Identification of Microbial and Rat Metabolites of Triflusulfuron Methyl, a New Sugar Beet Herbicide

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Triflusulfuron methyl (methyl 2-[[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]-amino]carbonyl]amino]sulfonyl]-3-methylbenzoate) is a new selective low use rate sulfonylurea herbicide for the control of many broad-leaved weeds and grasses in sugar beets. Our studies show the compound undergoes rapid microbial metabolism using *Streptomyces griseolus* and that a mixture of similar metabolites is excreted by triflusulfuron methyl-dosed rats. Nine intact sulfonylurea metabolites were tentatively identified in microbial studies, and five of these were later identified in urine or feces of rats dosed with radiolabeled triflusulfuron methyl. Metabolic reactions include alkyl and/or aryl oxidation, N-dealkylation, and ester hydrolysis. All identifications were made using liquid chromatography/mass spectrometry (LC/MS) with fast atom bombardment (FAB) ionization. FAB mass spectra gave strong protonated molecular ions and structurally useful fragment ions of these extremely thermally labile metabolites.

Keywords: *Sulfonylurea herbicide; microbial metabolism; rat metabolism; triflusulfuron methyl metabolites; liquid chromatography/mass spectrometry (or LC/MS)*

INTRODUCTION

Triflusulfuron methyl (formerly DPX-66037) (methyl 2-[[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,2,3-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methyl-benzoate) is the active ingredient in a new low use rate sulfonylurea herbicide for postemergence weed control in sugar beets (Peeples et al., 1991). Use rates are approximately 15 g of AI/ha for each application with up to three applications per year.

In an attempt to generate preliminary information regarding the metabolic fate of triflusulfuron methyl, its metabolism was investigated in submerged cultures of *Streptomyces griseolus* ATCC 11796. Previous studies showed *S. griseolus* ATCC 11796 contains two inducible cytochrome P-450 monooxygenases which metabolize sulfonylureas and generate oxidative metabolites from a variety of sulfonylurea herbicides (Romesser and O'Keefe, 1986; O'Keefe et al., 1988; Stieglitz and Scott, 1990; Reiser and Stieglitz, 1990). *Streptomyces griseolus* ATCC 11796 sulfonylurea biotransformation cultures can be extracted with solvent and analyzed directly by liquid chromatography/mass spectrometry (Reiser and Stieglitz, 1990).

In this study, metabolites tentatively identified from *S. griseolus* ATCC 11796 biotransformations of triflusulfuron methyl were compared with those isolated from rat excreta. The latter were obtained from rats dosed with radiolabeled triflusulfuron methyl. Results of these investigations are reported in this paper.

EXPERIMENTAL PROCEDURES

Materials. [*phenyl*-U-¹⁴C]- and [*triazinyl*-U-¹⁴C]triflusulfuron methyl (DuPont NEN Research Products, Boston, MA) were diluted with nonradiolabeled analytical standard grade triflusulfuron methyl (DuPont Agricultural Products, Wilmington, DE) to give a final specific activity of ~0.45 μCi/mg and chemical and radiochemical purity >95%.

Methods. *Cell Growth, Enzyme Induction, and Triflusulfuron Methyl Biotransformation.* Cell growth, cytochrome P-450 enzyme induction, and sulfonylurea biotransformation

were carried out as described previously (Romesser and O'Keefe, 1986; Stieglitz and Scott, 1990). For biotransformations in C-salts chemically defined medium (Rhodes et al., 1959), the induced cells were washed once in C-salts and then resuspended in fresh medium. Triflusulfuron methyl biotransformation cultures were incubated at 30 °C for 4, 24, and 48 h.

Analysis of Biotransformation Broths. Cell-free biotransformation broths adjusted to pH 7.0 were analyzed by HPLC with a Perkin-Elmer Series 4 liquid chromatograph system including a UV–visible spectrophotometer detector (Model LC-95) set at 230 nm. A Zorbax ODS semipreparative column (6.4 mm × 25 cm) with a flow rate of 2.5 mL/min at room temperature was used. The mobile phase consisted of a linear gradient of acetonitrile–water–phosphoric acid from 10:90:0.1 to 75:25:0.1.

