Cytochrome P450-Dependent Hydroxylation of Prosulfuron (CGA 152005) by Wheat Seedling Microsomes†

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Microsomes isolated from shoot tissues of etiolated wheat seedlings (*Triticum aestivum* L. var. Olaf) oxidized the sulfonylurea herbicide prosulfuron (CGA 152005). One major and two minor enzymatic oxidation products were isolated and identified by negative ion FAB/MS and cochromatography (TLC and HPLC) with reference standards. Identification of the major oxidation product (**I**) as 1-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl)-5-hydroxyphenylsulfonyl]-urea was confirmed by proton NMR spectroscopy. Minor oxidation products were tentatively identified as 1-[4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2-yl]-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea (**II**) and an intermediate oxidation product 1-[4-[(hydroxymethyl)oxy]-6-methyl]-1,3,5-triazin-2-yl]-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea (**III**) that degraded to 1-(4-hydroxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea (**IV**). Microsomal oxidation of prosulfuron required NADPH and molecular oxygen. Constitutive enzyme activity was increased 5−28-fold by induction with ethanol and with the herbicide safeners naphthalic anhydride or cloquintocet-mexyl (CGA 185072) and by combinations of naphthalic anhydride or cloquintocet-mexyl with ethanol. Inhibition of enzyme activity by CO in the dark was reversible by light. Other inhibitors of prosulfuron oxidation included tetcyclacis, piperonyl butoxide, cytochrome *c*, polyclonal antibodies raised against wheat cytochrome *c* (P450) reductase, and the herbicides bifenox and linuron. Kinetic studies established that the apparent K_m for prosulfuron was 12.6 ± 1.1 μM and that bifenox and linuron were noncompetitive and mixed-type inhibitors of prosulfuron oxidation, respectively, with apparent K_i values of 210 and 59 μM.

Keywords: *Wheat; microsomes; prosulfuron oxidation; cytochrome P450*

INTRODUCTION

Prosulfuron is a new sulfonylurea herbicide developed for selective postemergence broadleaf weed control in corn and other cereals (Kupatt *et al.*, 1993; Peek and Porpiglia, 1993; Schulte *et al.*, 1993). The tolerance of cereals to sulfonylurea herbicides is associated with differential oxidative metabolism and the compartmentation of oxidized intermediates as conjugated polar metabolites (Beyer *et al.*, 1988; Blair and Martin, 1988; Sweetser *et al.*, 1982).

Microsomal cytochrome P450 monooxygenases responsible for the oxidative metabolism and detoxification of a number of different sulfonylurea herbicides in cereals have been reported (Barrett, 1995; Fonné-Pfister *et al.*, 1990; Frear *et al.*, 1991; Moreland *et al.*, 1993; Thalacker *et al.*, 1994). Recent studies have shown that prosulfuron is also oxidized by microsomes from etiolated corn, grain sorghum, and wheat seedlings (Frear and Swanson, 1994; Moreland *et al.*, 1993, 1994).

The objectives of this study were to (a) use wheat seedling microsomes for the *in vitro* biosynthesis, isolation, and identification of oxidized prosulfuron metabolites in wheat and (b) further characterize the cytochrome P450 monooxygenase system responsible for prosulfuron oxidation and detoxification in wheat.

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MATERIALS AND METHODS

Chemicals. Prosulfuron [1-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea], [*phenyl*-U-^{14}C]prosulfuron (17.89 μCi/μmol), and cloquintocet-mexyl [1-methylhexyl (5-chloroquinolin-8-yloxy)acetate] were provided by the Ciba-Geigy Corp. Radiolabeled prosulfuron was purified by TLC to >99% radiochemical and chemical purity. The following reference compounds were provided by Ciba-Geigy: 1-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl)-5-hydroxyphenylsulfonyl]urea; 1-[4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2-yl]-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea; and 1-(4-hydroxy-6-methyl-1,3,5-triazin-2-yl)-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea. Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] was provided by DuPont Co., and bifenox [methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate] was provided by Mobay Chemical Co.

Instrumentation. Negative ion fast atom bombardment mass spectra (FAB/MS) were obtained with a Varian Model CH-5 DF mass spectrometer interfaced with a Model SS-200 data system (Frear *et al.*, 1991). Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM 400 (400 MHz) spectrometer with an Aspect 3000 processing system (Frear *et al.*, 1993). Gradient HPLC separations were obtained with a Waters Model 660 solvent programmer. Separated reference compounds and microsomal oxidation products were detected by UV absorption at 254 nm on a Varian Vari-Chrom UV−vis monitor, and radioactivity was determined with a Packard Trace 7140 radioactivity monitor (RAM).

Spectrophotometric Assays. Cytochrome P450, NADPH cytochrome *c* (P450) reductase activity, and microsomal protein concentrations were determined as reported (Frear *et al.*, 1991).

