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Epoxide hydrolase activities in the microsomes and the soluble fraction from *Vicia sativa* seedlings

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Abstract Epoxide hydrolases (EC 3.3.2.3) in the microsomal and soluble fractions of 4 d old *Vicia sativa* seedlings hydrolyze 9,10-epoxystearic acid to 9,10-dihydroxystearic acid. No alteration of epoxide hydrolase activity was observed after treatment of the seedlings with 8 mM phenobarbital or 0.5 mM 2,4-dichlorophenoxyacetic acid compared to control plants. Treatment with 1 mM clofibrate decreased the activity in the microsomes by 37%, but had no effect on the activity in the soluble fraction. Four different inhibitors of mammalian epoxide hydrolases were tested. Preincubation of either subcellular fraction with 4-fluorochalcone oxide had no effect. Weak inhibition (25% in microsomes and 16% in the soluble fraction) was observed following preincubation with 1,1,1-trichloropropene-2,3-oxide. After preincubation with (2S,3S)-(-)-3-(4-nitrophenyl)-glycidol, we measured a 45% decrease in soluble epoxide hydrolase activity whereas no change was observed in the microsomal epoxide hydrolase activity. The 2R, 3R enantiomer did not affect activity in either fraction. Lowering the pH from 9 to 7.4 stimulated the activity by 366% in the cytosol but only by 72% in the microsomes. After incubation of a racemic mixture of 9,10-epoxystearic acid with the microsomal fraction, the chirality of the residual epoxide was 69/31 in favor of the 9S, 10R enantiomer. We determined K_m of 5.2±0.5 and 2.5±0.4 μM and $V_{max\ (observed)}$ of 198±4.7 and 404±10 pmol min^{-1} mg^{-1} for the 9S, 10R and 9R, 10S enantiomers, respectively.

Key words Epoxide hydrolase, plant, induction, inhibition, cutin, xenobiotic, *Vicia sativa*.

Abbreviations EH, epoxide hydrolase; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; SS-NPG, (2S,3S)-(-)-3-(4-nitrophenyl)-glycidol; RR-NPG, (2R,3R)-(-)-3-(4-nitrophenyl)-glycidol; TCPO, 1,1,1-trichloropropene-2,3-oxide; TLC, thin layer chromatography; 2,4-D, 2,4-dichlorophenoxyacetic acid; 4-FCO, 4-fluorochalcone oxide.

INTRODUCTION

Epoxide hydrolases (EHs) (EC 3.3.2.3) convert epoxides to diols *via* addition of water. These enzymes have been found in all mammalian species tested and in most organs. Based on substrate specificity and subcellular location, five distinct enzyme groups have been described in mammals: (1) microsomal EH, (2) soluble EH (formally called cytosolic EH), (3) cholesterol EH, (4) leukotriene A_4 EH, and (5) hepoxilin EH. However, research has mainly focused on the microsomal and soluble EHs. Microsomal and soluble EHs from different origins have been cloned and based on sequence homology, they have been classified as members of the α/β hydrolase fold family (Arand *et al.*, 1994; Beetham *et al.*, 1995). Members of this family are hydrolytic enzymes and all have a catalytic triad consisting of a nucleophile, a histidine and an acid. During our study of the catalytic mechanism of the mouse soluble EH, we identified the amino acids involved in catalysis (Pinot *et al.*, 1995). These amino acids are conserved in all known EHs sequences (Beetham *et al.*, 1995) suggesting that the catalytic mechanism is also conserved.

