Microbial Transformations of Prosulfuron

Kerry Kulowski,[†] Eric L. Zirbes,[†] Bruce M. Thede,[‡] and John P. N. Rosazza*[,†]

Division of Medicinal and Natural Products Chemistry, Center for Biocatalysis and Bioprocessing, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242, and Ciba Crop Protection, Greensboro, North Carolina 27420

Microbial transformations were conducted with prosulfuron {1-(4′-methoxy-6′-methyltriazin-2′-yl)-3-[[2″-(3‴,3‴,3‴-trifluoropropyl)phenyl]sulfonyl]urea} to identify pathways by which the sulfonylurea herbicide is metabolized by microorganisms. *Streptomyces griseolus* produced four new hydroxylated metabolites which were isolated, chromatographically purified, and characterized by mass spectrometry and NMR spectroscopy as the following: a benzyl alcohol from methyl hydroxylation on the triazine ring, phenolic and catechol metabolites by 3″ and subsequent 4″ hydroxylation on the carbocyclic aromatic ring, and a second benzylic alcohol obtained by microbial hydroxylation of the trifluoropropyl side chain. The new metabolites were used as analytical standards for soil incubation studies.

Keywords: *Prosulfuron; sulfonylurea; oxidative biotransformations; microbial metabolites; benzylic and aromatic hydroxylations; O-demethylation; hydrolysis*

INTRODUCTION

During the development of new pesticides, environmental considerations require an understanding of how new agents may be distributed and persist in nature. In soil, for example, xenobiotics may undergo chemical, physical, and/or biological transformation, all of which control their environmental persistence. When bioavailability in soil, sewage, or water is adequate, pesticides may undergo microbial transformation resulting in their conversions into altered chemical structures, ultimately leading to complete mineralization. Identification of such microbial metabolites becomes a crucial step in evaluating the ultimate potential for xenobiotic toxicity and environmental persistence. Thus, metabolite identification may provide crucial information required for new pesticide design and discovery.

Soil studies remain essential in establishing kinetics of degradation and, where possible, the identification of major breakdown products. However, levels of some pesticides used in the environment, such as the sulfonylureas, may be extremely low, rendering the production, isolation, and identification of metabolic, physical, or chemical breakdown products difficult. Radiolabeled pesticide substrates may be used in soil to demonstrate pesticide degradability versus time. Although they are essential to new pesticide licensing and use, typical soil persistence studies are usually time consuming, and they may not permit the identification of potential metabolites formed by microbial metabolism. Analyses of soil extracts containing radioactive substrates usually rely on synthetic metabolite standards which may be difficult to prepare and may not be representative of the complete range of metabolites possible by microbial transformation. In addition, for complete structure elucidation, it ultimately becomes necessary to isolate and purify sufficient quantities of soil degradates for full characterization, structure elucidation, and biological evaluation.

Soils contain many types of bacteria, yeasts, and fungi that are well-known for their individual or collective abilities to catalyze nearly every type of metabolic transformation or degradative reaction sequence with natural or xenobiotic organic compounds (Charney and Herzog, 1967; Gibson, 1984; Iizuka and Naito, 1967; Kieslich, 1976, 1984; Laskin and Lechevalier, 1984; Rosazza and Duffel, 1986; Sariaslani and Rosazza, 1984; Sebek, 1974; Sebek and Rosazza, 1995; Wallen et al., 1959). Herbicides added to mixed culture consortia found in the environment may undergo single or multiple metabolic transformations, resulting in either complete mineralization or the accumulation of metabolites that are recalcitrant to further microbial transformation (Cork and Krueger, 1991). The idea of using microorganisms as models for the metabolism of xenobiotics in mammals was set forth by Smith and Rosazza (1975, 1977, 1979, 1982, 1983) to address significant problems associated with studying drug metabolism in mammals. This approach has been successfully applied and exploited in metabolism studies of alkaloids, aromatics, various classes of drugs, and other compounds (Beukers et al., 1972; Cerniglia, 1983; Clark and Hufford, 1991; Davis, 1987; Griffiths et al., 1991; Wallen et al., 1959).

We recently described the use of selected pure cultures of representative soil microorganisms cultivated in the laboratory as "models" to elucidate metabolic pathways of the herbicide clomazone in soils (Liu et al., 1996). Screening experiments using HPLC analyses identified strains of *Aspergillus niger* and *Cunninghamella echinulata* that were capable of metabolizing Clomazone, and preparative scale incubations with these microorganisms rapidly provided sufficient amounts of metabolites for spectral and chromatographic identification. Microbial transformation reactions observed with clomazone included aliphatic and aromatic mono- and dihydroxylations, cleavage of an isoxazolidone N−C bond, and complete removal of the isoxazolidone ring to form chlorobenzyl alcohol.

Dietrich et al. recently reported the results of microbial transformations of a sulfonylurea, trisulfuron methyl (Dietrich et al., 1995). Identified metabolites resulted

[†] University of Iowa.
[‡] Ciba Crop Protection.
* Corresponding author. Phone: (319) 335-4902. FAX: (319) 335-4901. E-mail: john-rosazza@uiowa.edu.

