Microbial Transformations of Clomazone

Song Yu Liu,[†] Mark Shocken,[‡,§] and John P. N. Rosazza*[,†]

Division of Medicinal and Natural Products Chemistry and Center for Biocatalysis and Bioprocessing, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242, and FMC Corporation, Agricultural Chemicals Group, Box 8, Princeton, New Jersey 08543

Common soil fungi and bacteria were screened for their abilities to biotransform the herbicide clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) into metabolites. Preparative-scale incubations were conducted with *Aspergillus niger* (UI-X172) and *Cunninghamella echinulata* (NRRL-3655) to obtain metabolites in sufficient quantities for spectral and chromatographic identification. HPLC analyses were used to assess the capacities of 41 microorganisms to metabolize the herbicide. Based on comparisons with authentic standard compounds and on the structures of metabolites ascertained by ^{1}H NMR and mass spectrometry, major microbial transformation reactions involved hydroxylation at the 5-methylene carbon of the isoxazolidone ring, hydroxylation of a methyl group on the oxazolidone ring, and aromatic hydroxylation at position 3′. Minor metabolic routes included dihydroxylations of clomazone on the aromatic ring, cleavage of the isoxazolidone N−C bond, or complete removal of the isoxazolidone ring to form chlorobenzyl alcohol.

Keywords: *Clomazone; metabolism; microbial models of soil metabolism; metabolite identification*

INTRODUCTION

Microorganisms are well known for their abilities to catalyze practically every type of oxidative, reductive, conjugative or degradative reaction sequence with nearly every class of natural or xenobiotic organic compound (Wallen et al., 1959; Charney and Herzog, 1967; Iizuka and Naito, 1967; Sebek, 1974; Kieslich, 1976; Gibson, 1984; Laskin and Lechevalier, 1984; Rehm and Reed, 1984; Sariaslani and Rosazza, 1984; Rosazza and Duffel, 1986). Microorganisms and their enzymes are widely used in the synthesis of organic compounds, in the modification of the structures of abundantly available prototype compounds, and as new "reagents" in synthetic organic chemistry.

The development of new biologically active agents by industry requires an understanding of how xenobiotics may be distributed throughout the food chain and in water, as well as their persistence and potential biodegradability in the environment. In soil, for example, xenobiotics may be subject to a combination of chemical, physical, and/or biological factors all of which control their persistence. Depending on bioavailability in soil, sewage, or water, xenobiotics may be subject to metabolic transformation by microbial enzymes resulting in their conversions into altered structures. The identification of such microbial metabolites becomes a crucial step in evaluating the ultimate potential for toxicity of any new xenobiotic as well as its potential for persistence in the environment. Understanding metabolic pathways and fate in the environment may also be a crucial step in the design and discovery of new herbicides.

Because of the complexities of potential interactions of xenobiotics in the environment, soil studies remain crucial in establishing kinetics of degradation, and, where possible, the identification of major breakdown products. However, levels of some pesticides used in the environment may be extremely low, rendering the production, isolation, and identification of metabolic, physical, or chemical breakdown products difficult. Studies using radiolabeled pesticide substrates in the soil provide valuable information about pesticide degradability versus time. However, these types of studies are, by nature, time consuming and they do not always offer sufficient information to directly identify the structures of potential metabolites formed by microbial metabolism. Furthermore, the analysis of soil extracts containing radioactive substrates often relies on the availability of synthetic standard compounds, which may be difficult to prepare and which may not be representative of the complete range of metabolites possible by microbial transformation.

Clomazone (**1**; 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) is a herbicide produced by FMC Corp. for use against species of annual broadleaf weeds and grass. Clomazone is currently used for weed control in the cultivation of soybeans, cotton, tobacco, and various vegetable crops under the trademark Command (Warfield et al., 1985; Chang et al., 1987). Clomazone selectively blocks both diterpene and tetraterpene synthesis in weeds (Duke et al., 1985). The metabolism of clomazone has been studied both in rats (Wu et al., 1983) and in soybean plants grown in the greenhouse (ElNaggar et al., 1992). Mervosh et al. (1995a,b) recently addressed the fate of clomazone in soil with attention to aspects of microbial activity, temperature, soil moisture, and bioavailability versus time. ^{14}C-Labeled clomazone was used in a clay loam soil to demonstrate that clomazone degradation was biologically dependent. After 84 days, 59% of applied clomazone was extracted unchanged, 12% of the applied radioactivity remained unextractable, and a single detectable metabolite representing <5% of the applied clomazone was obtained only under conditions of low temperature or low soil moisture content. The structure of the metabolite was not determined.

* Author to whom correspondence should be addressed.

[†] Division of Medicinal and Natural Products Chemistry.

[‡] FMC Corporation.

[§] Present address: Springborn Laboratories, Inc., 790 Main St., Wareham, MA 02571.

