Inhibitory Activity of Sulfentrazone and Its Metabolic Derivatives on Soybean (*Glycine max*) Protoporphyrinogen Oxidase

Franck E. Dayan,*,† Brian M. Armstrong,‡ and John D. Weete‡

Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 8048, University, Mississippi 38677, and Department of Botany and Microbiology, Auburn University, Auburn, Alabama 36849

The biological activities of sulfentrazone and its metabolic derivatives were investigated in an effort to elucidate the basis for soybean tolerance to this herbicide. All of the metabolic derivatives were less toxic than sulfentrazone as measured by electrolyte leakage from soybean leaf disks. Their in vivo activity correlated with the amount of protoporphyrin IX (Proto) accumulating in herbicide-treated tissues (i.e., more Proto accumulated in tissues treated with sulfentrazone than with the metabolites). I_{50} values for protoporphyrinogen oxidase (Protox) inhibition were 1.2, 0.35, 10, and 37 μM for sulfentrazone and its 3-hydroxymethyl, 3-demethyl, and 3-carboxylic acid metabolic derivatives, respectively, and their binding affinities were related to their relative inhibitory potency. Oxidative degradation of sulfentrazone did not have a great influence on the overall shape of the molecule but affected the steric and electronic environment surrounding the methyl group on the triazolinone ring.

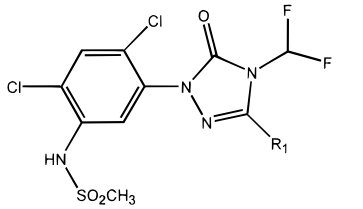
Keywords: *Binding affinity; cellular leakage; computer modeling; herbicide; metabolites; phytotoxicity; protoporphyrin*

INTRODUCTION

The experimental phenyl triazolinone herbicide sulfentrazone, *N*-[2,4-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide (Figure 1), has recently been marketed by FMC Corp. (Theodoridis et al., 1992) for broad spectrum preemergence weed control in soybean (Hancock, 1992).

The molecular site of inhibition of sulfentrazone was determined to be protoporphyrinogen oxidase (Protox) (Nandihalli and Duke, 1993; Dayan et al., 1997c). Inhibition of this highly regulated enzymatic conversion of protoporphyrinogen IX (Protogen) to protoporphyrin IX (Proto) leads to an unregulated extraplastidic accumulation of Proto (Matringe and Scalla, 1988; Becerril and Duke, 1989a,b; Sherman et al., 1991). Accumulation of this photodynamic chlorophyll precursor was responsible for the light-dependent herbicidal action of Protox-inhibiting herbicides (Scalla et al., 1990). All of the known Protox inhibitors apparently compete with Protogen at or near the catalytic site on the enzyme (Matringe et al., 1989, 1992; Witkoski and Halling, 1989; Camadro et al., 1991; Nandihalli et al., 1992a,b; Nandihalli and Duke, 1994; Scalla and Matringe, 1994; Dayan et al., 1997a,b).

We have shown previously that sulfentrazone was sufficiently absorbed by the roots and translocated to induce phytotoxic damage to the foliage of soybean (Dayan et al., 1997c). We have also reported that sulfentrazone was metabolized via a stepwise oxidation of the methyl group on the triazolinone ring to form the 3-hydroxymethyl and 3-carboxylic acid derivatives (Figure 1) (Dayan et al., 1996, 1997c). These initial metabolites were rapidly transformed into conjugated metabolites that accounted for 90% of all the herbicide 24 h after application.



Name	R_1
Sulfentrazone	CH_3
3-hydroxymethyl	$-CH_2OH$
3-carboxylic acid	-COOH
3-demethyl	-H

Figure 1. Structure of sulfentrazone and derivatives.

Metabolic transformation of xenobiotic compounds usually leads to the formation of biologically less active derivatives; however, the reverse effect (bioactivation) has been observed in some cases (Hathway, 1989; Jeffcoat and Harries, 1975; Shimabukuro et al., 1979). This represents a potential problem with fast-acting herbicides such as Protox inhibitors because plants may experience permanent damage due to transient accumulation of either the herbicide or toxic metabolites within a few hours following application. It has been inferred that plants were relatively tolerant to Protox-inhibiting herbicides because of their ability to rapidly metabolize these compounds (Frear et al., 1983; Dayan

* Author to whom correspondence should be addressed [fax (601) 232-1035; e-mail fdayan@ag.gov].
† U.S. Department of Agriculture.
‡ Auburn University.

S0021-8561(97)00988-6 CCC: $15.00
Published on Web 04/09/1998

et al., 1996, 1997a,c). However, we have reported substantial uptake and translocation of sulfentrazone by soybean that may lead to a potentially harmful transient accumulation of the herbicide and/or phytotoxic metabolites (Dayan et al., 1997c). This condition would explain the temporary nature of injury that has been observed on the foliage of soybean (Vidrine et al., 1994; Walker et al., 1992; Dayan et al., 1997c). To date, a limited amount of information is available on the phytotoxicity of herbicide metabolic byproducts (Duke et al., 1991).