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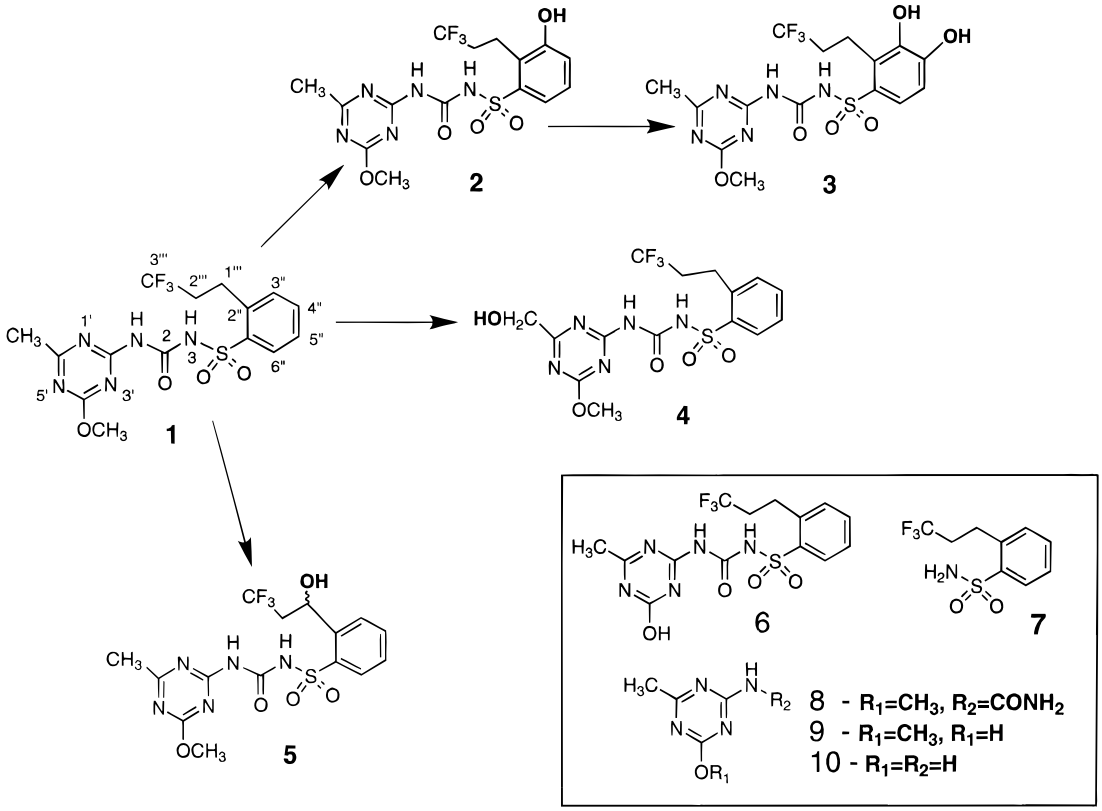

Figure 1. Structures of metabolites **2**−**5** isolated from *S. griseolus* transformation of prosulfuron (**1**). Structures of standard compounds **6**−**10** are shown in the box.

from combinations of metabolic transformations including oxidative N-dealkylation, saponification, and benzylic methyl oxidation of trisulfuron methyl. Information from microbial transformation of trisulfuron methyl enabled the chemical and microbial preparation of metabolite standards for use in metabolism and residue studies. Romesser and O'Keefe (1986) identified metabolites of sulfmeturon methyl produced via hydroxylation of a pyrimidine methyl group and subsequent oxidation to the carboxylic acid by unique, inducible cytochrome P-450 enzyme systems in *S. griseolus* (Romesser and O'Keefe, 1986). O-Dealkylation and benzylic methyl-group oxidation gave alcohol products of a triazine containing sulfonylurea (chlorsulfuron), and O-dealkylation and ethyl ester cleavage of a pyrimidine-containing sulfonylurea (chlorimuron ethyl) have been observed (Sariaslani, 1991).

This paper describes the first microbial transformation experiments of the herbicide prosulfuron. This work was conducted to identify prosulfuron metabolite producing microorganisms and to scale up fermentations to rapidly produce metabolites for spectral and chromatographic characterization.

MATERIALS AND METHODS

Chemicals. The herbicide prosulfuron, 1-(4-methoxy-6-methyltriazin-2-yl)-3-[[2-(3,3,3-trifluoropropyl)phenyl]sulfonyl]-urea (**1**), and other standard compounds **6**−**10** were obtained from Ciba Crop Protection, Greensboro, NC. The purity of each compound was established by HPLC analysis, and the structure of **1** was confirmed by nuclear magnetic resonance spectroscopy (^{1}H, ^{13}C NMR) and chemical ionization mass spectrometry (CI-MS). NMR solvents were from Sigma Chemical (St. Louis, MO) or Cambridge Isotope Laboratories (Woburn, MA). Filtered and degassed OmniSolv acetonitrile (EM Science, Gibbstown, NJ) and double distilled water were used for HPLC. Other reagents were analytical reagent grade (Fisher Scientific, Springfield, NJ).