Plant Materials. Wheat (*Triticum aestivum* L. var. Olaf) seeds were germinated and grown in the dark at 21 °C for 72−90 h (Thalacker *et al.*, 1994). Etiolated shoots (1.0−1.8

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cm) were excised and used directly or separated into coleoptile and leaf tissues for the preparation of microsomes.

Induction Treatments. Microsomal prosulfuron hydroxylase activity and cytochrome P450 were induced with naphthalic anhydride and ethanol (Frear *et al.*, 1991). Naphthalic anhydride was applied directly as a seed dressing (0.25% w/w), and ethanol was applied by subirrigation as a 10% solution in 0.5 mM $CaSO_4$ 24 h before shoot tissues were excised. Induction with cloquintocet-mexyl (25% wettable powder) was by direct application as a seed dressing (2.0 g/kg of seed).

Microsome Isolation. Microsomes were isolated from tissue homogenates by differential centrifugation and stored as previously described (Frear *et al.*, 1993). Etiolated shoot, leaf, and coleoptile tissues were homogenized with 1.5 volumes of 0.1 M potassium phosphate buffer (pH 7.5) containing 10% glycerol, 0.1% globulin-free bovine serum albumin (BSA, Sigma No. A-7638), 5 mM dithiothreitol (DTT), and 1 mM $LiCO_3$. Microsomal pellet fractions (100 000 g) were resuspended in 0.1 M potassium phosphate buffer (pH 7.5) with 10% glycerol and 2.5 mM DTT and either stored at −80 °C or used directly for enzyme and spectrophotometric assays after gel filtration on PD-10 Sephadex G-25 (Pharmacia) columns with 0.1 M potassium phosphate buffer (pH 7.5) containing 2.5 mM DTT.

Prosulfuron Hydroxylase Assays. Standard prosulfuron hydroxylase assays contained 100 μM [^{14}C]prosulfuron (ca. 2.7 $\times$ 10^5 dpm) with a specific activity of 1.2 μCi/μmol together with 0.5−1.0 mg of microsomal protein, 0.1 M potassium phosphate buffer (pH 7.5), 2.5 mM DTT, 1 mM NADPH, and 1% (w/v) globulin-free BSA in a final volume of 1.0 mL.

Reactions were initiated by the addition of substrate and NADPH and were incubated at 25 °C for 15 or 30 min. Control reactions were run without NADPH. Under these assay conditions, enzyme activity exhibited steady-state kinetics, was proportional to microsomal protein concentration, and was linear with respect to time.

Reactions were terminated by rapid freezing in a dry ice/acetone bath and lyophilized. Metabolites were dissolved in MeOH and analyzed by TLC on 250 μm Whatman LK6F silica gel plates. The benzene/dioxane/formic acid solvent system (90:10:2 v/v, 2×) used for the TLC analysis of microsomal oxidation products was developed twice. Separated ^{14}C oxidation products were located under ultraviolet light or by scanning with an image-analyzing radioactivity detector (Bioscan System 200 imaging scanner, Bioscan, Inc., Washington, DC). The radioactive zones were scraped off the TLC plates and quantitated by liquid scintillation spectrometry with Universol cocktail (ICN Biomedicals, Inc.).

Isolation and Identification of Microsomal Oxidation Products. Microsomes from etiolated seedlings treated with a combination of naphthalic anhydride and ethanol as inducers of cytochrome P450 monooxygenase activity were used for the *in vitro* biosynthesis, isolation, and identification of prosulfuron oxidation products. The standard microsomal assay system was scaled up 50-fold, incubated at 25 °C for 60 min, terminated by freezing in a dry ice/acetone bath, and lyophilized. An aqueous 80% methanol extract of the lyophilized reaction mixture was filtered, concentrated *in vacuo* on a rotary evaporator at 40 °C to remove the MeOH, and loaded on a 1.5 × 5.0 cm C_{18} silica gel column. The column was washed with 30 mL of distilled water, and the oxidation products, together with unreacted prosulfuron, were eluted with 30 mL of aqueous 10% acetonitrile. The eluate was concentrated to dryness on a Speed Vac (Savant Instruments, Inc.) at 40 °C, dissolved in MeOH, and rechromatographed three times by TLC with benzene/dioxane/formic acid (90:10:2 v/v, 3×). Separated ^{14}C oxidation products were located under UV light, scraped off the TLC plate, and placed directly on PrepSep C_{18} extraction columns (Fisher Scientific). The extraction columns were washed with 6−8 mL of distilled water and the oxidation products eluted with 3−5 mL of aqueous 40% acetonitrile. The acetonitrile eluates were concentrated to dryness at 40 °C on a Speed Vac and the purified oxidation products were transferred to small vials in acetone for FAB/MS and NMR analysis.

