Geranyl Derivatives as Inhibitors of the Carotenogenesis in *Synechococcus* PCC 6911 (Cyanobacteria)

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The ability of several geranyl derivatives to inhibit carotenoid synthesis in *Synechococcus* (Cyanobacteria) was measured using a cyanobacterial bioassay. The inhibitory activity of different molecules varied according to chain length and substituents. Pseudoionone, farnesyl acetate, geranyl acetate, geranial and farnesol proved to be the most active derivatives tested. 6-Methylhept-5-en-2-one, farnesyl acetone, linalool and geranyl linalool exhibited no effect when employed in concentrations as high as 100 ppm. Besides phytofluene, ζ-carotene was accumulated after application of sublethal amounts of active geranyl derivatives. Cell growth and chlorophyll *a* synthesis were only slightly affected under conditions were carotenoid synthesis was markedly inhibited.

While studying the biological effects of different volatile algal excretion products, we found that geranyl acetone exhibits a strong inhibitory effect on the growth of cyanobacteria [1]. In a subsequent, more detailed investigation a massive reduction in carotenogenesis was found when geranyl acetone was employed in sublethal amounts [2]. Since several constituents or excretion products of cyanobacteria and algae have been characterized in recent years which are structurally related to this compound [3], we have identified the essential structural elements of this compound which are responsible for its inhibitory action. The variation of the molecular structure revealed a new family of inhibitors characterized as geranyl derivatives. The chemical nature of these compounds is significantly different from that of those molecular species which have hitherto been shown to influence the process of carotenogenesis [4, 5].

Materials and Methods

Cultivation of Synechococcus

Synechococcus PCC 6911 was obtained from the Pasteur Culture Collection, Paris. 300 ml Erlenmeyer flasks containing 100 ml of suspension were used for its cultivation. The flasks were incubated in a controlled environment incubation shaker (New Brunswick G 27) at 27 °C, illuminated with 3 fluorescent tubes (1400 lx), shaken at 120 strokes/min and indirectly supplied with a 0.27% (v/v) CO_2/air mixture (450 ml/min). The medium used and the procedure for checking sterility of *Synechococcus* after each transfer have been published [6].

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Cyanobacterial bioassay

Synechococcus cultures at the end of the exponential growth stage were diluted with fresh medium supplemented with 20 mM $NaHCO_3$ to give a final chlorophyll *a* concentration of 0.3 µg/ml. 100 ml samples of the diluted suspension were transferred to Erlenmeyer flasks with ground glass stoppers under axenic conditions and incubated as above. Various amounts of 10% stock solutions of inhibitor in ethanol were added to the experimental cultures. The highest equivalent amount of ethanol alone was included in untreated reference cultures. The growth rates were determined by reading the optical density of samples at 550 nm in a spectrophotometer (Zeiss PM 2 K) twice a day.

Separation and quantitative determination of pigments

Chlorophyll *a* and the total carotenoids were determined in an ethanolic extract obtained from 5 ml of a cyanobacterial suspension. The molar extinction coefficient given by Seely and Jensen [7] and the equation carotenoid (nmol/ml) = $8.27 \times A_{477} - 0.19 \times A_{665}$ were used for the quantitative determination of chlorophyll *a* and the total carot-

enoids, resp. For the determination of individual carotenoids, 195 ml samples were necessary. The extraction procedures and the separation on Kieselgel G (Merck) plates with light petroleum as the solvent were essentially the same as previously described [2].

Determination of phytofluene

Phytofluene was determined by absorption spectrometry or by measurement of its fluorescence on a Perkin Elmer fluorescence spectrophotometer (model MPF-3). Excitation wavelength was 366 nm, the emission wavelength 490 nm [8]. 100 ml of cyanobacterial suspension were extracted with ethanol, the pigment solution was brought to dryness in a rotary evaporator and the carotenes redissolved in light petroleum. This solution was applied to a Al_2O_3-column and the carotene fraction eluted with light petroleum containing 2% diethyl ether. Phytofluene in this fraction was determined by the fluorometric method without any further treatment.

