Microbial Transformation of the Herbicide Metolachlor by a Soil Actinomycete

Adam Krause, W. Gregory Hancock, Robert D. Minard, Alan J. Freyer, Richard C. Honeycutt, Homer M. LeBaron, Donald L. Paulson, Shu-Yen Liu, and Jean-Marc Bollag*

Transformation of metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)-acetamide] was studied with an actinomycete strain isolated from metolachlor–contaminated soil. Eight metabolites were obtained and identified by mass and NMR spectral analysis. Benzylic hydroxylation of the aralkyl side chains and/or demethylation at the *N*-alkyl substituent appeared to be the only transformations involved. All metabolites had a monochlorine isotopic pattern, indicating that no dehalogenation of the chloroacetyl moiety occurred.

INTRODUCTION

The biological decontamination of pesticide wastes or spills has become an increasingly important area of research (Finn, 1983; Kobayashi and Rittmann, 1982), and it would be desirable if a microbial or biological method of degradation was available for the various pesticidal compounds. Therefore, we studied the possible transformation and degradation of the widely used herbicide metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide]. Metolachlor is an important selective herbicide used for the control of several annual grass weeds, yellow nutsedge, and certain broadleafed weeds in corn, soybeans, peanuts, and other crops.

Several researchers have investigated metolachlor metabolism in soil. Ellgehausen (1976a,b) found that 12 weeks after application of the herbicide, 30% of metolachlor was dechlorinated under aerobic sterile conditions, while under aerobic or anaerobic nonsterile conditions about 18% of the radioactivity was oxidized at the acetyl group to form an oxalic acid derivative. In a separate study (1976b,c), the same author reported a slow but steady evolution of $^{14}CO_2$ in nonsterile soil reaching 4.8% of the applied radiolabeled metolachlor after 12 weeks. Analysis of the soil indicated that only 10% of the residual activity in nonsterile soil was the parent compound vs. 65% of metolachlor remaining in the sterile soil. Sumner et al. (1976) identified five metabolites from soil, but altogether these comprised less than 4% of the total radioactivity.

McGahen (1982) studied the anaerobic metabolism of metolachlor with lake sediment and found two products, the dechlorinated metolachlor with and without a thiomethyl moiety. McGahen and Tiedje (1978) investigated metabolism of metolachlor by resting cells of *Chaetomium globosum* and found 45% disappearance of the herbicide after 144 h of incubation. Eight extractable products were found, and four were identified as 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-hydroxy-1-methylethyl)acetamide, 2-chloro-*N*-(2-ethyl-6-methylphenyl)acetamide, 8-ethyl-3-hydroxy-*N*-(2-methoxy-1-methylethyl)-2-oxo-1,2,3,4-tetrahydroquinoline, and 2-hydroxy-*N*-(2-methyl-6-vinylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide.

In studies of microbial degradation of other chloroacetanilides, propachlor (Kaufman et al., 1971; Lee et al., 1982), butachlor (Chen and Wu, 1978; Lee, 1978; Chahal et al., 1976), and alachlor (Tiedje and Hagedorn, 1975; Smith and Phillips, 1975), transformation reactions included dechlorination, dehydrogenation, dealkylation, hydroxylation, and indoline ring formation.

In this paper we report the identification of eight hydroxylated metabolites of metolachlor produced by a soil-isolated strain of actinomycete and present a scheme of degradation.

MATERIALS AND METHODS

Metolachlor (technical grade) of 95.4% purity and uniform ring-labeled [^{14}C]metolachlor with specific activity of 26.1 μci/mg were supplied by Ciba-Geigy Corp., Agricultural Division, Greensboro, NC. Soil samples collected from railroad beds in North White Plains, NY, were also provided by Ciba-Geigy. The soil was previously treated to metolachlor at 4 lb per acre active ingredient in May, 1981, and again in May, 1982.

A strain of actinomycete active in the transformation of metolachlor was isolated from an enrichment culture from the aforementioned soil by a dilution plating technique. The culture of the actinomycete was grown in a basal medium containing 0.5 g of NH_4Cl, 0.5 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 3 g of Na_2HPO_4, 2 g of KH_2PO_4, 4 g of sucrose, and 0.4 g of yeast extract per liter of distilled water (pH 6.8). Metolachlor dissolved in ethanol was sterilized by passing through a 0.45-μm pore size millipore filter (Millipore Corp., Bedford, MA) and was added aseptically to the autoclaved medium for a final concentration of 50 mg/L. All cultures were incubated at 25 °C on a rotary shaker (140 oscillations/min).

