Plant Physiol. (1994) 106: 1429–1433

Inhibition of Barnyardgrass 4-Hydroxyphenylpyruvate Dioxygenase by Sulcotrione

Jacob Secor*

DowElanco, 9410 Zionsville Road D-1, Indianapolis, Indiana 46268–1053

4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) was partially purified from barnyardgrass (*Echinochloa crus-galli* L.) leaves and assayed by high-performance liquid chromatography analysis of product formation or by the capture of released $^{14}CO_2$. The bleaching herbicide sulcotrione [2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione] was shown to be a potent, linear competitive inhibitor of 4-hydroxyphenylpyruvate dioxygenase. Kinetic analyses determined that the K_m for the substrate, 4-hydroxyphenylpyruvate, was 4.3 μM, and the K_i value was 9.8 nM for sulcotrione.

Sulcotrione [(2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione; Fig. 1] is a member of the triketone class of herbicides discovered by Zeneca (Michaely and Kratz, 1985, 1988) and is being marketed under the trademarked name of Mikado in Europe. Members of this class of herbicides cause plants to bleach, but the bleaching pattern is unlike that caused by other bleaching herbicides such as norflurazon. One striking difference is that the triketones affect new growth, whereas other bleaching herbicides affect older growth (Mayonado et al., 1989). Also, unlike the other bleaching herbicides, there are indications that light is required for the triketones to cause the bleaching effect (Sandman et al., 1990; Nandihalli and Bhowmik, 1992).

Early investigations aimed at elucidating the mode of action of triketone herbicides led to conflicting conclusions. Most investigators had observed that these compounds caused a decrease in carotenes, carotenoids, and Chl with a concomitant increase in phytoene, a precursor to the synthesis of these pigmented compounds. Based on pigment patterns in treated radish (*Raphanus sativus* L.) plants, Soeka and Uchida (1987) concluded that the triketones were affecting pigment biosynthetic steps but in a manner unlike that of other bleaching herbicides such as norflurazon. In contrast, Mayonado et al. (1989) showed that sulcotrione caused the same pattern of pigment changes in soybean (*Gly max* L.) treated with norflurazon. Kawakubo et al. (1979) concluded that 1,3-dimethyl-4-(2,4-dichlorobenzoyl)-5-hydroxypyrazole blocked synthesis of Pchlide. Sandman et al. (1990) reported that 2-(4-chloro-2-nitrobenzoyl)-5,5-dimethylcyclohexane-1,3-dione had no effect on colored carotenoids in soybean cell culture or unicellular organisms.

The observations of the changes in pigments and intermediates led investigators to propose that the enzyme phytoene desaturase is the site of action of the triketones. However, Sandman et al. (1990) showed that concentrations of up to 10^{-5} M of 2-(4-chloro-2-nitrobenzoyl)-5,5-dimethylcyclohexane-1,3-dione did not inhibit phytoene desaturase from maize (*Zea mays* L.) chloroplasts or from chromoplasts of petals of *Cheiranthus cheiri*, whereas norflurazon did inhibit this enzyme. These results led them to speculate that the compound may need to undergo bioactivation before becoming herbicidal.

In late 1992 a report was published indicating that a triketone (NTBC) was a potent inhibitor of mammalian HPPD (EC 1.13.11.27) (Lindstedt et al., 1992). Subsequent reports indicated that structurally related herbicidal triketones inhibit HPPD in plants (Prisbylla et al., 1993; Schulz et al., 1993). The purpose of this report is to present detailed evidence that sulcotrione is a potent inhibitor of HPPD in plants.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise stated all chemicals were reagent grade or better and purchased from Sigma. Radiolabeled Tyr was purchased from Dupont Medical Products. Sulcotrione was synthesized at DowElanco.

Substrate Preparation

Radiolabeled HPPA was synthesized from either [1-^{14}C]-Tyr or [U-^{14}C]Tyr by a modification of the method of Buckthal et al. (1987). Fifty microliters (5 μCi) of radiolabeled Tyr (60 Ci/mol) was dried under a stream of N_2 in a 7-mL glass vial. To the residue was added 193 μL of K-phosphate buffer (100 mM, pH 6.5), 5 μL of catalase (Sigma C-100, 6100 units/μL), and 2 μL of L-amino acid oxidase (Sigma A-9370, 72 units/mL). The 200-μL mixture was shaken at 30°C for at least 30 min. Product formation was checked by HPLC as described below. At the conclusion of the reaction, the product, HPPA, was separated from any remaining Tyr by passing the reaction mixture through a column containing approximately 400 μL of Dowex AG 50W-X8 resin equilibrated with 0.1 N HCl. The column was washed with approximately 1.5 mL of water to collect the radiolabeled HPPA. More than 95% of the

* Fax 1–317–337–3546.