Microbial Metabolite Extraction. Following biotransformation, the cells were removed by centrifugation and membrane filtration, and the supernatant was adjusted to pH 3 with 3M H₂SO₄ and extracted with 2 volumes of methylene chloride. The extract was evaporated to dryness and the residue was dissolved in acetonitrile/water/formic acid (50:50:0.1) for analysis by LC/FAB MS.

Rat Dosing and Excreta Collection. This study was designed to generate metabolites and characterize the qualitative nature of triflusulfuron methyl metabolism in rats. Two young adult Sprague-Dawley rats per sex per radiolabeled form of triflusulfuron methyl (eight rats total) were each given a single oral dose of radiolabeled triflusulfuron methyl. The dose was equivalent to ca. 235 mg of [¹⁴C]-triflusulfuron methyl/kg of body weight. Urine and feces were collected separately for 0–24 h and 24–48 h following dosing. Excreta samples were stored frozen until extraction and/or analysis.

Excreta Extractions. Feces samples were extracted five times with acetone/0.1 M ammonium carbonate (50/50, volume/volume) in an ultrasonic bath at ambient temperature. After each extraction, the mixture was centrifuged and the supernatant decanted. The combined extracts were concentrated *in vacuo*, and an aliquot was diluted in HPLC mobile phase prior to chromatographic analysis. Urine samples were diluted in HPLC mobile phase and analyzed without further workup.

Chromatographic Methods for Analysis of Rat Metabolites. Urine samples and feces extracts were analyzed by HPLC on a Zorbax R$_x$ C-8 column (4.6 mm × 25 cm) with a Zorbax R$_x$

Table 1. Comparison of Triflusulfuron Methyl Metabolites Detected in Microbial Biotransformation and Rat Metabolism Studies[a]

	metabolite detected in					
	microbial biotransformation		rat metabolism			
metabolite no.	spor[b]/4 h	C-salt[c]/48 h	urine	feces	MH[+]	m/z triazine fragments
1	+	−	+	−	509	254, 280, 297
2	+	−	+	+	479	224, 250, 267
3	+	+	+	+	495	240, 266, 283
4	+	+	−	+	465	210, 236, 253
5	+	+	−	−	479	238, 264, 281
6	+	+	−	−	465	224, 250, 267
7	−	+	+	+	495	224, 250, 267
8	−	+	−	−	511	240, 266, 283
9	−	+	−	−	481	210, 236, 253
triflusulfuron methyl	+	+	−	−	493	238, 264, 281

[a] See Figure 6 for proposed metabolite structures. [b] O'Keefe et al. (1988) and Reiser et al. (1991). [c] Rhodes et al. (1959).

guard column (4.6 mm × 10 mm) maintained at 40 °C. The column was eluted with water containing 0.2% formic acid for 1 min followed by a linear gradient to 60% acetonitrile containing 0.2% formic acid over the next 30 min (flow, 2 mL/min). Radioactive components were detected with a Ramona LS radiochemical detector, equipped with a 0.2 mL calcium fluoride flow cell.

Purification of Rat Metabolites. Radiolabeled metabolites were purified using a Rainin Rabbit HPLC system equipped with a 21.4 mm × 25 cm Dynamax-60A, 8 μm, C_{18} column with a similar 21.4 mm × 5 cm guard column at ambient temperature. The column was eluted at 20 mL/min with water containing 0.1% formic acid for 5 min followed by a rapid linear gradient to 45% acetonitrile containing 0.1% formic acid, and the mobile phase composition was held constant for 30 min. Radiolabeled components were hand collected, on the basis of the response of a Raytest Ramona 90 radiochemical flow detector. In addition, some samples were subjected to a final purification using the system described above for chromatographic analysis of urine and feces extracts [Zorbax R_x C-8 column (4.6 mm × 25 cm) with a Zorbax R_x guard column (4.6 mm × 10 mm) maintained at 40 °C].

LC/MS-FAB Analyses. A packed capillary LC column, 25 cm × 0.25 mm i.d. fused silica tubing packed with 3 μ Spherisorb ODS2, was used with a Valco C14W submicroliter injection valve. A typical mobile phase was 50% CH_3CN/45% H_2O (pH 3, formic acid)/5% glycerol (FAB matrix), and flow rate was 1 μL/min. An Isco CV4 UV detector was used on line at a 238 nm wavelength.