Gas–liquid chromatographic determinations of metolachlor were done on a Packard Model 7424 gas chromatograph equipped with a flame-ionization detector. A 1.2-m, 2-mm i.d. glass column, packed with 10% SP-2100 on 80–100 mesh Supelcoport was used. Operating temperatures: injection port 240 °C, column 210 °C, and detector 230 °C. Nitrogen (carrier gas) flow rate was 40 mL/min; hydrogen and air were 40 and 400 mL/min, respectively.

High-performance liquid chromatography (HPLC) was carried out on a Waters Associates (Milford, MA) instrument equipped with two Model 6000A solvent delivery systems, a U6K septumless injector and a Lambda Max Model 480 LC spectrophotometer operated at 220 nm. A radial compression unit (RCM-100) with a 5-μm Radial-Pak A cartridge (8 mm i.d. × 10 cm) was used. The mobile phase (1.5 mL/min) was composed of 42% methanol and 58% water for the initial 20 min. For the next 15 min, the methanol content was increased linearly up to 80%.

Radioactivity was measured with a Beta Trac 6895 Liquid Scintillation Counter (Tracor Analytic, Elk Grove

Laboratory of Soil Microbiology, Department of Agronomy (A.K., S.-Y.L., and J.-M.B.) and Department of Chemistry (G.H., R.D.M., and A.J.F.), The Pennsylvania State University, University Park, Pennsylvania 16802, and Agricultural Division, CIBA-GEIGY Corporation, Greensboro, North Carolina 27419 (R.C.H., H.M.L., and D.L.P.).

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Village, IL). Samples were counted in ScintiVerse universal liquid scintillation cocktail (Fisher Scientific Company, Fair Lawn, NJ).

Gas chromatography–mass spectrometry (GC–MS) was performed on a Finnigan Model 3200 equipped with a 30 m × 0.25 mm i.d. DB-5 (phenylmethyl silicone similar to SE-54) fused silica capillary column (J and W Associates, San Francisco, CA) operated with split injection. Operating temperatures were injection port 250 °C, column programmed from 100 to 250 °C at a rate of 10 °C per min. Hydrogen was used as the carrier gas. For the purpose of molecular weight determination GC–MS was operated in the chemical ionization mode with methane as a reagent gas.

Low- and high-resolution electron impact mass spectra were taken with Kratos MS 9/50 mass spectrometer at an ionization potential of 70 eV by using a direct insertion probe for sample introduction. Nuclear Magnetic Resonance data were obtained with a Bruker 360-MHz instrument with a Fourier transform system with deuteriochloroform as solvent.

To assess the disappearance of metolachlor, the culture of actinomycete was grown in 250-mL Erlenmeyer flasks containing 100 mL of medium. Five-milliliter samples were removed from the growth medium at regular intervals and centrifuged at 5000*g* for 10 min to remove the cells. Four milliliters of supernatant were transferred into 25-mL glass-stoppered test tubes and extracted with an equal volume of methylene chloride. Two microliters of methylene chloride extract were analyzed by gas chromatography by using an external standard quantitation method. The calibration curve of metolachlor was linear in the range between 0 and 150 μg/mL.

In order to produce sufficient quantities of metabolites for identification, the culture of actinomycete was grown in 2-L flasks containing 1 L of medium. When the concentration of metolachlor remaining reached approximately 30% of the original, the cultures were filtered through a Whatman No. 4 filter paper, and the filtrate was extracted twice with half its volume of methylene chloride. The methylene chloride extract was evaporated to dryness on a rotary evaporator and the residue was dissolved in 4 mL of HPLC grade methanol. Samples in methanol were filtered through 0.45-μm millipore membrane with a Swinney filter unit and 20-μL portions were injected onto HPLC. Each metabolite fraction separated by HPLC was collected separately and diluted with Milli-Q water to achieve approximately 8% of the methanol content. The sample in the 8% methanol was then passed through a Sep-Pak C_{18} cartridge. Metabolites retained in the cartridge were subsequently eluted with 100% methanol and prepared for spectrometric analysis.

Quantitative analysis of metabolites was achieved by incorporating radioactive metolachlor (2 μCi/L) into the growth medium. A radioactivity profile of the metabolites was obtained by collecting 0.75-mL fractions of the HPLC eluate into scintillation vials containing 5 mL of ScintiVerse.