Inhibitors

Origin, trivial and systematic names of the compounds tested are given below: mesityloxide (4-methylpent-3-en-2-one), Fluka; citronellol (3,7-dimethyloct-6-en-1-ol), Roth/Karlsruhe; geraniol (3,7-dimethylocta-*trans*-2,6-dien-1-ol), Roth/Karlsruhe; methyl geranate (methyl 3,7-dimethylocta-2,6-dienoate, mixture of *cis*/*trans*-isomers), Firmenich Genève; geranyl acetate (3,7-dimethylocta-*trans* 2,6-dienyl acetate), Fluka; alloocimene (2,6-dimethylocta-2, *trans* 4, *trans* 6-triene), Fluka; neo-alloocimene (2,6-dimethylocta-2, *trans* 4, *cis* 6-triene), Fluka; *trans*-geranyl acetone (6,10-dimethylundeca-*trans* 5,9-dien-2-one), Fluka; 2,6-dimethylocta-2, *trans* 6-diene, Pfaltz and Bauer; 2,6-dimethylocta-2, *cis* 6-diene, EGA-Chemie/Steinheim. The following substances were obtained from BASF/Ludwigshafen: farnesyl acetone (6,10,14-trimethylpentadeca-5,9,13-trien-2-one, mixture of *cis*/*trans*-isomers); pseudoionone (6,10-dimethylundeca-3,5,9-trien-2-one, mixture of *cis*/*trans*-isomers); linalool (3,7-dimethylocta-1,6-dien-3-ol); geranonitrile (3,7-dimethylocta-2,6-diene nitrile, mixture of *cis*/*trans*-isomers); nerolidol (3,7,11-trimethyldodeca-1,6,10-trien-3-ol, mixture of *cis*/*trans*-isomers); geranyl linalool 3,7,11,15-tetramethylhexadecan-1,6,10,14-tetraen-3-ol, mixture of *cis*/*trans*-isomers); 6-methylhept-5-en-2-one; farnesol (3,7,11-trimethylundeca-2,6,10-trien-1-ol, mixture of *cis*/*trans*-isomers; farnesyl acetate (3,7,11-trimethylundeca-2,6,10-trienyl acetate, mixture of *cis*/*trans*-isomers). Geranial (3,7-dimethylocta-*trans* 2,6-dien-1-al) was obtained after oxidation of geraniol with activated MnO_2 and separated on a silica gel column.

Results

Bioassay with Synechococcus

The degree of growth retardation of the cyanobacterium *Synechococcus* PCC 6911 was used to determine the biological potency of different terpenoid inhibitors. Under axenic conditions, increasing amounts of each compound to be tested were added to exponentially growing/shaking cultures of this organism. To prevent a loss of the more volatile inhibitors by evaporation, closed vessels were used. During the period of the assay, no difference in growth was noticed under these conditions as compared to open shaking cultures when bicarbonate was added as the carbon source. The growth was followed over 3–4 generations by reading the optical density at 550 nm. Inactive compounds, such as 6-methylhept-5-en-2-one, mesityloxide, farnesyl acetone, linalool and geranyl linalool, could be added in concentrations as high as 100 ppm without significant effect on the growth (Fig. 1 A and B). When active compounds (*e.g.* geraniol, pseudoionone, farnesol, geranyl acetate) were applied, low concentrations did not affect the growth rate during the first two generations. However, high degrees of inhibition were noted at later stages (Fig. 1 C and D). By increasing the inhibitor concentrations, the inhibition stage was shifted to earlier times. The activities of the different derivatives tested are listed in Table I. In each case, a range between the highest concentration tested which had no effect and the lowest concentration tested that measurably inhibited the growth of *Synechococcus* is stated. Low concentrations of the active compounds resulted in retarded growth rates. Higher concentrations caused bleaching of the cultures and cell death.

