Hodgson, H. H.; Mahadevan, A. P.; Ward, E. R. 1,4-Dinitronaphthalene. In *Organic Syntheses*; Horning, E. C., Ed.; Wiley: New York, 1960; Collect. Vol. III, pp 341–343.

Huntress, E. H.; Shriner, R. L. 4-Nitrophthalimide. In *Organic Syntheses*; Blatt, A. H., Ed.; Wiley: New York, 1959; Collect. Vol. II, p 459.

Jacobson, A. R.; Makris, A. N.; Sayre, L. M. Monoacylation of Symmetrical Diamines. *J. Org. Chem.* **1987**, *52*, 2592–2594.

Kornblum, N.; Cheng, L.; Kerber, R. C.; Kestner, M. M.; Newton, B. N.; Pinnick, H. W.; Smith, R. G.; Wade, P. A. Displacement of the Nitro Group of Substituted Nitrobenzenes-a Synthetically Useful Process. *J. Org. Chem.* **1976**, *41*, 1560–1564.

Newman, M. S.; Cella, J. A. Studies on the Monoalkylation of Hydroquinone. *J. Org. Chem.* **1974**, *39*, 214–215.

Rigaudy, J.; Klesney, S. P. *Nomenclature of Organic Chemistry*; IUPAC, Pergamon: New York, 1979.

Schoutissen, H. A. J. The Diazotization of Very Weakly Basic Amines. *J. Am. Chem. Soc.* **1933**, *55*, 4531–4534.

Shimabukuro, R. H.; Walsh, W. C.; Hoerauf, R. A. Metabolism and Selectivity of Diclofop-Methyl in Wild Oat and Wheat. *J. Agric. Food Chem.* **1979**, *27*, 615–623.

Smith, A. E. Esterification of the Hydrolysis Product of the Herbicide Dichlorfop-Methyl in Methanol. *J. Agric. Food Chem.* **1976**, *24*, 1077–1078.

Tanaka, F. S.; Wien, R. G. Specific ^{14}C-Labeled Surfactants. The Addition of Homogeneous Polyoxyethylene Glycols to p-(1,1,3,3-Tetramethylbutyl)phenol. *J. Labelled Compd. Radiopharm.* **1976**, *12*, 97–105.

Tanaka, F. S.; Wien, R. G.; Stolzenberg, G. E. Specific ^{14}C-Labeled Surfactants. The Addition of Homogeneous Polyoxyethylene Glycols to 2,6,8-Trimethyl-4-Nonanol. *J. Labelled Compd. Radiopharm.* **1976**, *12*, 107–118.

Tanaka, F. S.; Yoshimoto, T.; Yamada, T.; Tomizawa, C. Synthesis of ^{14}C-Labeled 2,4,6-Trichlorophenyl 4-Nitrophenyl Ether and 4-(Acetylamino)phenyl 2,4,6-Trichlorophenyl Ether. *J. Agric. Food Chem.* **1979**, *27*, 75–79.

Tanaka, F. S.; Hoffer, B. L.; Shimabukuro, R. H.; Walsh, W. C. Identification of Ring Hydroxylated Metabolites of Diclofop-Methyl in Wheat. *Abstract of Papers*, The Sixth International Congress of Pesticide Chemistry, Ottawa, Canada; IUPAC: Ottawa, 1986; 7A-03.

Tarbell, D. S.; Wilson, J. W.; Fanta, P. E. 2,6-Dichlorophenol. In *Organic Syntheses*; Horning, E. C., Ed.; Wiley: New York, 1960; Collect. Vol. III, pp 267–270.

Vyas, G. N.; Shah, N. M. Quinacetophenone Monomethyl Ether. In *Organic Syntheses*; Rabjohn, N., Ed.; Wiley: New York, 1967; Collect. Vol. IV, pp 836–838.

Williard, P. G.; Fryhle, C. B. Boron Trihalide-Methyl Sulfide Complexes as Convenient Reagents for Dealkylation of Aryl Ethers. *Tetrahedron Lett.* **1980**, *21*, 3731–3734.

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Identification of the Isomeric Hydroxylated Metabolites of Methyl 2-[4-(2,4-Dichlorophenoxy)phenoxy]propanoate (Diclofop-Methyl) in Wheat

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The major metabolic pathway for detoxication of diclofop-methyl in tolerant plant species is by ring hydroxylation followed by glucoside conjugation. This investigation reports on the conclusive identification of the three isomeric hydroxylated metabolites of diclofop-methyl obtained from wheat after acid hydrolysis of the glucoside conjugates. Identified as metabolites were the 2,3-dichloro-4-hydroxy, 2,5-dichloro-4-hydroxy, and the 2,4-dichloro-5-hydroxy isomers. The first two metabolites were formed via the NIH shift.

The herbicide diclofop-methyl has been developed for the selective control of undesired grasses (Hoechst AG, 1973). This herbicide also shows considerable promise for the control of wild oat in cereal crops (Miller and Nalawaja, 1974; Friesen et al., 1976). One reason for this herbicidal selectivity may be due to the differences in root growth inhibition that was observed by Shimabukuro et al. (1978). When intact plants are root-treated with diclofop-methyl, root growth of wild oat is severely inhibited while inhibition of wheat root growth is only slight. However, the primary cause for diclofop-methyl selectivity appears to result from the significant differences in the metabolic detoxication pathways of wild oat and wheat (Shimabukuro et al., 1979; Gorecka et al., 1981; Jacobson and Shimabukuro, 1984).

When plants are treated with diclofop methyl, rapid hydrolysis occurs to afford diclofop acid, 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid, in both resistant and susceptible plants. The detoxication of diclofop acid by resistant plants, such as wheat, occurs by hydroxylation of the dichlorophenoxy moiety of diclofop acid (Gorbach et al., 1977; Shimabukuro et al., 1979). This hydroxylated metabolite is rapidly conjugated with glucose; hence, very little hydroxylated diclofop acid in its

free form remains available in plant systems for further metabolic reaction (Shimabukuro et al., 1979). Gorbach and co-workers (1977) were able to separate the three isomers of ring-hydroxylated diclofop acid by gas–liquid chromatography as their methylated derivatives. They reported the identification of the most abundant isomer as 2-[4-(2,4-dichloro-5-hydroxyphenoxy)phenoxy]propanoic acid, but no attempt was made to identify the remaining two isomers.