For mycelial dry weight determination, triplicate samples of the culture were harvested separately by suction filtration through a Whatman No. 4 filter paper and washed with equal volumes of water. The filter papers containing mycelium were dried overnight in the oven at 85 °C and weighed.

RESULTS AND DISCUSSION

A strain of actinomycete isolated from soil rapidly transformed metolachlor in the liquid medium. We investigated the relationship between the growth of the actinomycete and the disappearance of metolachlor, and found that all the herbicide was transformed in the growth medium, while no decrease in the concentration of metolachlor was observed in the control medium. The actinomycete biomass rapidly increased within the first five days, remained almost constant for the next few days, and then slowly declined.

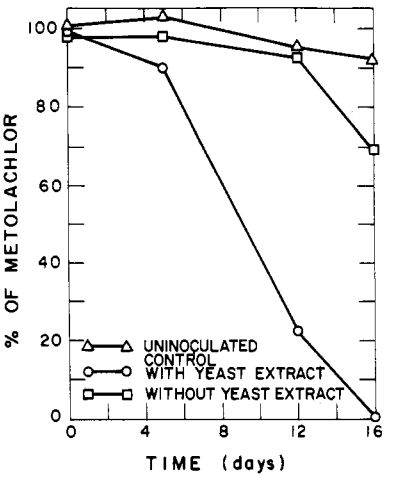

Figure 1. The effect of yeast extract (0.04%) on the degradation of metolachlor by the actinomycete.

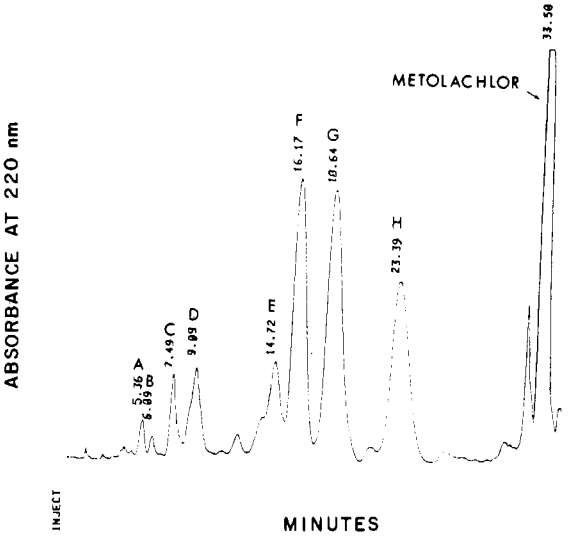

Figure 2. HPLC analysis of metolachlor and its metabolites produced by the actinomycete (peaks A through H refer to metabolites listed in Table 1) after 13 days of incubation.

The disappearance of metolachlor seemed to be affected by the concentration of sucrose. Over 50% of the metolachlor had disappeared from the medium containing 0.4% of sucrose after 24 days. However, after the same period of time, 94% of the metolachlor still remained in the medium with 0.05% sucrose. These results indicated the inability of the actinomycete to utilize metolachlor as the sole carbon source. Addition of a 0.04% yeast extract clearly had a stimulatory effect on the degradation of metolachlor, evidently due to its promotion of growth. As shown in Figure 1 the disappearance of metolachlor from the medium containing yeast extract was almost complete after 16 days as compared to only a 30% loss from the medium without yeast extract.

An HPLC separation profile of the product mixture showed the presence of about 10 peaks eluting before the metolachlor. Subsequent investigation with radioactive substrate indicated that only eight of the peaks which eluted prior to metolachlor contained radioactivity and