This paper reports the results of a comparative study of the physiological and biochemical activities of a Protox inhibitor, sulfentrazone, and its metabolic derivatives. We also discuss the consequences of the oxidative degradation of sulfentrazone on the structure−activity relationship at the molecular level.

MATERIALS AND METHODS

Materials. Sulfentrazone and related metabolic derivatives provided by FMC Corp. (Agricultural Products Group, Philadelphia, PA) were, with purities in parentheses, as follows: technical grade sulfentrazone (98.2%); 3-hydroxymethyl (97.2%), *N*-[2,4-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-(hydroxymethyl)-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide; 3-carboxylic acid (95.2%), *N*-[2,4-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-carboxyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-phenyl]methanesulfonamide; and 3-demethyl (95.6%), *N*-[2,4-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-5-oxo-1*H*-1,2,4-triazol-1-yl]phenyl]methanesulfonamide. Refer to Figure 1 for structures.

Cellular Damage. Tissues were treated with 100 μM sulfentrazone or the metabolites as described by Kenyon et al. (1985). Fifty 4-mm soybean leaf disks (≈0.1 g fresh weight) were placed in a 6-cm-diameter disposable Petri dish containing 5 mL of 1% sucrose and 1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.5) with or without the test compounds. Sulfentrazone and its metabolites were dissolved in methanol. Control tissues were exposed to the same amount of methanol as treated tissues but without the test compounds. The final concentration of methanol in the dishes was 1% (v/v). The leaf disks were incubated at 25 °C in darkness for 20 h and then exposed to 500 μmol m^{-2} s^{-1} photosynthetically active radiation for 24 h. Cellular damage was determined by measuring electrolyte leakage into the bathing medium with a conductivity meter capable of assaying 1 mL of bathing medium (Kenyon et al., 1985).

Conductivity was monitored for 20 h in darkness, followed by 24 h of continuous light. Because of differences in background conductivity between different treatment solutions, results are expressed as a change in the conductivity after initial measurement at the beginning of the dark period. All treatments for electrolyte leakage measurements were in triplicate, and the experiment was repeated.

Protox Inhibition. Crude etioplast preparations, obtained from 10-day-old dark-grown soybean seedlings according to the method of Sherman et al. (1991), were further purified by centrifugation on a Percoll gradient at 6000*g* for 6 min followed by ultracentrifugation on a sucrose step gradient (30−52%) at 80000*g* for 60 min. The yellow band containing purified etioplasts was collected and placed in 10 mL of cold resuspension buffer [50 mM HEPES (pH 7.8) and 330 mM sucrose] and pelleted by centrifugation at 6000*g* for 15 min. The etioplasts were resuspended in 2 mL of buffer and kept at −80 °C until used. No loss of activity was observed in samples stored under these conditions (up to 1 month). All solutions contained 10 μg mL^{-1} phenylmethanesulfonyl fluoride (PMSF), and all centrifugations were performed at 4 °C. Protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin as the standard.

The procedures for Protogen preparation and Protox enzyme assays were derived from Jacobs and Jacobs (1982). Briefly, Protogen was generated by reducing Proto in the presence of sodium amalgam. The assay mixture consisted of 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), and 20 μL of substrate. The I_{50} values were determined under saturated substrate conditions (2 mM Protogen) in the presence of 0, 0.01, 0.1, 1, 10, 100, 1 000, and 10 000 μM technical grade sulfentrazone or 3-hydroxymethyl, 3-carboxylic acid, or 3-demethyl metabolites of sulfentrazone.

The reaction was initiated by adding 100 μL of etioplasts, which had been preincubated with or without the inhibitors for 15 min on ice prior to the assay, to 900 μL of assay mixture. The rate of formation of Proto was measured continuously for 90 s at 30 °C using a spectrofluorometer (model RF-5000, Shimadzu, Japan) with excitation and emission wavelengths set at 395 and 626 nm, respectively. The excitation and emission bandwidths were set at 1.5 and 30 nm, respectively, and an electronic emission filter with cutoff at 430 nm was used to reduce background noise.

Binding Studies. Binding of [^{14}C]acifluorfen (AF) to soybean etioplasts in the presence or absence of sulfentrazone or the metabolites was determined according to the method of Tischer and Strotmann (1977). Etioplasts (0.6 mg of protein) were suspended in a reaction solution consisting of 330 mM sorbitol, 100 mM HEPES (pH 7.7), 1 mM EDTA, and 1 mM $MgCl_2$. Various concentrations (2−100 nM) of ^{14}C-labeled AF (specific activity of 18.03 mCi/mmol, uniformly ring labeled) plus 100 nM of nonlabeled sulfentrazone or the metabolites were added. The suspensions were thoroughly mixed and incubated for 30 min on ice. The samples were centrifuged for 6 min at 12000*g* at 4 °C. The supernatant was transferred to vials and mixed with 12 mL of premixed scintillation cocktail (Ecolume) for radioactivity measurements. The inner walls of tubes were wiped dry with cotton swabs without disturbing the pellets to remove excess ^{14}C. A 100 μL aliquot of tissue solubilizer (Protosol) was added to the pellets and heated in a water bath at 50 °C for 15 min. The slurry was neutralized with 50 μL 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) at pH 7.0 and transferred to vials. Ethanol (50 μL) used to wash the inner walls of each tube was combined to the slurry before radioactivity measurements. The amount of bound [^{14}C]AF was calculated from the radioactivity in the pellets.

