) strain of *Phycomyces blakesleeanus* was obtained from the culture collection of the Departamento de Genética, Universidad de Sevilla, Spain. Its growth and maintenance conditions have been described previously [9].

The preparation of cell-free extract, incubation conditions, and the extraction and purification of radioactive carotenes have been described in detail in earlier publications [10, 11]. Radioassay of the purified carotenes was by liquid scintillation counting [12]. Inhibitors (10 µl) were added in methanol solutions prior to addition of cell extracts to the incubations. Methanol (10 µl) was similarly added to control incubations.

Results and Discussion

As inhibitors of carotene formation, neither fluridone nor J852 were effective *in vitro*, as they are in photosynthetic tissues. In both cases, their I_{50} values are significantly (−) lower in higher plants than the concentrations used in this study. Difunon was more inhibitory and caused a significant accumulation of phytoene, while amitrole prevented the formation of all carotenes, but apparently stimulated squalene biosynthesis (Table I). None of these four herbicides caused an accumulation of ζ-carotene, in contrast to reports of the effect of both

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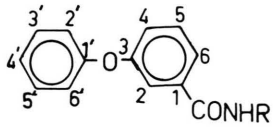
Table I. The effect of certain bleaching herbicides on carotene formation in cell extracts of *Phycomyces*.

Treatment	% of Total lipid incorporation		Ratio phytoene/ β-carotene	Reported effect in plants [see ref. 6]
	phytoene	β-carotene		
Control	10.3	10.4	0.99	–
I Fluridone				
10^{-6} M	10.8	9.3	0.87	accumulation
10^{-5} M	14.6	10.6	10.3	of phytoene
10^{-4} M	25.1	4.9	12.0	
II Difunon				
10^{-6} M	35.2	11.5	3.07	accumulation of phytoene
III Amitrole				
10^{-5} M	0.31	0.26	0.06 *	accumulation of acyclic carotenes
IV J852				
10^{-5} M	12.7	11.0	1.15	accumulation of
10^{-4} M	20.6	12.5	1.65	ζ-carotene

All incubations were carried out with 1 μCi DL-[2-^{14}C]MVA.
* Incorporation into squalene increased 2.5-fold compared to the control.

Table II. Structure-activity relationships of N-alkylphenoxybenzamides (60 μM) on carotenogenesis in *Phycomyces* cell extracts.



Substituent	Incorporation [dpm]*		Ratio phytoene/β-carotene
	phytoene	β-carotene	
Control	8922	31281	0.29
R = $-CH_2CH_3$	18257	30838	0.59
R = $-CH_2CH_2CH_2CH_3$	44822	14810	3.0
R = $-CH_2CH_3$; 2′-Cl; 4′-CF_3	35944	17213	2.1
R = $-CH_2CH_3$; 2′-Cl; 4′-CF_3; 6-NO_2	21423	17795	0.77

* From 0.25 μCi [1-^{14}C]IPP.
Data are averages of 3 experiments, s.d. < 10%.

amitrole [13] and J852 [6] in higher plants. These differences between *in vitro* and *in vivo* sensitivites may be either a result of the carotenogenic enzymes being fundamentally different in *Phycomyces* and photosynthetic tissues, or because the principal target site of these herbicides is other than binding to the enzyme responsible for the metabolism of phytoene.

Another group of bleaching herbicides, the N-alkylphenoxybenzamides, were found to inhibit *in vitro* carotenogenesis (Table II). Their relative effectiveness, however, was related to the substi-

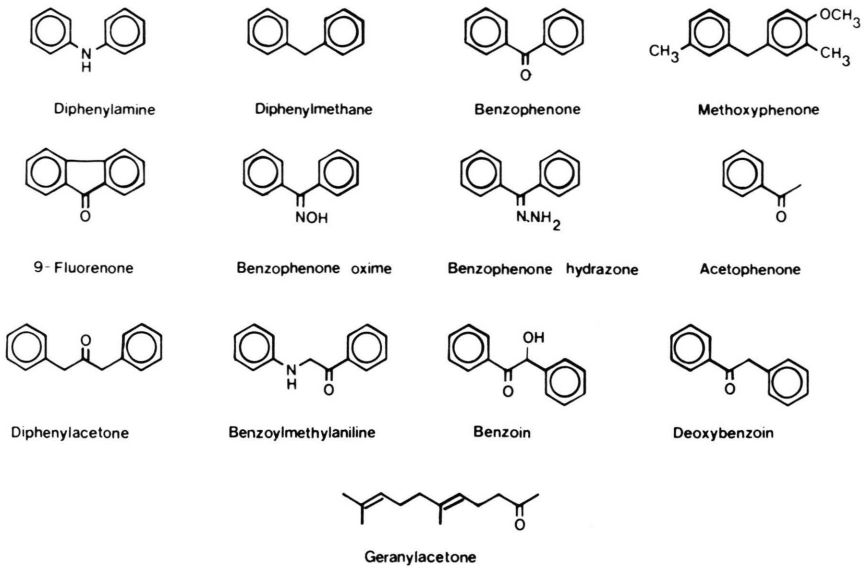


Fig. 1. Structures of biphenyl compounds, acetophenone and geranylacetone.