Chromatography. Thin layer chromatography (TLC) was performed on silica gel 60 GF_{254} (EM Science, Gibbstown, NJ) coated glass plates, 0.25 mm thick for analytical TLC and 1.0 mm for preparative TLC. Silica plates were prepared with a Quickfit Industries (London, U.K.) spreader, air-dried, and activated by oven heating for 30 min at 120 °C. TLC plates were developed with CH_2Cl_2:CH_3CN:HCOOH (86:14:0.25, v/v/v), which gave an R_f of 0.5 for **1**. Metabolites were detected under 254 nm UV light as quenching spots, followed by consecutive spraying and heating with ceric ammonium sulfate and Dragendorff's reagent (Kirchner, 1978) to give yellow or orange spots. A second solvent system, CH_2Cl_2:CH_3CN:HCOOH (75:25:1, v/v/v), resolved nearly all the metabolites and gave an R_f of 0.68 for **1** and R_f values for analytical standards and metabolites **2**−**10** as follows: **2**, 0.6; **3**, 0.3; **4**, 0.3; **5**, 0.5; **6**, 0.2; **7**, 0.8; **8**, 0.25; **9**, 0.1; **10**, 0. Flash silica gel 40 μm (J. T. Baker, Phillipsburg, NJ) was used for flash column chromatography (FCC).

Purified metabolites, and crude extract residues from screening scale incubations of 16 microorganisms and a soil incubation, were analyzed with a modular HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The HPLC system consisted of dual LC-6A pumps, an SCL-6B system controller, and an Econosil C-18 reverse phase column (10 μm, 25 cm × 4.6 mm i.d., Alltech, Deerfield, IL) with a matching guard column. Eluting peaks were detected at 260 nm with a model SPD-6AV, UV−vis detector and recorded with a CR-501 Chromatopac. The solvent system began with water (pH 2.5, H_3PO_4) and, after following the step gradient as in Figure 2, changed at 120 mL to an exponential gradient until 100% CH_3CN was reached at 160 mL. The column was then equilibrated with water for 5 min before the next injection. A flow rate of 2 mL/min with a maximum operating pressure of 120 kg/cm^2 was used. A retention volume of 115 mL for **1** and retention volumes for consecutive metabolites were as follows: **2**, 103 mL; **3**, 91 mL; **4**, 84 mL; **5**, 83 mL; **6**, 66 mL; **7**, 60 mL; **8**, 29 mL; **9**, 27 mL; **10**, 4 mL.

Incubations. All cultures are held in the University of Iowa, College of Pharmacy collection and were grown from slants maintained previously at 4 °C on Sabouraud maltose or nutrient agar (Difco Laboratories, Detroit, MI). The liquid

medium composition (pH 7.0) used for screening and preparative incubations consisted of 20 g of dextrose, 5 g of yeast extract, 5 g of soybean meal, 5 g of NaCl, 5 g of K_2HPO_4, and 1 L of distilled water. The medium was sterilized by autoclaving at 15 lb/in.^{2} and 121 °C for 15 min (Amsco, Apex, NC). Screening was performed using a two-stage incubation protocol where growth from slants was suspended in medium and then added to 25 mL of sterile medium held in stainless steel-capped, 125 mL DeLong flasks (Bellco, Vineland, NJ). Stage I flasks were incubated for 72 h in a model G-25 shaker at 250 rpm, 28 °C (New Brunswick Scientific Co., Edison, NJ). A 2.5 mL aliquot from a stage I flask was used as inoculum for each stage II flask. Substrate was added to each flask 24 h later as 100 μL dimethylformamide (DMF) aliquots each containing 6.25 mg of **1** in solution to give a final concentration of 0.25 mg of **1**/mL of culture. Control flasks were prepared without addition of substrate. Experimental and control flasks were sampled for TLC analysis at 24, 48, 72, and 144 h. Samples of 4 mL of culture were adjusted to pH 3 and extracted with 1 mL of ethyl acetate (EtOAc), and 30 μL of EtOAc extract was spotted at the origin of a silica gel TLC plate.

An HPLC method was used to confirm initial screening results for 16 microorganisms (see Results and Discussion). Stage II incubations were prepared as above in triplicate. Substrate **1** was added to two experimental flasks at 24 h, while the third flask with no substrate served as a control. At 72 h the contents of each flask was transferred to a 250 mL polyethylene centrifuge bottle containing 25 mL of EtOAc. After manual shaking followed by centrifugation at 4000*g* for 3 min in a Model RC5B centrifuge with GSA rotor (Sorvall, DuPont, Newtown, CT), the organic layer was collected and concentrated under vacuum. Extract residues were dissolved in enough CH_3OH to give a final solution concentration of 1 mg/mL, and 5 μL samples were injected into the HPLC system.