Proto Determination. All extractions for HPLC determinations of Proto were made under dim, green light after 20 h of incubation in a 100 μM technical grade sulfentrazone solution in darkness at 25 °C. Samples (≈0.1 g of soybean leaf disks) were homogenized in 2 mL of HPLC grade methanol/0.1 N NH_4OH (9:1 v/v) with a Brinkman Polytron at full speed for 15 s. The homogenates were washed four times with 2 mL volumes of hexane and centrifuged at 6000*g* for 10 min at 4 °C. The supernatants were filtered through a 0.2-μm nylon syringe filter. Samples were stored in light-tight glass vials at −20 °C until analysis by HPLC.

The HPLC system was composed of Waters Associates components, which included a model 510 pump, a model 712 autosampler, a Maxima 820 controller, and models 470 fluorescence and 990 photodiode spectrophotometric detectors. The column used was a 250 × 4.6 mm (i.d.) Spherisorb 5-μm ODS-1 reversed-phase column preceded by a Bio-Rad ODS-5S guard column. The solvent system consisted of 80% HPLC grade methanol with 20% PIC A (paired-ion chromatography) reagent (Waters Corp., Milford, MA). The injection volume was 50 μL. Proto concentration in samples was calculated from a standard curve determined using an authentic Proto standard. Proto was detected with the fluorescence detector with excitation and emission wavelengths set at 400 and 630 nm, respectively. The data are expressed on a molar basis per gram of fresh weight. All treatments were triplicated.

Molecular Properties. The molecular properties of sulfentrazone and its metabolites were calculated using Spartan 4.0 molecular modeling software (Spartan 4.0, Wavefunction Inc., Irvine, CA). The initial structures were constructed using Spartan's builder program, which contains standardized mo-

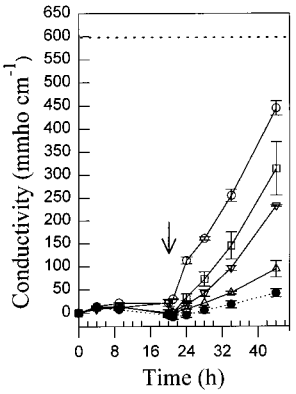


Figure 2. Electrolyte leakage from cells treated with sulfentrazone and its derivatives as measured by change in conductivity in the bathing medium. Soybean leaf disks were incubated in the presence of the herbicidal compounds for 20 h in darkness and then exposed to continuous light: (○) sulfentrazone; (□) 3-hydroxymethyl derivative; (▽) 3-demethyl derivative; (△) 3-carboxylic acid; (●) control without test compound. Arrow indicates beginning of light exposure. Dotted line represents maximum conductivity obtained 24 h after samples were boiled.

lecular fragments and atoms. These preliminary structures were optimized by a molecular mechanics program contained within the builder program. The structures minimized by molecular mechanics were then submitted for full geometry optimization by semiempirical calculation using an AM1 Hamiltonian (Dewar et al., 1985). To minimize the chance of generating molecular geometries that were only local energy minima, rotational isomers were also calculated. The lowest energy structures generated for sulfentrazone and its metabolites were then used to calculate molecular properties. Calculations were performed on the 3-carboxylate rather than the 3-carboxylic acid metabolite because this intermediate is likely to exist predominantly in its ionized form under the conditions in the cells. Structures of sulfentrazone and the metabolites were also generated using Sybyl molecular modeling software (Sybyl 6.0, Tripos Inc., St. Louis, MO), minimized using Powell, and optimized with Mopac running AM1. Electrostatic maps were generated to illustrate the steric and electrostatic changes occurring in the region of the methyl group on the triazolinone ring.

RESULTS

Electrolyte Leakage. Cellular damage, as measured by electrolyte leakage from soybean leaf disks and expressed as conductivity in the bathing medium, was greater with sulfentrazone than with the metabolites tested. Electrolyte leakage induced by sulfentrazone after 24 h of light exposure was 300% greater than in the control treatment (Figure 2) and represented 74% of the amount of electrolyte released by boiled leaf disks (dotted line on graph). Photobleaching symptoms were observed on the sulfentrazone-treated leaf disks after 8 h of exposure to light. The metabolites were less active than sulfentrazone. Conductivity of the bathing medium of tissues exposed to 3-hydroxymethyl and 3-demethyl metabolites was 150% greater than the control, and the 3-carboxylic acid had no activity with electrolyte levels being similar to the control; leakage measured by conductivity as a percent of maximum decreased progressively when exposed to these metabolites, respectively (Table 1). Evidence of photobleaching was not detected on leaf disks treated with the metabolites

Inhibition of Protox. Protox activity in soybean etioplast suspensions was 47.6 nmol h^{-1} (mg of protein)$^{-1}$.