Occurrence of carotene precursors

Among the active geranyl derivatives the inhibiting effect of pseudoionone was studied more

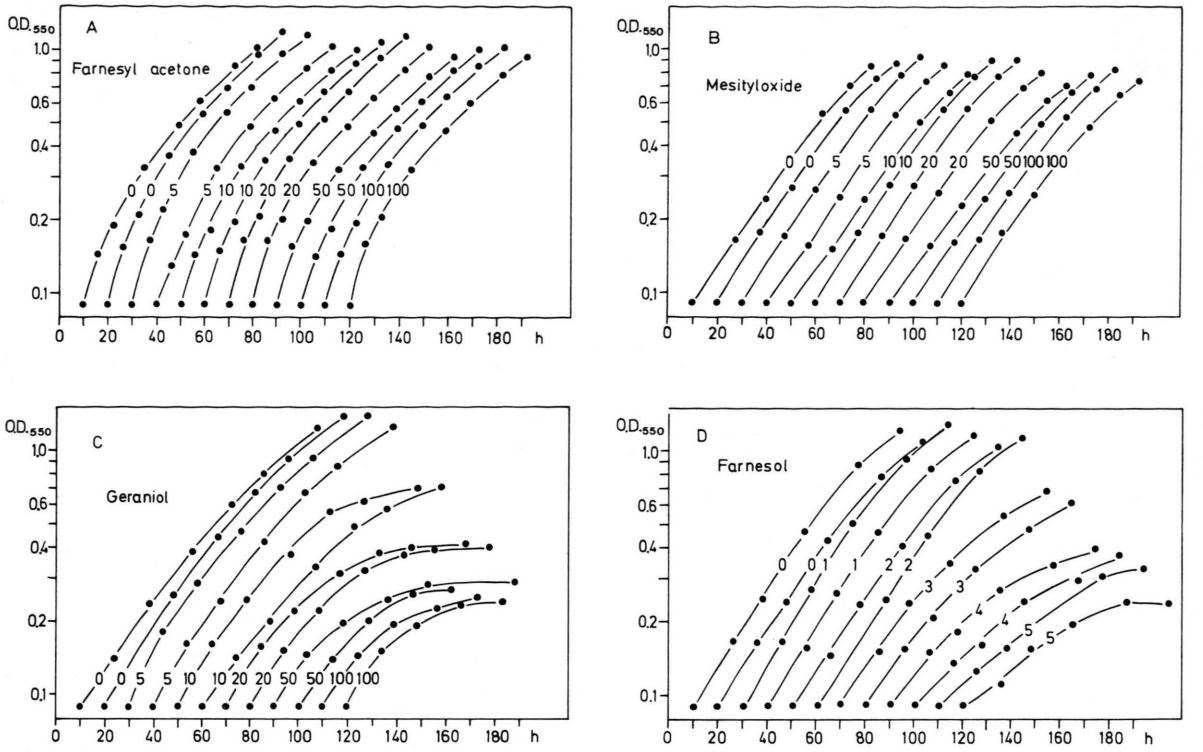

Fig. 1. Growth curves of *Synechococcus* as determined by optical density at 550 nm after addition of different concentrations (ppm) of farnesyl acetone (A), mesityloxide (B), geraniol (C) and farnesol (D). The reference samples contained the highest amount of ethanol added to the experimental cultures. Each curve represents a single culture. Cultures with different concentrations of a compound, each in duplicate, were started at the same time, but for reasons of clarity, the curves are shown staggered at intervals of 10 h on the time axis.

closely. Since geranyl acetone was known to result in accumulation of phytofluene in the cells of *Synechococcus* [2], experiments were performed to look for carotene precursors. Besides phytofluene ζ-carotene was also detected in pseudoionone-inhibited cells. Both pigments were undetectable in exponentially growing cultures. The identity of these carotenes was deduced from their electronic spectra, their R_f-values on thin layer plates and, in the case of phytofluene, from the fluorescence spectrum. Reference compounds used were isolated from carrots. The accumulation of these two carotene precursors was also noted during growth inhibition with *trans*-dimethyloctadiene, *cis*-dimethyloctadiene, geranial, geraniol, *trans*-geranyl acetone and farnesyl acetate.

