during microbial metabolism of PBAl in soil. As organic acids, both PBAc and DCVA could be expected to be somewhat mobile through soil. The extent of their mobility, however, is determined by the soil pH, as well as by the rate of their degradation in soil. Both compounds are degraded in soil (Kaneko et al., 1978; Roberts and Standen, 1977a,b), and neither can be considered persistent. Only trace amounts of PBAc were eluted from soils which had been incubated for several weeks prior to leaching (Kaneko et al., 1978). Based on the results obtained with the few compounds examined in this investigation, it seems that soil TLC and soil columns provide a comparable measure of the mobility of chemicals in soil. Due to its ease in handling, processing, reproducibility, ease of analysis, and limited space requirements, soil TLC should be the method of preference.

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Metabolism of *N*-Nitrosopendimethalin and *N*-Nitropendimethalin by a *Streptomyces* Isolated from Soil

William R. Lusby,* James E. Oliver, Richard H. Smith, Jr.,[1] Philip C. Kearney, and Harold Finegold

[^{14}C]-*N*-Nitrosopendimethalin (**1**) [*N*-(1-ethylpropyl)-*N*-nitroso-3,4-dimethyl-2,6-dinitrobenzenamine], a contaminant of the dinitroaniline herbicide pendimethalin, was metabolized to several products by a *Streptomyces* sp. isolated from soil. Identifications were made by chemical ionization and electron impact mass spectrometry, chromatographic and spectral comparisons, and synthesis. Reduction of a nitro group was characteristic of all metabolites; hydroxylation of ring methyl groups was also present in some metabolites. Metabolism of [^{14}C]-*N*-nitropendimethalin (**2**) [*N*-(1-ethylpropyl)-3,4-dimethyl-*N*-2,6-trinitrobenzenamine] yielded two hydroxymethyl metabolites.

Alkyl nitrosamines and nitrosated herbicides have been detected in certain herbicide formulations. These disclosures have prompted research on the environmental fate and possible human exposure to these nitrosamines. *N*-Nitrosopendimethalin (**1**; Figure 1) (Bontoyan et al., 1978, 1979) and *N*-nitropendimethalin (**2**) (Bontoyan, 1979) were detected as contaminants of the dinitroaniline herbicide pendimethalin (**3**) [*N*-(1-ethylpropyl)-3-4-dimethyl-2,6-dinitrobenzenamine]. Radiolabeled **1** was included in a study of aerobic soil degradation of herbicide-related nitrosamines (Oliver et al., 1979); in one experiment, 50% of the radioactivity was recovered as intact **1** after 4 months. Thin-layer chromatography of the soil extract revealed a few additional bands; however, the amounts of material were too small for isolation and identification. Both **1** and **2** were found to degrade more rapidly than pendimethalin, **3**, in flooded anaerobic soil (Smith et al., 1979). This paper describes the isolation and identification of metabolites from the degradation of **1** and **2** by an aerobic soil microorganism.

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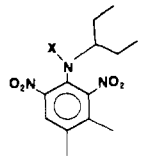
MATERIALS AND METHODS

Reagents. All solvents were glass distilled and used without further purification except for diethyl ether which was redistilled just prior to use. Pendimethalin-*1-ethyl*-^{14}C (0.043 μCi/mg) was supplied by American Cyanamid, Agricultural Division, Princeton, NJ. The labeled material was >99% pure by thin-layer chromatography (TLC) and autoradiography. We are also indebted to American Cyanamid for supplying samples of 3-[(1-ethylpropyl)-amino]-6-methyl-2,4-dinitrobenzenemethanol (**17**) and 4-[(1-ethylpropyl)amino]-2-methyl-3,5-dinitrobenzenemethanol (**13**). Syntheses of **1** (Oliver et al., 1979) and **2** and N^2-(1-ethylpropyl)-4,5-dimethyl-3-nitro-1,2-benzenediamine (**5**) (Smith et al., 1979) have been described.



1 x = NO
2 x = NO_2
3 x = H

Figure 1. Structures of pendimethalin (3) and contaminants (1 and 2).

Thin-layer chromatography was performed on E. Merck 0.25 mm silica gel 60 F-254 plates. TLC solvent systems employed were as follows: (A) toluene; (B) benzene–ethyl acetate (1:1); (C) toluene–ethyl acetate–acetic acid (60:40:1); (D) benzene–ethyl acetate (3:1).

Soil Microbiology. The isolation of the *Streptomyces* used for the metabolism of the starting material has been previously described (Lusby et al., 1980). Into a 2.8-L Fernbach flask was placed 1 g of yeast extract and 1 L of mineral salt solution containing 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5 g of $(NH_4)_2SO_4$, and 0.1 g of $CaSO_4$. After the mixture was autoclaved and while it was still warm, 2.88 mg (0.124 μCi) of 1 in ~1mL of ethanol was added, and the flask was inoculated with actively growing cells. Ten flasks were agitated at 85–100 rpm for 14 days at room temperature in subdued light. No products were detected in uninoculated controls. An increase in turbidity was observed in inoculated flasks, but no quantitative measurements of growth were taken.