Inhibition Studies. The oxygen requirement and light-reversible CO inhibition studies were conducted as reported (Frear *et al.*, 1993). In other studies, microsomes were preincubated for 10 min at 4 °C with selected cytochrome P450 and herbicide inhibitors (Frear *et al.*, 1991). Reactions were then initiated by the addition of NADPH and substrate and incubated at 25 °C for 30 min. Inhibition studies with polyclonal antibodies to wheat NADPH cytochrome *c* (P450) reductase were performed as reported (Thalacker *et al.*, 1994). Induced microsomes were preincubated with purified antibodies for 1 h at 4 °C prior to enzyme assays.

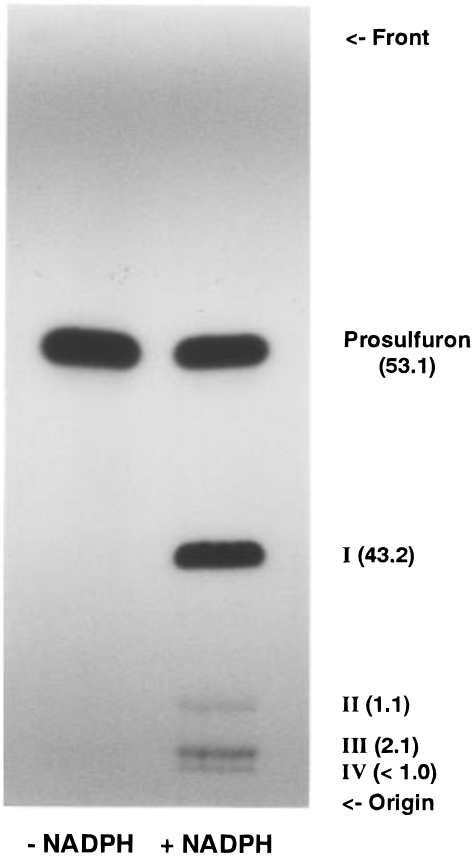

Figure 1. Autoradiograph of TLC-separated [^{14}C]prosulfuron oxidation products from a standard microsomal assay system incubated for 60 min at 25 °C. Chromatogram was developed twice (15 cm) with benzene/dioxane/formic acid (90:10:2 v/v, 2X). The percent distribution of ^{14}C is in parentheses.

Enzyme Kinetic Studies. Lineweaver−Burk, Hanes−Woolf, and Dixon plots were used for kinetic analysis and the determination of apparent K_m and K_i values. Data were analyzed with Enzfitter, a nonlinear regression analysis program developed for the IBM PC (Biosoft, Cambridge, U.K.).

RESULTS AND DISCUSSION

Separation of Microsomal Oxidation Products. A typical TLC separation and autoradiograph of [^{14}C]-prosulfuron oxidation products from a microsomal reaction is shown in Figure 1. One major oxidation product (**I**) and two minor products (**II** and **III**) were separated, isolated, and purified for identification. Cochromatography (TLC and HPLC) with synthetic standards (Table 1) suggested that the major oxidation product (**I**) was hydroxylated on the phenyl ring and that the minor products were formed by the oxidation of either the methyl (**II**) or the methoxy (**III**) substituents on the *s*-triazine ring. An additional slightly more polar trace product **IV** that separated from **III** was also detected by autoradiography (Figure 1).

Identification of Isolated Oxidation Products. Characteristic negative FAB/MS molecular and fragment ions for isolated prosulfuron oxidation products

Table 1. Chromatographic Separation of Prosulfuron Oxidation Products and Reference Standards

oxidation product	reference standard	TLC[a] R_f (×100)	HPLC[b] R_t (min)
	prosulfuron	53	10.70
I	(5-hydroxyphenyl)prosulfuron	28	9.60
II	[4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2yl]prosulfuron	10	9.00
III		5	
IV	(4-hydroxy-6-methyl-1,3,5-triazin-2yl)prosulfuron	3	7.95

[a] Benzene/dioxane/formic acid solvent system developed twice (90:10:2 v/v, 2×) with a 15 cm solvent front on a 250 μm Whatman LK6F silica gel plate. [b] Whatman C_{18} Partisil 10 ODS-3 column (9 mm × 25 cm) with a linear 15 min gradient from 25% to 100% (v/v) CH_3CN in 1% (v/v) aqueous HOAC at 5 mL/min.

(**I**−**III**) are shown in Figure 2. Mass spectra of the major oxidation product (**I**) were comparable to those of a (5-hydroxyphenyl)prosulfuron reference standard. A molecular ion (M − 1) at *m*/*z* 434 together with ion fragments *m*/*z* 268 and 294 (296 − 2) suggested that the phenyl ring was oxidized, while the ion fragment at *m*/*z* 139 indicated that the substituent methyl and methoxy groups on the *s*-triazine ring were unchanged. Induced microsomes isolated from shoots of corn, grain sorghum, barley, oat, rice, and wheat seedlings also have been reported to hydroxylate the phenyl ring of prosulfuron as a primary oxidation product (Moreland *et al.*, 1996).