For incubations of soil suspensions, topsoil was collected from a corn field 2 mi east of Tiffin, IA, near Highway 6. The soil was a silt-loam composed of sand (6%), course silt (43%), fine silt (35%), and clay (16%). Soil was collected in Fall 1991 and Spring 1992. A soil suspension was made by adding 50 g of air-dried soil to 50 mL of double distilled water and shaking for 30 min at 250 rpm on a model G-10 shaker (New Brunswick Scientific Co., Edison, NJ). Then, 5 mL aliquots of soil suspension were used to inoculate stage I flasks. Incubation procedures and TLC analyses were performed for fall soil sample incubations as described above. The metabolism of **1** in incubations of spring soil samples at days 6, 10, and 24 was analyzed by HPLC.

Preparative Transformation of 1 by *Streptomyces griseolus*. *S. griseolus* (ATCC 11796) was grown in 10, 1 L stage II flasks each containing 200 mL of medium. A total of 500 mg of **1** was dissolved in 2 mL of DMF and distributed among the 10 flasks. At 72 h the cultures were pooled, acidified to pH 4 with 6 N HCl, and extracted with EtOAc (1.5 L × 3). Emulsions split into two layers by centrifugation at 4000*g* for 10 min. Extracts were dehydrated using anhydrous Na_2SO_4 and concentrated with a rotary evaporator (Büchi, Flawil, Switzerland) under vacuum to give a few grams of a brown, oily liquid. A portion of the liquid was filtered through a cotton plug in a Pasteur pipet to remove lipid-like solids before chromatography.

FCC and preparative TLC were used to isolate metabolites of **1**. A 2.0 × 20 cm^2 column was packed dry with 40 μm flash silica (J. T. Baker). Solvent (CH_2Cl_2:CH_3CN, 95:5 v/v) was forced through the column with nitrogen at 10 kPa. Crude extract residue (350 mg) was dissolved in 0.5 mL of CH_2Cl_2 and layered on the packing. The initial solvent system (CH_2Cl_2:CH_3CN, 95:5 v/v, 500 mL) was eluted in 10 mL fractions at a flow rate of 15 mL/min at 10 kPa. When a more polar system (CH_2Cl_2:CH_3CN, 90:10 v/v, 500 mL) was used, fractions 1−4 contained 26 mg of **1**, fraction 5 contained **1** and **2**, and fractions 6 and 7 contained 7 mg of **2**. The column was completed as a 5% step gradient of 100 mL portions until 100% CH_3CN was reached. Each 100 mL step was collected as one fraction in a round-bottom flask. Fractions were analyzed by TLC, combined, and further purified by preparative silica TLC to give analytical samples (1−2.5 mg) of **3**−**5**.

Spectroscopy. UV−visible spectra were recorded in methanol (MeOH) using a Model UV-160 scanning spectrophotometer (Shimadzu). NMR spectra were obtained at 360.13 MHz for ^{1}H NMR or 90.56 MHz for ^{13}C NMR with a WM-360 FTQ NMR spectrometer with an Aspect 2000 computer (Bruker Instruments, Billerica, MA). A 5 mm broadband C/H probe was used with 5 mm premium NMR tubes (Sigma, St. Louis, MO) containing samples dissolved in 0.35 mL of either $CDCl_3$, CD_3OD (Sigma, St. Louis, MO), or CD_3CN (Cambridge Isotope Laboratories, Woburn, MA). For HMQC and HMBC reversed-probe NMR experiments performed on an AMX-600 NMR spectrometer (Bruker Instruments, Billerica, MA), Gold Label tubes (Sigma, St. Louis, MO) and 0.5 mL of lock solvent were required. The pulse sequence parameters were the same for both HMQC and HMBC acquisitions: initial delay = 0.06 s, 90° pulse = 7.5 μs, mixing time = 0.003 125 s, relaxation delay = 3.75 s, recovery delay = 0.06 s, scan number = 64. Tetramethylsilane or solvent peaks served as chemical shift internal references. Chemical shift values (δ) are given in parts per million, and coupling constants (J) are in hertz. Abbreviations for NMR signals are as follows: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, ddd = triplet of doublets, and m = multiplet. Low-resolution chemical ionization mass spectrometry (CIMS) data were obtained on a Nermag model R 10-10C (Paris, France) using a desorption probe, with ammonia as the chemical ionization reagent. High-resolution fast atom bombardment mass spectrometry (HRFABMS) data were obtained on a VG Analytical model ZAB-HF (Fisons, Beverly, MA) in the positive ion mode. Either thioglycerol or 3-nitrobenzyl alcohol was used as the matrix. Duplicate mass determinations were averaged.

