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SC-0051, a 2-benzoyl-cyclohexane-1,3-dione bleaching herbicide, is a potent inhibitor of the enzyme *p*-hydroxyphenylpyruvate dioxygenase

Arno Schulz[a], Oswald Ort[a], Peter Beyer[b] and Hans Kleinig[b]

[a]*Hoechst AG, 6230 Frankfurt/Main 80, Germany* and [b]*Institut für Biologie II, Universität Freiburg, D-7800 Freiburg i.Br., Germany*

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Growth inhibition of *Lemna gibba* plantlets by the bleaching herbicide, SC-0051 (2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione)) was alleviated by the addition of homogentisic acid to the growth medium. Homogentisic acid is a key intermediate in the biosynthesis of tyrosine-derived plant quinones as well as in tyrosine metabolism. The herbicide prevented the incorporation of radioactivity from [^{14}C]tyrosine into lipophilic plant metabolites and, in rat liver extracts, the herbicide inhibited the conversion of tyrosine to homogentisic acid. The enzyme *p*-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) from both *Zea mays* seedlings and liver tissues, was found to be subject to strong inhibition by SC-0051. Inhibition of plant quinone biosynthesis is a new mode of herbicidal action. One of the consequences of quinone depletion in plants in vivo is apparently an indirect inhibition of phytoene desaturation. The enzyme phytoene desaturase itself, however, is not afflicted by the herbicide.

p-Hydroxyphenylpyruvate dioxygenase; Phytoene desaturase; Enzyme inhibition; Herbicide; SC-0051

1. INTRODUCTION

The experimental compounds of the benzoyl cyclohexane-1,3-dione type, such as SC-0051 (2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione; proposed common name 'sulcotrione') comprise a relatively new class of herbicides for selective use in corn (*Zea mays* L.) and other crops ([1,2] and references therein). In susceptible plant species these herbicides cause strong bleaching effects which are accompanied by a decrease in chlorophyll and carotenoid levels as well as by a massive accumulation of phytoene [1,2]. These symptoms led to the hypothesis that the benzoyl cyclohexane-1,3-diones might inhibit the enzyme, phytoene desaturase [2], which is the target enzyme for most of the bleaching herbicides known so far (for review see [3]). In tests in vitro, however, even high concentrations of the herbicides showed no interference with the phytoene desaturase reactions [4], and it was speculated that metabolization of the herbicides by the plant might be necessary to render them active [4].

Some recent evidence suggests that the phytoene desaturation is a complex redox reaction in which quinones and factors regulating the redox state of quinones may play a major role [5,6]. In the present communication we provide evidence that the benzoyl cyclohexane-1,3-dione herbicides inhibit the enzyme, *p*-hydroxyphenylpyruvate dioxygenase (*p*-hydroxyphenylpyruvate:oxygen oxidoreductase EC 1.13.11.27), a key-enzyme in the biosynthesis of plastoquinones and tocopherols. The possible relationship between quinone biosynthesis and phytoene desaturation will be discussed.

Correspondence address: A. Schulz, Hoechst AG, Biol. Forsch. C, H 872N, 6230 Frankfurt/M 80, Germany. Fax. (49) (69) 305 7342.

2. MATERIALS AND METHODS

The herbicide, 2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexandione, was synthesized at Hoechst AG 15-*cis*-[^{14}C]phytoene was biosynthesized from [1-^{14}C]isopentenyl diphosphate as described [5]. L-[carboxyl-^{14}C]tyrosine (2.03 GBq/mmol) and L-[U-^{14}C]tyrosine (16.9 GBq/mmol) were purchased from Amersham Buchler, Braunschweig, Germany.

p-Hydroxy[1-^{14}C]phenylpyruvate was prepared from L-[carboxyl-^{14}C]tyrosine by L-amino acid oxidase catalysis as follows [7]: 0.185 MBq of tyrosine were incubated with 1 mg of rattle-snake venom (*Crotalus adamanteus*) and 1,000 U of catalase in 3 ml of 0.1 M phosphate buffer. The reaction was stopped by the addition of 0.6 ml of 1 N H_2SO_4, transferred to a 3 ml column of Dowex 50 WX8 (equilibrated with 0.1 M HCl) and eluted with either 100 ml 0.1 N HCl or 100 ml 2 mM *p*hydroxyphenylpyruvate (pH 3.2) carrier. The labeled *p*hydroxyphenylpyruvate was adjusted to pH 7 and stored at −80°C for no longer than three days.