In mammals, hydrolysis of epoxides to diols is thought to be a detoxifying reaction because the product is more water soluble and can be easily excreted. Induction of hepatic microsomal EH in mice and rats by a number of foreign compounds suggests an involvement in xenobiotic metabolism (Oesch, 1973). The soluble form of the enzyme hydrolyzes a large number of different substrates, also suggesting a detoxifying function (Wixtrom and Hammock, 1985). However, soluble EH hydrolyses epoxides of fatty acids more rapidly than many other substrates, indicating a possible involvement in the metabolism of endogenous compounds (Nourooz-Zadeh *et al.*, 1992). A large number of studies have thoroughly characterized these enzymes in mammals (Oesch, 1973; Lu and Miwa, 1980; Wixtrom and Hammock, 1985) particularly experiments assessing the induction and inhibition of these enzymes. Less information is available on plant EHs despite the fact that the first report of EH activity in a plant occured 20 years ago (Croteau and Kolattukudy, 1975). This EH activity was observed in a $3000 \times g$ particulate fraction prepared from homogenates of the skin of young apples. To study this enzymatic system, the authors measured the hydrolysis of 18-hydroxy-9,10-epoxystearic acid to the corresponding triol which is a major component of plant envelopes (Kolattukudy, 1981). Almost two decades later, a soluble EH from soybean was purified and characterized using 9,10-epoxystearic acid as a substrate (Blée and Schuber, 1992*a*) This enzyme was shown to be regio- and enantioselective (Blée and Schuber, 1992*b*). More recently, soluble EHs were cloned from potato (*Solanum tuberosum*) (Stapleton *et al.*, 1994) and *Arabidopsis thaliana* (Kiyosue *et al.*, 1994). The mRNA coding for the EH from *S. tuberosum* was shown to accumulate on wounding and application of exogenous methyl jasmonate. In *A. thaliana*, drought stress or treatment with auxin also led to the accumulation of the coding mRNA. All these recent publications concern soluble EHs. A few reports mention the presence of EH activity associated with membranes (Blée and Schuber, 1992*a*; Stark *et al.*, 1995*a*). However, there is no work, to our knowledge, concerning a microsomal EH in plants.

In this paper we study, for the first time, a microsomal epoxyde hydrolase in a plant. We performed experiments in order to distinguish the microsomal and soluble enzymes in *Vicia sativa*. Using 9,10-epoxystearic acid, a precursor of cutin monomers as a substrate, we measured EH activities in both subcellular fractions after treatment with different xenobiotics known to induce microsomal or soluble EHs in mammals or soluble EHs in plants. We also measured the effect of EH inhibitors and the effect of pH on these activities. We investigated the enantioselectivity of the microsomal EH. Our results strongly suggest the existence of two distinct epoxide hydrolases in *V. sativa*. Different subcellular locations of EHs may reflect different physiological roles. We discuss the significance of these enzymes, in particular their possible involvement in the synthesis of cutin monomers.

RESULTS

Effects of inducers

In order to investigate the effect of different compounds on the EHs from *V. sativa*, seedlings were treated with 1 mM clofibrate, 8 mM phenobarbital or 0.5 mM 2,4-D. The hydrolysis of 9,10-epoxystearic acid was measured in the microsomal and soluble fractions of treated seedlings and compared with activity in untreated seedlings (H_2O). To minimize contamination of microsomes by the soluble enzyme, the microsomes were washed by resuspending in high ionic strenght buffer (100 mM pyrophosphate). Results are presented in figure 1. The specific activity is approximately 10 fold higher in the soluble fraction than in microsomes. The same difference was also found in terms of the total activity (232 ± 14, 224 ± 9, 237 ± 5 and 240 ± 12 μmol min^{-1} in the soluble fraction and 27 ± 2, 17 ± 4, 37 ± 1 and 30 ± 2 μmol min^{-1} in the microsomes of 50 g of shoots treated with H_2O, clofibrate, phenobarbital or 2,4 D respectively). Phenobarbital did not alter the EH activity in either fractions. As a positive control for the treatment with clofibrate and 2,4-D, we measured the ω-hydroxylation of oleic acid which was shown previously to be induced under these conditions (Salaun *et al.*, 1986; Pinot *et al.*, 1992). Clofibrate treatment resulted in a 25 fold stimulation of this activity (3090 pmol min^{-1} mg^{-1} versus 122 pmol min^{-1} mg^{-1} in control plants) and 2,4-D treatment caused a 13 fold stimulation (1590 pmol min^{-1} mg^{-1} versus 122 pmol min^{-1} mg^{-1}). However, treatment with 2,4-D had no effect on EH activity in microsomes or in the soluble fraction. The only change in EH activity was a 37% decrease in the microsomal activity of plants treated with clofibrate, whereas soluble activity was not altered.