The purpose of this research was to determine why one isomer was being produced in approximately twice the abundance of the other two isomers. If direct hydroxylation of the chlorinated ring were to take place, we would expect to observe the 3-OH, 5-OH, and 6-OH isomers as the three hydroxylated metabolites of diclofop-methyl and these isomers would be expected to be produced in approximately equal abundance. Since identification of all the isomeric hydroxylated metabolites of diclofop-methyl was not accomplished by Gorbach et al. (1977), this study was also undertaken to identify the two remaining isomeric metabolites derived from ring hydroxylation. However, in the preliminary report of our investigations, we found that the most abundant metabolite was not the 5-OH isomer (Tanaka et al., 1986). Furthermore, our final results show that two of the hydroxylated metabolites were formed via the NIH shift reaction (Tanaka et al., 1988). Therefore, the complete data for positive identification of the three hydroxylated isomeric metabolites of diclofop-methyl are now being reported.

EXPERIMENTAL SECTION

Materials. [*2,4-dichlorophenoxy*-U-^{14}C]Diclofop-methyl was purchased from New England Nuclear Corp. with a specific activity of 2 mCi/mmol. Unlabeled analytical-grade diclofop-methyl was prepared from a commercial emusifiable concentrate by standard extraction and purification procedures in the laboratory. In this study, the specific activity of [^{14}C]diclofop-methyl was reduced to 0.28 mCi/mmol by addition of unlabeled material to the radioactive substrate. Syntheses of the authentic standards that were used for identification of the unknown isomeric hydroxylated metabolites of diclofop-methyl are reported in the preceding paper in this issue. (Tanaka et al., 1990).

Plant Treatment. Wheat (*Triticum aestivum* L.) was seeded in vermiculite in perforated metal trays, and the seeds were subirrigated with one-third strength Hoagland's nutrient solution (Blankendaal et al., 1972). The seedlings were allowed to grow for 11 days in the greenhouse before harvest. Shoots at the 1.5- to 2-leaf stage were immersed in water and excised at the vermiculite level. The excised shoots were placed into 20-mL vials containing 15 mL of aqueous 10 μM [^{14}C]diclofop-methyl (0.28 mCi/mmol) in 1% acetone. Fifty vials were prepared with approximately 20 shoots/vial. The shoots were incubated for 24 h in a controlled environmental chamber at 20 °C, 40% relative humidity, and continuous light of 14-klx intensity. Following this incubation period, the shoots were rinsed thoroughly with distilled water. Fresh weight of the upper shoots (portion of shoots extending above the treating solution) was 89.7 g, and fresh weight of the lower shoots (portion of shoots immersed in the treating solution) was 76.7 g. The two fractions of treated shoot material were frozen for subsequent extraction procedures.

Plant Extraction. The frozen lower shoot material was homogenized in a blender with 80% methanol (5 mL/g fresh weight). The homogenate was filtered, and the residue was extracted two additional times. The extracts were combined, and the methanol was removed by rotary vacuum evporation. During concentration of the methanol extract, chlorophyll and other nonpolar, natural products precipitated from solution to form a tacky insoluble mass. The remaining aqueous liquid was decanted from the precipitate, and the precipitate was washed with additional portions of water. The glucoside conjugates of the three isomers of ring-hydroxylated diclofop (HO-diclofop) were in the aqueous fraction.

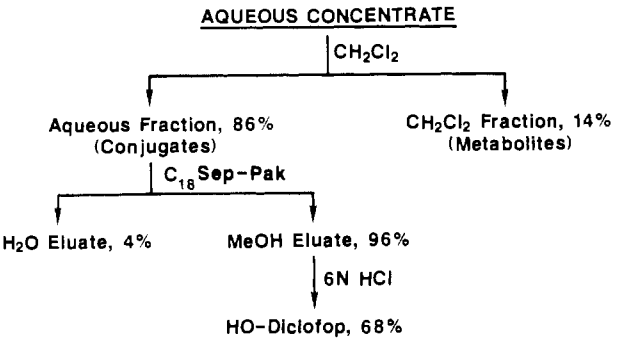

Figure 1. Flow scheme for isolation of the hydroxylated metabolites of diclofop-methyl.

Analysis of the distribution of radioactivity after plant treatment showed the posttreating solution to contain 15.8%, the insoluble residue to contain 3.8%, and the lower-shoot extract to contain 79.8% of the radioactivity that was initially introduced as [^{14}C]diclofop-methyl. Only 0.6% of the radioactivity was translocated to the upper shoots; therefore, the upper-shoot fraction was not extracted.

Isolation of Hydroxylated Metabolites. The aqueous fraction obtained from concentration of the methanolic plant extract was further purified by the scheme given in Figure 1. Extraction with dichloromethane afforded separation of the glucoside conjugates from the other metabolites (diclofop-methyl, diclofop, hydroxylated diclofop). The aqeous phase containing the conjugated metabolites was passed through a reversed-phase C_{18} Sep-Pak (Waters Associates). The adsorbed glucoside conjugates were eluted from the Sep-Pak with methanol. The methanol solution of the conjugates was taken to approximate dryness, and the residue was treated with 6 N HCl at 70 °C for 16 h under a nitrogen atmosphere. After hydrolysis, the aqueous phase was taken to dryness in vacuo to remove the HCl and the partially purified mixture of isomeric HO-diclofop was dissolved in methanol for final purification by high-performance liquid chromatography (HPLC).

