# The Algal Excretion Product, Geranylacetone: A Potent Inhibitor of Carotene Biosynthesis in *Synechococcus*

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The algal excretion product geranylacetone was proved to be an effective and novel inhibitor of carotene synthesis in *Synechoccus* 6911. The application of geranylacetone resulted in an accumulation of phytofluene, indicating an inhibition of the conversion of this compound into $\zeta$-carotene. The *cis*-isomer of geranylacetone was as effective as the natural *trans*-isomer. The chlorophyll synthesis was only slightly affected.

Algae liberate a certain part of the incorporated $CO_2$ as organic material into the environment, the amount of which is greatly variable. Little is known on the mode of action of these substances. That primary metabolites as carbon sources can effect the growth of organisms in freshwater ecosystems has been demonstrated for cyanobacteria and heterotrophic bacteria [1–3]. Secondary metabolites can act as stimulators [4] or inhibitors [5–8]. The structures of a few of these compounds have been elucidated but there are no reports on their effector sites in the metabolism. During the course of studies of volatile excretion products of *Cyanidium caldarium* [9] we found a strong inhibitory effect of most of these compounds determined on the growth of microalgae. One of these nor-carotenoids, geranylacetone, showed to be an effective inhibitor of the carotene synthesis in *Synechococcus* 6911.

## Materials and Methods

### *Origin and cultivation of algae*

*Synechococcus* 6911 was a kind gift of Prof. R. Y. Stanier, Institut Pasteur, Paris, and *Anacystis nidulans* of Dr. N. G. Carr, Department of Biochemistry, Liverpool. The axenic nature during cultivation was controlled after each inocculation by plating on DEV nutrient agar (No. 11471, Merck/Darmstadt). 110 ml of algal suspension were cultivated in 300 ml Erlenmeyer flasks under the same conditions in a controlled environment incubator as previously stated [9].

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### *Separation of pigments*

The pigments were separated on modified 0.5 mm thin layer plates according to Hager and Meyer-Bertenrath [10] (Kieselgur G/Kieselgel G/$CaCO_3$, 12/6/3, w/w/w; solvent: petroleum ether (80–100 °C)/propanol-2/$H_2O$, 100/11/0.25, v/v/v). Phytofluene was further characterized on 0.5 mm Kieselgel G plates with the solvent petroleum ether (60–80 °C), purified on 0.5 mm MgO/Kieselgur G (1/1, w/w) plates with the same solvent [11] and detected by the greenish fluorescence by exposure to 366 nm UV light. The thin layer materials were purchased from Merck/Darmstadt.

### *Quantitative determination of pigments*

After addition of 10 ppm geranylacetone (1 : 10 diluted in ethanol) aliquots were removed from the Erlenmeyer flasks to give a total volume of 85 ml algal suspension. The algal pellet which was obtained after centrifugation for 20 min at $1700 \times g$ was successively extracted with 5 ml methanol and 5 ml methanol/petroleum ether 40–60 °C (10/3, v/v) [12]. The extracts were brought to dryness in a rotary evaporator at 25 °C under light protection. The residue was dissolved in a small amount of methanol/$CHCl_3$ (2/1, v/v) [13], streaked on a $20 \times 20$ cm thin layer plate covered with 0.5 mm reversed phase Kieselgur G [14]. The solvent system used was methanol/acetone/$H_2O$ (20/100/15, v/v/v). The separated pigments were eluted with ethanol. The molar extinction coefficient given by Seely and and Jensen [15] was used for quantification of chlorophyll a and that of $\beta$-carotene given in Davies [16] for cyclic carotenoids. Phytofluene was re-

corded on a Cary 14 spectrophotometer. The mean value of the 338 and 358 nm troughs in the absorption spectra was used to determine the height of the 347 nm peak. The conversion factor based on the value given by Davies [16] was adjusted to this procedure.