RESULTS AND DISCUSSION

Prosulfuron (**1**) was screened for biotransformations using 72 species of 19 genera: *Aspergillus* (seven species), *Bacillus* (two), *Candida* (five), *Corynebacterium* (one), *Cunninghamella* (five), *Curvularia* (one), *Cylindrocarpon* (one), *Fusarium* (one), *Hansends* (one), *Microsporum* (one), *Mucor* (three), *Mycobacterium* (one), *Nocardia* (two), *Penicillium* (11), *Pseudomonas* (five), *Rhizopus* (three), *Streptomyces* (20), *Stysanus* (one), and *Zygosaccharomyces* (one).

Because apparent metabolites of **1** were observed in initial screening experiments by TLC, the following microorganism extracts were subsequently analyzed by HPLC: *Cylindrocarpon radicicola* (ATCC 11011), *Cunninghamella echinulata* (NRRL 3655), *Microsporum gypseum* (ATCC 11395), *Mucor mucedo* (ATCC 4605, ATCC 20094), *Nocardia aurantia* (ATCC 12674), *Penicillium madriti* (ATCC 18233), *Penicillium purpurogenum* (ATCC 9777), *Pseudomonas testosteroni* (ATCC 11996), *Rhizopus* species (MR 224), *Streptomyces flocculus* (ATCC 25453), *S. griseolus* (ATCC 11796), and *S. griseus* (ATCC 13273). Six of these cultures and the incubation inoculated with growth from the Spring 1992 soil sample formed prosulfuron metabolites by HPLC (see Table 1).

The most commonly occurring metabolites identified in incubations with cultures listed in Table 1 were intact metabolites **3** and **5** and fragment metabolites **6**, **7**, **9**, and **10**. Control incubations without **1** added showed no coincidental peaks eluting with identified metabolites. *S. griseolus* was most prolific in forming all compounds, **2**−**10,** as metabolites (Figure 1). Of the remaining cultures, *P. testosteroni* formed metabolites **3**, **6**, **7**, and **10**, *P. purpurogenum* formed metabolites **6**, **9**, and **10**, *S. flocculus* formed metabolites **4**, and **6,** and an incubation using the sample of Spring soil formed metabolites **3**, **5**, **7**, and **9**. Single products were formed

Table 1. Biotransformation of (1) As Determined by HPLC Analysis of Culture Extracts

		metabolites observed in culture extracts by HPLC								
microorganism	strain	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Cylindrocarpon radicicola	ATCC 11011	−	−	−	+	−	−	−	−	−
Penicillium purpurogenum	ATCC 9777	−	−	−	−	+	−	−	+	+
Pseudomonas testosteroni	ATCC 11996	−	+	−	−	+	+	−	−	+
Rhizopus sp.	MR 224	−	−	−	−	−	−	−	+	−
Streptomyces flocculus	ATCC 25453	−	−	+	−	+	−	−	−	−
Streptomyces griseolus	ATCC 11796	+	+	+	+	+	+	+	+	+
soil		−	+	−	+	−	+	−	+	−

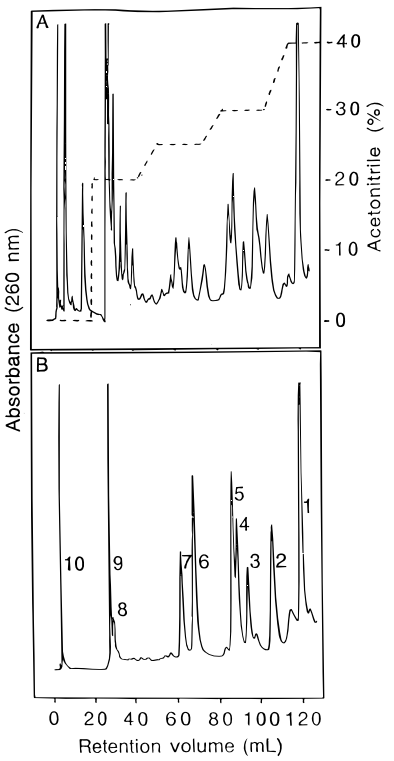

Figure 2. (A) HPLC chromatogram of crude extract from a preparative biotransformation of **1** with *S. griseolus*. (B) Analytical mixture of synthetic standards and purified metabolites with HPLC gradient curve shown as percent of acetonitrile.

by *Cylindrocarpon* (metabolite **3**) and *Rhizopus* sp. (metabolite **9**). The most common metabolites produced by all cultures were **3**, **5**−**7**, **9**, and **10**.

S. griseolus was chosen for a preparative incubation to produce sufficient amounts of metabolites for isolation and structure determination. An HPLC chromatogram of crude extract residue from *S. griseolus* at 72 h is depicted in Figure 2a. The HPLC chromatogram that resulted from a mixture of analytical standards is shown as Figure 2b. Identities of eluting peaks were confirmed by co-injection of pure metabolites or synthetic standards with a solution of crude extract residue. On the basis of peak area comparisons with standards, *S. griseolus* produced four major metabolites, **2**, **3**, **4**, and **5**, in yields of 14%, 12%, 19%, and 13%, respectively. These metabolites were isolated by extensive silica gel chromatography and then analyzed by UV, MS, and NMR analyses.