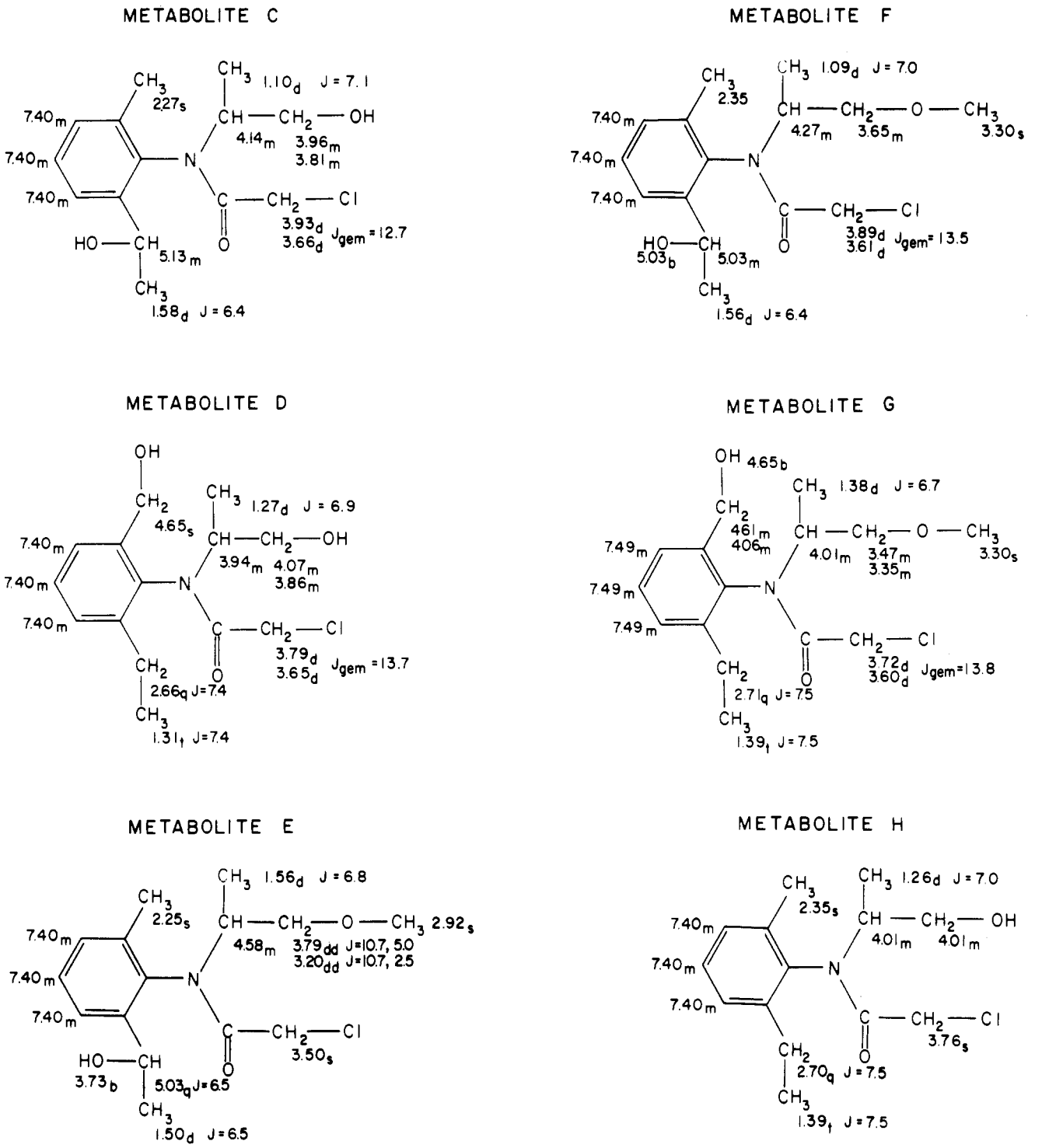

Figure 3. Suggested chemical structures and NMR data of metabolites C, D, E, F, G, and H.

were metabolites, designated A through H according to their sequence of elution (Figure 2). Over 99% of the added radioactivity was extracted into methylene chloride. The total radioactivity recovered from HPLC, including 32.3% of untransformed metolachlor, was 90.9% of that injected onto HPLC. Radioactivity associated with the eight metabolites (A through H) accounted for 58.6% of that recovered. The distribution of radioactivity is presented in Table I. Metabolites F, G, and H were major products comprising 47% of the radioactivity recovered. The eight metabolites isolated from HPLC were again subjected to HPLC until a high-purity sample was obtained. All metabolites isolated were colorless, solid, and quite stable. Mass spectra of all metabolites showed typical monochlorine isotopic patterns, indicating that no dehalogenation took place. The high-resolution mass and NMR spectral data are presented in Table II and Figure 3, respectively.