Corn (*Zea mays* L.) seeds were surface sterilized and planted on sterile filter paper in the dark for 6 days at 25°. Sterile cultures of duckweed (*Lemna gibba* L.) were maintained at room temperature in 200 ml Erlenmeyer flasks on nutrient solution [8]. All other plants used in the experiments were grown in a greenhouse.

Plant pigments were analyzed by HPLC as described [2].

In vitro phytoene desaturation was measured with daffodil (*Narcissus pseudonarcissus* L.) chromoplasts. Chromoplasts were isolated on sucrose gradients as described [9], suspended in incubation buffer (Tris-HCl, pH 7.2, 100 mM; $MgCl_2$ 10 mM; dithioerythrol 2 mM) and homogenized with a French press. The homogenates were fractionated into membranes and stroma by centrifugation (180,000 × *g*, 2 h), the membrane pellet was washed in incubation buffer and, after a second centrifugation, resuspended in buffer. The desaturation assay was carried out in the dark at 27°C in a total volume of 0.5 ml containing

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ca. 3.2 mg membrane protein and 833 Bq [^{14}C]phytoene as described [5].

*p*Hydroxyphenylpyruvate dioxygenase (pOHPP-DO) was extracted from rat liver acetone powder [10] by mixing 600 mg of acetone powder with 5.6 ml H_2O. The slurry was stirred for 5 min at 4°C and then centrifuged for 10 min at 10,000 × *g*. The supernatant was collected and 0.1 mg/ml glutathione was added. For the preparation of *p*hydroxyphenylpyruvate dioxygenase from corn, etiolated plants were homogenized with 0.6 vols. of extraction buffer (20 mM phosphate buffer, pH 7.0; 0.14 M KCl; 0.1 mg/ml glutathione and 1% insoluble PVP) for 3 × 10 s in a Waring blendor (all steps performed at 4°C). The extract was filtered through miracloth and centrifuged for 20 min at 10,000 × *g*. The supernatant was used as a crude enzyme preparation and was eventually further purified by fractionated $(NH_4)_2SO_4$ sulfate precipitation. pOHPP-DO precipitated in the 20–40% saturation fraction. After dialysis, it could be stored at −80°C for several days.

For the TLC analysis of the transformation of pOHPP to homogentisic acid by rat liver extracts, 60 μl [^{14}C]tyrosine were first reacted with 6 mg of L-amino acid oxidase in 6.6 ml 0.1 M phosphate buffer (pH 7.2) plus 850 U catalase for 45min at 37°C. To 1 ml of this solution, 1 ml (16 mg protein) of rat liver extract was added, together with 1.4 mg sodium ascorbate, 0.3 mg dipyridyl and inhibitors dissolved in acetone or acetone alone. After 90 min at 37°C the reaction was stopped with 500 μl 3 N H_2SO_4, and 1 mg of unlabelled homogentisic acid was added. The reaction mixture was extracted with diethylether and analyzed on silica gel TLC plates with benzene:isoamyl alcohol:acetic acid (40:20:1.5) followed by autoradiography.

pOHPP-DO activity was measured by two different methods:

1. Continous measurements of activity were made with the enol-borate method [11]. To 8.5 ml borate buffer (0.42 M boric acid adjusted to pH 6.2 with 0.25 M Na_2HPO_4) 600 μl of 1.8 mM *p*hydroxyphenylpyruvate (HPP), 300 μl of a mixture of 0.1 ml 166 mM glutathione and 1 ml 3.3 mM 2,6-dichlorophenol-indophenol (freshly prepared) and 10 μl of phenylpyruvate tautomerase (Sigma) were added. The reaction mixture was kept at room temperature until the tautomerization of the HPP (measured as increase in absorbance at 308 nm) was completed. To 950 μl of the reaction mixture, 25 μl of acetone (or compound to be tested, dissolved in acetone) and 25 μl of enzyme preparation were added. The reaction was followed at 25°C by measuring the decrease in absorbance at 308 nm.
2. Endpoint measurements were made by the [^{14}C]CO_2 method [12] where the radioactive CO_2 released from [^{14}C]HPP during the enzyme reaction was trapped and measured. Enzyme assays were performed in 20 ml polyethylene scintillation vials. Two single use syringe needles (0.9 × 40 mm) were stuck through the vial cap. One of the needles was bent to form a hook and a 10 mm disk of filter paper was placed on the hook and moistened with 25 μl of methylbenzethonium hydroxide (Sigma). The second needle was used for injecting the start and stop solution into the vial.

The reaction mixture contained 1 ml 0.1 M phosphate buffer, pH 7.3; 2 g/l bovine liver catalase; 100 μl of a mixture of 3 mM 2,6-dichlorophenol-indophenol and 150 mM glutathione; 25 μl acetone (or compound to be tested dissolved in acetone) and enzyme in a total volume of 1635 μl. After 5 min preincubation at 37°C (liver enzyme) or 30°C (plant enzyme), 800 μl of [^{14}C]HPP were added to start the reaction. The reaction was stopped by adding 600 μl of 1 N H_2SO_4, incubated for another 15 min and radioactivity on the filter disks was counted in a liquid scintillation counter.

3. RESULTS

Cyperus monti plants were treated with SC-0051 (0.008 kg/ha), and pigments were analyzed by HPLC 4 weeks after treatment. The results obtained confirmed previous reports [1,2] that the bleaching effect of the herbicide (reduction of carotenoid and chlorophyll content) was due to an in vivo inhibition of phytoene desaturation: whereas in untreated control plants phytoene was detectable only in trace amounts, a massive accumulation of phytoene was observed in the bleached tissues. In order to detect a possible interference of the herbicide with phytoene desaturase in vitro, we measured the enzyme from *Narcissus pseudonarcissus* chromoplast membranes using [^{14}C]phytoene as substrate. As can be seen in Table I, SC-0051 did not inhibit this reaction, whereas the typical phytoene desaturase inhibitor, Fluridone, blocked phytoene desaturation completely, even though it was administered as a much lower concentration.

The possible involvement of quinones in the phytoene desaturase reaction prompted us to carry out ^{14}C labeling studies with tyrosine, which is the common precursor of plastoquinones and tocopherols in plants. Wheat seedlings (2 weeks old) were treated either with SC-0051 (0.1 kg/ha) applied as a standard screening formulation or with the formulation alone. After 1 h from each treatment, nine seedlings were cut off just above the root and allowed to incorporate [^{14}C]tyrosine (83330 Bq) from 2 ml of a phosphate-buffered solution (pH 6.5) for 15 h. After extraction ($CHCl_3$/MeOH, 2:1), TLC analysis of the lipophilic extracts revealed incorporation of radioactivity from tyrosine into two major non-polar compounds in the control plants, as well as in the plants treated with the formulation alone; in contrast, the herbicide treatment prevented this incorporation (Fig. 1). Band 2 co-migrated with an authentic plastoquinone reference, indicating that quinone-formation may indeed be affected by SC-0051.

In the course of quinone biosynthesis, tyrosine is first either transaminated or oxidatively deaminated to *p*hydroxyphenylpyruvate, which is subsequently transformed to homogentisic acid. We tested the effect of both of these intermediates on the growth of the water plant duckweed (*Lemna gibba*) in the presence of SC-0051. The herbicide severely inhibited the growth of

Table I

Effect of SC-0051 and Fluridone on the desaturation of [^{14}C]phytoene in isolated chromoplast membranes

Incubation conditions	Products		
	Phytoene	Phytofluene	δ-Carotene
control	32.7%	trace	67.3%
SC-0051	34.2%	trace	65.8%
Fluridone	100.0%	trace	n.d.