The percent distribution of radioactivity after each purification step is shown in Figure 1. Thin-layer chromatography (TLC) of the conjugated metabolites from the 80% methanol extract in toluene–acetic acid (50:8, v/v) indicated that 72% of the radioactivity was conjugated material (R_f 0.14). After acid hydrolysis, TLC (toluene–acetic acid) indicated that 68% of the ^{14}C was ring-hydroxylated diclofop (R_f 0.35). These results showed that about 42% of the original diclofop-methyl from the treating solution was converted into HO-diclofop metabolites. The recovered mixture of HO-diclofop metabolites had a total activity of approximately 10^6 dpm and a specific activity of 1600 dpm/μmol. Therefore, the total weight of the mixture of the three hydroxylated metabolites was about 625 μg.

HPLC Purification. Chromatography was conducted on a Beckman HPLC system that was previously described (Tanaka et al., 1985). Separations were accomplished at a flow rate of 0.5 mL/min on an Altex Ultrasphere ODS-C_{18} column (4.6 mm (i.d.) × 15 cm) containing 5-μm spherical particles. A two-reservior system was employed, with solvent A being composed of CH_3CN–HOAc–H_2O (10:1:89, v/v) and solvent B being 100% CH_3CN. HPLC was conducted in the isocratic mode with the eluting solvent consisting of 55% solvent A and 45% solvent B. The chromatographic separation achieved for the three isomeric hydroxylated metabolites of diclofop-methyl is shown in Figure 2. The retention times for hydroxylated metabolites as their methyl esters were 18.2, 19.5, and 27.1 min, respectively, for isomers I–III. The percentage distributions of metabolites I–III were determined to be 21, 55, and 24%, respectively. The percent distributions of the three isomers were quantitated by both ultraviolet light absorption (254 nm) and liquid scintillation counting of radioactivity, and both methods gave identical results.

Gas–Liquid Chromatography. GLC was conducted on a Hewlett-Packard Model 5790A chromatograph equipped with

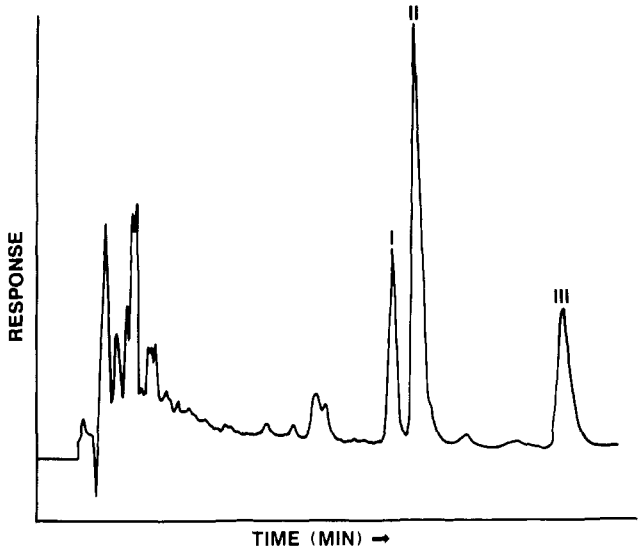

Figure 2. HPLC separation of the three isomeric hydroxylated metabolites of diclofop-methyl.

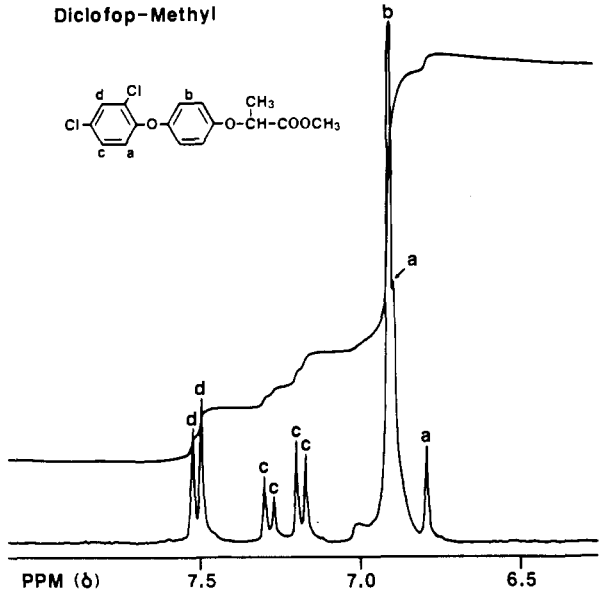

Figure 3. NMR spectrum of the aromatic region of diclofop-methyl.

a flame ionization detector. Separations were accomplished on a 0.22 mm (i.d.) × 9.5 m Ultra Performance capillary column (HP 19091A) coated with a 0.33-μm film of cross-linked methyl silicone. The column temperature was programmed with an initial 2-min hold at 80 °C followed by a temperature increase at a rate of 10 °C/min to a final temperature of 275 °C. Helium carrier gas was maintained at a flow rate of 1.5 mL/min. For GLC analysis, both the hydroxyl and carboxyl groups of HO-diclofop were methylated with diazomethane. The retention times of methylated isomers I–III were 18.3, 17.7, and 17.6 min, respectively.

Gibbs Reagent Test. The Gibbs reagent (2,6-dichloroquinone 4-chloroimine) reacts with phenols to form indophenols that afford an intense blue color upon exposure to ammonia vapors (Feigl, 1966). A solution of Gibbs reagent was prepared at 0.1% concentration in ethanol. Test samples of metabolites I–III were spotted onto filter paper, and each sample was overspotted with Gibbs reagent. After the reaction was allowed to take place, the material on the filter paper was exposed to ammonia vapors. Metabolite I showed a pale blue spot (positive result); II showed a very intense blue color (positive result); II showed a very intense blue color (positive result); and III produced a very pale blue ring on the edge of the test spot (negative result).

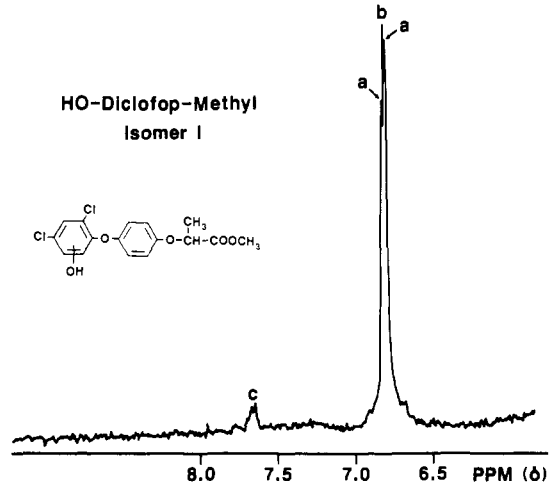

Figure 4. NMR spectrum of the aromatic region of unknown isomer I.