During the active growth phase metabolism of 1, 10-mL aliquots of the culture were withdrawn after 1, 2, 4, 8, and 14 days. The aliquots were extracted with ethyl acetate, and the ethyl acetate was concentrated and spotted onto two TLC plates. The plates were eluted with solvents A and B, respectively, and exposed to X-ray film (Kodak Type NS-5T) for 2 weeks.

Isolation. After fourteen days the pooled culture was filtered through a pad of Celite, and 1500-mL portions of the filtrate were extracted with ethyl acetate (3 × 200 mL). The Celite pad was extracted with methanol (800 mL) by stirring for 3 h. The methanol extract, the combined ethyl acetate extracts, and the aqueous phase were made up to specific volumes and aliquots counted. The ethyl acetate and methanol extracts were combined, further concentrated, and evaporated onto a few cubic centimeters of silica gel which was placed on top of a 90 × 2 cm i.d. silica gel (Silica Woelm TLC) dry column. The column was eluted with benzene and then cut into 6-cm fractions, except for bands 1 and 2 which were respectively the origin band and the first 2.5 cm beyond the origin. Each fraction was extracted with ethyl ether, and the ether solutions were concentrated to specific volumes and counted.

Those fractions which contained 4% or more of the recovered ^{14}C were further purified either by silica gel TLC using solvent system C, by high-pressure liquid chromatography (LC), or by a combination of both chromatographic methods. All bands or peaks were assayed for radioactivity, and purified radiolabeled materials were analyzed by both electron impact (EI) and chemical ionization (CI) mass spectrometric methods. When sufficient sample was available and additional confirmation needed, infrared spectroscopy and proton nuclear magnetic resonance spectroscopy were utilized.

Instrumentation. Radioactivity was measured on a Nuclear-Chicago Mark I liquid scintillation system. Liquiscint (Natural Diagnostics) was used as the scintillation cocktail. Samples for counting were evaporated under nitrogen before the addition of cocktail, and corrections were made for background. Low-resolution mass spectral data were obtained from a Du Pont 491-B mass spectrometer operated at a resolution of 600 and fitted with Du Pont 094 data system. Isobutane, ammonia, water, and deuterium oxide (Hunt et al., 1972; Hunt, 1973) were used as reagent gases for chemical ionization. The source block temperature was 135–150 °C. Infrared spectra were obtained from 1.5-mm KBr pellets by using a Perkin-Elmer Model 621 infrared spectrophotometer fitted with a beam condenser. High-pressure liquid chromatography was performed (1) on a Du Pont 830 liquid chromatograph with a Zorbax ODS column and methanol–water or methanol–water–acetic acid or (2) on a Waters Model 244/w liquid chromatograph with a Zorbax-CN column using isooctane–2-propanol.

Synthesis of Metabolites. *1-(1-Ethylpropyl)-5,6-dimethyl-7-nitrobenzotriazole* **(6).** A solution of **5** (100 mg) in acetic acid (5 mL) was treated with a solution of $NaNO_2$ (55 mg) in H_2O (~0.5 mL). After 1 h the solution was concentrated to near dryness, water was added, and the solid was collected and recrystallized from wet methanol to give **6** as small gold needles; mp 106–109: mass spectrum, electron impact, *m/e* 262 (100, $M^+\cdot$), 159 (95); mass spectrum, chemical ionization, isobutane, *m/e* 263 [100, $(M + H)^+$].

N^2-(1-Ethylpropyl)-4,5-dimethyl-3-nitro-N^1-[(pentafluorophenyl)methylene]-1,2-benzenediamine **(7).** A solution of diamine **5** (Smith et al., 1979; 200 mg) and pentafluorobenzaldehyde (220 mg) in benzene (2 mL) containing 0.1 μL of trifluoroacetic acid was allowed to stand at room temperature for 45 min. A little anhydrous $MgSO_4$ was added, the solution was filtered, and the filtrate was concentrated to ~1 mL. A small amount of hexane was added, and the resulting deep red solution was stored at 2 °C for 2 days to give a red-orange solid. Recrystallization from *i*-PrOH gave two crops of **7**, 108 mg, mp 127–128 °C, and 52 mg, mp 124–127 °C; mass spectrum, chemical ionization, isobutane, *m/e* 430 [100, $(M + H)^+$], 428 (50, $M + H^+ - H_2$).