Mass spectra of minor oxidation product **II** were comparable to the mass spectrum of the [4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2-yl]prosulfuron reference standard and showed that the substituted phenyl ring was unchanged (ion fragments *m*/*z* 237 and 252). Instead, the molecular ion (M − 1) at *m*/*z* 434 and the fragment ion at *m*/*z* 155 indicated that either the substituent methyl or methoxy group on the *s*-triazine ring was oxidized. Since the [4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2-yl]prosulfuron reference standard also cochromatographed (TLC and HPLC) with oxidation product **II** (Table 1), this oxidation product was identified as 1-[4-(hydroxymethyl)-6-methoxy-1,3,5-triazin-2yl]-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]-urea (Figure 2).

Mass spectra of minor oxidation product **III** were comparable to the mass spectra of the (4-hydroxy-6-methyl-1,3,5-triazin-2yl)prosulfuron reference standard and showed that the phenyl ring was not changed (ion fragments *m*/*z* 237 and 252). A molecular ion (M − 31) at *m*/*z* 404 and ion fragments at *m*/*z* 125 and 151 (153 − 2) suggested that the methoxy group on the *s*-triazine ring was oxidized to an unstable hydroxymethyloxy (−OCH_2OH) intermediate (**III**) that degraded during FAB/MS and, to a limited extent, during TLC with a loss of formaldehyde (HCHO) to form **IV** (Figure 1), a slightly more polar product that cochromatographed (TLC) with the reference standard, 1-(4-hydroxy-6-methyl-1,3,5-triazin-2yl)-3-[2-(3,3,3-trifluoropropyl)-phenylsulfonyl]urea (Table 1). Similar intermediate oxidation products of *O*-demethylation have been reported in rice as metabolites of the herbicides chlomethoxynil (Niki *et al.*, 1976) and bensulfuron (Beyer *et al.*, 1988). Prosulfuron *O*-demethylation and the formation of 1-(4-hydroxy-6-methyl-1,3,5-triazin-2yl)-3-[2-(3,3,3-trifluoropropyl)phenylsulfonyl]urea (**IV**) as a major oxidation product have been reported with induced microsomes isolated from shoots of tolerant grain sorghum seedlings (Moreland *et al.*, 1996).

Identification of the major oxidation product **I** as 1-(4-methoxy-6-methyl-1,3,5-triazin-2yl)-3-[2-(3,3,3-trifluoropropyl)-5-hydroxyphenylsulfonyl]urea was confirmed by comparison of its proton NMR spectrum (Figure 3) with