Mass Spectrometry. MS analysis was performed with a Varian MAT CH-5DF spectrometer, and sample ionization was accomplished by electron impact (70 eV). Derivatization of the isolated HO-diclofop with diazomethane afford the methyl ether of HO-diclofop-methyl. The methylated derivatives of metabolites I–III all gave the same mass spectral fragmentation pattern [m/z (relative intensity)]: 370 (M^+, 67), 311 (33), 283 (100), 191 (14), 120 (35). The chlorine isotopic ratio indicated the presence of two chlorines (McLafferty, 1967).

Nuclear Magnetic Resonance Spectrometry. The NMR spectra (90 MHz) were obtained on a JEOL FX-90Q Fourier transform spectrometer. The hydroxylated metabolite spectra were taken in 1.8-mm-o.d. capillary tubes held in the standard JEOL microprobe. Metabolites were dissolved in about 30 μL of methanol-d_4 (99.6%), and tetramethylsilane was used as the internal reference.

Diclofop-methyl standard: ^{1}H NMR (MeOH-d_4) δ 1.56 (d, J = 7.0 Hz, 3 H, CH_3), 3.74 (s, 3 H, COOCH_3), 4.77 (q, J = 7.0 Hz, H, CH), 6.85 (d, J = 9.2 Hz, H, ArH-6), 6.91 (s, 4 H, OArO), 7.23 (dd, J = 9.2, 2.6 Hz, H, ArH-5), 7.50 (d, J = 2.6 Hz, H, ArH-3). Figure 3 shows the aromatic region of this spectrum.

Metabolite I: ^{1}H NMR (MeOH-d_4) δ 1.53 (d, J = 7.0 Hz, 3 H, CH_3), 3.70 (s, 3 H, COOCH_3), 6.82 (d, J = 2.2 Hz, 4 H, -OArO-), 6.82 (s, 2 H, ArH-5,6). The CH quartet of the propionic acid moiety was under the OH peak of the MeOH-d_4 solvent peak at δ 4.86. Decoupling by irradiation at δ 4.86 caused the CH_3 doublet (δ 1.55) that was spin-coupled with the CH group to collapse into a singlet. Figure 4 shows the aromatic region of this spectrum.

Metabolite II: ^{1}H NMR (MeOH-d_4) δ 1.55 (d, J = 7.0 Hz, 3 H, CH_3), 3.74 (s, 3 H, COOCH_3), 6.84 (s, 4 H, OArO), 6.96 (s, H, ArH-6), 7.01 (s, H, ArH-5). The CH quartet of propionic acid was under the OH peak of the solvent. Figure 5 shows the aromatic region of this spectrum.

Metabolite III: ^{1}H NMR (MeOH-d_4) δ 1.56 (d, J = 7.0 Hz, 3 H, CH_3), 3.71 (s, 3 H, COOCH_3), 6.42 (s, H, ArH-6), 6.95 (s, 4 H, OArO), 7.39 (s, H, ArH-3). The CH quartet of propionic acid was under the OH peak of the solvent. Figure 6 shows the aromatic region of this spectrum.

RESULTS AND DISCUSSION

In this study, the isomeric HO-diclofop acids were isolated from excised wheat shoots as their glucoside conjugates. The conjugates were hydrolyzed with HCl to yield the free HO-diclofop acids, which were then taken to dryness and subsequently dissolved in methanol. Upon being allowed to stand at room temperature in methanol, however, these metabolites were readily esterified to afford the isomers of HO-diclofop-methyl rather than the expected methanol solution of the HO-diclofop acids. At the time the HPLC separations were accomplished, we were unaware that diclofop acid had previously been shown to undergo esterification upon being allowed to

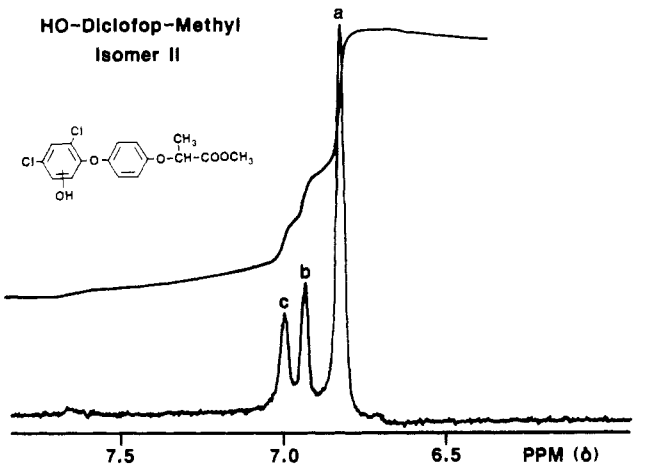

Figure 5. NMR spectrum of the aromatic region of unknown isomer II.

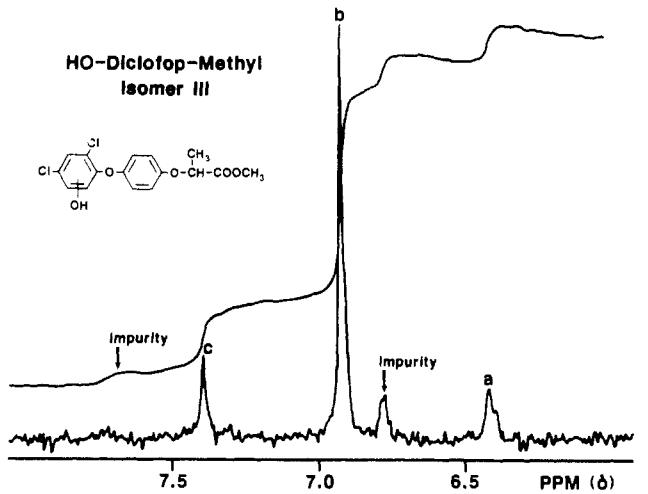

Figure 6. NMR spectrum of the aromatic region of unknown isomer III.

stand in methanol at room temperature to yield diclofop-methyl (Smith, 1976). However, mass spectrometry of the three isomers isolated by HPLC afforded a molecular ion at m/z 356 to verify that methylation of the carboxyl group had occurred. Therefore, Figure 2 is the HPLC separation of the isomers of HO-diclofop-methyl rather than HO-diclofop acid.

