Inhibition of Carotenoid Biosynthesis by Interaction of 2,6-Diphenylpyridine Derivatives with Phytoene Desaturation

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Physiological and biochemical properties of bleaching 2,6-diphenylpyridines have been investigated. Treatment of cress plantlets and *Anacystis* cultures resulted in decreased formation of colored carotenoids and a concurrent accumulation of 15-*cis*-pytoene. The direct interaction of this new class of herbicidal inhibitors on cell-free phytoene desaturation was demonstrated with a carotenogenic in vitro system using *Anacystis* membranes. Structure–activity studies with five derivatives showed for these pyridines the electronic equivalence of the ring–N=imine with the C=O element present in many other herbicidal phytoene desaturase inhibitors.

INTRODUCTION

Different heterocyclic compounds have been functionally characterized as bleaching agents in plants. Their primary mode of action is direct inhibition of phytoene desaturase, which results in decreased biosynthesis of colored carotenoids and subsequent photooxidation of chlorophyll in the light (Sandmann and Böger, 1989). Herbicidal inhibitors exhibiting this mode of action are, e.g., the phenylpyridazinone norflurazon (Sandmann et al., 1989), the phenylfuranone flurtamone (Sandmann et al., 1990), or tetrahydropyrimidinones (Babczinski et al., 1990). For a new class of herbicidal compounds, the 4-substituted-2,6-diphenylpyridines, bleaching activity was recently proposed (Kawamura et al., 1991).

MATERIALS AND METHODS

Cress plantlets (*Lepidium sativum*) grown in vermiculite under hydroponic conditions were treated postemergence (after 2 days of germination) for 14 days under continuous illumination (3000 lux) with bleaching compounds. Secondary leaves were extracted by homogenization in 3% (w/v) MgO/acetone. Pigments from filtrates were separated by HPLC on a Nucleosil RP-18 5 μm, 120 mm column using a linear gradient of (A) methanol/water (90/10 v/v) and (B) ethyl acetate (% B = 10–65 within 13.5 min). The mass spectrum was recorded on a Finnigan CH 5DF mass spectrometer under the following conditions: introduction of the sample by direct inlet (sample temperature 150 °C), ionization with an electron energy of 70 eV, and a source temperature of 200 °C. Photomixotrophic plant cell cultures of *Catharanthus roseus* (kindly provided by Prof. M. Zenk, University of Munich) were grown in Gamborg B5 medium containing 2,4-D (1 mg L^{-1}) and sucrose (20 g L^{-1}). Cultures were grown in 18 mL of medium (50-mL flasks, 2 mL of inoculum) at 24 °C illuminated with 3000 lux and shaking with 120 rev min^{-1}. Inhibitors were applied in acetone (100 μL). Packed cell volume was determined after centrifugation in graduated vials, and pigment absorbance was measured after extraction of culture (2 mL) by ultrasonication in 2 mL of petroleum ether/1-propanol (1:1 v/v) at 450 nm or by HPLC as described above.

Anacystis R2 (=*Synechococcus* PCC7942) and the *Fusarium* mutant SG4 were grown for 2 or 5 days, respectively, as previously described (Sandmann and Kowalczyk, 1989). *Anacystis* cells were harvested by centrifugation (12000*g*, 10 min). For determination of carotenoids, these cells were extracted in methanol containing 6% KOH by partitioning into 10% (v/v) diethyl ether/petroleum ether. Separation was by HPLC on a Spherisorb ODS-1, 5 μm, 250 mm column with acetonitrile/methanol/2-propanol (85:10:5 v/v/v) and a flow of 1 mL min^{-1}. For in vitro carotenogenesis, *Anacystis* cells were resuspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris-HCl) buffer, pH 8.0, containing 5 mM dithiothreitol (DTT), and broken in a French press at 500 bar. After centrifugation (12000*g*, 15 min), the membrane pellet was resuspended in the same buffer. *Fusarium* SG4 extracts, which form [^{14}C]geranylgeranyl pyrophosphate from [^{14}C]mevalonic acid, were obtained by suspending 0.1 g of powdered material in 0.8 mL of 0.4 M Tris-HCl buffer, pH 8.0, containing 5 mM DTT, and centrifugation (10000*g*, 10 min). The incubation mixture contained 0.2 mL of this supernatant, 0.1 mL of *Anacystis* thylakoid membranes equivalent to 0.15 mg of chlorophyll, ATP (5 μmol), NAD^+ (1 μmol), Mn^{2+} (3 μmol), and Mg^{2+} (2 μmol) made up to a total volume of 0.5 mL with water. The reaction was terminated after 2 h by addition of 2.5 mL of methanol containing 6% KOH. After saponification for 20 min at 60 °C, the carotenoids were partitioned into 10% (v/v) diethyl ether/petroleum ether and separated by HPLC (as above). Radioactivity of the elution peaks was determined by a radioactive flow detector (Ramona LS, Raytest, Straubenhardt, Germany).