*Separation of geranylacetone isomers*

A mixture of *cis/trans*-geranylacetone was a kind gift of the BASF/Ludwigshafen. The isomers were separated by gas chromatography on a preparative column (1.8 m length, 10 mm i. d.) packed with 3% OV-17 on Chromosorb W AW DMCS (80 – 100 mesh) in a Hewlett Packard gas chromatograph 5750 G with a temperature program (110 – 180 °C, 4 °C/min). The nitrogen carrier gas flow was 120 ml/min, the split ratio 1 : 30. A total of 1.6 µl of the *trans*- and 2.8 µl of the *cis*-isomer was trapped after separation with a purity of 94.7% and 92.6%, resp. as determined with a WCOT glass capillary column coated with OV 101.

## Results

The test organism for the excretion product geranylacetone was the axenic cyanobacterium *Synechococcus* 6911. This alga forms a homogeneous and stable suspension. Its pigments can easily be extracted and separated.

*Synechococcus* 6911 is closely related to *Anacystis nidulans* [17]. In *Synechoccus*, β-carotene (β,β-carotene), β-cryptoxanthin (β,β-carotene-3-ol), zeaxanthin (β,β-carotene-3,3′-diol), caloxanthin (β,β-carotene-2,3,3′-triol) and nostoxanthin (β,β-carotene-2,3,2′,3′-tetrol) were found. Their identity was confirmed by co-chromatography with authentic pigments determined in *Anacystis* [18, 19] and their electronic absorption spectra. Quantitative determinations of the pigments revealed great differences in their percentage distribution during the growth phase. Old standing cultures (90 days) showed a much higher percentage of hydroxylated carotenoids than cultures of declining logarithmic growth. Table I presents the percentage distribution of pigments extracted from both types of cultures.

For inhibition experiments, geranylacetone was added to fast growing (declining logarithmic growth phase) cultures. Two days after addition of the inhibitor the color of the inhibited cultures changed to a more blue-green tone. The total amount

Table I. Percentage distribution (weight) of individual carotenoids in cultures of *Synechococcus* 6911 of stationary and declining logarithmic growth phase.

| Growth phase | Stationary | Declining logarithmic |
|---|---|---|
| Carotenoids | % weight | % weight |
| β-Carotene | 24.1 | 37.4 |
| β-Cryptoxanthin | 3.5 | 1.5 |
| Zeaxanthin | 39.8 | 37.9 |
| Caloxanthin | 18.6 | 15.7 |
| Nostoxanthin | 14.0 | 7.5 |

of carotenoids was reduced in comparison to that of chlorophyll a. If 20 ppm geranylacetone was added to a culture, the carotenoid synthesis was nearly quantitatively inhibited since chlorophyll a synthesis continued with only a slight decrease. Addition of 10 ppm geranylacetone resulted in a partial inhibition of the carotenoid synthesis. The rate of chlorophyll a synthesis remained nearly unchanged. The separation in individual carotenoids showed the inhibitor to effect in different ways the rate of synthesis of single carotenoids (Fig. 1). The synthesis of the main pigments β-carotene and zeaxanthin markedly decreased, but no significant change was noticed in the rate of synthesis of the more hydroxylated caloxanthin and nostoxanthin, in the first hours. In addition, a new carotene was found which was not present in growing cultures, however detectable in low concentrations in old