0021-8561/96/1444-0313$12.00/0

We have conducted microbial transformation studies of clomazone in an effort to identify the structures of microbial metabolites of the herbicide and to determine possible pathways of biodegradation in the environment. In this paper, we develop the concept of "microbial models of soil metabolism", with clomazone as an example. The concept of using microorganisms as models for the metabolism of xenobiotics, such as drugs, in mammals was set forth by Smith and Rosazza (Smith and Rosazza, 1975, 1982, 1983; Smith et al., 1977; Rosazza and Smith, 1979) to address significant problems associated with studying drug metabolism, which are similar to those found in studying xenobiotic metabolism in soils. This concept has been successfully applied and exploited in metabolism studies of alkaloids, aromatics, and various classes of drugs and other compounds (Wallen et al., 1959; Beukers et al., 1972; Cerniglia, 1983; Davis, 1987; Clark and Hufford, 1991; Griffiths et al., 1991). In essence, microorganisms can mimic expected metabolic transformations in mammals, soil and water microorganisms, and higher plants. Although a single microorganism might not be able to mimic all of the biotransformations performed by other living systems, a selected group of microorganisms can be used to produce metabolites of a xenobiotic. Metabolites observed in culture extracts can be obtained easily by incubation scaleup for identification and biological evaluation. The microbial models approach can be used as a supplement to required soil studies, to produce microbial metabolites for structure elucidation and use as analytical standards, and to predict pathways of metabolism in a more complex mileux like the soil. The nature of microbial transformation products of clomazone is discussed in this paper.

MATERIALS AND METHODS

Chemicals. Clomazone (**1**) and analytical samples of presumed clomazone metabolites (except **4**; Figure 1) were supplied by FMC Corp., Chemical Research and Development Center, Princeton, NJ. These compounds were prepared in conjunction with mammalian metabolism studies conducted earlier (Wu et al, 1983). The identities of clomazone and other standards were confirmed by ^{1}H NMR and MS, and their purities were assessed by TLC and HPLC before use. Acetonitrile was purchased from EM Sci. All other inorganic reagents used were of HPLC reagent grade from Fisher Scientific.

Chromatography. TLC was performed on 0.25-mm layers of SiO_2 GF_{254} (EM Sci.) and Al_2O_3 GF_{254} (ICN Biomedicals). Plates were air dried and activated at 120 °C for 1 h before use. Solvent systems used were CH_2Cl_2:EtOH (9:1, v/v) for Al_2O_3 GF_{254} TLC analysis, and CH_2Cl_2:MeOH (98:2, v/v; solvent system 1) and CH_2Cl_2:MeOH:HCOOH (98:2:1, v/v/v; solvent system 2) for SiO_2 TLC analysis. Preparative TLC plates consisted of 1-mm layers of SiO_2 GF_{254} developed with CH_2Cl_2:EtOH:HCOOH (80:5:1, v/v/v). All plates were visualized under 254 and 366 nm UV light before being sprayed with Pauly's reagent. Pauly's reagent (diazotized sulfanilic acid) is useful to visualize phenols that give yellow, orange, or red colors (Axelrod et al., 1960). The final alkaline spray reagent consisted of 5% NaOH in 50% ethanol (w/v).

Flash column chromatography (FCC) columns (J. T. Baker Inc.) were packed with Al_2O_3 (50−200 mesh, activity grade II) for preparative scale separations of metabolites from crude culture extracts.

HPLC analyses were performed with a Shimadzu LC-6A instrument, a SCL-6B System Controller, and a CR-501 Chromatopac data processor equipped with a Whatman Partisil 5 ODS-3, RP C-18, 5 mm, 25 cm × 4.6 mm i.d., analytical column preceeded by a Partisil ODS-3, 5-mm guard column cartridge. Eluting compounds were detected with a Shimadzu SPD-6AV UV−vis detector at 214 nm. A gradient of acetonitrile-water was applied according to Shimadzu HPLC gradient curve 6 (a concave gradient) at a flow rate of 1 mL/min. HPLC pure solvent water was adjusted to pH 5.9 with H_3PO_4 and filtered through a 5 mm membrane filter (Micro Sep), and organic components were filtered through a 0.45-mm filter (Millipore Type HV). Solvents were deaerated by house vacuum. The gradient composition is shown in Figure 1 along with elution profile for clomazone and various metabolites.

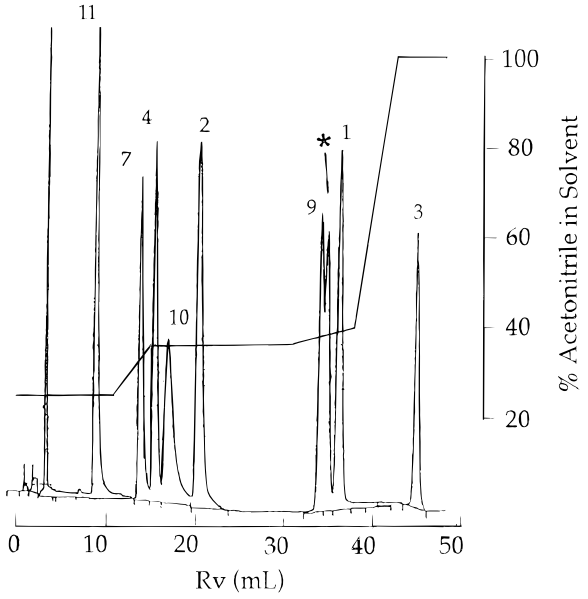

Figure 1. HPLC chromatogram of clomazone (**1**), standard compounds, and metabolites. Numbers at the tops of peaks correspond to structures in Figure 2, except for (*) which indicates the peak for 4′-acetoxyclomazone, a synthetic standard not observed in incubations. The HPLC gradient ranged from 30% CH_3CN in H_2O to 100% CH_3CN as shown. Linear gradients were used throughout, except during the period 12−16 min where a concave gradient was produced with curve 6 of the Shimadzu system controller software and solvent program.