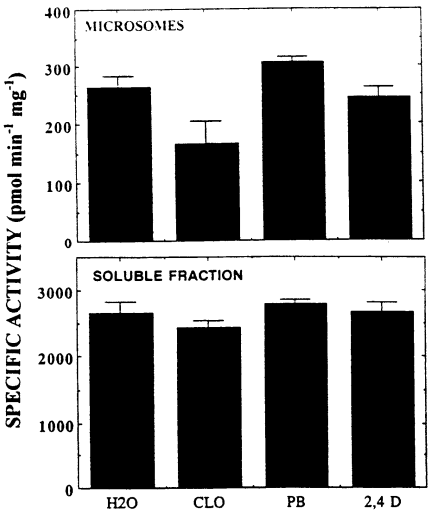

Figure 1. *Effect of* Vicia sativa *treatment with xenobiotics on hydrolysis of 9,10-epoxystearic acid in microsomes and the soluble fraction.* Seedlings (4 days old) were treated for 48 h with H_2O for control, or with 1 mM clofibrate (CLO), 8 mM phenobarbital (PB) or 0.5 mM 2,4-D. Epoxide hydrolase activity was measured in microsomes and the soluble fraction with 20 mM phosphate buffer pH 7.4. Data are expressed as the mean with standard deviation of activity assays in triplicate.

Inhibition studies

We measured the effect of different mammalian EH inhibitors on EH activity from *V. sativa* microsomal and soluble preparations. As shown in table 1, preincubation of microsomes and of the soluble fraction with 4-fluorochalcone oxide (4-FCO) did not inhibit EH activity. When these two fractions were preincubated with 1,1,1-trichloropropene-2,3-oxide, weak inhibition of EH activity was measurable in both fractions (25% in microsomes and 16% in the soluble fraction). Hydrolysis of 9,10-epoxystearic acid was partially inhibited (45%) after a preincubation of the soluble fraction with SS-NPG, whereas microsomal EH activity was not affected by this compound. No alteration of activity was observed after preincubation of either fraction with the RR-NPG enantiomer.

Effect of pH

We compared EH activity in microsomes and soluble fractions at pH 9 and 7.4. These are optimum pH for the microsomal and soluble EHs from mammals respectively (Wixtrom and Hammock, 1985). Lowering the pH from 9 to 7.4 led to a drastic 366% increase of activity in the cytosol

Table 1. *Inhibition of the hydrolysis of 9,10-epoxystearic acid in microsomes and the soluble fraction from* Vicia sativa. Microsomes and the soluble fraction were preincubated with 500 μM TCPO, 100 μM 4-FCO, 500 μM SS-NPG, or 500 μM RR-NPG. Controls were preincubated with solvent only. After a 10 min preincubation, hydrolysis of 9,10-epoxystearic acid was measured as described in Methods. Determination of activities was carried in 20 mM phosphate buffer pH 7.4. Data for 4-FCO and TCPO are means of duplicate experiment, data for SS-NPG and RR-NPG are means ± SD of triplicate experiment.