Identification of Metabolites. Establishment of proton and carbon chemical shift assignments for the substrate **1** was essential for the ultimate characterization of the new metabolites depicted in Figure 1. Initial resonance assignments from 1-D NMR spectra were confirmed by HMQC (Bax and Subramanian, 1986) and HMBC (Bax and Summers, 1986) experiments. The

Table 2. ^{1}H and ^{13}C NMR Chemical Shifts from HMQC and HMBC Spectra of Prosulfuron (1) in CD_3OD

position	^{1}H, ppm ($J_{H,H}$, Hz)	^{13}C, ppm	^{13}C signals correlated by HMBC
4'-OCH_3	4.02 s	55.6	
6′-CH_3	2.50 s	25.3	
1″		136.8	
2″		138.4	
3″	7.50 dd (1.2, 7.7)	131.9	1″, 5″, 1‴
4″	7.64 ddd (1.3, 7.5)	134.3	2″, 6″
5″	7.47 ddd (1.3, 8.0)	127.3	1″, 3″
6″	8.05 dd (1.3, 8.0)	131.5	2″, 4″
1‴	3.29 m	26.1	1″, 2″, 3″, 2‴
2‴	2.56 m	35.2 q[a]	1‴
3‴		170.5[b]	

[a] J_{CF} = 19 Hz. [b] Very weak, broad signal observed.

structures of metabolites of **1** were characterized by comparison with available standards and by NMR and CI-MS analysis.

Structure determination of new metabolites was usually straightforward because of characteristic parent and fragment ions by CI-MS, and chemical shifts and splitting patterns exhibited in ^{1}H NMR spectra of **1**. Aromatic hydroxylated metabolites gave mass spectra with molecular ions with additional 16 amu or 32 amu (metabolite **3**) and simplified aromatic proton splitting patterns. Metabolites containing unaltered triazine rings always exhibited a major ion fragment of *m*/*z* 141. Thus, metabolic transformation of the triazine ring such as O-demethylation or methyl group hydroxylation could be readily assessed by a combination of MS and ^{1}H NMR. Positions of aromatic hydroxylation in metabolites were determined from results of HMQC and HMBC NMR experiments.

The substrate (**1**) showed the following spectral properties: UV−vis (200−400 nm) λ_{max} 208; CI-MS, *m*/*z* (% relative abundance) 420 (37), 313 (3.9), 190 (5), 184 (5), 167 (3), 141 (100); ^{1}H NMR (360 MHz, CD_3CN) δ 2.46 (s, 3, CH_3), 2.57 (m, 2, 2‴), 3.26 (t, 2, 1‴), 3.97 (s, 3, OCH_3), 7.47−7.49 (m, 2, 3″,5″), 7.64 (t, 1, J = 9 Hz, 4″), 8.13 (d, 1, J = 9 Hz, 6″). The presence of fluorine in the trifluoromethyl substituent produced diagnostic splitting patterns and chemical shifts in the ^{1}H NMR spectrum. Thus, the methylene resonance at δ 2.57 which showed complex fluorine and hydrogen splitting could be readily assigned to the 2‴ position. Long-range fluorine splitting of the benzylic methylene proton signals at δ 3.26 distorted the predominant triplet pattern expected from proton splitting and was assigned the 1‴ position. Other NMR experiments, including 1-D homonuclear decoupling, 1-D broadband decoupled ^{13}C, and 2-D HMQC and HMBC experiments, gave the chemical shift assignments listed in Table 2. Thus, from HMBC spectra, correlations were observed between the 1‴ side chain proton signal and aromatic carbons at 1″, 2″, and 3″, of which only one had an attached proton. This proton-bearing carbon was assigned as the 3″

Table 3. Essential ^{1}H and ^{13}C NMR Chemical Shifts from HMQC and HMBC Spectra for Metabolite 2 in CD_3OD

position	^{1}H, ppm ($J_{H,H}$, Hz)	^{13}C, ppm	^{13}C signals correlated by HMBC
1″	—	139.8	—
2″		126.0	
3″	—	158.5	—
4″ [a]	7.64 dd (1.1, 8.0)	123.4	2″, 6″
5″	7.30 t (8.0)	129.0	1″, 3″
6″ [a]	7.14 d (8.0)	121.8	2″, 4″
1‴	3.24 m	21.0	1″, 2″, 3″, 2‴
2‴	2.43 m	34.0	1‴

[a] Chemical shift assignments for 2″ and 4″ may be switched.

carbon by HMQC results. Similarly H-5″ was correlated with C-1″ and C-3″. Other aromatic carbons and protons were assigned, with confirming proton coupling established from homonuclear decoupled ^{1}H NMR spectra.