Chromoplast membranes were incubated for 5 h with 15-*cis*-[^{14}C]phytoene (833 Bq = 100%) in the presence or absence (control) of bleaching herbicides. Formation of phytofluene and δ-carotene was monitored by TLC analysis. The concentration of the herbicides in the desaturation reaction were 10^{-4} M (SC-0051) and 10^{-6} M (Fluridone). All other conditions were as described in Materials and Methods. n.d., not detectable.

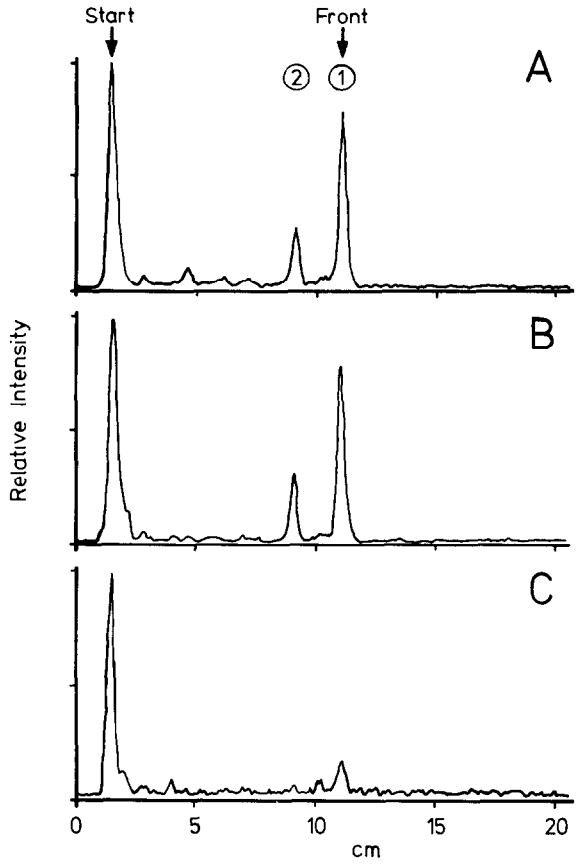

Fig. 1. TLC analysis of incorporation of radioactivity from [^{14}C]tyrosine into lipophilic metabolites by wheat seedlings. Plants were either untreated (A), or treated with a standard screening formulation (B) or with 100 g/ha SC-0051 in screening formulation (C) and incubated with [^{14}C]tyrosine. Lipophilic metabolites were extracted with chloroform, separated on a silica gel 60 HPTLC plate with chloroform:diethylether (99:1), and the radioactivity was analyzed with a TLC scanner.

Lemna at a concentration of 10^{-7} M. As is evident from Table II, *p*-hydroxyphenylpyruvate had no effect on this inhibition, whereas homogentisic acid was clearly able to abolish the herbicidal effect. It should be noted that both *p*-hydroxyphenylpyruvate and homogentisic acid per se produced slight phytotoxic symptoms, but this did not interfere with plant growth. The results strongly indicate that SC-0051 interferes with the conversion of *p*-hydroxyphenylpyruvate to homogentisic acid, catalyzed by the enzyme *p*-hydroxyphenylpyruvate dioxygenase.

Whereas little information is available on plant *p*-hydroxyphenylpyruvate dioxygenase [13], the enzyme has been well characterized from vertebrate liver (for review see [14] and citations therein) and microorganisms (for review see [7] and citations therein). We have therefore investigated the effect of SC-0051 on the conversion of hydroxyphenylpyruvate to homogentisic acid in a rat liver homogenate. To do this [^{14}C]tyrosine was first converted to *p*-hydroxyphenylpyruvate with the aid

Table II

Effects of *p*-hydroxyphenylpyruvate (pOHPP) and homogentisic acid (HGA) on the reproduction of SC-0051-treated *Lemna* plantlets

Intermediates added	Number of newly grown plants	
	Control medium	10^{-7} M SC-0051
None (control)	25	3
1 mM pOHPP	22	2
1 mM HGA	29	32

Three *Lemna* plantlets were inoculated in 100 ml of sterile medium for 6 days in the presence or absence (control) of filter-sterilized metabolites. SC-0051 was added from a 10^{-3} M acetone stock solution.

of L-amino acid oxidase from snake venom and subsequently incubated with the liver homogenate. In the TLC analysis the diethylether extracts from these incubations revealed in the control a radioactive band comigrating with homogenisic acid, whereas this band was absent in the herbicide-treated preparation (Fig. 2). In this cell-free liver system, SC-0051 clearly inhibited the conversion of *p*-hydroxyphenylpyruvate to homogentisic acid.