A portion of the hydroxylated metabolites was methylated with diazomethane to afford derivatives where both the hydroxyl and carboxyl groups of HO-diclofop acid were methylated. GLC analysis of this material gave an elution pattern that was the same as that reported by Gorbach et al. (1977). As expected, the order of elution by GLC was the reverse of that observed by HPLC; thus, isomer III was eluted first, and isomer I was eluted last from the methyl silicone column. The GLC data showed that our hydroxylated metabolites were the same as those isolated by Gorbach et al. (1977).

The Gibbs reagent reacts with test compounds at a free position para to a hydroxyl group on an aromatic ring to give an intense blue color for a positive test. About 0.3–2 μg of material is required for this test. When the Gibbs test was conducted on HPLC-purified fractions of the three isomeric metabolites, two positive tests and one negative test were obtained. The test results were in agreement with the assumption that hydroxylation was occurring at the 3-, 5-, and 6-positions on the chlorinated ring. However, some functional groups such as alkoxy, sulfate, or halogen can be displaced from the aromatic ring during reaction to give a color response (Feigl, 1966). The negative test showed a pale blue ring on the outer edge of the test spot. This result was apparently due to slow displacement of chlorine from the aromatic ring.

At the time the Gibbs tests were conducted, we were unaware that phenoxy groups could easily be displaced from an aromatic ring to give a positive test (Josephy and Van Damme, 1984). Therefore, the two positive test results led to the assumption that two of the metabolites had free positions para to the hydroxyl group. Also, the initial interpretation of the NMR data suggested that two of the unknowns might be the 3- and 6-hydroxylated metabolites. The rationale for these interpretations is given below. In the NMR spectrum of isomer I (Figure 4), a decomposition impurity (c) gave an integral equal to one proton and a coupling constant of about 2 Hz to make it appear that meta coupling was taking place. Hence, an observation of meta coupling would indicate the unknown to be the 6-OH metabolite. Isomer II gave two uncoupled peaks for the two protons on the chlorinated ring (Figure 5). If the two singlets (b and c) of the unknown were considered to be the two inner peaks of an AB case, the unknown would appear to be the 3-OH metabolite with strong ortho coupling. This interpretation appeared reasonable because AB cases involving one chemical shift and a large coupling constant can afford spectra where just the inner two peaks are observed (Wiberg and Nist, 1962). Therefore, on the basis of only their Gibbs test and NMR spectrum, the identity of the unknown metabolites appeared to be the 3-, 5-, and 6-hydroxylated isomers (Tanaka et al., 1986). Since our preliminary results showed that the 5-OH isomer was not the most abundant metabolite and the data were not in agreement with that reported by Gorbach et al. (1977), additional studies were necessary to verify our experimental data before publication. In order to obtain conclusive identification of the hydroxylated metabolites, authentic standards of all possible metabolites were synthesized and their NMR spectra were taken for comparison with spectra of the unknown metabolites (Tanaka et al., 1990). From these syntheses, authentic standards of the 3- and 6-hydroxylated isomers were prepared, and their NMR spectra clearly showed that the unknown metabolites were not the 3- and 6-OH isomers.

The NMR spectrum of the aromatic region of diclofop-methyl is shown in Figure 3. This spectrum was taken to obtain the chemical shifts and coupling constants of the diphenyl ether moiety prior to hydroxylation. In this spectrum, the four protons (b) of the nonchlorinated ring are all equivalent and yield a singlet while the three protons on the chlorinated ring have different chemical shifts. Proton a is a doublet due to ortho coupling with c. Proton c is a doublet of doublets due to ortho coupling with a and meta coupling with d. Proton d is a doublet due to meta coupling with c. An observed ortho coupling constant of 9.2 Hz and a meta coupling constant of 2.6 Hz are of the expected order of magnitude (Bible, 1965).

Identification of Isomer I. The NMR spectrum of the aromatic region of unknown isomer I is given in Figure 4. The two protons (a) of the chlorinated ring appear to have the same chemical shift and are observed as a singlet. The four protons of the nonchlorinated ring are an AB pattern where the four protons have approximately the same chemical shift (Zanger, 1972). Therefore, the two inner peaks of doublets (b) are large while the outer peaks are observed as rounded shoulders at the base of the inner peaks. Thus, the inner peaks are greatly amplified at the expense of the outer peaks and approach

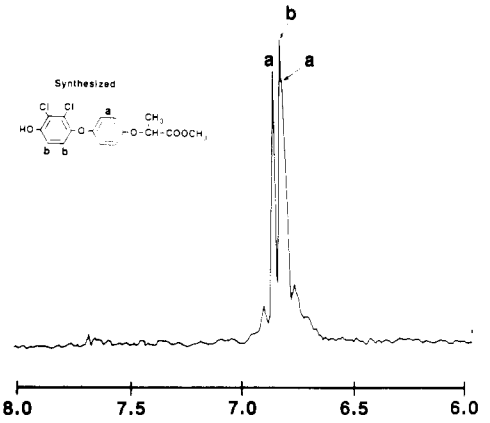

Figure 7. NMR spectrum of the aromatic region of the synthesized standard of the 2,3-dichloro-4-hydroxy isomer.