	Activity (*pmol min^{-1} mg^{-1}*)			
	Microsomes		Soluble fraction	
	Control*	Inhibitor	Control	Inhibitor
TCPO	269	204	2 420	2 027
4-FCO	306	349	2 899	2 608
SS-NPG	292 ± 6	279 ± 9	2 846 ± 197	1 576 ± 343
RR-NPG	306 ± 13	307 ± 28	2 409 ± 320	2 512 ± 317

(1020 ± 6 pmol min^{-1} mg^{-1} at pH 9 and 4760 ± 410 pmol min^{-1} mg^{-1} at pH 7.4). This pH effect was less pronounced in the microsomes where the decrease of pH led to 72% increase of activity (197 ± 13 pmol min^{-1} mg^{-1} at pH 9 and 339 ± 7 pmol min^{-1} mg^{-1} at pH 7.4). Experiments to determine the effect of pH and the effect of inhibitors (*tab.* 1) were performed with different preparations. Furthermore effect of pH was determined in 50 mM Tris buffer when other experiments were performed in 20 mM Na-phosphate buffer. This might explain the different values for the specific activities located in the soluble fraction (4760 ± 410 pmol min^{-1} mg^{-1} versus 2409 ± 320 pmol min^{-1} mg^{-1} or 2846 ± 197 pmol min^{-1} mg^{-1} in *tab* 1).

Enantioselectivity studies

Racemic 9,10-epoxystearic acid is a 50/50 mixture of the 9R, 10S and 9S, 10R enantiomers. Two different experiments were performed in order to determine whether the microsomal enzyme preferentially hydrolyzes one of these enantiomers. In the first experiment, the racemic mixture of the epoxide was incubated with the microsomal fraction. The reaction was stopped before more than 20% of the substrate was hydrolyzed and the chirality of the residual epoxide was determined as described in Methods. The residual epoxide was no longer racemic, being a 69/31 mixture with the 9S, 10R enantiomer in excess (*fig.* 2). This shows that the 9R, 10S was converted to the diol at a more rapid rate than the 9S, 10R enantiomer. In the second experiment, both enantiomers of 9,10-epoxystearic acid were separated

and purified by HPLC (see Methods). Different concentrations of each enantiomer (6 concentrations ranging from 4 to 70 μM) were incubated separately with the microsomes from the same preparation and we studied the kinetics of their hydrolysis. Figure 3 is a Lineweaver-Burk representation of these kinetics. From these kinetics, we determined a K_m and $V_{max(observed)}$ of 5.2±0.5 μM and 198±4.7 pmol min^{-1} mg^{-1} respectively for the 9S, 10R enantiomer and a K_m and $V_{max(observed)}$ of 2.5±0.4 μM and 404±10 pmol min^{-1} mg^{-1} for the 9R, 10S enantiomer. The EH has a higher affinity for the 9R, 10S enantiomer and hydrolyzes it twice as fast as the 9S, 10R enantiomer.

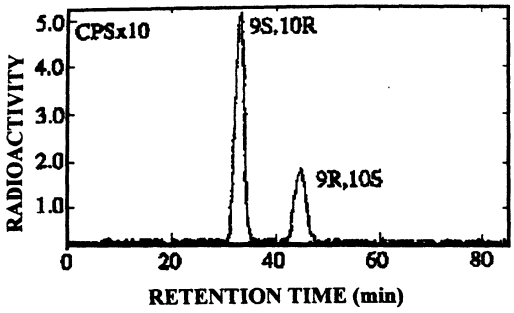

Figure 2. *HPLC elution profile of the enantiomers of the residual [1-^{14}C] 9,10-epoxystearic acid after incubation with microsomes of* Vicia sativa. Racemic [1-^{14}C]9,10-epoxystearic acid was incubated with the microsomal fraction of *Vicia sativa*. After separation from the diol by TLC, the residual epoxide was eluted from the silica and methylated. The epoxide was then subjected to HPLC analysis and the configuration of the residual epoxide was assigned using the optically pure methyl ester of 9R, 10S-epoxystearic. The racemic material showed peaks with equal area.

DISCUSSION

During a previous study, we described the metabolism of fatty acids by subcellular fractions of *V. sativa* and noticed the presence of an EH activity in the microsomes (Pinot *et al.*, 1992). In this paper, we use 9,10-epoxystearic acid, a precursor of cutin monomers, as a substrate to study for the first time a plant EH located in the membranes.