Spectral Methods. MS measurements on isolated metabolites were obtained using GC/MS on a VG Trio-1 GC/MS instrument equipped with a GC/MS data system. A glass column (150 cm × 0.320 mm i.d.) of 3% methylsilicone OV-1 was used, with He as the carrier gas at a flow rate of 20 mL/min. Temperature was programmed to increase from 50 to 250 °C at a rate of 20 °C/min, with an injection port temperature maintained at 250 °C. Direct inlet probe (DIP) MS analyses were also performed on the same instrument in an electron-impact mode, with an ionizing voltage of 70 ev.

NMR spectral measurements were made on solutions of metabolites in 99.96% acetone-d_6 or $CDCl_3$ in 5-mm NMR tubes. The ^{1}H NMR spectra were recorded with a Bruker WM-360 MHz NMR spectrometer at 360 MHz, whereas ^{13}C NMR spectra were recorded at 90 MHz. Chemical shifts were recorded as ppm (δ) downfield from tetramethylsilane (TMS) as an internal standard. In cases where metabolite quantities were very low, no TMS was added, and chemical shifts were based on those of specific solvent signals. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; dd, doublet of doublets; and m, multiplet. All metabolites were identified by comparison with authentic standards by HPLC, TLC, NMR, and MS, except for diphenols, which were identified only on the basis of GC/MS and TLC.

Microorganisms. For initial screening, 41 microorganisms were selected from the culture collection of the College of Pharmacy, University of Iowa. The following genera (number of organisms screened) were screened for their abilities to metabolize clomazone: *Absidia* spp. (2), *Aspergillus* spp. (6), *Bacillus* spp. (2), *Candida* spp. (2), *Corynebacterium* spp. (1), *Cunninghamella* spp. (4), *Curvularia* spp. (1), *Cylindrocarpon* spp. (1), *Helicostylum* spp. (1), *Mucor* spp. (2), *Mycobacterium*