Abbreviations: HA, homogentisic acid; HPPA, 4-hydroxyphenylpyruvic acid; HPPD, 4-hydroxyphenylpyruvate dioxygenase; I_{50}, concentration of inhibitor that reduces enzyme activity by 50%; NTBC, 2-(2-nitro-4-trifluromethylbenzoyl)cyclohexane-1,3-dione.

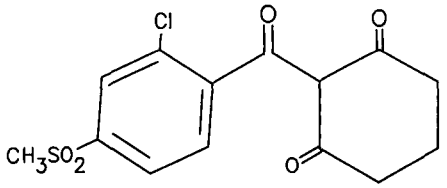

Figure 1. Chemical structure of sulcotrione (M_r = 329).

applied radioactivity passed through the column. Analysis by HPLC showed that the eluant contained at least 90% of the keto tautomer of HPPA. The eluant was stored undiluted at −80°C and was stable for at least 1 week.

Enzyme Preparation

All procedures were conducted at 4°C except weighing and cutting of leaf material, which was done at room temperature. At least 25 g fresh weight of barnyardgrass (*Echinochloa crus-galli* L.) leaves were cut into small (<2 cm) pieces and homogenized 3 × 20 s at high speed in a Waring Blendor in 3 volumes of grinding solution as described by Schulz et al. (1993). The homogenate was then filtered through two layers of Miracloth and four layers of cheesecloth. The filtrate was centrifuged for 15 min at 30,000*g*. Solid $(NH_4)_2SO_4$ was added to the resulting supernatant fraction to make 20% saturation. The solution was stirred for 20 min, followed by centrifugation for 20 min at 30,000*g*. The supernatant fluid was made to 50% saturation with solid $(NH_4)_2SO_4$, stirred for 20 min, and centrifuged as described above. The precipitated pellet was solubilized in 3 mL of K-phosphate buffer (50 mM, pH 7.3) and desalted through a Bio-Rad P6DG column (1.5 × 5 cm). The desalted solution was purified further through a DEAE-Sepharose Fast Flow (Pharmacia) column (1.6 × 14 cm) at a flow rate of 1.5 mL/min. The elution protocol used was 50 mL of K-phosphate buffer (50 mM, pH 7.3), followed by a 200-mL linear gradient from 0 to 0.3 M KCl in K-phosphate buffer (50 mM, pH 7.3). The HPPD activity eluted between 0.2 and 0.3 M KCl.

Enzyme Assay

HPPD activity was assayed either by loss of substrate (HPPA), as measured by the capture of released $^{14}CO_2$, or by the amount of product formed as quantified by HPLC analysis of [U-^{14}C]HA. For the assay of the release of CO_2, reactions were conducted in 20-mL glass scintillation vials, each capped with a serum stopper through which a polypropylene well containing 50 μL of 4 N KOH was suspended. The reaction mixture contained 10 μL of catalase (5000 units), 100 μL of a freshly prepared 1:1 (v/v) mixture of 150 mM GSH (Sigma G-4251) and 3 mM dichlorophenolindophenol (Sigma D-1878), 10 μL of inhibitor, 50 to 250 μL of enzyme, 100 μL of unlabeled HPPA, and K-phosphate buffer (50 mM, pH 7.3) to make 975 μL. Stock catalase was diluted to 500 units/μL and centrifuged before being added. A 2-mM solution of unlabeled HPPA was made fresh daily in K-phosphate buffer (50 mM, pH 7.3) and allowed to equilibrate for 2 h at room temperature before being diluted as needed for the kinetic experiments. The equilibration was necessary because freshly made solutions of HPPA are the nonsubstrate enol tautomer; at equilibrium the keto tautomer is the predominant (>95%) form. The reaction was initiated with 25 μL of undiluted [1-^{14}C]HPPA (45 nCi, 60 Ci/mol) and proceeded for 45 to 60 min at 30°C. Reactions were terminated by injecting 250 μL of 4 N H_2SO_4 through the serum stopper. The vials were further incubated for 30 min to ensure complete capture of released CO_2. The wells were then cut into scintillation vials containing 15 mL Hionic (Packard Instruments, Meriden, CT) scintillation fluid and ^{14}C radioactivity was measured.