A major property of EHs in mammals is inducibility by xenobiotics (Oesch, 1973; Lu and Miwa, 1980; Wixtrom and Hammock, 1985). Compounds that cause peroxisome proliferation, like clofibrate, induce soluble EH in rats or mice (Grant *et al.*, 1994). Furthermore, it was recently shown that treatment of *A. thaliana* with the herbicide 2,4-D led to

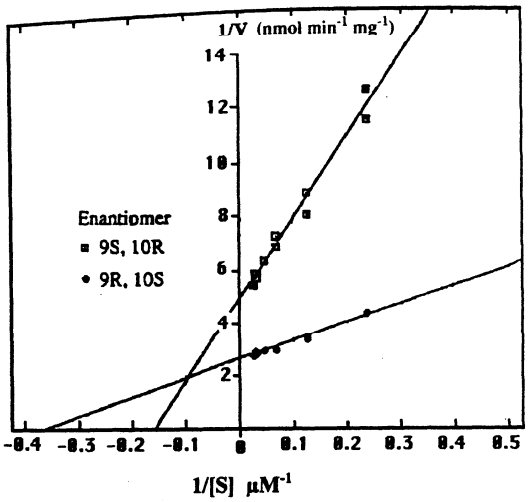

Figure 3. *Lineweaver-Burk reciprocal plot of hydrolysis of the enantiomers of 9,10-epoxystearic acid in* Vicia sativa *microsomes.* Racemic radiolabeled 9,10-epoxystearic acid was subjected to HPLC analysis and both enantiomers were purified. Enantiomers were then separately incubated at different concentrations of substrate (S) with microsomes of *Vicia sativa*.

accumulation of mRNA coding for soluble EH (Kiyosue *et al.*, 1994). We treated *V. sativa* seedlings with 2,4-D or with the peroxisome proliferator clofibrate. We used ω-hydroxylation of oleic acid as a positive control for these treatments (Salaun *et al.*, 1986; Pinot *et al.*, 1992). This activity was strongly stimulated, indicating that 2,4-D and clofibrate were taken up and reached their target sites. However they did not stimulate the EHs activities. Further investigations (*i.e.* time course, dose-response effect...) are needed before definitive conclusions can be drawn but our results suggest that neither soluble nor microsomal EHs are induced by clofibrate or 2,4-D in *V. sativa*. Surprisingly clofibrate treatment resulted in a decrease of EH activity in the microsomes, but not in the soluble fraction. This decrease was also observed when activity was expressed per unit wet weight (0.54+0.04 μmol min^{-1} g^{-1} and 0.34±0.08 μmol min^{-1} g^{-1}). Phenobarbital treatment was performed using the same conditions shown to cause induction of cytochrome P450 dependent reactions in plants (Pinot *et al.*, 1994). Phenobarbital, which is known to induce microsomal EH in mammals (Oesch, 1973; Lu and Miwa, 1980; Wixtrom and Hammock, 1985) had no effect on EH activities of *V. sativa* under these conditions.

We tested the effect of four inhibitors of mammalian EHs, a microsomal EH inhibitor, 1,1,1-

trichloropropene-2,3-oxide (TCPO) (Oesch, 1973) and three soluble EH inhibitors, 4-fluorochalcone oxide (4-FCO) and nitrophenylglycidols (SS-NPG and RR-NPG) (Mullin and Hammock, 1982; Dietze *et al.*, 1991 and 1993). Only weak inhibition of EH activity was observed in both fractions in the presence of TCPO. The mechanism of action of this inhibitor is not known, however some data suggest that it acts as a competing substrate with a low K_m (Wixtrom and Hammock, 1985). The poor inhibition that we observed could be explained by a low affinity of the plant EHs for this compound.