RESULTS AND DISCUSSION

All structures of the substituted diphenylpyridines used in the present study are listed in Table I. Their synthesis has been described (Kawamura et al., 1991). For the lead compound B, physiological investigations were carried out using cress plantlets. From its structural similarity to other well-known bleaching herbicides such as fluridone, norflurazon, flurtamone, fluorchloridone, and diflufenican, the compound was expected to induce chlorotic symptoms in this preemergence test system, which has been used extensively in other cases, e.g., substituted phenyltetrahydropyrimidinones (Babczinski et al., 1990). Surprisingly, chlorotic symptoms cannot be observed in primary leaves as usual; postemergence conditions have to be applied, and only secondary and later leaves become chlorotic. This behavior is unique within chlorosis-inducing classes of compounds investigated by using the cress test system. Obviously, very peculiar physiological

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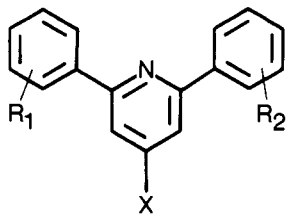
Table I. Pyridine Compounds Used in the Course of This Investigation



compd	R_1	R_2	X
A	3-Cl	H	SCH_3
B	3-CF_3	H	SCH_3
C	3-CF_3	3-Cl	SCH_3
D	3-CF_3	3-Cl	OC_2H_5
E	3-CF_3	2-Cl	OC_2H_5
F	3-Cl	4-Cl	SCH_3

Table II. Concentration of Chlorophylls and Carotenoids in Cress Leaves (A, nmol g^{-1} fw) of Control Plants or Seedlings Treated Shortly after Emergence with Compound F (1 g L^{-1}) and in *Anacystis* (B, μg/g dw) Grown in the Presence of 1 μM

pigments in	untreated	treated
(A) cress seedlings		
β-carotene	85.1	nd[a]
α-carotene	5.5	nd
neoxanthin	130.4	nd
violaxanthin	124.2	nd
lutein	358.9	6.1
chlorophyll *a*	352.6	26.0
chlorophyll *b*	285.8	7.9
phytoene	nd	747.7
(B) *Anacystis*		
zeaxanthin	4.20	0.43
β-cryptoxanthin	0.12	0.04
β-carotene	2.19	0.26
phytoene	<0.02	0.47

[a] nd, not detectable.

properties such as uptake, transport, or systemicity might cause this phenomenon.

The mode of action of diphenylpyridines has been investigated by looking for the accumulation of metabolic intermediates in chlorotic cress leaves. As revealed by HPLC of organic leaf extracts, a compound that cannot be observed in controls is drastically increased. It has been identified by UV spectroscopy and HPLC cochromatography with authentic phytoene. The latter has been produced by the application of the known carotenoid biosynthesis inhibitors of the tetrahydropyrimidinone type (Babczinski et al., 1990) and identified independently by UV and mass spectroscopy. A molecular ion of 544 was determined, and the spectrum of this compound resembles that of 15-*cis*-phytoene (Sandmann and Albrecht, 1990). From these results it can be assumed that chlorosis is caused by inhibition of carotenoid biosynthesis at the phytoene desaturase step. Loss of chlorophyll therefore should occur as a secondary effect.