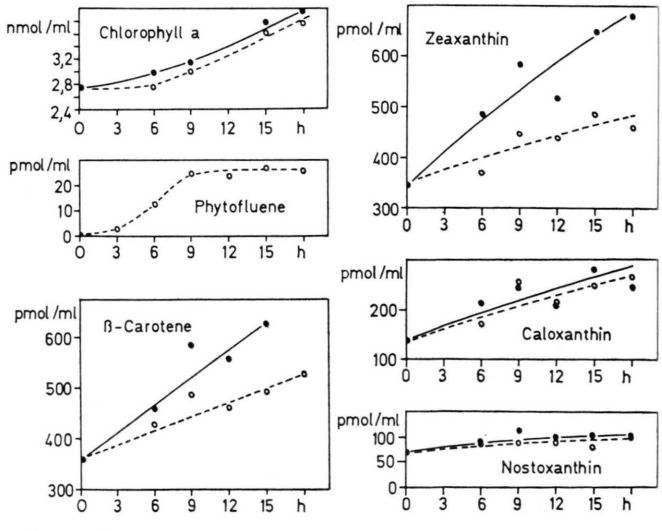


Fig. 1. Synthesis of carotenoids in untreated *Synechococcus* 6911 ●——● and during 10 ppm inhibition with geranylacetone ○ - - - ○. The inhibitor was added at 0 h.

Table II. Carotenoids (pmol/ml algal suspension) in *Synechococcus* after 24 h inhibition with *cis*-geranylacetone, *trans*-geranylacetone and a mixture of both.

| Hours<br>Inhibitors | 0<br>— | 24<br>— | 24<br>*cis* | 24<br>*trans* | 24<br>*cis/trans* |
|---|---|---|---|---|---|
| Phytofluene | — | — | 39 | 35 | 38 |
| β-Carotene | 509 | 643 | 501 | 463 | 485 |
| Zeaxanthin | 384 | 597 | 320 | 303 | 344 |
| Caloxanthin | 207 | 338 | 228 | 193 | 228 |
| Nostoxanthin | 108 | 144 | 132 | 123 | 136 |
| Chlorophyll a | 2550 | 4440 | 3980 | 3770 | 4230 |

stationary phase cultures. The TLC properties, the electronic absorption and fluorescence spectrum showed the characteristics reported for phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene). After 3 hours application of geranylacetone the accumulation of phytofluene can be measured and its amount increases until the 9th hour.

The geranylacetone used for the experiments was a mixture of the *cis/trans*-isomers (57/43, v/v). Since only the *trans*-geranylacetone is a natural product excreted by *Cyanidium* the isomers were separated by preparative gas chromatography and added to cultures of *Synechococcus* (each 10 ppm). Table II indicates the results obtained.

## Discussion

The strong inhibition of carotene synthesis in *Synechococcus* points to an unexpected feature of geranylacetone which was found to be an excretion product of the alga *Cyanidium caldarium* [9]. A partial inhibition of carotene synthesis was achieved with $5 \times 10^{-5}$ M and a complete inhibition with $10^{-4}$ M addition. The effective concentrations are within the same range as those for the well known inhibitors diphenylamine ($10^{-3} - 10^{-4}$ M in *Flavobacterium* [20]) or San 6706 ($10^{-4} - 10^{-6}$ M in *Triticum* [21], *Raphanus* and *Hordeum* [22]). The primary inhibition seems to be specific for the carotene synthesis. The formation of chlorophyll a is only slightly affected. The small decrease may be the result of the reduced light protection by the reduced level of carotenoids. There is strong evidence for geranylacetone to be an effective inhibitor of the conversion from phytofluene into ζ-carotene as indicated by the accumulation of the former product. The concentration of phytofluene is below the detection limit in fast growing cultures though in old stationary phase cultures it can be found in trace amounts. Only a very few inhibitors have the same inhibition site [23] as has been demonstrated for geranylacetone. The application of most inhibitors known, results in the accumulation of phytoene, lycopene or a series of acyclic carotene precursors [24 – 27]. Also a block in this step of biosynthesis is very seldom found in mutants [28]. The formation of polyhydroxycarotenes is not directly effected by geranylacetone. If the inhibition of total carotenoid synthesis is complete the formation of polyhydroxycarotenes continues for a certain time at the expense of β-carotene and zeaxanthin. The inhibition of the phytofluene turnover is not only due to the natural *trans*-geranylacetone but can be found with *cis*-geranylacetone, too. No significant differences in the effectivity were found for both.

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