A Finnigan MAT 8230 mass spectrometer was used with an Ion Tech FAB gun and xenon gas. The MS resolution was 1000, and the mass range scanned was 95−650. All FAB mass spectra were background subtracted to remove glycerol matrix ions. Additional details were previously described (Reiser et al., 1991).

RESULTS

Streptomyces griseolus **ATCC 11796 Triflusulfuron Methyl Biotransformation.** Triflusulfuron methyl is rapidly metabolized by *S. griseolus* ATCC 11796 in both nutrient-rich sporulation (spor) and minimal C-salt medium. A spor/4 h sample yielded a large number of polar metabolites as determined by HPLC (Figure 1A). A small peak at 28 min for triflusulfuron methyl showed that it was nearly completely metabolized. The C-salt/48 h biotransformation also showed complete triflusulfuron methyl metabolism and a slightly different pattern of more polar metabolites (Figure 1B), presumably, because of the longer incubation period. Also, the metabolite patterns of spor/24 h and C-salt/24 h differed only in the relative size of the individual peaks (results not shown). Control flasks containing only triflusulfuron methyl gave no metabolites in either sporulation or C-salt media (results not shown). Table
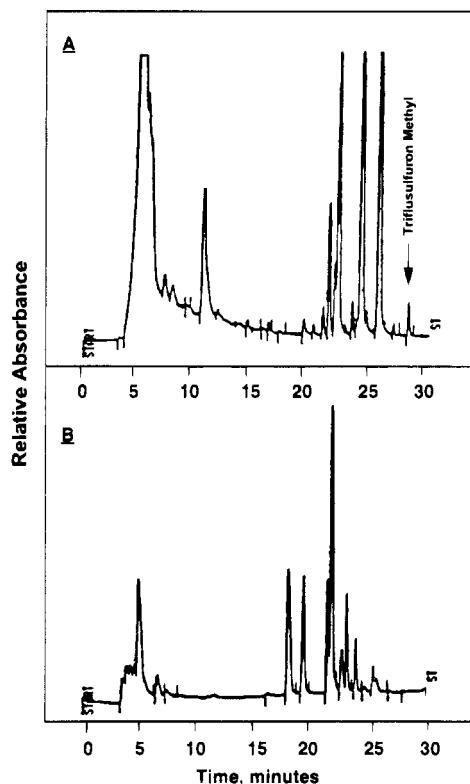

Figure 1. HPLC/UV analysis of *S. griseolus* ATCC 11796 triflusulfuron methyl bioconversion broths: (A) sporulation/4 h, and (B) C-salts/48 h.

1 lists the metabolites identified in each medium, along with supporting FAB mass spectral data.

Triflusulfuron Methyl Metabolism in Rats. Triflusulfuron methyl is converted to several more polar metabolites in rats dosed orally with radiolabeled triflusulfuron methyl. Figure 2 shows HPLC radiochromatograms comparing urine and feces extracts from [*triazinyl*-U-^{14}C]triflusulfuron methyl-treated rats. The chromatographic profile of radiolabeled components in the two samples differs both qualitatively and quantitatively. While triflusulfuron methyl is a major component in the feces sample (Figure 2B), it is not present in the urine (Figure 2A). Also, while metabolite 4 was found in feces, it was not detected in urine samples (Table 1). Metabolite 1 was a major component in urine, but is not present at significant levels in the feces extract. Only major metabolites from urine and feces extracts were purified by preparative HPLC and analyzed by FAB LC/MS. Individual components found in
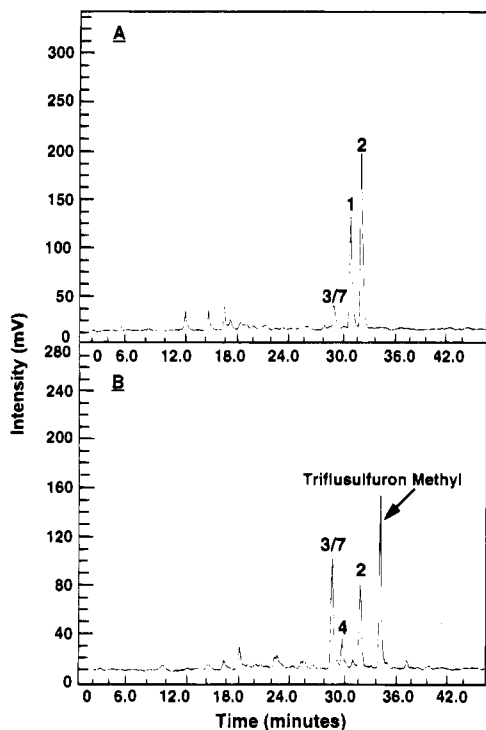
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Figure 2. HPLC radiochromatograms of urine (A) and feces (B) from rats dosed with [*triazinyl*-U-^{14}C]triflusulfuron methyl.