A systematic screening of over 150 compounds revealed that chalcone oxides are potent inhibitors of previously studied EHs (Mullin and Hammock, 1982). In collaborating work, we have shown that 4-FCO (62.5 μM) completely inhibited the soluble EHs from *A. thaliana* (Kiyosue *et al.*, 1994), *S. tuberosum* (Stapleton *et al.*, 1994) and the EH activity present in a crude extract from *S. tuberosum* (Stapleton *et al.*, 1994). Inhibition of EHs by chalcone oxides is thought to be a mechanism based inactivation (Borhan *et al.*, 1995). The lack of inhibition by 4-FCO that we observed with *V. sativa* may be explained as a difference in conformation of the active site preventing 4-FCO from binding. The different sensitivities of EHs from *A. thaliana, S. tuberosum* and *V. sativa* to 4-FCO support the hypothesis that different forms of EH exist in different plants.

Preincubation with the nitrophenylglycidol SS-NPG inhibited EH activity in the soluble fraction by 45% but did not affect activity in the microsomes. The enantiomer RR-NPG did not affect EH activity either in microsomes or in the soluble fraction. These results are consistent with those from Dietze *et al.*, (1991, 1993) who showed that nitrophenylglycidols inhibit mammalian soluble EHs from different sources, but the microsomal enzymes are either not, or poorly, inhibited. Furthermore, the SS-NPG is 750 fold more potent than RR-NPG (Dietze *et al.*, 1993).

Studies performed to investigate the effect of pH showed that lowering the pH from 9 to 7.4 stimulated the activity by 366% in the cytosol and by only 72% in the microsomes. Despite careful washing of the microsomes with high ionic strength buffer, we can not completely eliminate the possibility of soluble EH contamination of the microsomal fraction. However, the differences in sensibility to SS-NPG and to pH of microsomal and soluble EH, together with the decrease in microsomal EH activity following clofibrate treatment strongly suggest the existence of at least two different enzymatic systems in *V. sativa.*

The significance of plant EHs remains to be clarified. Different subcellular locations of EHs may reflect different physiological roles. Plants are isolated from the environment by cutin, a structural part of the cuticle which protects plants against attacks from pathogens (Kolattukudy, 1981). Cutin is a polymer of oxidized fatty acids (Kolattukudy, 1981). Due to its subcellular location, the microsomal EH is a good candidate for involvement in cutin monomer synthesis. Epoxides of fatty acids in plants can be generated by cytochrome P450 (Fahlstadius, 1991; Salaun *et al.*, 1992) or by peroxygenases that use hydroperoxides of fatty acids as cofactors (Blée and Schuber, 1990; Hamberg and Hamberg, 1990). All these enzymes are located in the microsomal membranes. The hydrophobic nature of fatty acids ensures that they are also mainly located in membranes. Therefore, the membranes possess both the enzymes and substrates necessary for the synthesis of epoxystearic acid. It is noteworthy that the microsomal EH that we describe here, is like the soluble EH from *Glycine max* (Blée and Schuber, 1992) enantioselective for the 9R, 10S-epoxystearic acid which is preferentially produced by a peroxygenase (Blée and Schuber, 1990). The resulting 9,10-dihydroxystearic acid is ω-hydroxylated by a cytochrome P450 (Pinot *et al.*, 1992) also located in the microsomes. The final product 9,10,18-trihydroxystearic acid is one of the major cutin monomers (Kolattukudy, 1981). Plant EHs might also be involved in resistance to disease *via* the production of diols of fatty acids which have been shown to inhibit the growth of pathogens (Kato *et al.*, 1985; Masui *et al.*, 1989; Ohta *et al.*, 1991). The stimulation of the mRNA coding for a soluble EH in potato by methyl jasmonate, a mediator in plant defence events, supports involvement of EHs in resistance. Plants, like animals are in continuous contact with epoxides which are ubiquitous and occur naturally, in industry, in the environment or in biochemicals pathways. Some of them are highly toxic (Manson, 1980). Contrary to animals, plants do not have an effective excretion pathway. Therefore plants must metabolize xenobiotics into more soluble products that can be easily stored in the vacuole (Sandermann, 1992). EHs would be the ideal enzymes to accomplish such reactions with epoxides. The recent finding of EH activities in 11 different plants is further evidence of the importance of these enzymes (Stark *et al.*, 1995 *b*). We are in the process of cloning EHs