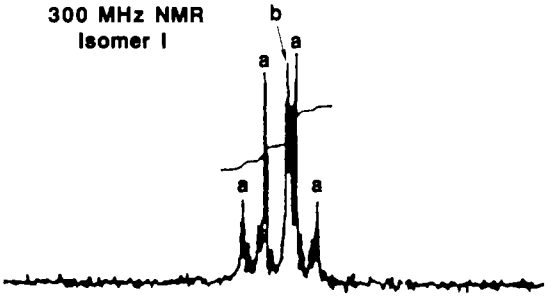

Figure 8. Aromatic region of the 300-MHz spectrum of unknown isomer I.

each other closely. Peak c is a decomposition product of isomer I.

When the NMR spectrum of the synthesized standard was obtained (Figure 7), considerable overlap of the protons of the chlorinated and nonchlorinated ring was also observed. Although the spectra of the authentic standard and unknown were similar and their HPLC retention times were the same, more evidence was needed to clearly establish the identity of isomer I. A 300-MHz spectrum had been obtained earlier of unknown isomer I at the USDA Regional Laboratory (Peoria, IL). This spectrum showed that considerable decomposition of the metabolite had occurred during transportation of the sample; however, the aromatic region still gave a clearly resolved spectrum of unknown isomer I (Figure 8). The peaks marked a show a standard para substitution pattern for the nonchlorinated ring. The integral shows that the two protons of the chlorinated ring are among the four protons of the para pattern. Therefore, peak b is a singlet accounting for the two protons on the chlorinated ring. Upon comparison with the synthetic standard shown in Figure 7, the spectra of the aromatic region of unknown isomer I given in Figures 4 and 8 verify that isomer I is methyl 2-[4-(2,3-dichloro-4-hydroxyphenoxy)phenoxy]propanoate and this metabolite is formed via the NIH shift reaction.

Identification of Isomer II. The NMR spectrum of the aromatic region of unknown isomer II is shown in Figure 5. The four protons of the nonchlorinated aromatic ring are equivalent and are observed as a singlet (a). Peaks b and c show integrals equivalent to one proton each, and they represent the two protons on the chlorinated ring. The spectrum of an authentic standard of the 2,5-dichloro-4-hydroxy isomer of HO-diclofop-methyl is shown in Figure 9. This spectrum is identical with that of unknown isomer II. Furthermore, the unknown and standard had identical HPLC retention

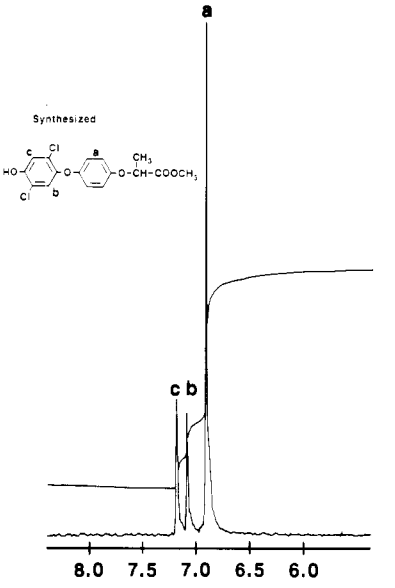

Figure 9. NMR spectrum of the aromatic region of the synthesized standard of the 2,5-dichloro-4-hydroxy isomer.

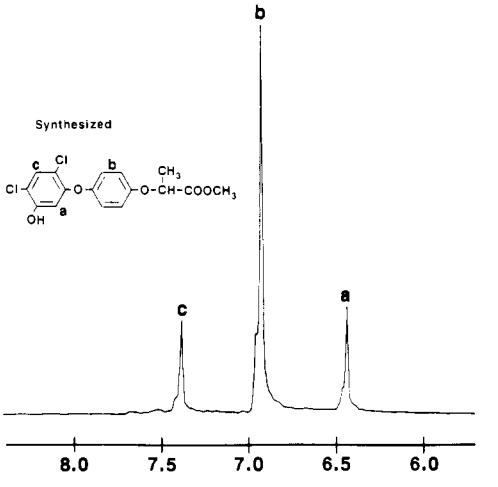

Figure 10. NMR spectrum of the aromatic region of the synthesized standard of the 2,4-dichloro-5-hydroxy isomer.

times. Therefore, isomer II is positively identified as being methyl 2-[4-(2,5-dichloro-4-hydroxyphenoxy)phenoxy]propanoate, which is the most abundant isomer produced by metabolic hydroxylation of diclofop-methyl. This metabolite is also formed by the NIH shift reaction.

Identification of Isomer III. The NMR spectrum of the aromatic region of unknown isomer III is shown in Figure 6. The four protons of the nonchlorinated ring are all equivalent and are observed as a singlet (b). Protons a and c on the chlorinated ring are observed as singlets with no evidence of para coupling. Impurity peaks can be seen at δ 6.77 and 7.68. Further purification of isomer III by HPLC removed the shoulder on peak a and caused the relative intensity of the impurity peak at δ 6.77 to decrease by half while the peak at δ 7.68 became much larger. Both peaks a and c became sharp singlets after repurification. The NMR spectrum of the aromatic region of the 5-OH synthetic standard is shown in Figure 10. The spectra shown in Figures 6 and 10 are identical, and both the standard and unknown exhibited identical HPLC retention times. Therefore, unknown isomer III is unequivocally identified as methyl 2-[4-(2,4-dichloro-5-hydroxyphenoxy)phenoxy]propanoate. As additional evidence, a synthetic standard of 5-OH diclofop acid was provided by Hoechst Chemical Co. (Sommer-

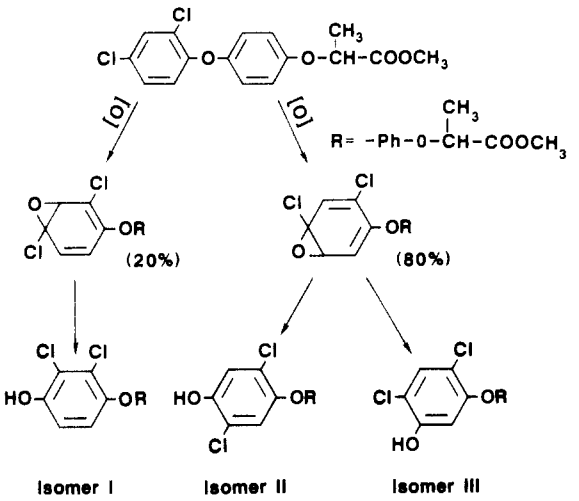

Figure 11. Scheme for arene oxide involvement in the NIH shift mechanism for formation of the hydroxylated metabolites.

ville, NJ) and the NMR spectrum of this compound was identical with our synthetic standard of 5-OH diclofop acid.