rat excreta and their corresponding mass spectral data are listed in Table 1.

Interpretation of FAB Mass Spectra of Triflusulfuron Methyl Metabolites. The FAB mass spectrum of triflusulfuron methyl (Figure 3) shows a prominent protonated molecule ion (MH$^+$) at m/z 493. Major fragment ions from the heterocycle (triazine) portion of the molecule are obtained at m/z 238, 264, and 281 due to the protonated heterocycle amine, isocyanate, and urea, respectively. The FAB mass spectrum of metabolite 2 (Figure 4) shows an MH$^+$ at m/z 479, 14 mass units below that of the parent which

indicates demethylation occurred. The heterocycle fragment ions are 14 mass units below those of the parent, showing demethylation occurred on the heterocycle portion of the molecule. Metabolite 2 is the least polar of the 9 metabolites identified, as determined by reversed-phase LC retention time. The FAB mass spectrum of metabolite 8 (Figure 5), which is the most polar metabolite, shows an MH$^+$ at m/z 511. The heterocycle fragment ions at m/z 240, 266, and 283 indicate this portion of the molecule was hydroxylated and demethylated. Additional fragment ions are obtained at m/z 222, 248, and 265 due to loss of water (18 mass units) from the heterocycle fragment ions. These dehydration fragments were also obtained in the FAB mass spectra of metabolites 1 and 3. The molecular weight of 510 indicates the benzene side of metabolite 8 is also hydroxylated.

The MH$^+$ and triazine fragment ions obtained with microbial and rat metabolites are listed in Table 1. Their proposed structures (shown in Figure 6) are based on similar interpretations for the above metabolites.

DISCUSSION

On the basis of results of this and previous work (Stieglitz and Scott, 1990; Reiser and Stieglitz, 1990), *S. griseolus* cultures provide a straightforward approach for generation of sulfonylurea metabolites. Tentative identification of nine different hydroxylated and/or dealkylated metabolites of triflusulfuron methyl was readily obtained from ethyl acetate extracts of the culture media using liquid chromatography/FAB mass spectrometry. No additional cleanup or isolation procedures were required. As noted in Table 1, all metabolites found in urine and/or feces from [^{14}C]-triflusulfuron methyl treated rats were also generated by *S. griseolus* in either C-salt or sporulation media.