Table 1. Biological Activities of Sulfentrazone and the Metabolites on Soybean

compound	I_{50}[a] (μM)	Proto[b] (nmol g^{-1} of FW)	conductivity[c] ($\mu\Omega$ cm^{-1})
sulfentrazone	1.2	1.1 ± 0.04	444 ± 16
3-hydroxymethyl	0.35	0.88 ± 0.04	313 ± 58
3-demethyl	10	0.54 ± 0.03	232 ± 20
3-carboxylate	37	0.09 ± 0	94 ± 18
control	n/a	0.07 ± 0	42 ± 8

[a] Inhibition of Protox. [b] Proto levels were determined after 20 h of dark incubation in the presence of 100 μM herbicides. [c] Conductivity of the bathing medium after 24 h of light exposure following a 12 h dark incubation period.

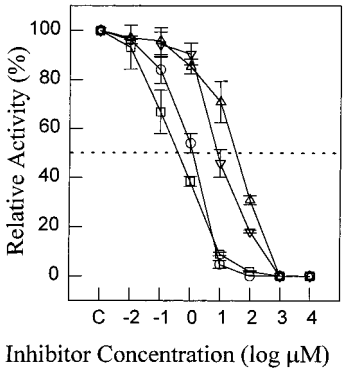


Figure 3. Effect of sulfentrazone and its derivatives on Protox activity of soybean etioplasts measured under initial velocity conditions: (○) sulfentrazone; (□) 3-hydroxymethyl derivative; (▽) 3-demethyl derivative; (△) 3-carboxylic acid. Dotted line represents 50% inhibition of activity.

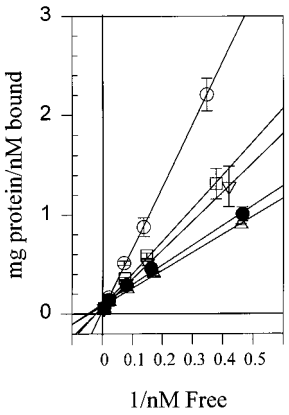


Figure 4. Binding of labeled acifluorfen (AF) in the presence of 1 mM unlabeled sulfentrazone or its derivatives: (●) acifluorfen alone; (○) sulfentrazone; (□) 3-hydroxymethyl derivative; (▽) 3-demethyl derivative; (△) 3-carboxylic acid derivative.

The I_{50} value for sulfentrazone against Protox was 1.2 μM (Table 1). While the 3-hydroxymethyl metabolite had slightly more in vitro activity than sulfentrazone with an I_{50} value of 0.35 μM, the 3-demethyl and 3-carboxylic acid metabolites were less active, with I_{50} values at 10 and 37 μM, respectively (Figure 3).

Sulfentrazone effectively displaced [^{14}C]AF from its binding site on Protox, and the 3-hydroxymethyl and 3-demethyl metabolites were less able to displace AF than sulfentrazone (Figure 4). The 3-carboxylic acid did not displace AF. Binding of sulfentrazone and the 3-hydroxymethyl and 3-demethyl metabolites was competitive with respect to the binding of [^{14}C]acifluorfen as the curves have similar *y*-intercepts in the double-reciprocal plot.

Table 2. Molecular Properties of Sulfentrazone and Its Metabolic Derivatives

parameter[a]	sulfentrazone	3-hydroxymethyl	3-carboxylate	3-demethyl
log *P* (calcd, Villar-AM1)	6.58	5.68	3.27	6.41
bulk descriptors				
VDW_{area}, Å^2	337.39	341.45	345.68	315.9
VDW_{volume}, Å^3	269.48	275.21	275.68	256.28
ovality	1.672	1.669	1.688	1.619
electronic descriptors				
dipole moment, D	3.8962	3.8412	18.3816	3.5341
molecular polarizability, Å^3	25.884	26.324	63.721	24.689
e_{HOMO}, eV	−9.3732	−9.4518	−6.1524	−9.4606
e_{LUMO}, eV	−1.0170	−1.0853	−6.1490	−1.0527
electronegativity	5.20	5.27	6.15	5.26
hardness	4.18	4.18	≈0	4.20
energy descriptors				
Q-minus	−0.952	−0.952	−0.9542	−0.9523
Q-plus	0.2628	0.2637	0.2579	0.2628

[a] VDW_{area} and VDW_{volume}, van der Waals area and volume, respectively; e_{HOMO}, energy of highest occupied molecular orbital; e_{LUMO}, energy of lowest unoccupied molecular orbital; *Q*-plus and *Q*-minus, largest positive and largest negative charge on hydrogen, respectively.