Pigment synthesis in pseudoionone inhibited cultures

After application of low amounts (3 ppm) of pseudoionone to cultures of *Synechococcus* characteristic patterns of inhibition could be observed. Only very slight effects on growth and chlorophyll *a* synthesis were observed in these experiments, in contrast to the marked inhibition of carotenoid synthesis (Fig. 2). The reduction in the amount of total carotenoids was mainly due to decreased synthesis of the hydroxylated compounds zeaxanthin and caloxanthin. The β-carotene concentration, on the other hand, differed only slightly from that of an untreated culture. Phytofluene and ζ-carotene were rapidly accumulated reaching constant levels after about 17–24 h (Fig. 3). The onset of phytofluene accumulation could be detected as early as 0.5 h after application of pseudoionone (Fig. 4). The reversibility of the inhibition was demonstrated when pseudoionone was washed out with new medium after a 30 h incubation. Growth and carotenoid synthesis in such cultures were essentially the same as in untreated cultures. Phytofluene and ζ-carotene were remetabolized within 5–10 h.

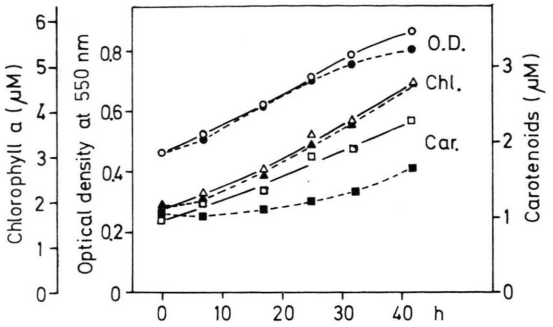

Fig. 2. The effect of pseudoionone (3 ppm) on the growth ●---● (determined by the optical density at 550 nm), synthesis of chlorophyll *a* ▲---▲ and total carotenoids ■---■ in *Synechococcus*. Pseudoionone was added at time zero. The data of the untreated reference culture are presented in open symbols.

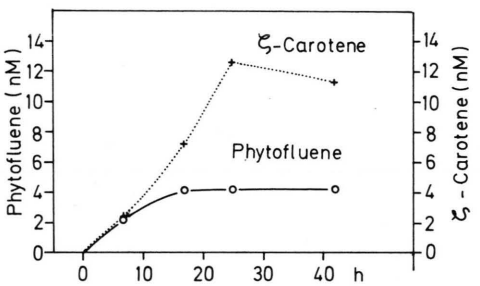

Fig. 3. The formation of ζ-carotene and phytofluene after application of 3 ppm pseudoionone (same experiment as stated in Fig. 2).

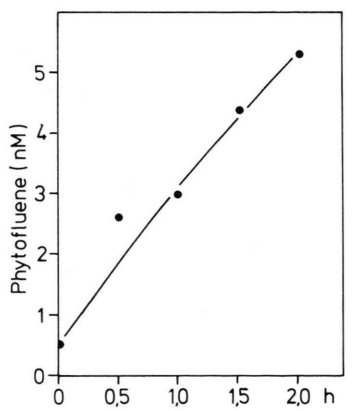

Fig. 4. Short time effect of the addition of pseudoionone on the accumulation of phytofluene. *Synechococcus* was precultivated for 12 h. At time zero 3 ppm pseudoionone were added to 100 ml cultures and the phytofluene concentrations determined by fluorescence spectrometry.

Table I. Effect of different terpenoid inhibitors on the growth rate of *Synechococcus*. The inhibitory potency is given as a concentration range from the highest inactive to the lowest active doses (in ppm) of the compounds assayed.

ppm	Inhibitor
> 100	Mesityloxide
> 100	6-Methylhept-5-en-2-one
0-5	trans-Geranyl acetone
> 100	Farnesyl acetone
> 100	Linalool
5-7.5	Nerolidol
> 100	Geranyl linalool
2-3	Farnesol
0-5	Farnesyl acetate
2-3	Pseudoionone
5-10	Geraniol
0-3	Geranial
0-5	Methyl geranate
0-2.5	Geranyl acetate
0-10	Geranonitrile
20-30	trans-Dimethyloctadiene
10-15	cis-Dimethyloctadiene
5-10	Alloocimene
5-10	neo-Alloocimene
20-50	Citronellol

However, small amounts of both compounds remained unchanged during the next 20 h indicating the possible existence of an inactive pool of these components.