Crude ethyl acetate extracts of the microbial culture media were examined directly by LC/MS-FAB. However, individual rat metabolites were isolated by pre-
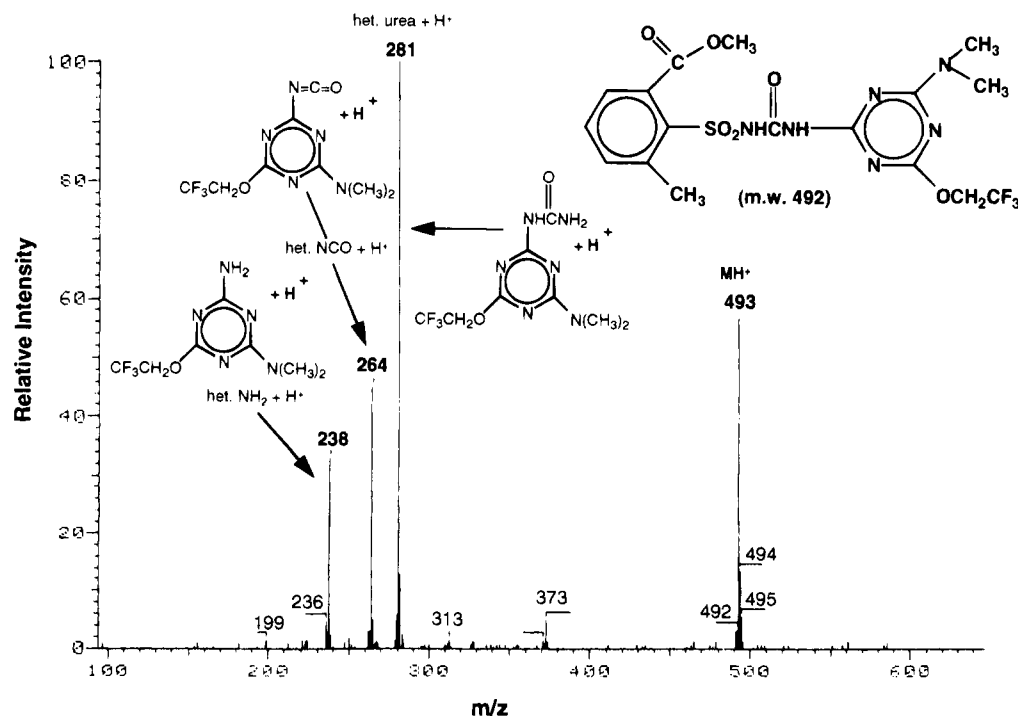

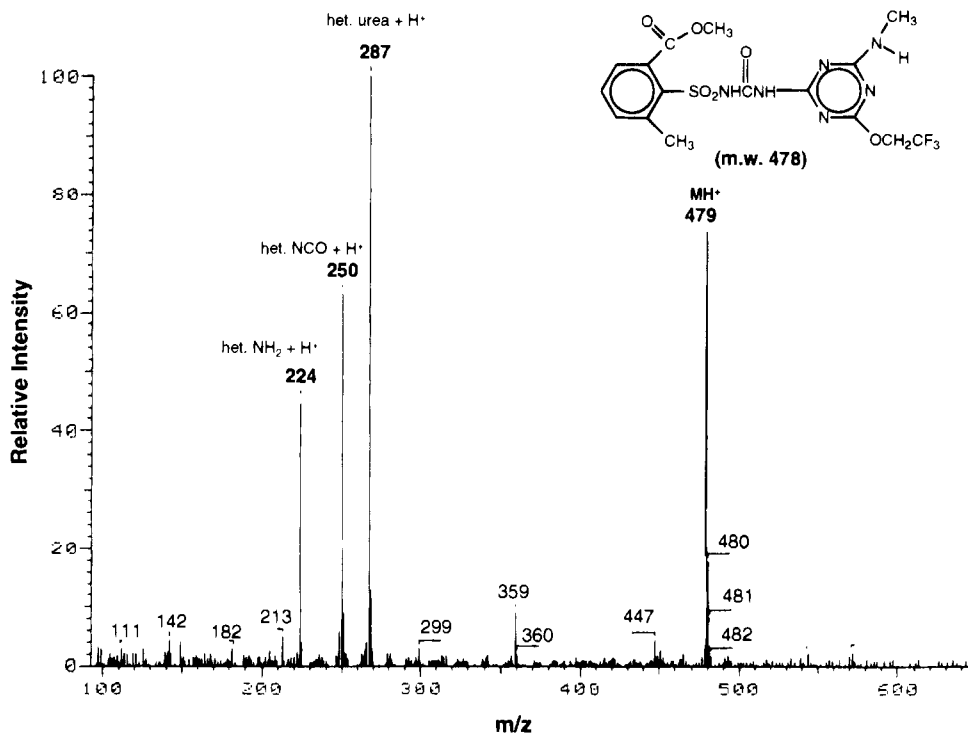**Figure 3.** FAB mass spectrum of triflusulfuron methyl.