Proto Accumulation. Sulfentrazone caused a >15-fold accumulation of Proto relative to controls (Table 1). The amounts of Proto in soybean tissues treated with the 3-hydroxymethyl and 3-demethyl derivatives were 12- and 7-fold greater than the control treatments, respectively, but were less than in sulfentrazone-treated tissues. The 3-carboxylic acid metabolite did not increase the level of Proto.

Molecular Properties. The metabolites of sulfentrazone did not differ from the parent compound in the geometric parameters measured (Table 2). There was a <10% difference between the smallest and largest van der Waals areas and volumes, respectively. Ovality, which is a computer-generated estimation of the sphericality of molecules according to

$$\text{ovality} = A/[4\pi(3V/4\pi)^{2/3}] \quad (1)$$

where *A* and *V* are the van der Waals area and volume, respectively, was not different between sulfentrazone and the metabolites. This indicates that no significant conformational changes resulted from the oxidation of the triazolinone methyl group.

On the other hand, values for the electronic descriptors changed during metabolism of sulfentrazone. Electronegativity and hardness properties, which were calculated according to eqs 2 and 3, respectively

$$\text{electronegativity} = -(e_{HOMO} + e_{LUMO})/2 \quad (2)$$

$$\text{hardness} = -(e_{HOMO} - e_{LUMO})/2 \quad (3)$$

and polarizability, which is related to lipophilicity, differed most in the case of the carboxylate anion. The parameters of the 3-hydroxymethyl metabolite were similar to those of sulfentrazone with the exception of the heat of formation (ΔH_f) and log *P*.

Examination of the steric changes occurring in the region of the triazolinone subject to metabolism show that the overall volumes delimited by the methyl, hydroxymethyl and carboxylate are relatively similar at 41.71, 52.92, and 41.83 Å^3, while the steric region occupied by the hydrogen of the 3-demethyl derivative was much smaller, at 4.44 Å^3. On the other hand, the range in electrostratic charges surrounding the site of metabolic oxidation was −0.96 to 218.19 for sulfentrazone, −64.80 to 166.86 for the 3-hydroxymethyl metabolite, −587.48 to −125.00 for the carboxylate anion, and 11.51−55.65 for the 3-demethyl metabolite (Figure 5).

DISCUSSION

As with most Protox inhibitors, sulfentrazone induced cellular damage as demonstrated by the leakage of electrolytes from herbicide-treated leaf disks. Electrolyte leakage after 24 h of continuous light exposure was 74% of potential maximum, indicating that a significant portion of the cell content had been released from the treated tissues. Although the 3-hydroxymethyl and 3-demethyl metabolites induced some electrolyte leakage, these compounds were much less toxic than sulfentrazone under these conditions (Figure 2). Treatment of the disks with the 3-carboxylic acid derivative resulted in no leakage. Our previous work indicated that the initial metabolites were rapidly converted into hydrophilic conjugated metabolites (Dayan et al., 1997c). Our current data suggest that the oxidative degradation of sulfentrazone occurring in soybean may not completely detoxify the molecule. Rather, the low in vivo activity observed with the metabolites is probably associated with their rapid conversion into inactive conjugated metabolites.

Treatment of the leaf tissue with sulfentrazone and metabolites resulted in Proto accumulation (Table 1), as might be expected on the basis of their site of action, and there was a strong correlation ($r^2 = 0.98$) between the activity of these compounds and their ability of induce Proto accumulation in treated tissues; that is, electrolyte leakage increased as more Proto accumulated. Our data agree with several studies which showed that the activity of structurally different groups of Protox inhibitors was correlated to Proto accumulation (Becerril and Duke, 1989b; Nandihalli et al., 1992a,b; Lee et al., 1995; Kojima et al., 1991). However, the putative detoxification of a Protox-inhibiting herbicide due to metabolic degradation is for the first time investigated.

The biochemical activity of sulfentrazone and its metabolites was investigated in our effort to explain the detoxification process. Nandihalli et al. (1993) have shown that sulfentrazone inhibited Protox in barley etioplast suspensions, and we have shown that sulfentrazone also inhibits this enzyme in soybean with an I_{50} of 1.2 μM (Figure 3), which is similar to the 1.1 μM reported for barley. The 3-hydroxymethyl metabolite had a lower I_{50} (0.35 μM) than sulfentrazone (Figure 3), although it caused less Proto accumulation (Table 2), and had a relatively low in vivo activity in inducing electrolyte leakage (Figure 2). In vivo herbicidal activity of Protox inhibitors does not always correlate well with their in vitro activity, indicating that factors other than the binding affinity of these compounds to the Protox receptor site may modulate phytotoxicity. The discrepancy between the in vivo and in vitro activities of the 3-hydroxymethyl derivative may be due to the fact that this metabolic intermediate is rapidly oxidized to the inactive carboxylic acid derivatives and/or is rapidly conjugated to inactive forms (Dayan et al., 1997c). While the hydroxymethyl functionality has a slightly lower log *P* value than sulfentrazone (Table 2), it was still able to reach membrane-localized Protox (Matringe et al., 1992; Lee et al., 1993) since it remained a potent Protox inhibitor (Table 1).