In order to monitor the enzyme activity in liver extracts directly, we used continuous measurements with the spectrophotometric enol-borate method according to Lin et al. [11]. SC-0051 interfered severely with *p*-hydroxyphenylpyruvate dioxygenase (Fig. 3), showing almost complete inhibition at a concentration of 10^{-6} M. Attempts to detect the enzyme activity by this method in plants failed. Therefore, a more sensitive radioactive assay was employed: endpoint-measurements based on the detection of [^{14}C]CO_2 released from [1-^{14}C]*p*-hydroxyphenylpyruvate in the course of the enzymatic reaction [12]. With this assay we were able to detect the enzyme activity in crude extracts from etio-

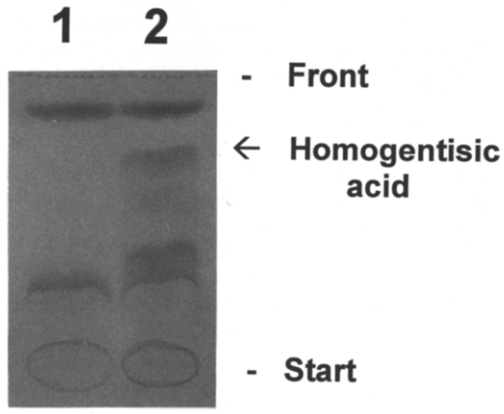

Fig. 2. TLC analyis of the inhibition of homogentisic acid formation by SC-0051 in rat liver extracts. ^{14}C-labeled homogentisic acid was formed from [^{14}C]tyrosine via hydroxyphenylpyruvate in the control (lane 2 of the autoradiograph), but was absent in the sample containing 10^{-4} M SC-0051 (lane 1 of the autoradiograph). The arrow indicates the position of an unlabeled homogentisic acid reference. The incubation conditions are outlined in Materials and Methods.

lated corn seedlings and to partially purify the protein, employing $(NH_4)_2SO_4$ precipitation followed by chromatography on Q-Sepharose (details on the purification will be published elsewhere). As is shown in Fig. 4, the activity of the enriched plant enzyme was severely inhibited by SC-0051. Complete inhibition was again observed at 10^{-6} M; the IC_{50} value was calculated to be 4.5×10^{-8} M. Clomazone, another herbicide with bleaching effects but with a hithero unknown site of action [15], was included in the investigations, but it did not interfere with *p*-hydroxyphenylpyruvate dioxygenase.

These results clearly demonstrate SC-0051 to be a potent inhibitor of the enzyme *p*-hydroxyphenylpyruvate dioxygenase, thereby preventing homogentisate formation.

4. DISCUSSION

The herbicidal compound SC-0051 causes strong bleaching effects as well as a massive accumulation of phytoene in treated plants [1,2]. These symptoms are well known from herbicides that block carotene biosynthesis by inhibiting the enzyme phytoene desaturase (for review see [3]). In vitro measurements of phytoene desaturase revealed that this enzyme clearly is not the target of SC-0051 ([4], Table I). If, however, the herbicide interferes with the formation of an essential cofactor of phytoene desaturase, this should result in an in vivo inhibition of the enzyme. Currently, two possible cofactors of phytoene desaturation are being discussed: the involvement of FAD was deduced from desaturase nucleotide sequence comparisons [16,17] and a quinone was postulated, acting as an electron-acceptor in the polyene formation [5].