Structure elucidation of 1-(4′-methoxy-6′-methyltriazin-2′-yl)-3-[[2″-(3‴,3‴,3‴-trifluoropropyl)-3″-hydroxyphenyl]sulfonyl]urea (**2**) was performed on a 5 mg analytical sample: UV−vis (200−400 nm) λ_{max} 211, 224 (sh), 294 nm; CI-MS, *m/z* 141 (100), 287 (1), 436 (49); HRFABMS calcd for $C_{15}H_{17}N_5O_5SF_3$ 436.0902, found 436.0916; ^{1}H NMR (360 MHz, CD_3CN) δ 2.42 (m, 2, 2‴), 2.46 (s, 3, CH_3), 3.19 (t, 2, J = 8.4 Hz, 1‴), 3.97 (s, 3, OCH_3), 7.14 (d, 1, J = 8 Hz, 6″), 7.30 (t, 1, J = 8 Hz, 5″), 7.64 (dd, 1, J = 1.1, 8 Hz, 4″). The prominent molecular ion at *m/z* 436 in the CI mass spectrum indicated that a single hydroxylation of prosulfuron had occurred, and the base peak ion fragment of *m/z* 141 indicated that the triazine ring of the metabolite was unaltered. The ^{1}H NMR spectrum for **2** showed one less aromatic proton resonance and a doublet−triplet−doublet ABC splitting pattern for three protons, indicating that aromatic hydroxylation could have occurred at either position C-3″ or C-6″, to leave three remaining protons adjacent to one another. Aromatic hydroxylation at C-3″ was assigned by analysis of HMQC and HMBC NMR spectra (Table 3). Specifically, signals at 4″ and 6″ were correlated only with each other and with the quaternary carbon at C-2″. If hydroxylation had occurred at position 6″, the only other possibility, then position 3″, would have been correlated with carbon signals at 1″, 1‴, and 5″ as observed in the HMBC spectrum of **1** (Table 2).

Structure elucidation of 1-(4′-methoxy-6′-methyltriazin-2′-yl)-3-[[2″-(3‴,3‴,3‴-trifluoropropyl)-4″,5″-dihydroxyphenyl]sulfonyl]urea (**3**) was performed using a 1.5 mg sample of 85% purity: UV−vis (200−400 nm) λ_{max} 210, 250, 288 nm; UV−vis ($AlCl_3$−methanol) λ_{max} 210, 225 (sh), 297 nm; CI-MS *m/z* 141 (100), 303 (1.2), 436 (1.5), 452 (2.2); HRFABMS calcd for $C_{15}H_{17}N_5O_6SF_3$ 452.0852, found 452.0847; ^{1}H NMR (360 MHz, CD_3OD) δ 2.39 (m, 2, 2‴), 2.45 (s, 3, CH_3), 3.17 (t, 2, J = 8.5 Hz, 1‴), 3.98 (s, 3, OCH_3), 6.76 (d, 1, J = 9 Hz, 5″), 7.50 (d, 1, J = 9 Hz, 6″). A molecular ion at *m/z* 452 suggested the incorporation of two oxygen atoms into the metabolite structure. No apparent change in the triazine portion of the metabolite was confirmed by the *m/z* 141 mass ion fragment and intact methyl singlets in the ^{1}H NMR spectrum. A simplified aromatic region in the ^{1}H NMR spectrum, consisting of two doublets each integrating as one proton with a coupling constant of 9 Hz, indicated two protons with an *ortho* arrangement in the aromatic ring.

The putative catechol metabolite bearing *ortho* hydroxyl groups at positions C-5″ and C-6″ was eliminated as a possibility by HMBC NMR analysis where the side chain 1‴ proton showed a three-bond correlation with one phenolic carbon. Neither **1** nor **2** ever showed four-bond correlations between C-6″ and the benzylic protons at H-1‴.

The two remaining possibilities were metabolites resulting from aromatic hydroxylation of the phenol **2** bearing a 3″-hydroxyl group. As shown in stepwise fashion in Figure 1, *ortho* hydroxylation of **2** would form a catechol metabolite **3** with hydroxyl groups at both positions C-3″ and C-4″. *Para* hydroxylation of **2** would give a hydroquinone derivative bearing hydroxyl groups at both positions C-6″ and C-3″. A method for the detection of *ortho* dihydroquinones (catechols) by UV spectroscopy was described by Markham and Mabry (1968). Addition of methanolic $AlCl_3$ to a methanol solution of **3** caused a bathochromic shift of the phenolic absorbance from 288 to 297 nm consistent with the structure of a catechol. The shift was reversible by acidification with methanol−HCl, supporting the *ortho* hydroquinone structure for metabolite **3**. Stepwise hydroxylation at C-3″ to form metabolite **2** followed by hydroxylation at position C-4″ would give metabolite **3**.