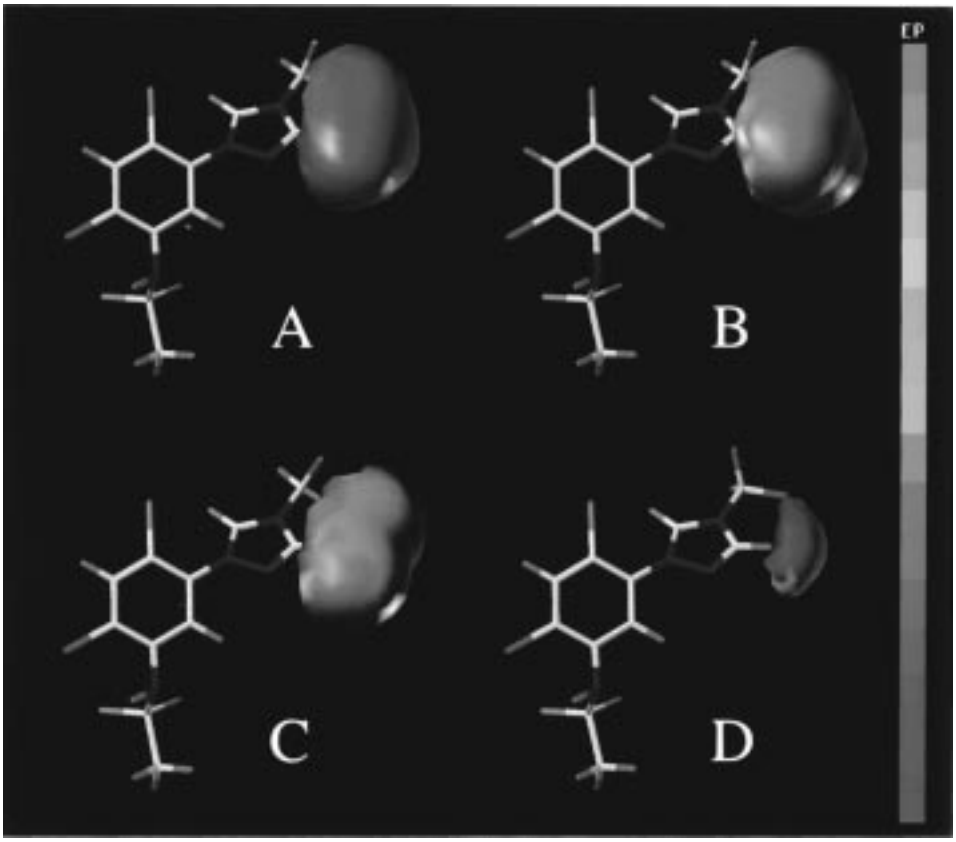


Figure 5. Comparison of the relative size and charge of the electrostatic maps in the region of the methyl group on the triazolinone ring: (A) sulfentrazone; (B) 3-hydroxymethyl; (C) 3-carboxylate; and (D) demethyl derivatives. The color ramp on the right illustrates the range in electrostatic potentials. Electrostatic potentials range from purple (more electropositive) to red (more electronegative).

The 3-demethyl was less toxic than sulfentrazone with an I_{50} value of 10 μM (Figure 3), providing an explanation for a previous paper indicating that the methyl group on the triazolinone ring is required for high activity of this herbicide class (Theodoridis et al., 1992). The relatively high I_{50} values of the 3-carboxylic acid metabolite (Table 1) combined with its rapid metabolic conjugation was accompanied with predictably low cellular leakage in the leaf disk assay (Figure 2).

Sulfentrazone competitively inhibited Protox, and all of the metabolites were less competitive toward the binding site on Protox relative to sulfentrazone (Figure 4). The two compounds with least herbicidal activity, that is, the 3-demethyl and 3-carboxylic acid derivatives, also exhibited the least binding affinity to Protox. At present, it is not clear why the 3-hydroxymethyl derivative (which is a slightly stronger Protox inhibitor than sulfentrazone) was not able to displace Protox-bound AF as readily as sulfentrazone. However, the catalytic site of Protox and the manner in which inhibitors compete for this site are unknown. Therefore, it is possible that the 3-hydroxymethyl metabolite can act as a potent Protox inhibitor while not readily displacing Protox-bound AF. This would be the case if the binding pocket of the metabolite on Protox does not exactly match that of AF. The two regions might overlap enough to allow the metabolite to displace AF, but not as commensurately for it to be able to compete for the binding of Protogen. Nonetheless, our research provides evidence that the 3-position on the triazolinone ring plays a significant role in the binding and inhibitory behavior of this molecule and that it is a key component providing selectivity to the herbicide, in that less tolerant plants do not readily oxidize the herbicide at this site (Dayan et al., 1996, 1997c).