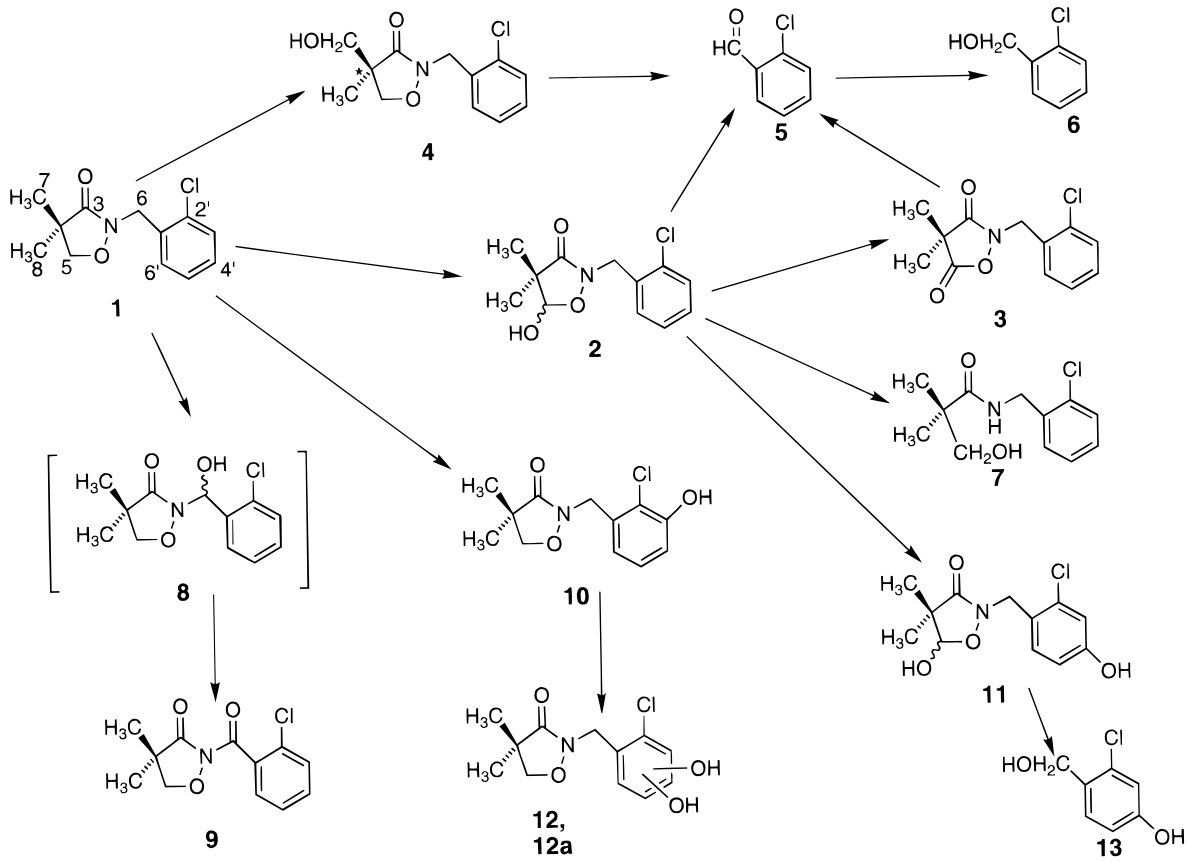

Figure 2. Pathways of clomazone metabolism in microorganisms.

sp. (1), *Nocardia* spp. (3), *Pseudomonas* spp. (4), *Rhizopus* spp. (3), *Rhodococcus* spp. (2), *Sepedonium* spp. (1), *Streptomyces* spp. (4), and *Syncephalastrum* spp. (1).

Incubation and Screening Procedure. The standard protocol for screening was our two-stage incubation procedure (Smith and Rosazza, 1975). This procedure was done to obtain sufficient biomass for biocatalysis and to ensure experimental uniformity and reproducibility. Microorganisms were maintained on Sabouraud-dextrose agar or sporulation agar (ATCC no. 5 medium) slants. Medium no. 5 slants consisted of 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1 mg of $FeSO_4 \cdot 7H_2O$ per liter, 1% glucose, and 1.5% agar, adjusted to pH 7.2 before autoclaving. The liquid screening medium was made with glucose (20 g), NaCl (5 g), K_2HPO_4 (5 g), yeast extract (5 g), soybean meal (5 g), and distilled water (1 L), and the final mixture was adjusted to pH 7.0 by addition of 6 N HCl before autoclaving at 121 °C for 15 min.

Fresh 1-week-old slants were flooded with sterile medium and agitated to suspend spores or vegetative growth. Stainless steel capped DeLong culture flasks (125 mL) containing 25 mL of sterile medium received slant inocula and were incubated in G-25 gyrotary shakers (New Brunswick Scientific, Edison, NJ) with shaking at 250 rpm and 28 °C for 72 h. The 72-h-old stage I culture was used to inoculate (10% inoculum) stage II cultures, which were incubated for 24 h before receiving of 10 mg of clomazone each as a concentrated solution in 0.1 mL of dimethylformamide (DMF). Controls consisted of sterile medium containing the same microorganisms and incubated under the same conditions without substrate. In addition, the stability of clomazone in buffer solutions at pH 3, 7, and 9 under the agitation conditions used during biotransformation was evaluated. Buffers used were: pH 3, 0.1 M potassium hydrogen phthalate/0.1 M HCl; pH 7, 0.1 M KH_2PO_4/0.1 M NaOH; and pH 9, 0.1 M Tris-HCl). Substrate-containing cultures and control flasks were sampled at various time intervals (24, 72, and 144 h) by removing 4-mL aliquots of the entire culture. Samples were adjusted to pH 3 with 6 N HCl and extracted with 1 mL of ethyl acetate, and the organic and aqueous layers were separated by centrifugation for 1 min in a desk-top centrifuge. The organic solvent layer was removed, and 30 μL was spotted onto TLC plates for analyses. A sample of soil taken from a corn field in Solon, IA, was also incubated with clomazone. The two-stage incubation procedure followed the standard protocol exactly, except 1 g of soil was used in the stage I incubation as the source of microorganisms.

A total of 12 clomazone metabolites were identified in these experiments. For simplicity in discussing the results, metabolites are numbered **2**−**13** with clomazone as **1**, and the structures of all compounds are shown in Figure 2.

RESULTS

Quantitative Analysis of Clomazone Metabolites in Culture Extracts. Culture extracts obtained from screening experiments were examined by HPLC to detect possible clomazone metabolites. For quantitative HPLC analyses, cultures containing 10 mg of clomazone substrate were harvested at 72 h and adjusted to pH 3 with 6 N HCl, then extracted twice with 40 mL each EtOAc:*n*-propanol (9:1, v/v). Extracts were dehydrated over anhydrous Na_2SO_4, and the crude extracts were dried in a rotary evaporator. Samples were then transferred into small vials and dried under a nitrogen stream. Extracts were reconstituted in 1 mL of acetone and filtered through a 0.45-mm Nylon acrodisc (Gelman Sciences, Ann Arbor, MI), and 2-μL samples were injected for HPLC quantitative analysis. Seventeen of the 41 cultures screened gave clomazone metabolites. The results of quantitative HPLC analyses of microbial transformation screening experiments are shown in Table 1.

Representative strains of *Aspergillus* and *Cunninghamella* were selected for preparative scale incubation to confirm the identities of metabolites formed in screening scale incubations and identified only by HPLC.