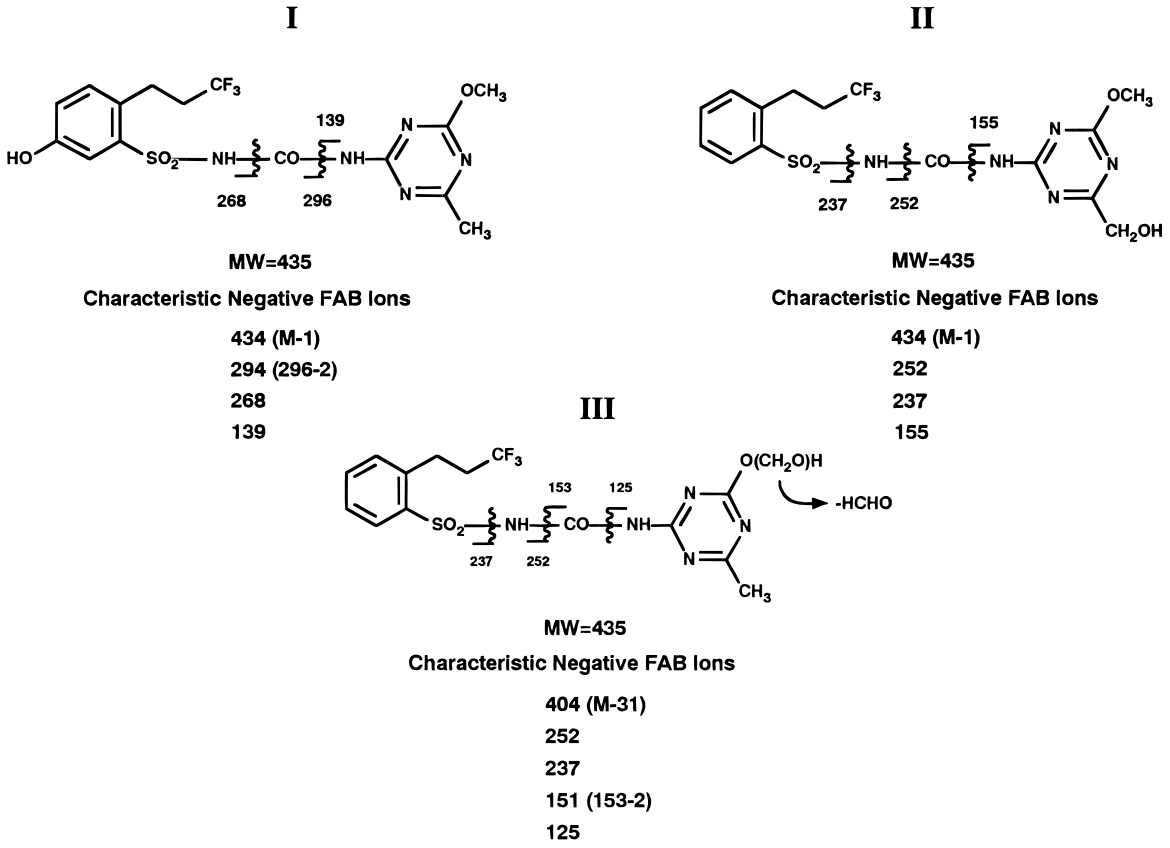

Figure 2. Characteristic molecular and fragment ions from negative FAB mass spectra of isolated microsomal oxidation products.

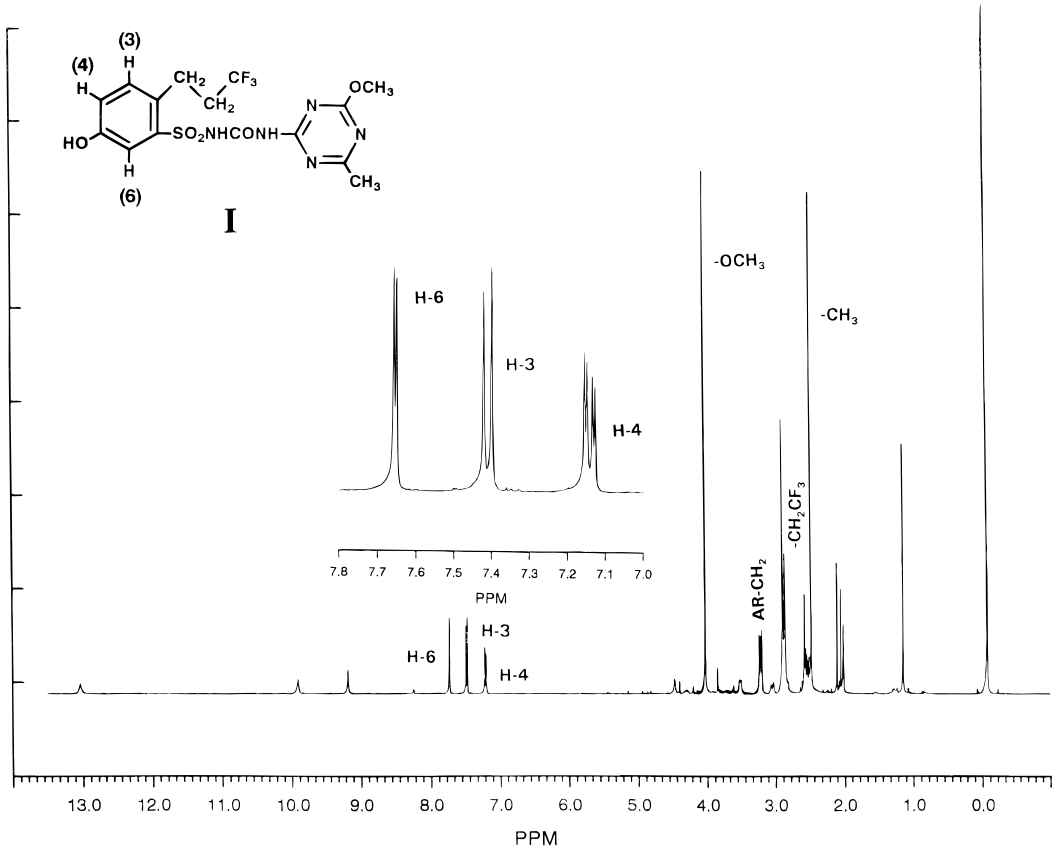

Figure 3. Proton NMR spectrum of isolated microsomal oxidation product **I**.