The magnitude of the structural changes occurring to sulfentrazone during metabolism is rather small when compared to the change in biological activity. On a molecular level, oxidation of the methyl moiety on the triazolinone ring did not affect the geometric parameters of the molecule (e.g., van der Waals areas and volumes and ovality) (Table 2). The volume occupied by the hydrogen atom of the 3-demethyl metabolite was the only major difference between the compounds (Figure 5). The electronic descriptors of the metabolites were fairly similar, except for the carboxylate anion. The relatively low log *P* values associated with the highly electronegative nature (dipole moment of 18.3 and polarizability of 63.7) of the carboxylate anion (Figure 5) may account for its high I_{50} value, as could be explained by this functionality preventing the metabolite from transcending membrane bilayers and hindering it from reaching the active site in Protox.

CONCLUSION

The phytotoxicity of sulfentrazone was associated with its ability to competitively inhibit Protox. The metabolic degradation of sulfentrazone via oxidation of the methyl substituent on the triazolinone ring system yielded derivatives with lower in vivo toxicity. However, the 3-hydroxymethyl derivative was more active than sulfentrazone on the molecular target site. The other metabolites were slightly less active but were not totally inactive. Therefore, the combined inhibitory potential of these metabolites and sulfentrazone may lead to a transient phytotoxic response that was responsible for

the temporary injury observed on the foliage of soybean plants treated with this herbicide. Oxidative degradation of sulfentrazone apparently initiates the detoxification of the molecule, but completely inactive forms of sulfentrazone may only be obtained following metabolic conjugation.

LITERATURE CITED

Becerril, J. M.; Duke, S. O. Acifluorfen effects on intermediates of chlorophyll synthesis in green cucumber cotyledon tissues. *Pestic. Biochem. Physiol.* **1989a**, *35*, 119−126.

Becerril, J. M.; Duke, S. O. Protoporphyrin IX content correlates well with activity of photobleaching herbicides. *Plant Physiol.* **1989b**, *90*, 1175−1181.

Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Camadro, J.-M.; Matringe, M.; Scalla, R.; Labbe, P. Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. *Biochem. J.* **1991**, *277*, 17−21.

Dayan, F. E.; Weete, J. D.; Hancock, H. G. Physiological basis for differential sensitivity to sulfentrazone by sicklepod (*Senna obtusifolia*) and coffee Senna (*Cassia occidentalis*). *Weed Sci.* **1996**, *44*, 12−17.

Dayan, F. E.; Duke, S. O.; Weete, J. D.; Hancock, H. G. Selectivity and mode of action of carfentrazone-ethyl, a novel phenyl triazolinone herbicide. *Pestic. Sci.* **1997a**, *51*, 65−73.

Dayan, F. E.; Duke, S. O.; Reddy, K. N.; Hamper, B. C.; Leschinsky, K. L. Effects of isoxazole herbicides on protoporphyrinogen oxidase and porphyrin physiology. *J. Agric. Food Chem.* **1997b**, *45*, 967−975.

Dayan, F. E.; Weete, J. D.; Duke, S. O.; Hancock, H. G. Soybean (*Glycine max*) cultivar differences in response to sulfentrazone. *Weed Sci.* **1997c**, *45*, 634−641.

Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* **1985**, *107*, 3902−3909.

Duke, S. O.; Moorman, T. B.; Bryson, C. T. Phytotoxicity of pesticide degradation products. *Am. Chem. Soc. Symp. Ser.* **1991**, *No. 459*, 188−204.

Frear, D. S.; Swanson, H. R.; Mansager, E. R. Acifluorfen metabolism in soybean: diphenyl ether bond cleavage and the formation of homoglutathione, cysteine and glucose conjugates. *Pestic. Biochem. Physiol.* **1983**, *20*, 299−310.

Hancock, H. G. Weed spectrum of F6285 in soybeans. *Proc. South. Weed Sci. Soc.* **1992**, *45*, 49.

Hathway, D. E. Herbicide metabolism in plants. In *Molecular Mechanisms of Herbicide Selectivity*; Hathway, D. E., Ed.; Oxford University Press: New York, 1989; pp 43−132.

Jacobs, N. J.; Jacobs, J. M. Assay for enzymatic protoporphyrinogen oxidase, a late step in heme synthesis. *Enzyme* **1982**, *28*, 206−219.

Jeffcoat, B.; Harries, W. N. Selectivity and mode of action of flamprop-isopropyl, isopropyl-2-*N*-(3-chloro-4-fluorophenyl)-benzoamidoproprionate, in the control of *Avena fatua* in barley. *Pestic. Sci.* **1975**, *6*, 283−296.

Kenyon, W. H.; Duke, S. O.; Vaughn, K. C. Sequence of herbicidal effects of acifluorfen on ultrastructure and physiology of cucumber cotyledons, *Pestic. Biochem. Physiol.* **1985**, *24*, 240−250.

Kojima, S.; Matsumoto, H.; Ishizuka, K. Protoporphyrin IX accumulation in *Lemna paucicostata* Hegelm. caused by diphenyl ether herbicides and their herbicidal activity. *Weed Res.* **1991**, *36*, 318−323.