Discussion

In the present study, the structural elements responsible for the inhibitory action of geranyl derivatives on cyanodacterial growth and carotenogenesis were elucidated. Chain length, as well as position and nature of the substituents influence the activity. *trans*-Geranyl acetone is an efficient inhibitor but neither farnesyl acetone, containing one additional isoprene unit, exhibited any activity nor did 6-methylhept-5-en-2-one which has one isoprene less. The same is true for the even smaller molecule of mesityloxide. A strong effect of chain length was also observed with linalool derivatives. Nerolidol exhibited an inhibition threshold at 5–7.5 ppm. However, both its lower and higher homologues, linalool and geranyl linalool, were inactive. In general, pure alkenes (alloocimene, dimethyloctadienes) were less efficient than those with a terminal substituent. A wide range of different substituents lead to an active substance, such as hydroxy, aldehyde, nitrilo and acetoxy groups or methyl esters. With the exception of *trans*- and *cis*-dimethyloctadiene, differences in the activity of *cis*/*trans*-isomers were within experimental error. Introduction of an additional double bond (pseudoionone) into geranyl acetone did not result in a change of activity. However, an increase of activity was observed with alloocimene in comparison to dimethyloctadiene which had only weak activity. Reduction of the ethyleniden group lead to marked loss of activity as demonstrated with citronellol.

As a result of these findings, predictions can be made for the activity of molecules not yet studied. This is of particular interest for algal constituents or excretes which exhibit similar structural elements. Geraniol and nerol have been detected as excretion products of *Synechococcus* [9], while *trans*-geranyl acetone and methyl geranate have been observed with *Cyanidium* [10]. The geranyl acetone derivative 6,10-dimethyl-5,9-undecadien-2,8-dione has been characterized as a constituent of the marine brown alga *Cystoseira crinita* [11].

The accumulation of phytofluene and ζ-carotene is a common feature of all active geranyl derivatives so far studied. An inhibition of the dehydrogenation steps from phytofluene to ζ-carotene and ζ-carotene to neurosporene can be postulated. The rapid onset of phytofluene accumulation after application of the inhibitor points rather to a direct interaction of this moleule with the enzymes involved than to an effect on the *de novo* synthesis of enzymes. Whether a modification of the enzymes or a competitive inhibition is responsible for the accumulation of phytofluene ad ζ-carotene can not be deduced from experiments performed with intact cells. It is noteworthy, however, that these intermediates accumulate only to small concentrations as compared with the total carotene pool and soon reach a constant level. This points to a regulation mechanism involving these intermediates. Though the synthesis of hydroxylated carotenoids is markedly inhibited, a direct involvement of pseudoionone in the hydroxylation steps cannot necessarily be assumed. The reduced amounts of β-carotene resulting from the action of inhibitor may be used preferentially for the formation of photosynthetic units, essential for continuous growth of the cells. On the other hand, the hydroxylated carotenoids can be diluted out to a certain extent without loss of viability of the cells.

The activity of geranyl derivatives is not restricted to cyanobacteria. Inhibitory effects of geranyl acetone have been observed in the aquatic bacteria *Arthrobacter* and *Chromobacterium* [12]. Most species of the chlorophycees were less sensitive to geranyl derivatives (unpubl. results). The same was observed for citral and pseudoionone in the fungus *Phycomyces blakesleeanus* [13] and for citral in the yeast *Rhodotorula gracilis* [14] where much higher concentrations (250 ppm) had to be applied to inhibit the carotenoid synthesis. Cyanobacteria are the most sensitive organisms so far tested for these inhibitors.

Acknowledgements

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