In this publication we have described the interference of SC-0051 with the plastoquinone and tocopherol biosynthetic pathway. The early steps of this pathway comprise the transamination or oxidative deamination of tyrosine to yield *p*-hydroxyphenylpyruvate, which is subsequently decarboxylated and hydroxylated by the enzyme *p*-hydroxyphenylpyruvate dioxygenase. This results in the formation of homogentisic acid, an important intermediate in the biosynthesis of plastoquinones, tocopherols and related compounds (for review see [18]).

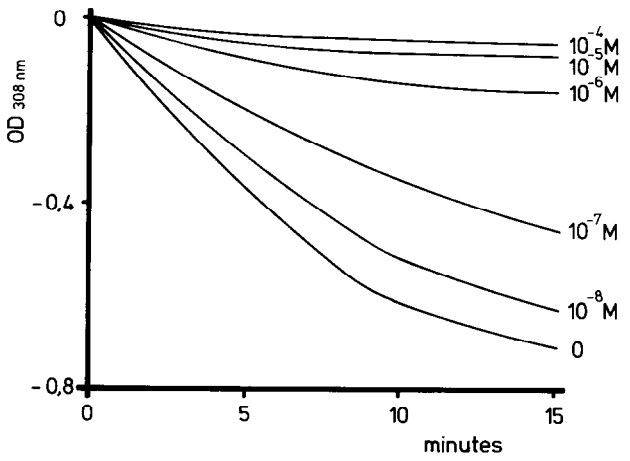

Fig. 3 Time-course of the reaction of rat liver *p*-hydroxyphenylpyruvate dioxygenase measured by the enol-borate method in the absence (0) and presence of increasing amounts of SC-0051. The protein concentration in the assay was 250 μg/ml.

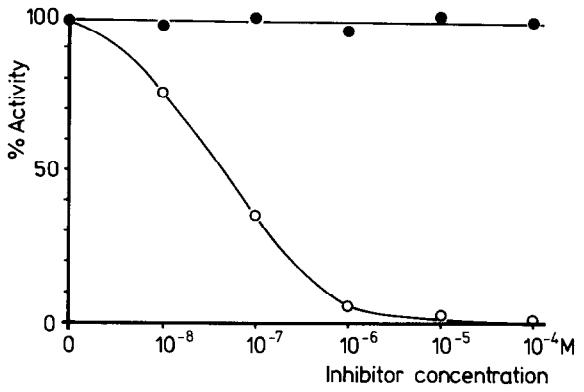

Fig. 4 Effect of the herbicides SC-0051 (○) and Clomazone (●) on partially purified corn *p*-hydroxyphenylpyruvate dioxygenase. Endpoint measurements of enzyme activity were performed by the $[^{14}C]CO_2$ release method as outlined in Materials and Methods. 100% activity = 208 Bq $[^{14}C]CO_2$ released from 1,333 Bq [1-^{14}C]*p*-hydroxyphenylpyruvate.

We have presented several lines of evidence that SC-0051 interferes with plastoquinone and tocopherol biosynthesis. First, the incorporation of radioactivity from tyrosine into the lipophilic quinone fraction of wheat was inhibited by the herbicide. Second, herbicide-mediated growth inhibition of *Lemna* plantlets could be reversed by supplementing the growth medium with homogentisic acid, whereas *p*-hydroxyphenylpyruvate was ineffective. This observation pointed to the enzyme *p*-hydroxyphenylpyruvate dioxygenase as a possible site of action. We therefore isolated and partially purified this enzyme from etiolated corn seedlings and were able to show that SC-0051 is indeed a potent inhibitor of this enzyme in vitro, with an IC_{50} value of 4.5×10^{-8} M. *p*-Hydroxyphenylpyruvate dioxygenases also exist in animal tissues and bacteria, where they are involved in tyrosine catabolism. The rat liver enzyme was shown here to be also very susceptible to SC-0051. Clomazone, another bleaching herbicide with unknown mode of action, did not inhibit the animal or the plant enzyme.

The results presented provide further evidence that a multi-component redox system in which quinones play an important role is involved in the reaction mechanism of phytoene desaturase [5,6,19]. We have shown here that the inhibition of a step in a quinone biosynthetic pathway leads to plant bleaching and subsequent plant death. This pathway therefore is a promising new target for herbicide design.

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