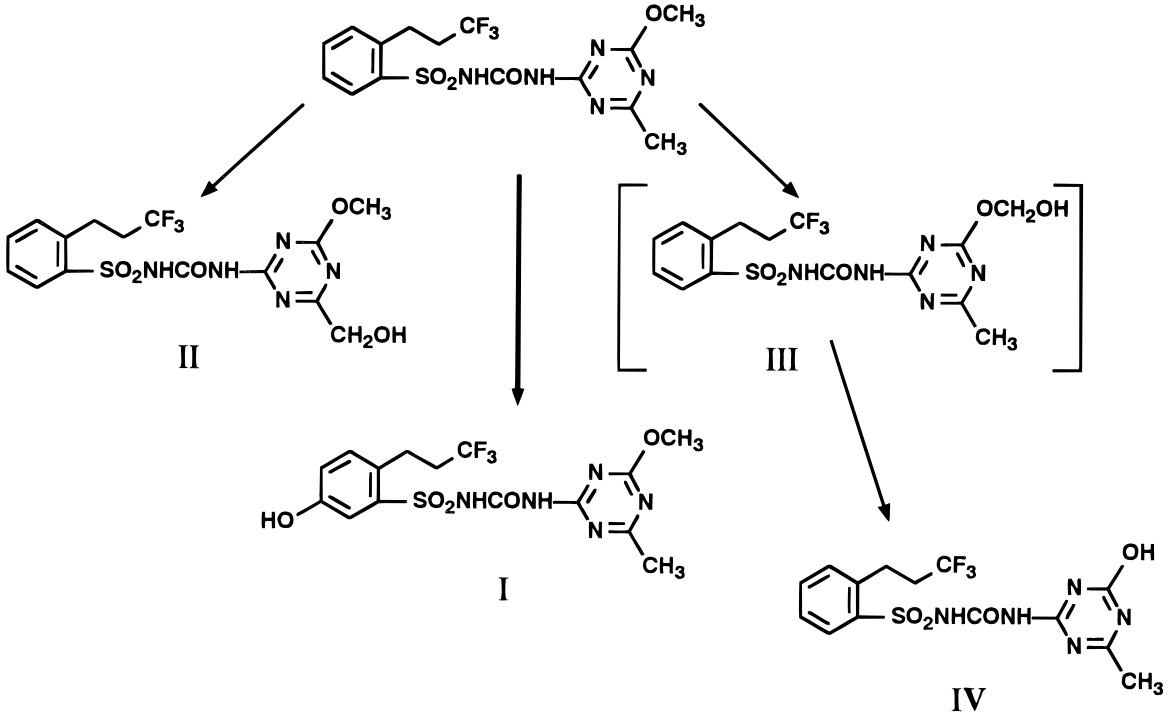

Figure 4. Proposed scheme for the oxidative metabolism of prosulfuron in wheat. Brackets indicate a proposed intermediate.

that of the (5-hydroxyphenyl)prosulfuron reference standard. Both spectra were identical. ^{1}H NMR (acetone-d_6) δ 2.51 (s, 3H, CH_3), 2.58 (m, 2H, CH_2CF_3), 3.24 (t, 2H, J = 8.4 Hz, $ArCH_2$), 4.02 (s, 3H, OCH_3), 7.14 (dd, 1H, J = 2.7, 8.4 Hz, ArH-4), 7.41 (d, 1H, J = 8.4 Hz, ArH-3), 7.65 (d, 1H, J = 2.7 Hz, ArH-6), 9.79 (s, 1H, NH-triazine), 12.87 (s, 1H, NH-SO_2). Proton NMR spectra of isolated minor oxidation products **II** and **III** were not obtained because of insufficient sample size for analysis.

On the basis of the identification of isolated microsomal oxidation products by comparative spectroscopy (FAB/MS and NMR) and cochromatography (TLC and HPLC) with reference standards, a proposed scheme for the oxidative metabolism of prosulfuron in wheat is shown in Figure 4.

Table 2. Induction of Microsomal Prosulfuron Hydroxylase Activity[a]

inducer[b]	cyt P450 [pmol (mg of protein)$^{-1}$]	NADPH cyt *c* red. [nmol min^{-1} (mg of protein)$^{-1}$]	prosulfuron hydroxylase act. [pmol min^{-1} (mg of protein)$^{-1}$]	rel prosulfuron hydroxylase act.
none	236 ± 6	91 ± 3	34 ± 7	1.0
naphthalic anhydride	254 ± 21	90 ± 5	197 ± 57	5.8
cloquintocet-mexyl	278 ± 5	93 ± 6	386 ± 35	11.4
EtOH	365 ± 12	97 ± 5	729 ± 50	21.4
naphthalic anhydride/EtOH	367 ± 15	93 ± 8	808 ± 53	23.8
cloquintocet-methyl/EtOH	416 ± 2	105 ± 1	969 ± 56	28.5

[a] Average values for two separate experiments with microsomal preparations from etiolated wheat seedling shoot tissues. [b] Naphthalic anhydride was applied as a seed dressing (0.25% w/w) prior to germination. Cloquintocet-methyl (25% wettable powder) also was applied as a seed dressing (2.0 g/kg of seed). Ethanol treatment was by subirrigation with a 10% (v/v) solution in 0.5 mM $CaSO_4$ for 24 h before shoot tissues were excised.