Metabolites A and B both had a protonated molecular ion at m/z 316 with C.I. E.I. produced major fragments at m/z 270, 243, 226, and 208. Concentrations of metabolites A and B were low, comprising only 0.4% and 0.3% of the recovered radioactivity, respectively; therefore, no attempts were made to isolate the compounds for NMR determination. However, based on high-resolution mass spectral data we assumed that metabolites A and B were isomers of 2-chloro-*N*-[2-(hydroxyethyl)-6-hydroxymethylphenyl]-*N*-(2-methoxy-1-methylethyl)acetamide. The suggested structure of both compounds having two hydroxyl groups at the aromatic side chains of metolachlor

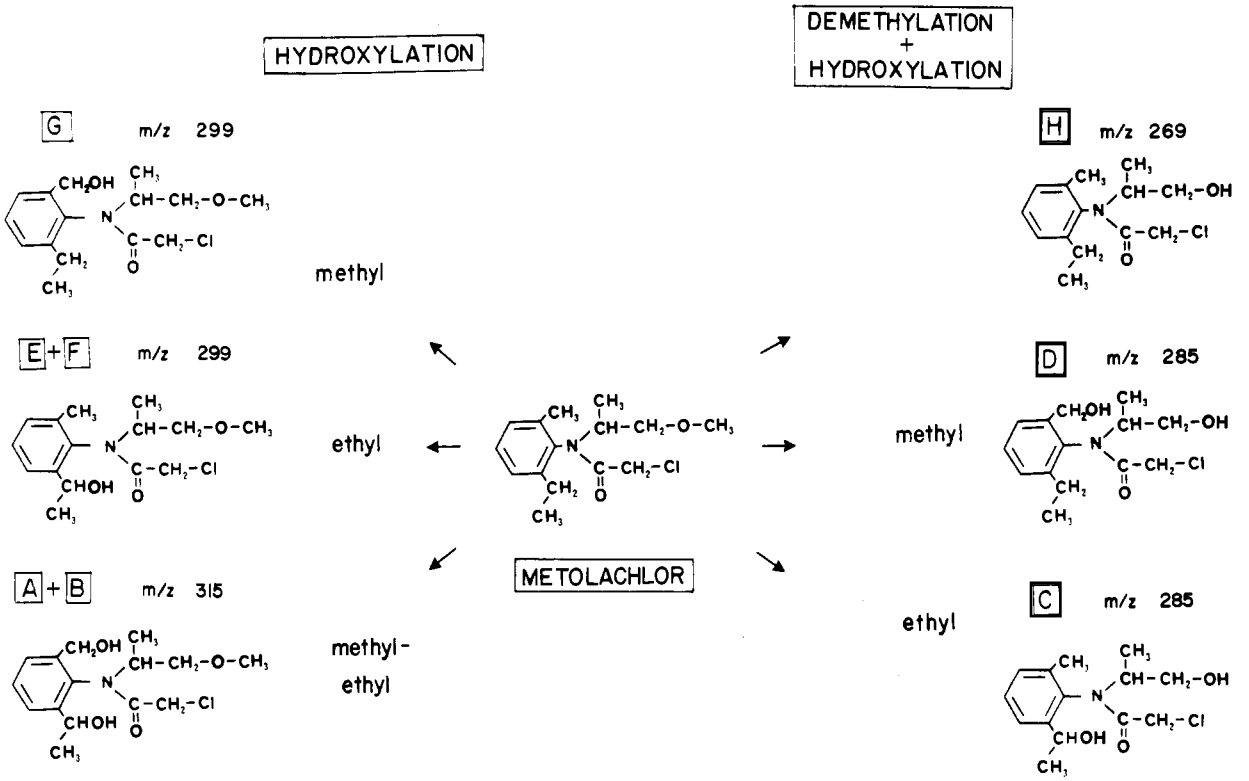

Figure 4. A scheme for metolachlor degradation by the actinomycete.

Table I. Distribution of Radioactivity in Metolachlor and Its Metabolites Produced by the Actinomycete[a]

metolachlor and metabolites	retention time, min	% of added ^{14}C
metolachlor	33.50	32.3
A	5.36	0.4
B	6.09	0.3
C	7.49	2.4
D	9.09	3.1
E	14.72	5.4
F	16.17	16.5
G	18.64	17.1
H	23.39	13.4
		total ^{14}C recovered 90.9

[a] Methylene chloride extract of a 13 day-old growth medium after separation by HPLC.

is indicative of compounds with higher polarity. The reverse-phase HPLC profile supported their polar nature, as both compounds were eluted early in comparison to other metabolites.