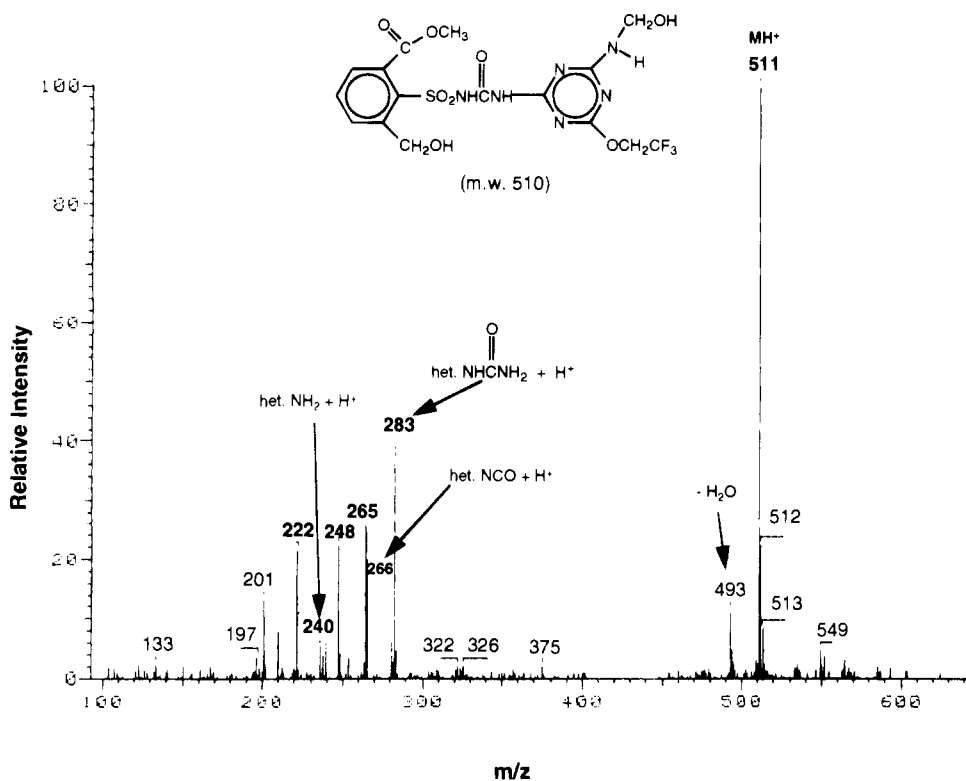**Figure 4.** FAB mass spectrum of metabolite 2.

Figure 5. FAB mass spectrum of metabolite 8.

parative HPLC prior to analysis by LC/MS-FAB as the objective of the rat study was to identify the major metabolites generated. Direct LC/MS-FAB analysis of ethyl acetate extracts of rat excreta may have demonstrated the existence of additional minor metabolites in common with those generated in the microbial system.

The metabolites identified arise from oxidative biotransformations at the four methyl groups in the molecule (Figure 6). There are 23 theoretically possible compounds from various combinations of these hydroxylated and/or demethylated metabolites. Nine microbial