Table 1. Results of HPLC Analyses of Cultures Containing Clomazone (10 mg/25 mL)

		mg of compound in flask[a]											
culture name	culture no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**13**
Absidia coerolia	UI-MR27b	4.75	1.65	1.20							0.10		
Absidia spinosa	UI-MR7600	4.35	0.99	0.20	0.28					0.03	0.28		
Aspergillus niger	UI-X172	0.05	2.10	0.01	1.55			0.06	0.02		0.60	0.32	0.26
Aspergillus niger	ATCC10581	9.15	0.12		0.78					0.01	0.13		
Bacillus megaterium	ATCC14581	6.33	1.46	0.07	0.19					0.03	0.07		
Cunninghamella echinulata	NRRL 3655	8.40	0.63	0.10	0.19		0.24			0.06	0.08		
Cunninghamella echinulata	ATCC8688a	5.30	0.32	0.15	0.06						0.19		
Cunninghamella elegans	NRRL 1393	6.15	0.53	0.35	0.38		0.17			0.02	0.11		
Curvularia lunata	NRRL 2178	4.15	0.48	0.03	0.10		0.10	0.38		0.04	0.14		
Mucor mucedo	UI-UC4605	4.50	0.20		0.06		0.02			0.05			
Mucor mucedo	ATCC20094	7.64	1.20	0.04			0.03			0.01			
Rhizopus species	UI-MR224	5.20	0.61	0.09	0.07			0.68					
Sepedonium chrysospermum	ATCC13378	2.60	4.85	0.04	0.28						0.10		
Streptomyces griseolus	ATCC11796	0.03	0.41	0.50	0.56	0.11		0.50		0.04		0.77	
Streptomyces griseus	NRRL8090	0.55	6.05		0.19		0.24	0.02		0.02	0.07	0.18	
Streptomyces griseus	ATCC13273	0.04	2.10	0.58	0.03	0.11	1.35			0.04		0.18	0.45
Streptomyces rimosus	ATCC23955	5.68	2.60	0.04	0.27		0.14			0.01		0.04	
soil samples from Solon, IA		9.96		0.05				0.33					
HPLC retention volumes		35.6	20.7	44.1	16.1	28.1	14.2	14.5	28.0	33.4	17.5	9.5	7.7

[a] The structures for **1**−**13** are shown in Figure 2.

Preparative Scale Biotransformations of Clomazone with *A. niger*. Two separate preparative incubations were conducted using *A. niger* (UI-X172) as the metabolizing culture. Stage II cultures were grown in 1-L DeLong flasks each holding 200 mL of culture medium. A total of 21 stage II flasks containing 4.2 L of culture and 2.22 g of clomazone were harvested between 48 and 72 h, depending on TLC analysis of the progress of the incubation. At harvest, the cultures were combined, adjusted to pH 3, and exhaustively extracted with ∼9 L of EtOAc. The extract was dehydrated over solid anhydrous Na_2SO_4 and concentrated under reduced pressure in a rotary evaporator. A total of 4.22 g of crude extract was obtained in this manner. HPLC analysis of the crude extract revealed the presence of peaks with retention volumes the same as metabolites **2**, **4**, **10**, and **12**. The metabolites were isolated by FCC.

A 2-g sample of the *A. niger* crude extract was fractionated by Al_2O_3 FCC (28 × 3 cm i.d.) by eluting fractions with solvents of increasing polarity including CH_2Cl_2, CH_2Cl_2:EtOH (9:0.5), CH_2Cl_2:EtOH (4:1), MeOH, and MeOH:88% HCOOH (5:1, v/v) while 20-mL/fractions were collected. Fractions 56−100 (30 mg) contained metabolites **10** and **12**, and metabolites **2** and **4** were isolated by preparative TLC (SiO_2 GF_{254}) from fractions 50−55 of the flash column.

Metabolite 2 Identified as 2-[(2-Chlorophenyl)-methyl]-5-hydroxy-4,4-dimethyl-3-isoxazolidinone (5-Hydroxyclomazone). Metabolite **2** (9 mg; R_v = 20.66 mL by HPLC and R_t = 9.62 min by GC/MS] was isolated as colorless crystals by preparative TLC over SiO_2 GF_{254} (R_f = 0.4, S 1) and displayed the following physical properties: EIMS *m*/*z* (% relative abundance) 257 (0.19, $M^+ + 2$), 255 (0.5, M^+), 220 (78.42, $M^+ - Cl$), 127 (33, C_7H_6Cl), 125 (100, C_7H_6Cl); ^{1}H-NMR (acetone-d_6) δ 1.16, 1.22 (2 × s, 6H, 2 × CH_3), 4.81 (s, 2H, 6-H), 5.35 (s, 2H, 5-H), 7.30−7.54 (m, 4H, ArH); specific rotation $[\alpha]_D^{25} = -2.3°$ (*c* 0.85, $CH_3OH-CH_2Cl_2$, 3:2).