The reactions in which [U-^{14}C]HA formation was measured were assayed in 20-mL scintillation vials as described above. The final assay volume was 500 μL with the same final concentrations of reagents as described above except that the amount of [U-^{14}C]HPPA was increased to 0.5 μCi (60 Ci/mol). After 60 min at 30°C, the reactions were terminated by freezing in solid CO_2 or putting them into a −80°C freezer. A 100-μL aliquot was removed for HPLC analysis.

HPLC Conditions

Analyses were performed with a Waters (Milford, MA) 625 HPLC system equipped with a Waters 486 UV detector set at 270 nm and an INUS β-Ram radiodetector with a liquid scintillation cell. The column used was a 4.6- × 250-mm Alltima 5-μm C_{18} (No. 88057; Alltech, Deerfield, IL). The separations were performed at ambient temperature at a flow rate of 1.0 mL/min with a gradient system composed of 1% (v/v) H_3PO_4 in H_2O (solvent A) and 1% (v/v) H_3PO_4 in methanol (solvent B). Separations were accomplished with a linear gradient of 25 to 50% B for 15 min, followed by 5 min of 50% B. Figure 2 shows a chromatogram of standards using this separation system. One or more of these unlabeled standards were included in samples to verify identification of radiolabeled peaks.

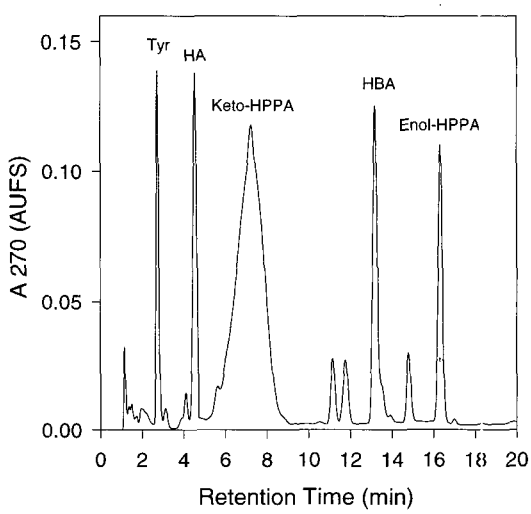

Figure 2. HPLC analysis of standards. Keto-HPPA and enol-HPPA are the respective keto and enol tautomers of HPPA. HBA is *p*-hydroxybenzaldehyde, a spontaneous decomposition product of HPPA. Minor peaks are contaminants or decomposition products of the standards. AUFS, Absorbance units, full scale.

Table I. *Effect of 250 nM sulcotrione on HPPD activity*

The data for HA and HPPA were derived from integration of peak areas of HPLC radiochromatograms. Data are means from two replications.

Component	No Enzyme	Control	Sulcotrione
		nCi	
HA	5.7	127.1	8.1
keto-HPPA	551.4	379.1	497.9
enol-HPPA	19.2	12.4	17.3
$^{14}CO_2$ captured	2.8	9.2	2.9

Statistical Treatments

All experiments were repeated at least twice. For some kinetics experiments in which logistical limitations prevented replications within an experiment, the experiment was repeated at least three times. Otherwise, there were at least two replications of treatments within each experiment. The data presented are from representative experiments.

RESULTS

Inhibition of the Formation of HA

By means of HPLC analysis, it was possible to detect and quantify the formation of the product of the HPPD-catalyzed reaction, HA, and the concomitant decrease in the substrate, HPPA. As shown in Table I, sulcotrione at 250 nM almost completely inhibited the formation of HA. These results provide clear evidence that sulcotrione is a potent inhibitor of this reaction. Also shown in Table I are the data for the $^{14}CO_2$ that was captured during the assay, indicating that the $^{14}CO_2$-capture assay accurately reflects the results of the HPLC assay.