Product C had a molecular ion at m/z 285 and major fragments at m/z 254, 227, 210, and 160. NMR spectra of product C showed the replacement of a methoxy group at the *N*-alkyl substituent by a hydroxyl moiety and a further hydroxylation of the ethyl group at the aromatic side chain. In addition, the mass fragment at m/z 73 ($CHCH_3CH_2OCH_3$) typical of metolachlor was no longer seen. Instead, a prominent fragment at m/z 59 ($CHCH_3CH_2OH$) was observed. This information, together with high-resolution mass spectral data, indicated that product C is 2-chloro-*N*-[2-(1-hydroxyethyl)-6-methylphenyl]-*N*-(2-hydroxy-1-methylethyl)acetamide.

Product D also had a molecular ion at m/z 285 and major fragments at m/z 254, 227, 210, and 160. As with product C, its NMR spectra showed the disappearance of the methoxy group at the *N*-alkyl substituent and its replacement by a hydroxyl group. However, NMR data indicated that in product D, the methyl rather than the ethyl group of the aromatic side chain was hydroxylated. Thus, product D was identified as 2-chloro-*N*-(2-ethyl-6-hydroxymethylphenyl)-*N*-(2-hydroxy-1-methylethyl)acetamide.

Products E and F had molecular ions at m/z 299 and major fragments at m/z 254, 227, 210, and 160. NMR data showed that the compounds had a hydroxyl group on the aromatic ethyl side chain. From these results and the high-resolution mass spectral data, we concluded that products E and F were diastereomeric isomers of 2-chloro-*N*-[2-(1-hydroxymethyl)-6-methylphenyl]-*N*-(2-methoxy-1-methylethyl)acetamide.

Product G also had a molecular ion at m/z 299 and major fragments at m/z 254, 227, 210 and 160. NMR data showed the presence of a hydroxyl group at the aromatic methyl side chain, with the rest of the spectra matching that of the metolachlor standard. Product G was therefore identified as 2-chloro-*N*-(2-ethyl-6-hydroxymethylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide.

Product H had a molecular ion at m/z 269 and major fragments at m/z 238, 211, and 162. NMR spectrum showed the disappearance of a methoxy group from the *N*-alkyl substituent. High-resolution mass spectrum indicated the elemental composition of $C_{14}H_{20}NO_2Cl$. This compound was previously reported by McGahen and Tiedje (1978); its structure was 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-hydroxy-1-methylethyl)acetamide.

Based on the metabolites produced by the actinomycete, a scheme of metolachlor transformation is presented in Figure 4. As shown in Figure 4, benzylic hydroxylation occurred at either the ethyl or methyl side chain of the benzene ring yielding metabolites E, F, and G, or at both side chains yielding metabolites A and B. No change in the *N*-alkyl substituent moiety nor the chloroacetyl group was found with these groups of metabolites. Products C and D, however, in addition to the presence of a hydroxyl group at the aromatic ethyl and methyl side chains, respectively, had the methoxyl group of the *N*-alkyl substituent demethylated.

Table II. Mass Spectral Data and Interpretation for Compounds A-H[a]

REASONABLE ION STRUCTURES	A	B	C	D	E	F	G	H
R =	OH	OH	H	CH_3	H	H	CH_3	H
R' =	CH_3	CH_3	CH_3	H	CH_3	CH_3	H	H
R" =	CH_3	CH_3	H	H	CH_3	CH_3	CH_3	H
M^+ =	315 (0)	315 (0)	285 (1)	285 (3)	299 (2)	299 (1)	299 (2)	269 (4) $C_{14}H_{20}NO_2Cl$
	270 (20)	270 (22)	254 (28)	254 (36)	254 (24)	254 (28)	254 (29)	238 (97) $C_{13}H_{17}NOCl$
	243 (3)	243 (2)	227 (3)	227 (3)	227 (11)	227 (11)	227 (10)	211 (7) $C_{11}H_{14}NOCl$
	—	—	—	—	—	—	—	212 (2) $C_{11}H_{15}NOCl$
	226 (52)	226 (25)	210 (100)	210 (99)	210 (100)	210 (100)	210 (100)	—
	208 (100)	208 (100)	—	—	—	—	—	—
	190 (37)	190 (99)	—	—	—	—	—	—
	176 (28)	176 (79)	—	—	—	—	—	—
	—	—	176 (11)	176 (7)	176 (5)	176 (9)	176 (30)	—
	—	—	—	—	—	—	—	162 (100) $C_{11}H_{16}N$
	—	—	160 (95)	160 (100)	160 (46)	160 (54)	160 (99)	—
?? (contains 1 Cl)	158 (60)	158 (96)	—	—	—	—	—	—
	146 (8)	146 (15)	146 (16)	146 (16)	146 (3)	146 (3)	146 (8)	146 (13)
	134 (26)	134 (63)	134 (19)	134 (7)	134 (10)	134 (10)	134 (5)	134 (9)
$+CCH_2Cl$	77 (7)	77 (10)	77 (8)	77 (8)	77 (3)	77 (4)	77 (6)	77 (4)
$-CH{=}CH_2OR'$	73 (23)	73 (20)	59 (9)	59 (5)	73 (11)	73 (13)	73 (12)	59 (7)