Metabolite 4 Identified as 2-[(2-Chlorophenyl)-methyl]-4-(hydroxymethyl)-4-methyl-3-isoxazolidinone [(Hydroxymethyl)clomazone]. Metabolite **4** (9 mg; R_v = 16.08 mL by HPLC) was isolated as a colorless oil by preparative TLC over SiO_2 GF_{254} and it displayed the following physical properties: EIMS *m*/*z* (% relative abundance) 257 (1.22, $M^+ + 2$), 255 (3.36, M^+), 220 (89.4, $M^+ - Cl$), 127 (71, C_7H_6Cl), 125 (100, C_7H_6Cl), ^{1}H NMR (acetone-d_6) δ 1.21 (s, 3H, 7 or 8-CH_3), 3.74, 3.50 (ABq, J = 10.77 Hz, 2H, 5-H), 4.04, 4.47 (ABq, J = 8.32 Hz, 2H, 7 or 8-H), 7.31−7.47 (m, 4H, Ar-H); ^{13}C NMR (acetone-d_6) δ 17.34 (C-7), 46.76 (C-6), 49.23 (C-4), 64.33 (C-8), 75.10 (C-5), 127.77, 129.79, 129.90, 130.11, 133.30, 134.01; specific rotation $[\alpha]_D^{25} = -11.4°$ (*c* 0.79, CH_3-OH).

Metabolite 10 Identified as 2-[(2-Chloro-3-hydroxyphenyl)methyl]-4,4-dimethyl-3-isoxazolidinone (3′-Hydroxyclomazone). Metabolite **10** (27 mg; R_v = 17.49 mL by HPLC) was isolated as a white crystalline solid from fractions 47−50, and displayed the following physical properties: TLC R_f = 0.44 (S 1), R_f = 0.60 (S 2), and R_f = 0.80 (Al_2O_3 GF_{254}, CH_2Cl_2:EtOH, 8:1); GC retention time R_t = 9.40 min; EIMS *m*/*z* (% relative abundance) 257 (17, $M^+ + 2$), 255 (45, M^+), 220 (100, $M^+ - Cl$), 143 (30, C_7H_6ClO), 141 (90, C_7H_6ClO); ^{1}H NMR ($CDCl_3$) δ 1.20 (s, 6H, 2 × CH_3), 4.11 (s, 2H, 5-H), 4.77 (s, 2H, 6-H), 6.93 (d, J = 3.6 Hz, 1H, 4′-H), 6.97 (d, J = 7.2 Hz, 1H, 6′-H), 7.15 (dd, J = 7.2 Hz, 7.2, 1H, 5′-H).

Metabolite 12. Metabolite **12** (<0.1 mg), with R_f = 0.2 by SiO_2 GF_{254} TLC (S1) and R_t = 10.22 min by GC/MS, was isolated by FCC over Al_2O_3 from fractions 43−46 of the original column and then by preparative TLC over SiO_2 GF_{254}. Metabolite **12** displayed the following EIMS s *m*/*z* (% relative abundance): 273 (5, $M^+ + 2$), 271 (15, M^+), 236 (15, $M^+ - Cl$), 159 (33, $C_7H_6ClO_2$), 157 (100, $C_7H_6ClO_2$).

Metabolite 12a. Metabolite **12a** (<0.1 mg) gave R_f = 0.1 (S 1) by SiO_2 GF_{254} TLC and R_t = 10.4 min by GC, and was isolated from fractions 43−46 of the original column and by subsequent Al_2O_3 FCC and preparative TLC over SiO_2. The EIMS *m*/*z* (% relative abundance): 273 (7, $M^+ + 2$), 271 (21, M^+), 236 (50, $M^+ - Cl$), 208 (9, $M^+ - Cl - CO$), 159 (17, $C_7H_6ClO_2$), 157 (50, $C_7H_6ClO_2$).

Preparative Biotransformation of Clomazone with *C. echinulata*. A *C. echinulata* (NRRL 3655) incubation consisting of 15 1-L DeLong flasks (3.0 L of culture medium) with 1.52 g of clomazone as substrate was accomplished with the same protocol as with *A. niger* except that cultures were harvested at 144 h. A total of 2.14 g of extract was obtained and subjected to

preparative TLC and Al_2O_3 FCC as before to give metabolite **2** (16 mg) and metabolite **10** (3 mg) and traces of **6**. These compounds exhibited spectral and chromatographic properties that were identical to those of **2** and **10** identified as metabolites of *A. niger*.

Metabolite 6 Identified as (2-Chlorobenzyl Alcohol). Metabolite **6** (<0.1 mg) with $R_t = 5.01$ min by GC, $Rf = 0.65$ (S1) and 0.61 (S2) by TLC, and $R_v = 14.2$ mL by HPLC, was isolated by preparative TLC over SiO_2 GF_{254} and identified by GC/MS and chromatographic comparisons with authentic **6**. The GC/MS gave m/z 144 (25, $M^+ + 2$), 142 (75, M^+), 125 ($M^+ - OH$), 107 ($M^+ - Cl$), 77 (100, $M^+ - Cl - CH_2O$). Identification of this metabolite as **6** was confirmed by matching the metabolite spectrum with the standard spectrum of **6** with the TRIO-1 GC-MS Data System.