Structure elucidation of 1-(6′-hydroxymethyl-4′-methoxytriazin-2′-yl)-3-[[2″-(3‴,3‴,3‴-trifluoropropyl)phenyl]-sulfonyl]urea (**4**) was performed on a 2.3 mg analytical sample: UV−vis (200−400 nm) λ_{max} 208, 248.8 nm; CI-MS *m/z* 141 (100), 157 (70), 436 (7.3); HRFABMS calcd for $C_{15}H_{17}N_5O_5SF_3$ 436.0902, found 436.0915; ^{1}H NMR (360 MHz, CD_3CN) δ 2.58 (m, 2, 2‴), 3.27 (m, 2, 1‴), 4.00 (s, 3, OCH_3), 4.54 (s, 2, CH_2OH triazine), 7.50 (s, 2, 3″, 5″), 7.66 (t, 1, J = 8 Hz, 4″), 8.13 (d, 1, J = 8 Hz, 6″). A molecular ion at *m/z* 436 and an ion fragment at *m/z* 157 suggested hydroxylation of the triazine ring. Loss of the methyl singlet signal at δ 2.46 for **1** and appearance of a two-proton singlet signal at δ 4.54 supported microbial hydroxylation at the triazine methyl group.

Structure elucidation of 1-(4′-methoxy-6′-methyltriazin-2′-yl)-3-[[2″-(1‴-hydroxy-3‴,3‴,3‴-trifluoropropyl)-phenyl]sulfonyl]urea (**5**) was performed using a 1 mg analytical sample: UV−vis (200−400 nm) λ_{max} = 207, 226 nm; CI-MS *m/z* 141 (100), 184 (1.9), 436 (9.2); HRFABMS calcd for $C_{15}H_{17}N_5O_5SF_3$ 436.0902, found 436.0890; ^{1}H NMR (360 MHz, CD_3OD) δ 2.49 (s, 3, CH_3), 2.57 (m, 2, 2‴), 3.98 (s, 3, OCH_3), 5.83 (dd, 1, J = 1, 9.7 Hz, 1‴), 7.47 (t, 1, J = 8 Hz, 4″), 7.69 (t, 1, J = 8 Hz, 5″), 7.85 (d, 1, J = 8 Hz, 3″), 8.09 (d, 1, J = 8 Hz, 6″). The metabolite was not phenolic (by UV). A prominent molecular ion at *m/z* 436 indicated one additional oxygen atom in the metabolite structure, likely introduced by a single hydroxylation step. The ^{1}H NMR spectrum indicated hydroxylation of the benzylic 1‴ methylene group. The characteristic triplet signal of **1** at 3.26 ppm disappeared while a doublet of doublets at 5.83 ppm integrated for one proton. Finally, the complex multiplet signal for the 2‴ methylene group was simplified in the spectrum where it resembled a quartet. The small amount of **5** precluded a determination of the stereochemistry of the hydroxyl group.

Results of preliminary soil biotransformation studies with **1** appeared to vary depending upon the season of the year when soil samples were collected. Incubations using soil collected in the fall gave an apparently robust degradation of **1** while no metabolites accumulated (by TLC). In contrast, an incubation using a soil sample collected at the same site in the spring appeared by HPLC analysis to degrade **1** relatively slowly, while

producing small amounts of metabolites **3**, **7**, and **9** by the 10th day of incubation with later formation of **5** by day 24. Repeat incubations of prosulfuron with the same fall and spring soils using HPLC analysis for the detection of metabolites confirmed the initial experimental results.

In this study, *S. griseolus* ATCC 11796 transformed prosulfuron into metabolites **2**−**10** (in analytical studies) within 72 h. All of these metabolites were also detected in the extract (Figure 2A) from the preparative incubation, and four of these, **2**−**5**, were characterized by spectral analysis. Species of *Streptomyces*, *Pseudomonas*, *Cylindrocarpon*, *Rhizopus*, and *Penicillium* produced and accumulated one or more metabolites each. All four of the metabolites **3**, **5**, **7**, and **9** formed in a soil incubation of **1** were common metabolites formed by several of the pure cultures (Table 1). This indicates the potential predictive value of the microbial models approach.

The major route of prosulfuron biotransformation by *S. griseolus* occurs by introduction of oxygen to form polar metabolites **2**−**5**, while minor metabolic pathways gave metabolites such as **6** and **7**. In contrast, the major products of aerobic soil studies with labeled **1** resulted from O-demethylation to form **6** and bridge cleavage to form the sulfonamide **7** (Thede, B. C., unpublished data), although these were minor metabolites in *S. griseolus* incubations. In our studies, **6** was a common metabolite in *Penicillium*, *Pseudomonas*, and two *Streptomyces* species although not in soil at day 6, 10, or 24. However, metabolite **6** is relatively unstable (i.e., in solvents such as methanol) and may not accumulate in incubation media in detectable levels—even if formed. Metabolites **2**, **4**, and **8** produced by pure cultures of streptomycetes were not formed by the soil culture. This emphasizes that some differences can be expected when comparisons are made in the metabolism of xenobiotics in pure cultures and complex soil microcosms.

S. griseolus catalyzed O-dealkylation and benzylic oxidation reactions with prosulfuron similar to those observed with other sulfonylurea substrates. This is the first time that aromatic hydroxylation of a sulfonylurea has been observed where prosulfuron was metabolically transformed to both phenol and catechol metabolites. The present work sheds new light on pathways involved in the biotransformation and ultimate mineralization of sulfonylurea pesticides.

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