[a] The m/z values and intensities (in parenthesis) are given for structurally significant ions. The ion elemental compositions shown were confirmed by high-resolution mass measurement.

There was no noticeable decline in the amount of radioactivity in the growth medium, even after complete transformation of metolachlor. This information indicated that degradation of the aromatic moiety of metolachlor did not occur. No proof of metolachlor ring cleavage has been reported by other researchers with pure cultures.

The HPLC profile showed the following general trends with regard to structures and polarities of the metabolites: metabolites containing two hydroxyl groups were more polar than those with only one hydroxyl group; metabolites with a hydroxyl group at the aromatic side chain appeared to be more polar than those with their hydroxyl moiety at the *N*-alkyl substituents; metabolites with a hydroxyl group at the aromatic ethyl side chains were more polar than those with hydroxylated methyl side chains.

The actinomycete readily degraded metolachlor and complete transformation could be achieved in the growth medium in 16 days. This capacity, however, is not widespread among soil microorganisms. In separate experiments, we screened various soil samples by enrichment techniques and tested a number of bacterial and fungal cultures isolated from soils previously exposed to metolachlor. The results were negative until now in the selected metolachlor concentration of 50 mg/L.

From the present study, it is clear that metolachlor was not utilized as a sole source of carbon or energy. It was found that an increase in the concentration of sucrose and the addition of yeast extract (0.04%) to a basal medium containing metolachlor increased the growth of the microorganism and therefore the rate of metolachlor degradation. As with many aerobic degradations of pesticides, the transformation of metolachlor by this actinomycete is due to cometabolism.

Earlier studies identified 2,6-diethylaniline among the metabolites of alachlor degradation by *Chaetomium globosum* (Tiedje and Hagedorn, 1975) and of butachlor by *Mucor sufui* (Chen and Wu, 1978). No release of an aniline moiety was found in degradation of metolachlor by *Chaetomium globosum* (McGahen and Tiedje, 1978; McGahen, 1982) and by the actinomycete in the present study.

Hydroxylation appeared to be the important mechanism involved in the transformation of metolachlor by the actinomycete. Hydroxylated compounds usually have higher solubility in water and are therefore more accessible to degradation by organisms. Thus, hydroxylation seems to be associated with detoxification processes in nature. It is interesting to note that dechlorination in conjunction with hydroxylation of the chloroacetyl moiety, a common mechanism in microbial metabolism of acetanilide herbicides, was not observed in the present study.

Because our experiments were conducted in batch cultures, the exhaustion of nutrients in the growth medium might have hindered further degradation of the metabolites. It will be of interest to follow the fate of the metabolites in a continuous culture system, particularly after introducing specific microorganisms or mixed cultures.

To our knowledge, there are no publications that have reported the microbial hydroxylation of methyl or ethyl side chains on the benzene ring of metolachlor or other chlorinated acetanilide pesticides. However, similarly hydroxylated products were found as metabolites of the fungicide metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)alanine methyl ester], in a tobacco plant and in the rat (Ciba-Geigy, unpublished data). The significance of this type of transformation mechanism is yet to be evaluated.

Registry No. Metolachlor, 51218-45-2; metolachlor metabolite C, 96394-95-5; metolachlor metabolite D, 96411-89-1; metolachlor metabolite E/F (isomer 1), 96394-96-6; metolachlor metabolite E/F (isomer 2), 96444-41-6; metolachlor metabolite G, 96394-97-7; metolachor metabolite H, 65513-61-3; metolachlor metabolite A/B (isomer 1), 96532-12-6; metolachlor metabolite A/B (isomer 2), 96532-11-5.

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