DISCUSSION

Previous metabolism studies of clomazone in mammals and in plants revealed that the herbicide was susceptible to monohydroxylations at positions 5, 7 or 3′, 4′, 5′; dihydroxylations at positions 5, 4′ and 4′, 5′; and trihydroxylations at positions 5, 4′, 5′ (Figure 2). In plants, clomazone is cleaved to yield 2-chlorobenzyl alcohol as an additional biotransformation reaction.

In this study, microorganisms typical of those found in the soil and a mixed culture prepared by inoculation of medium with a soil sample were evaluated for their abilities to biotransform clomazone into metabolites. The availability of standard compounds permitted the establishment of a reproducible HPLC system that could be used to resolve mixtures of expected metabolites in extracts of cultures inoculated with clomazone (Figure 1).

Screening experiments revealed that many microorganisms metabolized clomazone. TLC and HPLC analyses of incubation samples revealed the presence of many common products found by several of the producing organisms as shown in Table 1. Quantitative HPLC analyses of culture extracts showed that metabolites **2**, **3**, **4**, **9**, and **10** (see Figure 2) were formed from clomazone by the majority of the organisms. Metabolites **5**, **8**, and **13** were sparingly formed by only a few of the metabolizing cultures. Metabolites **6**, **7**, and **11** were formed by at least five of the clomazone metabolizing organisms. The most metabolically proficient organisms in biotransforming clomazone to at least five metabolites were *Absidia spinosa var biappendiculata* (MR7600) (5 metabolites), *A. niger* (X-172) (8 metabolites), *Bacillus megaterium* (ATCC 14581) (5 metabolites), *C. elegans* (NRRL 1393) (6 metabolites), *C. echinulata* (NRRL 3655) (6 metabolites), *Curvularia lunata* (NRRL 2178) (7 metabolites), *S. griseus* (NRRL 8090) (7 metabolites), *S. griseus* (ATCC 13273) (8 metabolites), *S. griseolus* (ATCC 11796) (7 metabolites), and *S. rimosus* (ATCC 23955) (6 metabolites). Preliminary results obtained by a soil culture involving culture medium inoculated with soil indicated that ~1% of clomazone substrate was converted to traces of metabolites **3** and **7** (Table 1). This result indicates the likelihood that pure cultures have the potential to form metabolites of clomazone similar to those formed by organisms in natural soil environments.

Mass spectrometry is very useful in the structure elucidation of clomazone metabolites, not only in the characteristic $m/z + 2$ isotope peaks of single chlorine-containing fragments, but also in demonstrating whether metabolic transformations occurred on either the aromatic or isoxazolidinone rings or on both of these moieties. Cleavage of the benzyl moiety is a major fragmentation observed in all metabolites of clomazone.

Analytical experiments with HPLC analysis revealed that *A. niger* strain X172 converted 95% of the clomazone substrate into a mixture of metabolites. The major products observed in analytical experiments were metabolites **2** (21% yield), **4** (15.5% yield), **10** (6% yield), **11** (3.2% yield), and **13** (2.6% yield). In the preparative scale incubation, quantities of metabolites **2**, **4**, **10**, and **12** could be obtained for structure elucidation. Metabolite **2** was identical in chromatographic mobility (R_v = 20.66 mL) and MS to authentic **2**. The M^+ at m/z 255 indicated the presence of one additional oxygen atom in the clomazone structure, and the fragment at m/z 125 indicated that hydroxylation had occurred on the isoxazolidone ring. In the ^1H NMR spectrum, the C-4-methyl groups were resolved into two distinct 3H-singlets at 1.16 and 1.22 ppm, and all aromatic and benzylic proton signals of clomazone were clearly observable in the metabolite spectrum. Similarities of the metabolite with authentic **2** in chromatographic mobilities, ^1H NMR, MS properties confirmed the metabolite structure as **2**. Furthermore, the resolution of the two CH_3-group signals that resulted from the anisotropic effect introduced by the hydroxyl group, and the measured $[\alpha]_D^{25}$ of −2.3° (*c* 0.85; $CH_3OH-CH_2Cl_2$, 3:2) indicate that enzymatic hydroxylation favored the formation of one chiral isomer with *A. niger*, the absolute configuration of which is unknown.

The structure of metabolite **4** obtained from *A. niger* biotransformation of clomazone was determined by spectral and chromatographic comparisons with authentic **4**. The mass spectrum at m/z 255 was consistent for a metabolite with empirical formula $C_{12}H_{14}ClNO_3$ or a metabolite containing one additional oxygen atom compared with clomazone. The key fragment at m/z 125 indicated that hydroxylation had occurred on the oxazolidone portion of the clomazone structure. The ^1H NMR spectrum was identical to that of clomazone except for the absence of a singlet for one of the *gem*-dimethyl groups at δ 1.21, and its replacement with a new signal at δ 4.47 for R-CH_2OH. The ^{13}C NMR spectrum of metabolite **4** confirmed the replacement of one of the *gem*-dimethyl groups (C7 or C8) at δ 21.71, with a new signal at δ 64.33 for an oxygenated carbon atom. All of these results indicate the structure of the metabolite as **4.** The carbon to which the *gem*-dimethyl groups of clomazone are attached is prochiral. Thus, enzymatic hydroxylation of one of the *gem*-dimethyl groups can induce chirality into the metabolite structure. The specific rotation $[\alpha]_D^{25}$ of −11.4° indicates that the enzymatic hydroxylation reaction favored the formation of one chiral isomer, the absolute configuration of which is uncertain. Microbial transformation readily afforded **4**, which is difficult to prepare synthetically.