Table II shows the carotenoid pattern of either cress leaves or *Anacystis* cells treated with compound F. In the case of cress, a comparably high dose was applied to achieve a high degree of bleaching, whereas *Anacystis* was grown in a concentration which resembles about twice the I_{50} value. In both photosynthetic organisms the effect was very similar. Xanthophylls as well as colored carotenes strongly decreased during treatment. Instead, large amounts of phytoene accumulated.

To exclude the possibility that inhibition of phytoene desaturation is caused by indirect or regulatory mechanisms, direct interaction of derivative B of Table I with phytoene desaturase was demonstrated by in vitro studies.

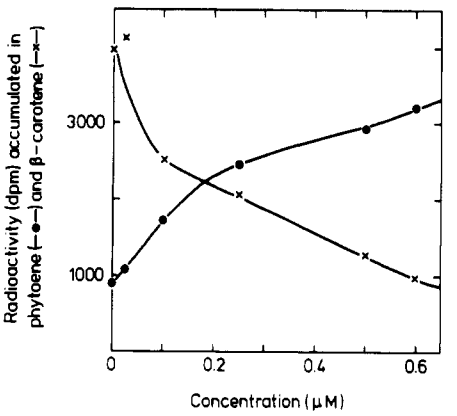


Figure 1. Interference of diphenylpyridine B with in vitro formation of phytoene and β-carotene from [^{14}C]geranylgeranyl pyrophosphate by *Anacystis* membranes.

Table III. Pigment Discoloration of Green Plant Cell Suspension Cultures of *C. roseus*

compd	concn, ppm	visual color	*E* (450 nm)	cell growth, mL pcv[a]
control		green	0.474	5.9
B	100	white-yellow	0.066	5.0
	10	yellow	0.107	5.3
	1	yellow-green	0.200	5.6
fluridone	100	white	0.041	1.0
	10	yellow	0.152	5.4
	1	yellow-green	0.423	5.7

[a] pvc, packed cell volume.

Thylakoid membranes from *Anacystis* convert [^{14}C]-geranylgeranyl pyrophosphate via [^{14}C]phytoene through the carotene biosynthetic pathway into [^{14}C]-β-carotene (Sandmann and Kowalczyk, 1989). Under our reaction conditions, accumulation of radioactivity in other carotene intermediates was negligible. Therefore, the in vitro synthesized β-carotene is an indicator for the activity of phytoene desaturase. Figure 1 shows the dependency of β-carotene formation on the CF_3-substituted pyridine derivative. With increasing concentrations, more phytoene is retained, which means that less phytoene is desaturated. Therefore, less β-carotene, the end product of this in vitro biosynthetic chain, is formed. Interaction of this pyridine derivative with phytoene desaturase is very similar to inhibition of this enzyme by, e.g., flurtamone (Sandmann et al., 1990) or norflurazon (Sandmann et al., 1989). For the latter herbicide it was shown that it is a reversible noncompetitive inhibitor of phytoene desaturase.

A green photomixotrophic *C. roseus* plant cell suspension culture has been used previously for structure–activity investigations (Babczinski and Zelinski, 1991). As compared to the phytoene desaturase inhibitor fluridone, which was introduced as standard herbicide, the dose–activity bleaching responses of compound B exceed those of the standard inhibitor, although the latter is more phytotoxic at high concentrations (Table III). Using a series of five compounds of diphenylpyridines (A–E of Table I), the results of structure–activity relationship studies with *Catharanthus* cells were compared to in vivo data from *Anacystis* (Table IV). Responses of four different parameters (i.e., pigment coloration at 450 nm, cell growth, contents of carotene and phytoene) demonstrate that substitution at C-4 of the pyridine ring with ethoxy (compound D) is slightly more active than 4-methylthio (C). Furthermore, meta substitution in both phenyl rings (C) is superior over single substitution (A, B), and meta substitution in the second phenyl ring (D) is more active than ortho substitution (E). In general,