from *V. sativa*. Comparison of their sequences with sequences from soluble EHs already reported (Kiyosue *et al.*, 1994; Stapleton *et al.*, 1994) will show if there is any prenylation or palmitoylation sites which would suggest a membrane localization. As expected, a search with the Prosite data base (Bairoch, 1991) showed no such prenylation or palmitoylation site in the soluble EHs from *A. thaliana* and *S. tuberosum*. In mammals (Oesch, 1973; Wixtrom and Hammock, 1985, Nourooz-Zadeh, 1992) studies suggest that EHs with different subcellular localization have different physiological roles. We are also in the process of purifying non recombinant and recombinant plant EHs. Enzymologic studies with different substrates and inhibitors, performed with pure enzymes should help to gain a better understanding of their physiological roles.

METHODS

Chemicals. [1-^{14}C] Oleic acid (2.0 GBq mmol^{-1}) was purchased form DuPont-New England Nuclear (Boston, MA, USA). Racemic [1-^{14}C] 9,10-epoxystearic acid was synthesized from [1-^{14}C]oleic acid using m-chloroperoxybenzoic acid. Clofibrate (ethyl *p*-chlorophenoxyisobutyrate) and phenobarbital were purchased from Sigma (St Louis, MO). Thin-layer chromatography plates (Silica gel G60 F254 0.25 mm) were purchased from Merck (Darmstadt, Germany). The EH inhibitors TCPO, SS-NPG and RR-NPG were purchased from Aldrich Chemical Co., INC (Milwaukee, WI, USA). The 4-FCO was prepared as reported previously (Mullin and Hammock, 1982).

Preparation of plant subcellular fractions. Four days old etiolated *V. sativa* L. (Var minor) were aged for 48 h in distilled water or in solutions containing 1 mM clofibrate, 8 mM phenobarbital or 0.5 mM 2,4-D. Soluble and microsomal fractions were isolated as previously described (Benveniste *et al.*, 1977) with the addition of the following step: after the first 100 000 × *g* centrifugation, microsomes were carefully washed by resuspending in 100 mM pyrophosphate, 10 mM β-mercaptoethanol buffer in order to eliminate contamination from the soluble fraction. For each treatment, 50 g of shoots were homogenized in a final volume of 100 ml. Microsomes were resuspended in 10 ml buffer. Protein concentration of the microsomal and soluble fractions were estimated with BCA reagent from Pierce and a microassay procedure from Biorad, respectively, using bovine serum albumin as a standard.

Enzyme activity measurement and inhibition studies. EH activity was measured using the radiolabeled substrate 9,10-epoxystearic acid as previously described (Pinot *et al.*, 1992). Briefly, the standard assay contained 307 or 40 μg of protein from microsomal or soluble fraction, respectively, in a final volume of 0.2 ml of 20 mM Na-phosphate buffer (pH 7.4). The reaction was initiated by the addition of 2 μl of a 10 mM epoxystearic acid solution in ethanol, using a Hamilton repeating syringe and stopped with 0.2 ml acetonitrile (0.2% acetic acid) after a 15 min incubation period at 27°C. The residual substrate and hydrolysis product were extracted twice with diethyl ether (400 μl) and resolved by TLC using a mixture of diethyl ether/light petroleum (bp 40-60°)/formic acid (50/50/1, v/v/v). The residual radioactivity in the aqueous phase after extraction was at background levels. The plates were scanned with a System 200 imaging scanner from Bioscan. The area corresponding to 9,10-dihydroxystearic acid (Rf 0.1) was scraped into counting vials. For inhibition studies, the fractions were preincubated with the tested compounds: 100 μM 4-FCO, 500 μM TCPO, 500 μM SS-NPG, 500 μM RR-NPG, all in ethanol, or only with ethanol (control). After 10 min, the substrate was added and the reaction was allowed to proceed for 15 min and stopped as described above. For kinetic studies, 6 data points (ranging from 4 to 70 μM) were used to determine K_m and $V_{max(observed)}$ by the computational method of Wilkinson (1961) using the Wilman4 computer program. We knew from previous work that clofibrate and 2,4-D induce ω-hydroxylation of fatty acids in microsomes from *V. sativa* (Salaun *et al.*, 1986; Pinot *et al.*, 1992). As a positive control for the induction experiments, ω-hydroxylation of oleic acid was measured according to the procedure previously described (Pinot *et al.*, 1992). To determine pH effect, incubations of microsomal and soluble fractions were performed in 50 mM Tris buffer at pH 7.4 and 9. All assays were shown to be linear for both protein and time.