Metabolite **10** was determined by chromatographic behavior and spectral comparisons with authentic **10**. The MS spectral ions at m/z 141 and 143 indicated that one additional hydroxyl group was located in the aromatic ring. The ^1H NMR spectrum of both the metabolite and authentic **10** exhibited signals at δ 6.93, 6.97, and 7.15 as an AB_2 splitting pattern, which clearly demonstrated the presence of three adjacent protons on the aromatic ring.

Metabolites **12** and **12a** have essentially identical mass spectra, but different TLC mobilities and retention

times by GC-MS. Molecular ions at *m/z* 271 and 273 in the spectra of both compounds were consistent for metabolites with empirical formulae of $C_{12}H_{14}ClNO_4$, each containing two additional oxygen atoms compared with the substrate clomazone. Fragment ions at *m/z* 157 and 159 indicated that these two hydroxyl groups were located on the aromatic rings. On TLC plates, **12a** was orange, whereas **12** was scarlet after visualizion by Pauly's reagent. These results indicate the possibility that the metabolites consisted of a 3′,4′-catechol metabolite and a 3′,6′-*p*-hydroquinone metabolite, each arising from *ortho* or *para* hydroxylation of **10** as a precursor (Smith and Rosazza, 1974). These studies confirmed the structures of metabolites **2**, **4**, **10**, **12**, and **12a** as microbial transformation products of clomazone formed by *A. niger*.

In the preparative scale incubation, *C. echinulata* transformed clomazone to metabolites **2**, **6**, and **10**. These metabolites were formed in 6.3, 1.9, and 0.8% yields, respectively, in analytical scale experiments (Table 1). Metabolites **2** and **10** isolated from *C. echinulata* culture extracts gave identical properties to those obtained for standards and metabolites formed by *A. niger*. The structure of metabolite **6** formed by *C. echinulata* was confirmed by both GC-MS spectral comparisons with standard **6** and with data for **6** in the GC-MS library.

Based on the HPLC results (Table 1) and those from the isolation and actual identification of metabolite structures, we have proposed the pathway shown in Figure 2 for the metabolic disposition of clomazone (**1**) by microorganisms. Microbial transformation reactions include aromatic ring hydroxylation forming metabolites **10**, **11**, **12**, and **12a**; benzylic hydroxylation to form **8** and subsequent dehydrogenation to form the amide **9**; hydroxylation at position-5 to form **2**, and subsequent dehydrogenation to form **3**, an active herbicide metabolite (Chang et al., 1987); and methyl group hydroxylation to form **4**. Clomazone metabolites formed in soybeans include **2**, **3**, **4**, **8**, and 5′-hydroxyclomazone, a phenolic metabolite not formed by microorganisms we examined (ElNaggar et al., 1992). Many of the hydroxylated metabolites are glycosylated in soybeans, and similar oxygenated metabolites are found in rats (unpublished results). The results of this work underline the value of model microbial transformations as a tool in elaborating the pathways of metabolism of compounds like clomazone in plants, mammals, and in soil.

ABBREVIATIONS

Al_2O_3, aluminum oxide; ATCC, American Type Culture Collection, Rockville, MD; DIP, direct insert probe; DMF, dimethylformamide; EIMS, electron-impact mass spectrum; FCC, flash column chromatography; GC-MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; i.d., internal diameter; M^+, molecular ion; NMR, nuclear magnetic resonance; NRRL, Northern Regional Research Laboratories, Peoria, IL; RP, reversed phase; Shimadzu HPLC curve 6 is a concave gradient used in Shimadzu system controller software and solvent programmers; R_t, retention time; R_v, retention volume; TLC, thin-layer chromatography; TMS, tetramethylsilane; UC, Upjohn Co., Kalamazoo, MI; UI, University of Iowa; UV−vis, ultraviolet−visible.

ACKNOWLEDGMENT

We thank Larry Dostal, Sherry L. Rose, and Eric Zirbes for technical assistance.

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Received for review May 2, 1995. Accepted September 27, 1995.[⊗] The financial support from FMC Corporation for this research is gratefully acknowledged.

Registry No. Supplied by the Author: Clomazone, 81777-89-1; 5-ketoclomazone, 80959-17-7; 2-chlorobenzyl alcohol, 17849-38-6; 5-hydroxyclomazone, 81778-16-7; hydroxymethylclomazone, 140360-36-7.

JF9502663

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.