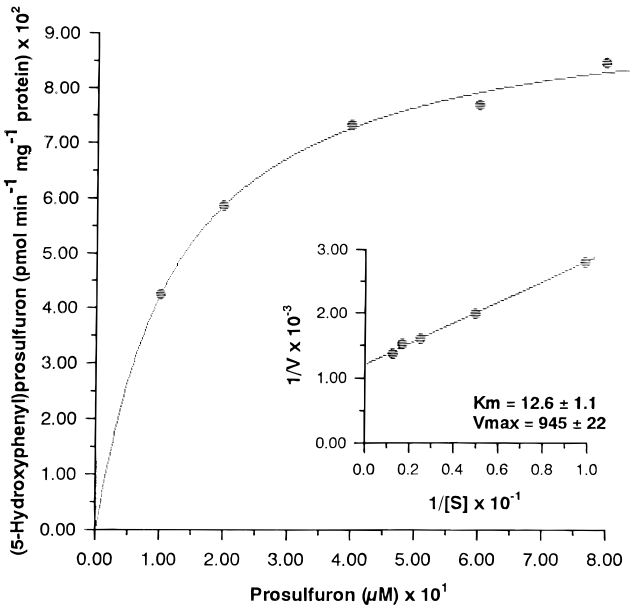

Figure 5. Michaelis−Menten kinetics and Lineweaver−Burk plot of microsomal prosulfuron hydroxylase reaction.

Enzyme Induction. Constitutive levels of microsomal prosulfuron hydroxylase activity were increased by treatment of etiolated wheat seedlings with herbicide safeners and/or ethanol (Table 2). Ethanol was the most effective single inducer of enzyme activity (21.4-fold) followed by cloquintocet-mexyl (11.4-fold) and naphthalic anhydride (5.8-fold). Cloquintocet-mexyl has been reported to be an effective herbicide safener that increases the cytochrome P450-dependent oxidation of the aryloxyphenoxypropanoate herbicide clodinafop-propargyl in wheat (Kreuz *et al.*, 1991). Combination treatments of etiolated seedlings with ethanol and either naphthalic anhydride or cloquintocet-mexyl were not additive, but did increase prosulfuron hydroxylase activity more than ethanol treatments alone (23.8- and 28.5-fold, respectively). Under standard 15 min assay conditions with five different prosulfuron concentrations (10−80 μM), apparent K_m and V_{max} values for prosulfuron were 12.6 ± 1.1 μM and 945 ± 22 pmol min^{-1} (mg of protein)$^{-1}$, respectively (Figure 5). Similar K_m values (13−14 μM) and hydroxylation reactions (5-hydroxyphenyl) were reported with the sulfonylurea herbicides triasulfuron and chlorsulfuron and either noninduced (constitutive) or induced (naphthalic anhydride) wheat seedling microsomes (Frear *et al.*, 1991).

In contrast to marked increases in prosulfuron hydroxylase activity, induced cytochrome P450 levels increased <2-fold and NADPH cytochrome *c* (P450) reductase activities were increased to only a limited extent (Table 2). Similar results with induced wheat seedling microsomes that oxidize triasulfuron (Thalacker *et al.*, 1994) and other herbicides (Frear *et al.*, 1991; Zimmerlin and Durst, 1990) suggest that the cytochrome P450 isoforms responsible for the oxidation of prosulfuron, triasulfuron, and other herbicides may represent a relatively small portion of the total microsomal cytochrome P450.

Table 3. Differences in Microsomal Enzyme Activities and Cytochrome P450 Levels in Etiolated Wheat Seedling Shoot Tissues[a]

tissue[b]	cyt P450 [pmol (mg of protein)$^{-1}$]	NADPH cyt *c* (P450) red. [nmol min^{-1} (mg of protein)$^{-1}$]	prosulfuron hydroxylase [pmol min^{-1} (mg of protein)$^{-1}$]
intact shoot	428	98	852
coleoptile	482	129	1550
leaf	190	59	426

[a] Naphthalic anhydride/EtOH-induced etiolated wheat seedlings. [b] Intact shoot tissues were excised and used directly or separated into coleoptile and leaf tissues for the isolation of microsome fractions.

Tissue Differences in Microsomal Cytochrome P450 Levels and Enzyme Activity. Cytochrome P450 levels, prosulfuron hydroxylase activities, and NADPH cytochrome *c* reductase activities were highest in microsomes isolated from coleoptile tissues compared to excised leaf tissues or intact shoots. Results of a typical study are shown in Table 3. Microsomes from coleoptile tissues contained more than twice the level of cytochrome P450 and NADPH cytochrome *c* (P450) reductase activity and over 3 times the level of prosulfuron hydroxylase activity compared to microsomes isolated from leaf tissues. Coleoptile microsomes also contained slightly higher levels of cytochrome P450 and NADPH cytochrome *c* (P450) reductase activity and nearly twice the level of prosulfuron hydroxylase activity relative to microsomes from excised shoots (coleoptile and leaf tisssues). However, since it was difficult and time-consuming to isolate coleoptile tissues, etiolated shoot tissues were used for routine microsome isolation. Lower levels of prosulfuron hydroxylase activity in leaf tissues and intact shoots may be associated with the presence of interfering pigments and endogenous inhibitors in leaf tissues or differences in the inducibility and expression of cytochrome P450 isoforms between coleoptile and leaf tissues (Thalacker *et al.*, 1994).