Previously, Gorbach and co-workers (1977) reported the 5-hydroxylated isomer as being the most abundant hydroxylated metabolite, and they further speculated the identity of the remaining hydroxylated metabolites as being the 3-OH and 6-OH isomers. Unfortunately, this work was later cited in a literature review by Menzie (1980), which stated that the 3-OH and 6-OH isomers were actually identified by GC–MS. Our results show unequivocally that the 5-OH isomer is not the most abundant hydroxylated metabolite and the remaining hydroxylated metabolites are not the 3-OH or 6-OH isomers but rather products formed via the NIH shift.

Two of the unknown metabolites (isomers I and II) were formed via the NIH shift reaction (Guroff et al., 1967; Daly et al., 1968). In the NIH shift mechanism, arene oxide intermediates are involved, which can spontaneously isomerize to a phenol or undergo enzymatic hydration to a *trans*-dihydrodiol (Daly et al., 1972). Therefore, in the oxidative metabolism of diclofop-methyl in tolerant plant species, arene oxide intermediates are formed as shown in Figure 11. The 3,4-arene oxide represents about 20% of the intermediates, and the 4,5-arene oxide represents about 80%. The 3,4-arene oxide appears to open in only one direction to yield isomer I, and this apparently occurs because of steric and electronic effects provided by both chlorine groups. On the other hand, the 4,5-arene oxide intermediate opens in both directions to yield isomers II and III. Since the yield of isomer II is twice that of isomer III, epoxide ring opening with concomitant NIH shift is the preferred direction of reaction for the 4,5-arene oxide.

Bristol and co-workers (1977) investigated the metabolism of 2,4-D in wheat cell suspension cultures. From this study, metabolites formed via the NIH shift were isolated as their sugar conjugates. After hydrolysis, the metabolites were identified as 4-HO-2,5-D, 4-HO-2,3-D, and 4-hydroxy-2-chlorophenoxyacetic acid. Additional 2,4-D metabolism studies were cited in the pesticide metabolism review by Menzie (1980) where species of plants other than wheat demonstrated the NIH shift. Since diclofop-methyl has a 2,4-dichloro ring substitution pattern, the identification of NIH shift metabolites from diclofop-methyl would not be entirely unexpected.

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LITERATURE CITED

Blankendaal, M.; Hodgson, R. H.; Davis, D. G.; Hoerauf, R. A.; Shimabukuro, R. H. *Growing Plants Without Soil for Experimental Use*; USDA/ARS Miscellaneous Publication No. 1251; U.S. Government Printing Office: Washington DC, 1972; pp 1–17.

Bible, R. H., Jr. *Interpretation of NMR Spectra*; Plenum: New York, 1965; p 39.

Bristol, D. W.; Ghanuni, A. M.; Oleson, A. E. Metabolism of 2,4-Dichlorophenoxyacetic Acid by Wheat Cell Suspension Cultures. *J. Agric. Food Chem.* **1977**, *25*, 1013–1015.

Daly, J.; Guroff, G.; Jerina, D.; Udenfriend, S.; Witkop, B. Intramolecular Migrations during Hydroxylation of Aromatic Compounds - The NIH Shift. In *Oxidation of Organic Compounds - III*; Gould, R. F., Ed.; Advances in Chemistry 77; American Chemical Society: Washington, DC, 1968; pp 279–289.

Daly, J. W.; Jerina, D. M.; Witkop, B. Arene Oxides and the NIH Shift: The Metabolism, Toxicity and Carcinogenicity of Aromatic Compounds. *Experientia* **1972**, *28*, 1129–1264.

Feigl, F. *Spot Tests in Organic Analysis*, 7th ed.; Elsevier: New York, 1966; pp 185, 279.

Friesen, H. A.; O'Sullivan, P. A.; VandenBorn, W. H. HOE 23408, a New Selective Herbicide for Wild Oats and Green Foxtail in Wheat and Barley. *Can. J. Plant Sci.* **1976**, *56*, 567–578.

Gorbach, S. G.; Kuenzler, K.; Asshauer, J. On the Metabolism of Hoe 23408 OH in Wheat. *J. Agric. Food Chem.* **1977**, *25*, 507–511.

Gorecka, K.; Shimabukuro, R. H.; Walsh, W. C. Aryl Hydroxylation: A Selective Mechanism for the Herbicides, Diclofop-Methyl and Clofop-Isobutyl, in Gramineous Species. *Physiol. Plant.* **1981**, *53*, 55–63.

Guroff, G.; Daly, J.; Jerina, D.; Renson, J.; Witkop, B. Hydroxylation-Induced Migration: The NIH shift. *Science* **1967**, *157*, 1524–1530.

Hoechst AG. Deutsche Patentoffenlengungsschrift No. 2223894, 1973.

Jacobson, A.; Shimabukuro, R. H. Metabolism of Diclofop-Methyl in Root-Treated Wheat and Oat Seedlings. *J. Agric. Food Chem.* **1984**, *32*, 742–746.

Josephy, P. D.; Van Damme, A. Reaction of Gibbs Reagent with Para-Substituted Phenols. *Anal. Chem.* **1984**, *32*, 813–814.

McLafferty, F. W. *Interpretation of Mass Spectra*; Benjamin: New York, 1967; p 22.

Menzie, C. M. *Metabolism of Pesticides Update III*; U.S. Government Printing Office: Washington, DC, 1980.

Miller, S. D.; Nalewaja, J. D. HOE 23408 for Post-Emergence Wild Oat and Foxtail Control. *Proc. North Cent. Weed Control Conf.* **1974**, *29*, 38–39.