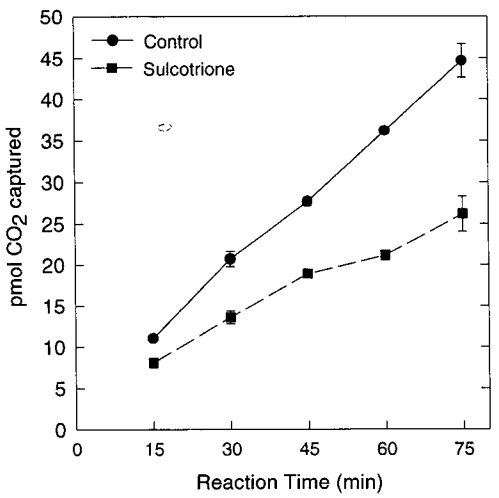

Figure 3. Time dependency of the reaction in the presence and absence of 15 nM sulcotrione. HPPD activity was measured at 0.7 μM HPPA using the $^{14}CO_2$-capture method. Each point is the mean ± SE of two replications.

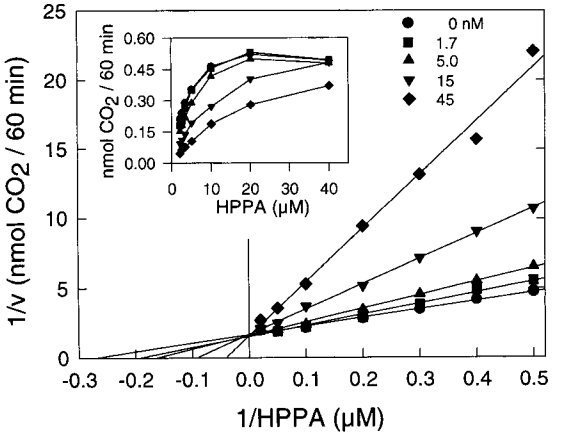

Figure 4. Double-reciprocal plot of the inhibition of barnyardgrass leaf HPPD by sulcotrione with HPPA as the varied substrate. Inset shows assay data used for the double-reciprocal plots. HPPD activity was assayed using the $^{14}CO_2$-capture method. The nM values are the concentrations of sulcotrione used in the assays.

Kinetic Studies

Experiments in which the $^{14}CO_2$-capture assay was used were conducted to determine kinetic and inhibition parameters and the type of inhibition by sulcotrione. Reaction rates in the presence and absence of sulcotrione were linear over the time course used for the kinetics studies (Fig. 3). The effects of substrate and inhibitor concentrations on the reaction rate are shown in Figure 4. The K_m for HPPA is 4.3 μM. The double-reciprocal plot clearly indicates that sulcotrione is a linear, competitive inhibitor with respect to HPPA. A K_i of 9.8 nM was determined graphically from the abscissa intercept of the linear plot of K_m/V_{max} versus inhibitor concentration. The inset in Figure 4 shows that at high concentrations HPPA slightly inhibited the reaction. Although this inhibition was not large, it was consistent among experiments, with inhibition varying from slightly less than 10% to about 20%.

DISCUSSION

Several studies aimed at elucidating the mode of action of triketones in plants showed that these compounds caused an accumulation of Tyr with a concomitant decrease in plastoquinone levels. Addition of HA to the growth medium alleviated these effects and prevented plant necrosis (Prisbylla et al., 1993; Schulz et al., 1993). These observations led the investigators to suspect that HPPD was the target site of these compounds. It is interesting to note that HPPD was postulated to be a potential herbicide target site in 1985 (Pascal et al., 1985).

The inhibition of HPPD by a triketone, NTBC, was first reported in late 1992 by Lindstedt et al. (1992), who were investigating the use of this drug for the treatment of tyrosinemia type I in humans. It was unfortunate that, since the study was conducted under clinical conditions, no direct measurements of enzyme inhibition could be done. However,

the authors stated that, based on indirect measurements, they believed that nearly total inhibition of HPPD was achieved.

The data presented in this paper and in other recent reports clearly indicate that triketones are potent inhibitors of HPPD extracted from mammals and plants. Schulz et al. (1993) showed that the rat liver enzyme is completely inhibited at sulcotrione concentrations between 1 and 10 μM. This is supported by the statement of Prisbylla et al. (1993) that the I_{50} of NTBC for the rat liver enzyme was 40 nM. The maize enzyme is equally sensitive, with I_{50} values of 45 nM for sulcotrione (Schulz et al., 1993) and 50 nM for NTBC (Prisbylla et al., 1993). These values compare closely with the results reported in this paper for the barnyardgrass enzyme. One must keep in mind, however, that I_{50} values are dependent on assay conditions and various kinetics parameters. For competitive inhibitors, such as sulcotrione (Fig. 4), I_{50} values will increase with increasing concentrations of the competitive substrate. This can be clearly demonstrated by calculating I_{50} values based on the relationship, $I_{50} = (1 + [S]/K_m)K_i$. For the data in the present paper, the I_{50} values range from 14.4 to 101 nM, depending on the HPPA concentration in the assay.