Table IV. Structure–Activity Relationship Studies with *C. roseus* Cell Suspension Culture and *Anacystis* Suspension Cultures

compd	concn, ppm	*E* (450 nm)	cell growth, mL pcv[a]	carotene, %	phytoene, %	rank	*Anacystis* I_{50}, M	*Anacystis* rank
control		1.795	4.4	100	100			
D	100	0.007	1.9	0	18194	1	1.9×10^{-8}	1
	10	0.018	2.5	2	15849			
	1	0.289	3.4	40	11743			
	0.1	0.740	4.1	76	1791			
C	100	0.014	2.0	2	16524	2	1.6×10^{-7}	3
	10	0.019	2.7	3	15755			
	1	0.555	3.8	nd[b]	nd			
	0.1	0.804	4.1	87	1234			
A	100	0.016	2.1	2	16017	3	8.8×10^{-8}	2
	10	0.024	3.0	4	13715			
	1	0.741	3.9	nd	nd			
	0.1	1.058	4.2	101	313			
E	100	0.016	2.5	3	13939	4	5.5×10^{-7}	5
	10	0.030	3.0	4	9724			
	1	0.804	4.0	75	6383			
	0.1	1.304	4.5	103	240			
B	100	0.019	2.5	3	12987	5	2.5×10^{-7}	4
	10	0.102	3.5	17	8209			
	1	0.976	4.0	nd	nd			
	0.1	1.867	4.5	114	199			

[a] pcv, packed cell volume. [b] nd, not determined.

these results resemble the *Anacystis* data quite well. However, ranking positions of compounds A and C or B and E are exchanged. This ranking based on *Anacystis* I_{50} values corresponds exactly with the ranking based on biological structure–activity responses obtained in greenhouse experiments (data not shown). The structural variations indicate that in *Catharanthus* cells as well as in *Anacystis* pyridines with a OC_2H_5 group at position 4 of the phenyl ring are superior to those carrying SCH_3. A 3-Cl group (A) is more favorable than 3-CF_3 (B), and a second substituent such as Cl at position 3′ of the second phenyl ring (C) enhances this effect. Furthermore, herbicidal activity is decreased when one of the substituents is moved from position 3 or 3′ to 2 (D, E).

The new carotenoid biosynthesis inhibitors of the diphenylpyridine type fit into the well-known pattern of diphenyl-substituted heterocycles, e.g., fluridone [1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(*H*)-pyridone], tetrahydropyrimidinones, flurtamone [5-(methylamino)-2-phenyl-4-[3-(trifluoromethyl)phenyl]-3(2*H*)-furanone], or monophenyl-substituted heterocycles such as norflurazon [4-chloro-5-(methylamino)-2-[3-(trifluoromethyl)phenyl]pyradizin-3(*H*)-one] or fluorchloridone [1-(3-trifluoromethylphenyl)-3-chloro-4-(chloromethyl)-2-pyrrolidone]. Also ring-opened structures such as fluometuron [1,1-dimethyl-3-[3-(trifluoromethyl)phenyl]urea] have to be mentioned in this context. Chemical structures of all these bleaching herbicides are shown in Sandmann and Böger (1989) and Babczinski et al. (1990). Common to all of them is a carbonyl element either in the central position of the heterocyclic ring or in its equivalent position in the opened structure. Within the diphenylpyridines this element apparently is replaced by a ring–imine sp^2 nitrogen. Its free electron pair mimics the =O substituent at the sp^2 carbon atom within the former compounds. A similar structural replacement can be observed in different classes of electron flow inhibitors at photosystem II. In particular, *s*-triazine (e.g., atrazine) and *s*-triazinone herbicides (e.g., metribuzin) exhibit similar structural changes and have been proposed to use either the C=O or the –N= structural element for electronic interaction within the binding niche of PSII (Tietjen et al., 1991).

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Registry No. A, 116610-61-8; B, 116610-67-4; C, 144320-18-3; D, 144320-19-4; E, 144320-20-7; F, 116579-59-0; β-carotene, 7235-40-7; α-carotene, 7488-99-5; neoxanthin, 14660-91-4; violaxanthin, 126-29-4; lutein, 127-40-2; chlorophyll *a*, 479-61-8; chlorophyll *b*, 519-62-0; phytoene, 13920-14-4; zeaxanthin, 144-68-3; β-cryptoxanthin, 472-70-8; phytoene desaturase, 107544-21-8.