metabolites were identified and five of these were later identified as rat metabolites (Table 1). No metabolites were found involving biotransformation of the OCH_2-CF_3 substituent. The *N*-hydroxymethyl metabolites (1, 3, and 8, Figure 6) are chemically unstable, but were identified as metabolites due to the mild sample workup and analytical techniques employed. The position of the hydroxyl on the benzene side in metabolites 7–9 (Figure 6) cannot be determined from the mass spectra, and its assignment on the methyl group rather than the benzene ring is based on previous studies of *S. griseolus*
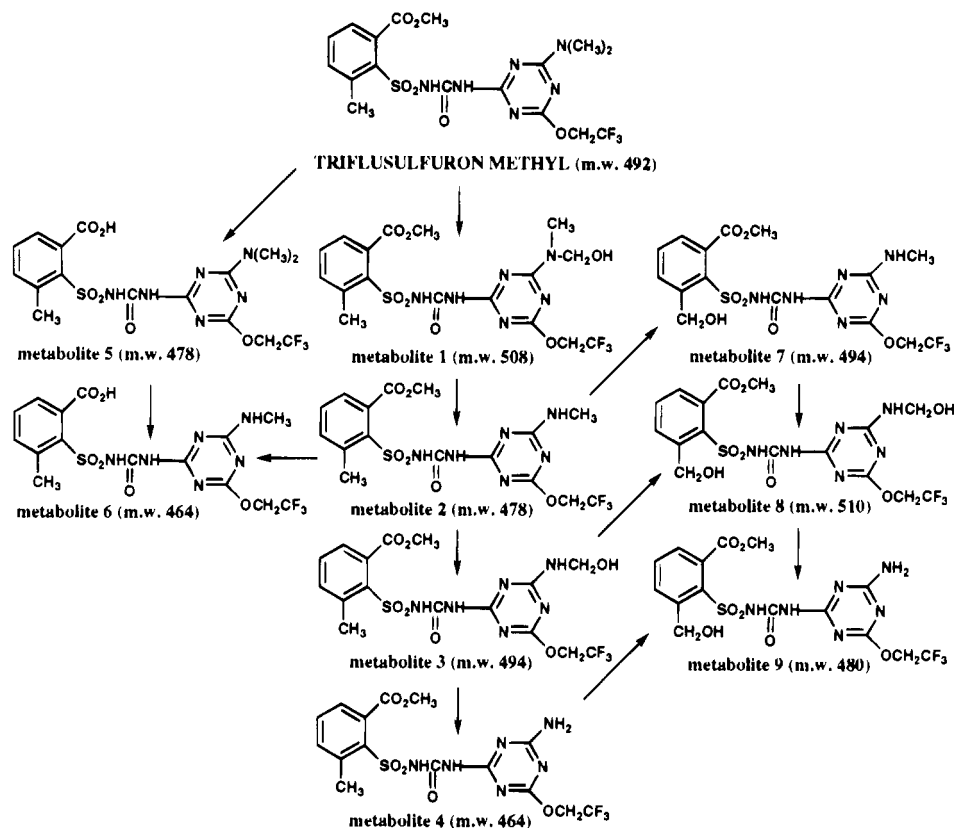
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Figure 6. Proposed pathways for *S. griseolus* ATCC 11796 metabolism of triflusulfuron methyl.

bioconversion of sulfonylureas (J. A. Romesser, internal DuPont Communications).

Synthetic samples of metabolites 1, 2, and 4–6 were prepared for structure confirmation and for use as standards in subsequent work for comparison with metabolites by cochromatography or for quantitative analyses. Metabolite 1 is unstable and degrades on storage into metabolite 2.

Several of the metabolites were not completely separated on the C_{18}-packed capillary LC column used for LC/MS analyses. Metabolites 3 and 7 eluted very close together, metabolites 1 and 5 coeluted and metabolite 4 eluted just before these. However, examination of the mass spectra and mass chromatograms and analysis of samples that had varying amounts of these metabolites (Table 1) allowed us to distinguish and identify them.

Figure 6 depicts the proposed pathways for triflusulfuron methyl metabolism based on observed metabolites and known metabolic reactions. Metabolites identified from both systems are consistent with the major pathway being oxidative N-dealkylation of triflusulfuron methyl to the mono- and bis-N-desmethyl derivatives (metabolites 2 and 4, respectively). As shown in Figure 6, N-hydroxymethyl intermediates (metabolites 1 and 3) are the presumed metabolic precursors of these dealkylated derivatives. Additional oxidative transformations of the phenyl moiety resulted in metabolites 7–9 and conversion of the methyl ester moiety to the corresponding carboxylic acid leads to formation of metabolites 5 and 6.

These results demonstrate that triflusulfuron methyl biotransformations obtained with *S. griseolus* ATCC 11796 are very similar to those obtained in the rat. LC/MS-FAB has proven to be a very powerful analytical technique for identification of the unknown metabolites generated in this study, either by analysis of mixtures in microbial broth extracts or individually purified rat metabolites. This information allowed the preparation of triflusulfuron methyl metabolite standards by both chemical syntheses and preparative microbial biotransformation for use in metabolism and residue studies required by government agencies for registration of herbicides.

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