Lee, H. J.; Duke, M. V.; Duke, S. O. Cellular localization of protoporphyrinogen-oxidizing activities of etiolated barley (*Hordeum vulgare* L.) leaves. *Plant Physiol.* **1993**, *102*, 881−889.

Lee, H. J.; Duke, M. V.; Birk, J. H.; Yamamoto, M.; Duke, S. O. Biochemical and physiological effects of benzheterocycles and related compounds. *J. Agric. Food Chem.* **1995**, *43* (3), 2722−2727.

Matringe, M.; Scalla, R. Effects of acifluorfen-methyl on cucumber cotyledons: Porphyrin accumulation. *Pestic. Biochem. Physiol.* **1988**, *32*, 164−172.

Matringe, M.; Camadro, J.-M.; Labbe, P.; Scalla, R. Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem. J.* **1989**, *260*, 231−235.

Matringe, M.; Camadro, J.-M.; Block, M. A.; Joyard, J.; Scalla, R.; Labbe, P.; Douce, R. Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenyl ether-like herbicides. *J. Biol. Chem.* **1992**, *267*, 4646−4651.

Nandihalli, U. B.; Duke, S. O. The porphyrin pathway as a herbicide target site. *Am. Chem. Soc. Symp. Ser.* **1993**, *No. 524*, 62−78.

Nandihalli, U. B.; Duke, S. O. Structure−activity relationships of protoporphyrinogen oxidase inhibiting herbicides. *Am. Chem. Soc. Symp. Ser.* **1994**, *No. 559*, 133−146.

Nandihalli, U. B.; Duke, M. V.; Duke, S. O. Relationships between molecular properties and biological activities of *O*-phenylpyrrolidino- and piperidinocarbamate herbicides. *J. Agric. Food Chem.* **1992a**, *40*, 1993−2000.

Nandihalli, U. B.; Duke, M. V.; Duke, S. O. Quantitative structure−activity relationships of protoporphyrinogen oxidase-inhibiting diphenyl ether herbicides. *Pestic. Biochem. Physiol.* **1992b**, *43*, 193−211.

Reddy, K. N.; Nandihalli, U. B.; Lee, H. J.; Duke, M. V.; Duke, S. O. Predicting activity of protoporphyrinogen oxidase inhibitors by computer-aided molecular modeling. *Am. Chem. Soc. Symp. Ser.* **1995**, *No. 589*, 211−224.

Reddy, K. N.; Dayan, F. E.; Duke, S. O. QSAR analysis of protoporphyrinogen oxidase inhibitors. In *Comparative QSAR*; Devillers, J., Ed.; Taylor and Francis: London, 1998; pp 197−233.

Scalla, R.; Matringe, M. Inhibitors of protoporphyrinogen oxidase as herbicides: diphenyl ethers and related photobleaching herbicides. *Rev. Weed Sci.* **1994**, *6*, 103−132.

Scalla, R.; Matringe, M.; Camadro, J.-M.; Labbe, P. Recent advances in the mode of action of diphenyl ether and related herbicides. *Z. Naturforsch.* **1990**, *45c*, 503−511.

Sherman, T. D.; Becerril, J. M.; Matsumoto, H.; Duke, M. V.; Jacobs, J. M.; Jacobs, N. J.; Duke, S. O. Physiological basis for differential sensitivities of plant species to protoporphyrinogen oxidase-inhibiting herbicides. *Plant Physiol.* **1991**, *97*, 280−287.

Shimabukuro, R. H.; Walsh, W. S.; Hoerauf, R. A. Metabolism and selectivity of dichlofop-methyl in wild oat and wheat. *J. Agric. Food Chem.* **1979**, *27*, 615−623.

Spartan 4.0. Wavefunction, Inc., Irvine, CA.

Theodoridis, G.; Baum, J. S.; Hotzman, F. W.; Manfredi, M. C.; Maravetz, L. L.; Lyga, J. W.; Tymonko, J. M.; Wilson, K. R.; Poss, K. M.; Wyle, M. J. Synthesis and herbicidal properties of aryltriazolinones. A new class of pre- and postemergence herbicides. *Am. Chem. Soc. Symp. Ser.* **1992**, *No. 504*, 135−146.

Tischer, W.; Strotmann, H. Relationship between inhibitor binding by chloroplasts and inhibition of photosynthetic electron transport. *Biochim. Biophys. Acta* **1977**, *460*, 113−125.

Vidrine, P. R.; Jordan, D. L.; Girlinghouse, J. M. Efficacy of F-6285 in soybeans. *Proc. South. Weed Sci. Soc.* **1994**, *47*, 62.

Walker, R. H.; Richburg, J. S.; Jones, R. E. F6285 efficacy as affected by rate and method of application. *Proc. South. Weed Sci. Soc.* **1992**, *45*, 51.

Witkoski, D. A.; Halling, B. P. Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl. *Plant Physiol.* **1989**, *90*, 1239−1242.

Received for review November 19, 1997. Revised manuscript received February 24, 1998. Accepted February 27, 1998. This research was supported, in part, by a grant from FMC Corp.

JF970988P