One shortcoming of previous reports of the mechanism of triketone herbicides is that they did not show that the formation of HA per se was inhibited by these compounds for enzyme extracted from plant tissue. Whereas other investigators have relied solely on spectrophotometric or CO_2-capture assays to study HPPD, the present report confirms the existence of HPPD in plants and demonstrates the effect of inhibitors on HPPD activity via methods that simultaneously measure the depletion of substrate and accumulation of product. Previous studies have only implicated the existence of HPPD in plants through substrate-feeding experiments (Löffelhardt and Kindl, 1979; Fiedler et al., 1982). Schulz et al. (1993), using TLC, showed that sulcotrione inhibited the formation of HA in rat liver extracts but did not demonstrate the effect on HA formation in plant extracts. The results reported in this paper confirm that the CO_2-capture assay is accurate in assessing plant HPPD activity, although one needs to be cognizant of conditions under which this assay method can be misleading. The details of some of these conditions have been cited in previous studies (Lindblad, 1971; Lin and Crawhall, 1976; Lindblad et al., 1977).

For barnyardgrass leaf HPPD, the K_m for HPPA was 4.3 μM, which is lower than values reported for the enzyme extracted from other sources. Examples of K_m values for HPPD extracted from other organisms are 30 μM for human liver (Lindblad et al., 1977), 20 to 50 μM for rat liver (Lin et al., 1958; Fellman et al., 1972), and 30 mM for *Pseudomonas* (Lindstedt et al., 1977). The K_m of the frog liver HPPD was originally reported to be 500 μM (Laskowska-Klita, 1969), but more recent results indicate that the value is 50 μM (Lindstedt et al., 1982).

High concentrations of both substrates, O_2 and HPPA, have been reported to inhibit the reaction. This effect has been observed for HPPD from rat (Fellman et al., 1972), frog (Lindstedt et al., 1982), human (Lindblad et al., 1977), and *Pseudomonas* (Lindstedt et al., 1977). HPPD is inhibited by relatively low substrate concentrations, but the amount of inhibition is not always large. Lindblad et al. (1977) showed that the human enzyme was inhibited by about 18% at an HPPA concentration of less than 7 times K_m. Frog HPPD was inhibited at lower HPPA concentrations, with 13% inhibition occurring at about 20 μM HPPA, a value less than 50% of the K_m measured in that study (Lindstedt et al., 1982). The barnyardgrass enzyme appears also to be sensitive to high concentrations of HPPA, with inhibition being seen at concentrations of less than 10 times K_m (Fig. 4).

It has been well documented that HPPD activity is inhibited by a variety of natural and synthetic aromatic α-keto acids, some of which are poor alternate substrates (Fellman et al., 1972; Rundgren, 1983; Pascal et al., 1985). However, none of these compounds have K_i values as low as the triketone tested in this study. For example, Pascal et al. (1985) reported that the best inhibitor that they tested was 2,6-difluoro-4-hydroxyphenyl pyruvate, which was a reversible, competitive inhibitor having a K_i of 1.3 μM, a value approximately 100-fold higher than the K_i for sulcotrione reported in this paper.

ACKNOWLEDGMENTS

The excellent technical skill of Jayne Koppatschek is greatly appreciated, especially her assistance in the preparation of radiolabeled substrates and protein extraction. The author also wishes to thank Drs. C. Cséke and T. Walsh for their helpful discussions and critical review of this manuscript.

Received June 13, 1994; accepted August 31, 1994.
Copyright Clearance Center: 0032-0889/94/106/1429/05.

LITERATURE CITED

Buckthal DJ, Roche PA, Moorehead TJ, Forbes BJ, Hamilton GA (1987) 4-Hydroxyphenylpyruvate dioxygenase from pig liver. Methods Enzymol **142:** 132–138

Fellman JH, Fujita TS, Roth ES (1972) Assay, properties and tissue distribution of *p*-hydroxyphenylpyruvate hydroxylase. Biochim Biophys Acta **284:** 90–100

Fiedler E, Soll J, Schultz G (1982) The formation of homogentisate in the biosynthesis of tocopherol and plastoquinone in spinach chloroplasts. Planta **155:** 511–515