Inhibition Studies. Microsomal prosulfuron hydroxylase activity required NADPH (Figure 1) and molecular oxygen. A typical study showed that enzyme activity was inhibited 94% in a N_2 atmosphere and 99% with added glucose oxidase as a dissolved oxygen scavenger (Table 4). Light-reversible CO inhibition, an established criteria for cytochrome P450 dependency,

Table 4. Oxygen Requirement and Light-Reversible CO Inhibition of Microsomal Prosulfuron Hydroxylase Activity[a]

condition	inhibition (%)	condition	inhibition (%)
air[b]		N_2 (40% v/v, light)[c]	
N_2	94	CO (40% v/v, dark)	80
N_2 + glucose oxidase	99	CO (40% v/v, light)	3

[a] Naphthalic anhydride/EtOH-induced microsomes from etiolated wheat seedling shoot tissues. [b] Control for oxygen requirement assays. [c] Control for CO inhibition and light reversal assays.

Table 5. Effect of Selected Cytochrome P450, NADPH Cytochrome *c* (P450) Reductase, and Herbicide Inhibitors on Microsomal Prosulfuron Hydroxylase Activity[a]

inhibitor	concentration (μM)	inhibition[b] (%)
piperonyl butoxide	100	46
tetcyclacis	100	38
cyt *c*	0.5	48
cyt *c* (P540) red. antibody[c]	50[d]	13
	100[d]	35
linuron	100	25
bifenox	100	24

[a] Naphthalic anhydride/EtOH-induced microsomes isolated from etiolated wheat seedling shoot tissues. [b] Average values for two or more assays with different microsome preparations. [c] Rabbit polyclonal antibodies raised against microsomal NADPH cytochrome *c* (P450) reductase from wheat (Thalacker *et al.*, 1994). [d] Micrograms.

was also demonstrated (Table 4). Inhibition by CO in the dark (80%) was almost completely reversed in the light.

In addition to light-reversible CO inhibition, microsomal prosulfuron hydroxylase activity was inhibited by the cytochrome P450 monooxygenase inhibitors piperonyl butoxide and tetcyclacis (Table 5). Cytochrome *c*, an effective competitive electron acceptor for NADPH cytochrome P450 reductase, and polyclonal antibodies to wheat seedling NADPH cytochrome *c* (P450) reductase also inhibited prosulfuron hydroxylase activity and established that enzyme activity was dependent on both cytochrome P450 and NADPH cytochrome P450 reductase. Similar results with induced wheat seedling microsomes and the inhibition of triasulfuron hydroxylase activity have been reported (Frear *et al.*, 1991; Thalacker *et al.*, 1994).

The phenylurea herbicide linuron and the diphenyl ether herbicide bifenox also inhibited prosulfuron hydroxylase activity (Table 5). Although linuron and bifenox represent different classes of herbicides with different sites of action compared to prosulfuron, both herbicides are oxidized by microsomes isolated from induced wheat seedling shoot tissues (Frear *et al.*, 1991; Frear, 1995). Both herbicides also have been reported to inhibit triasulfuron hydroxylation by isolated wheat microsomes (Frear *et al.*, 1991). Lineweaver–Burk, Hanes–Woolf, and Dixon plots (Segel, 1975) of kinetic studies with a range of different linuron or bifenox concentrations (0–120 μM) as prosulfuron hydroxylase inhibitors established that linuron was a mixed-type inhibitor (partially competitive and noncompetitive) with a K_i of 59 μM and that bifenox was a noncompetitive inhibitor with a K_i of 210 μM (Figure 6).

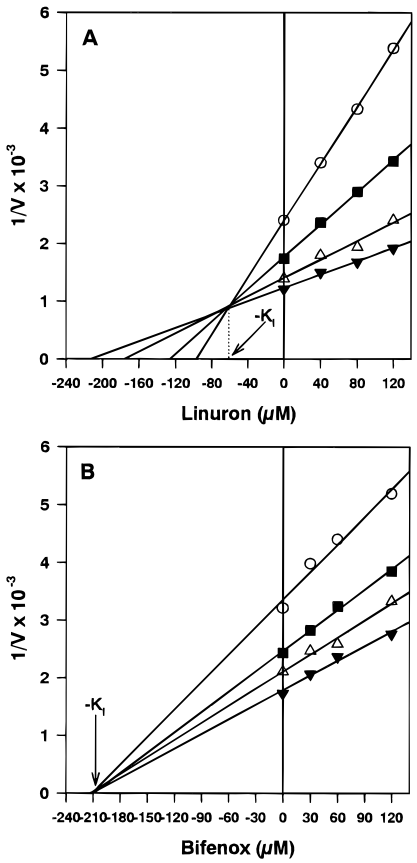

Figure 6. Dixon plots of prosulfuron hydroxylase inhibition by linuron (A) and bifenox (B). Prosulfuron concentrations were 10 (○), 20 (■), 40 (△), and 80 μM (▼). Velocity (*V*) was measured as the amount of (5-hydroxyphenyl)prosulfuron formed in picomoles per minute per milligram of microsomal protein.

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