Synthesis of methyl 9R, 10S-epoxystearate. 1,7 heptanediol was treated under liquid-liquid extraction conditions with hydrobromic acid to yield 7-bromoheptanol (87% yield). Oxidation with potassium permanganate in the presence of sulfuric acid provided 7-bromoheptanoic acid (81% yield). Sequential treatment of this bromo acid with 2-equivalents of the di-lithium salt of 2-propyn-1-ol (formed from 2 equivalents of lithium diisopropylamide) and then with diazomethane yielded the propynol ester (58% yield). There was about 10% O-alkylation of the 2-propyn-1-ol in this literature-precedented reaction. Cis hydrogenation over a Ni/P-2 catalyst yielded the olefinic ester (90% yield). Treatment of the alcohol, under Sharpless conditions in the presence of (+) diethyl tartrate produced the epoxy alcohol (85% yield). Collins oxidation (CrO_3Py_2) of the epoxy alcohol, in the presence of Celite yielded the epoxy aldehyde (62% yield). Attempts to perform this transformation using Swern conditions yielded the epoxy aldehyde in ~30% yield. The epoxy aldehyde was treated with heptyl triphenylphosphonium bromide under Wittig conditions (butyl lithium, tetrahydrofuran/hexamethylphosphoric triamide) to provide the olefin (54% yield). Treatment of the olefin with

2.4.6-tri-*iso*-propylbenzenesulfonyl hydrazide in refluxing THF yielded the final 9R, 10S-epoxystearate (90% yield). The overall yield for this synthesis was 9% and the enantiomeric excess was on the order of 95% as determined on a chiral HPLC column.

Chiral analysis. Radiolabeled enantiomers of epoxystearic acid were separated on HPLC (Waters equiped with 2 pumps 510 and a U6K injector from Waters) using a chiral column (Column Chiracel OB (4.6 x 250 mm) J.J. Baker Chemical Co.) Enantiomers were resolved using isocratic solvent: hexane/isopropanol/acetic acid (99.7/0.2/0.1, v/v/v) at a flow of 0.8 ml min^{-1}. Radioactivity of the HPLC effluent was monitored with a computerized on-line solid scintillation counter (Ramona-D RAYTEST, Germany). To ascertain the configuration of each peak, an amount of radiolabeled epoxide non detectable by ^{1}H-NMR in our conditions (0.5 μg), was injected onto the column, both enantiomers were collected and then, methylated with diazomethane. They were then coinjected onto the column with 1 mg of optically pure methyl 9R, 10S-epoxystearate. Both peaks were collected and analyzed by ^{1}H-NMR. Only one of them was shown to contain the pure epoxide. In our conditions of chiral analysis, the methyl esters of 9S, 10R- and 9R, 10S-epoxystearic acid have retention times of 33 and 45 min respectively, and the corresponding free acids have retention times of 61 and 68 min respectively. To determine the chirality of the residual epoxide after incubation, the epoxide and the diol were separated on TLC as described above. The area corresponding to the epoxide was scraped and the epoxide was eluted from the silica with 10 ml of diethyl ether/hexane (50/50, v/v) which was then removed by evaporation. The residual epoxide was dissolved in hexane (40 μl) after methylation with diazomethane and analyzed by HPLC as described above.

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