Kawakubo K, Shindo M, Konotsune T (1979) A mechanism of chlorosis caused by 1,3-dimethyl-4-(2,4-dichlorobenzoyl)-5-hydroxypyrazole, a herbicidal compound. Plant Physiol **64:** 774–779

Laskowska-Klita T (1969) Purification of *p*-hydroxyphenylpyruvate hydroxylase and its natural inhibitor from liver of the frog *Rana esculenta*. Acta Biochim Pol **16:** 35–44

Lin ECC, Pitt BM, Civen M, Knox WE (1958) The assay of aromatic amino transaminations and keto acid oxidation by the enol borate-tautomerase method. J Biol Chem **233:** 668–673

Lin K-T, Crawhall JC (1976) Purification of *p*-hydroxyphenylpyruvate hydroxylase from rat liver—requirements for cofactors. Can J Biochem **54:** 423–431

Lindblad B (1971) Radiochemical assays for *p*-hydroxyphenylpyruvate hydroxylase activity in human liver. Clin Chim Acta **34:** 113–121

Lindblad B, Lindstedt G, Lindstedt B, Rundgren M (1977) Purification and some properties of human 4-hydroxyphenylpyruvate dioxygenase (I). J Biol Chem **252:** 5073–5084

Lindstedt S, Holme E, Lock EA, Hjamarson O, Strandvik B (1992) Treatment of hereditary tyrosinaemia type 1 by inhibition of 4-hydroxyphenylpyruvate dioxygenase. Lancet **340:** 813–817

Lindstedt S, Odelhög B, Rundgren M (1977) Purification and some

properties of 4-hydroxyphenylpyruvate dioxygenase from *Pseudomonas* sp. PJ 874. Biochemistry **16:** 3369–3377

Lindstedt S, Odelhög B, Rundgren M (1982) On 4-hydroxyphenylpyruvate dioxygenase of adult frog liver. Comp Biochem Physiol **72B:** 537–541

Löffelhardt W, Kindl H (1979) Conversion of 4-hydroxyphenylpyruvic acid into homogentisic acid at the thylakoid membrane of *Lemna gibba*. FEBS Lett **104:** 332–334

Mayonado DJ, Hatzios KK, Orcutt DM, Wilson HP (1989) Evaluation of the mechanism of action of the bleaching herbicide SC-0051 by HPLC analysis. Pest Biochem Physiol **35:** 138–145

Michaely WJ, Kratz GW (1985) 2-(2-Substituted benzoyl)-1,3-cyclohexanediones. European Patent No. 0135191. European Patent Office, Vienna, Austria

Michaely WJ, Kratz GW (1988) Preparation of benzoylcyclohexanedione herbicides. US patent No. 4,780,127. U.S. Patent Office, Washington, DC

Nandihalli UB, Bhowmik PC (1992) Inhibition of chlorophyll and carotenoid biosynthesis by the herbicide ICIA-0051. Photosynthetica **26:** 355–362

Pascal RA, Oliver MA, Chen Y-CJ (1985) Alternate substrates and inhibitors of bacterial 4-hydroxyphenylpyruvate dioxygenase. Biochemistry **24:** 3158–3165

Prisbylla MP, Onisko BC, Shribbs JM, Adams DO, Liu Y, Ellis MK, Hawkes TR, Mutter LC (1993) The novel mechanism of action of the herbicidal triketones. *In* Brighton Crop Protection Conference: Weeds. British Crop Protection Council, Surrey, UK, pp 731–738

Rundgren M (1983) Some kinetic properties of 4-hydroxyphenylpyruvate dioxygenase from *Pseudomonas* sp. strain P.J. 874. Eur J Biochem **133:** 657–663

Sandman G, Böger P, Kumita I (1990) Atypical inhibition of phytoene desaturase by 2-(4-chloro-2-nitrobenzoyl)-5,5-dimethylcyclohexane-1,3-dione. Pest Sci **30:** 353–355

Schulz A, Ort O, Beyer P, Kleinig H (1993) SC-0051, a 2-benzoylcyclohexane-1,3-dione bleaching herbicide, is a potent inhibitor of the enzyme *p*-hydroxyphenylpyruvate dioxygenase. FEBS Lett **318:** 162–166

Soeka T, Uchida T (1987) Inhibition of pigment synthesis by 1,3-dimethyl-(2,4-dichlorobenzoyl)-5-hydroxypyrazole, norflurazon and new herbicidal compounds in radish and flatsedge plants. Pest Biochem Physiol **29:** 35–42