Table III. Inhibition of *in vitro* carotenogenesis by biphenyl derivatives and geranylacetone.

Compound [100 μM]	Incorporation [dpm]*		Ratio phytoene/β-carotene
	phytoene	β-carotene	
Control	43738	6240	7
Methoxybenzophenone	182825	144	1270
Diphenylamine	158603	304	522
Geranylacetone	175630	406	433
Diphenylmethane	144900	420	345
Benzophenone	127712	478	267
9-Fluorenone	172327	1373	126
Benzophenone oxime	223349	1110	93
Benzoylmethyl aniline	87351	1419	62
Deoxybenzoin	188869	3777	50
Diphenylacetone	168024	5310	32
Benzoin	48504	5426	9
Benzophenone hydrazone	70078	6492	9
Acetophenone	50386	6298	8

* From 0.5 μCi DL-[2-^{14}C]MVA.
See Fig. 1 for structures.

tuents attached to the amide group and to the aromatic rings. A butyl moiety on the amide group was the most effective, whilst the potency of ethylphenoxybenzamide was enhanced on addition of lipophilic groups to the 2'- and 4'-carbon atoms. Introduction of a 6-NO_2 group resulted in an approximately 3-fold decrease in inhibitory activity. These data confirm the results with both a thylakoid preparation of *Aphanocapsa* [14] and whole cells of *Scenedesmus* [15]. In this context, it is interesting to note that the 6-NO_2 derivative is still herbicidal *in vivo*, since it shows peroxidizing properties [16].

Of the 11 biphenyl compounds tested for inhibitory activity, 9 caused significant changes in the incorporation or radioactivity from [2-^{14}C]MVA into carotenes (Table III). The algal excretion product geranylacetone [2] was also a potent *in vitro*

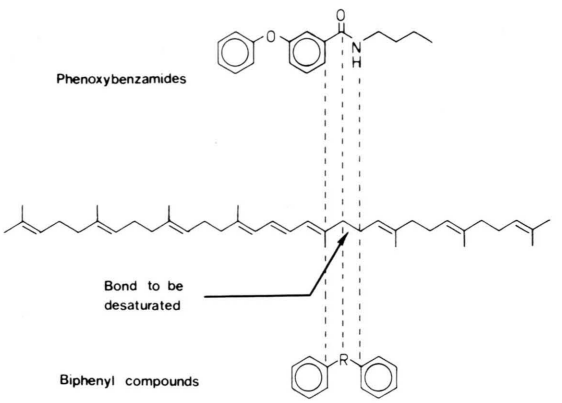


Fig. 2. Structural similarities between phytoene, alkylphenoxybenzamides and biphenyl compounds.

inhibitor of desaturation. A comparison of the structure-inhibitory activity relationships of these compounds with the C12–C8′ portion of the phytoene molecule (Fig. 2) provides support for the hypothesis that inhibitors of phytoene desaturation compete for the active site of the dehydrogenase [4, 17]. Diphenylamine has previously been shown to inhibit the activity, rather than the synthesis of phytoene desaturase [3]. Those compounds exhibiting maximum inhibitory activities fit most closely with this region of the phytoene molecule. They all contain at least one C=C bond, and in the case of the biphenyl compounds, have either 1 or 3 atoms between the rings (Fig. 1). Benzoin and deoxybenzoin, both of which have 2 inter-ring C atoms, and acetophenone which has a single aromatic ring, are poor inhibitors of phytoene metabolism. Apparently, the presence of either N-atoms or oxo groups has little effect on inhibitory activities, although a bulky, charged moiety such as a hydrazone presumable prevents efficient binding to the enzyme. The phenoxybenzamides can also be envisaged as competitive inhibitors of phytoene desaturase, due to the structural similarities between the C_{40} polyene chain and the side chain of the benzamide molecule (Fig. 2). The precise modes of action of these inhibitors must await a more detailed understanding of the enzymes involved in carotene formation.

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