Shimabukuro, M. A.; Shimabukuro, R. H.; Nord, W. S.; Hoerauf, R. A. Physiological Effects of Methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate on Oat, Wild Oat, and Wheat. *Pestic. Biochem. Physiol.* **1978**, *8*, 199–207.

Shimabukuro, R. H.; Walsh, W. C.; Hoerauf, R. A. Metabolism and Selectivity of Diclofop-Methyl in Wild Oat and Wheat. *J. Agric. Food Chem.* **1979**, *27*, 615–623.

Smith, A. E. Esterification of the Hydrolysis Product of the Herbicide Dichlofop-Methyl in Methanol. *J. Agric. Food Chem.* **1976**, *24*, 1077–1078.

Tanaka, F. S.; Hoffer, B. L.; Wien, R. G. Detection of Halogenated Biphenyls from Sunlight Photolysis of Chlorinated Herbicides in Aqueous Solution. *Pestic. Sci.* **1985**, *16*, 265–270.

Tanaka, F. S.; Hoffer, B. L.; Shimabukuro, R. H.; Walsh, W. C. Identification of Ring Hydroxylated Metabolites of Diclofop-Methyl in Wheat. *Abstract of Papers*, The Sixth International Congress of Pesticide Chemistry, Ottawa, Canada; IUPAC: Ottawa, 1986; 7A-03.

Tanaka, F. S.; Wien, R. G.; Hoffer, B. L.; Shimabukuro, R. H. Application of NMR Spectrometry in the Identification of

the Isomeric Hydroxylated Metabolites of Methyl 2-(4-[2,4-Dichlorophenyoxy]phenoxy)propanoate (Diclofop-Methyl) in Wheat Seedlings. *Abstracts of Papers*, 3rd Chemical Congress of North America and 195th National Meeting of the American Chemical Society, Toronto, Canada; American Chemical Society: Washington, DC, 1988; AGRO 166.
Tanaka, F. S.; Wien, R. G.; Hoffer, B. L. Synthesis of Possible Ring-Hydroxylated Metabolites of Diclofop-Methyl. *J. Agric. Food Chem.* **1990**, previous paper in this issue.
Wiberg, K. B.; Nist, B. J. *The Interpretation of NMR Spectra*; Benjamin: New York, 1962; pp 3–9.
Zanger, M. The Determination of Aromatic Substitution Patterns by Nuclear Magnetic Resonance. *Org. Magn. Reson.* **1972**, *4*, 1–25.

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Effect of Nitrogen Fertilization on Glycoalkaloid and Nitrate Content of Potatoes

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The effect of nitrogen fertilization on the total glycoalkaloid (TGA) and nitrate content of potato tubers was investigated on six cultivars in each of two consecutive years. Ammonium nitrate was applied to the soil at rates of 56, 168, and 224 kg/ha during the first year and 112, 168, and 224 kg/ha during the second year of the study. Significant increases in both TGA and nitrate nitrogen occurred in all six cultivars with nitrogen fertilization. Nitrogen fertilization at the rate of 224 kg/ha resulted in tubers containing the highest levels of both TGA and nitrate nitrogen.

Nitrogen is a major element required for plant growth, and large applications of this element are used to obtain maximum yield of tubers. Nitrogen fertilization promotes active growth and delays dry matter accumulation (Painter and Augustin, 1976). Some workers have reported an increase in dry matter with increased nitrogen levels (Eppendorfer et al., 1979; Talley, 1983), while some have reported no effect (Hartman, 1982; Kunkel, 1968). Generally, increasing the nitrogen fertilization resulted in an increase in the total yield (Dubetz and Bole, 1975; Talley, 1983), but in many cases a large percentage of malformed tubers resulted (Murphy and Govern, 1975). High levels of nitrogen generally tend to increase the total nitrogen content (Eppendorfer et al., 1979).

Glycoalkaloids are known to possess anticholinesterase activity (Orgell et al., 1958) and, hence, are potential toxicants. The glycoalkaloid molecule consists of a sugar moiety and an alkaloid, the aglycon. In the potato, α-solanine and α-chaconine are the chief glycoalkaloids. Researchers have disagreed as to the effect of nitrogen fertilization on glycoalkaloid synthesis in potatoes. Nowacki et al. (1975) found a decrease in the glycoalkaloid content with increasing nitrogen levels of fertilization, while Cronk et al. (1974) found an increase. However, Cronk et al. (1974) found that tuber response to nitrogen fertilization was also dependent on variety.

Nitrates are the precursors of nitrites, which oxidize ferrous hemoglobin to ferric hemoglobin, subsequently inhibiting oxygen transportation through the body and causing methemoglobinemia (Hartman, 1982). Infants are particularly susceptible to methemoglobinemia. Ingested nitrate can be reduced to nitrite, which reacts with secondary or tertiary amines to form carcinogenic and mutagenic *N*-nitroso compounds (Walters et al., 1979). Nitrosation reactions have been linked to cancers of the esophagus, stomach, large intestine, and bladder. According to White (1975), potatoes contribute approximately 14% of the per capita ingestion of nitrates in the United States. Increasing the rate of nitrogen fertilization has been shown to increase the nitrate content of both potato tubers (Ponnampalam and Mondy, 1985) and beets (Peck et al., 1971).

The objective of this study was to investigate the effect of nitrogen fertilization on both the glycoalkaloid and the nitrate nitrogen contents of potatoes.

MATERIALS AND METHODS

In the first year Katahdin, Chipbelle, and Rosa cultivars were used, and in the second year Russet Burbank, Lemhi Russet, and Shepody cultivars were studied. The potatoes were grown at the Cornell Vegetable Research Farm in Freeville, NY. Nitrogen, in the form of ammonium nitrate, was banded to the soil at rates of 50 (56), 150 (168), and 200 (224) lb/acre (kg/ha) during the first year and 100 (112), 150 (168), and 200 (224) lb/acre (kg/ha) during the second year of the study. The lowest rate applied during the first year was too low for normal growth so the level was increased during the second year of the study. Three replicates of each treatment were made. Different plots were used during each year to prevent any carryover of nitrogen from one year to the next. The tubers were mechanically harvested 18 weeks after planting and stored at 5 °C and

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