N^2-(1-Ethylpropyl)-4,5-dimethyl-3-nitro-N^2-nitroso-N^1-[(pentafluorophenyl)methylene]-1,2-benzenediamine **(8).** A solution of **7** (95 mg) in CH_2Cl_2 (2 mL) containing ~200 mg of suspended NaOAc was cooled to −78 °C and treated with a slight excess of 1.7 M N_2O_4 in CH_2Cl_2. The orange color of **7** was quickly discharged. Ice and water were then added, the mixture was brought to 0 °C, and the layers were separated. The aqueous phase was reextracted with CH_2Cl_2, and the combined extracts were washed with water, dried ($MgSO_4$), and concentrated to give a solid that was recrystallized from hexane to give 66 mg of a white solid, mp 144.5–146 °C; mass spectrum, chemical ionization, isobutane, *m/e* 515 [25, $(M + C_4H_9)^+$], 459 [15, $(M + H)^+$], 429 [100, $(M + H - NO)^+$].

N^2-(1-Ethylpropyl)-4,5-dimethyl-3-nitro-N^2-nitroso-1,2-benzenediamine **(4).** A suspension of **8** (52 mg) in ethanol (1 mL), water (0.5 mL), and HOAc (0.5 mL) was stirred at 0 °C and treated with 2 drops of 6 N HCl. No apparent change occurred in 15 min, and the ice bath was removed and 1 mL of ethanol and 2 drops of 6 N HCl were added to the flask. Similar additions of ethanol and HCl were performed twice more during 4 h. The white solid gradually dissolved and a yellow solution developed which was finally partitioned between CH_2Cl_2 and aqueous $NaHCO_3$. The CH_2Cl_2 was washed with H_2O, dried, and concentrated to give a yellow oil that was triturated with

warm hexane and then allowed to stand at room temperature. A bright yellow solid (24 mg; mp 108–110 °C) was obtained that was recrystallized from heptane, mp 113 °C: mass spectrum, chemical ionization, isobutane, *m/e* 337 [12, $(M + C_4H_9)^+$], 281 [12, $(M + H)^+$], 251 [100, $(M + H - NO)^+$]; mass spectrum, electron impact, *m/e* 250 [100, $(M - NO)^+$].

Conversion of **4** *to* **6**. A solution of **4** (0.75 mg) in toluene (0.5 mL) containing a tiny crystal of *p*-toluenesulfonic acid monohydrate was refluxed 2 h, and then the product was isolated by preparative TLC (solvent A). The only significant band (R_f 0.63) was scraped and the silica gel was extracted with ether. Evaporation of ether gave benzotriazole **6** whose mass spectrum was identical with that of the material prepared by direct nitrosation of **5**.

1-(1-Ethylpropyl)-5,6-dimethyl-7-nitro-2-(pentafluorophenyl)-1H-benzimidazole (**9**). A solution of **7** (21 mg) in HOAC (1 mL) was stirred at room temperature and treated 3 mg of $NaNO_2$ in ~5 drops of H_2O. The orange-red color of **7** was immediately replaced by yellow, and a bright yellow solid separated and was collected and recrystallized from EtOH–H_2O: mp 176–179 °C; mass spectrum, chemical ionization, isobutane, *m/e* 428 [100, $(M + H)^+$]; mass spectrum, electron impact, *m/e* 427 (58, $M^{+\cdot}$), 340 (100, $M^{+\cdot} - C_5H_{11}O$); ^{1}H NMR ($CDCl_3$) δ 0.78 (6 H, t), 1.66 (4 H, m), 2.31 (3 H, s), 2.45 (3 H, s), 3.83 (1 H, 5 peaks, J = 8.5 Hz), 7.72 (1 H, s).

Alternate Preparation of **9**. A solution of diamine **5** (19 mg) in CH_2Cl_2 (1 mL) was treated with 25 μL of pentafluorobenzoyl chloride. The color faded from red to yellow within 1 min. After ~10 min, 5% K_2CO_3 was added; within 5 min the layers were separated, the organic phase was dried, filtered, and then concentrated, and the residue was recrystallized from hexane plus a little EtOAc to give bright yellow needles (16 mg) of the pentafluorobenzanilide **10**: mass spectrum, electron impact, *m/e* 445 (13, $M^{+\cdot}$), 416 (100, $M^{+\cdot} - C_2H_5$).

Conversion of **10** *to* **9**. A mixture of **10** (6 mg) and $POCl_3$ (0.3 mL) was refluxed 1 h, and then excess $POCl_3$ was evaporated and the residue was partitioned between CH_2Cl_2 and 5% K_2CO_3. Analysis of the CH_2Cl_2 solution by TLC (A, R_f 0.20 and 0.40) and by GLC (20 × $^1/_8$ in. UCW column, 210 °C, 4.4 and 6.9 min) showed an approximately 3:1 mixture of unreacted **10** and benzimidazole **9**. The mixture was then recombined with 1 mL of $POCl_3$; after the mixture was refluxed another hour, GLC showed the product to consist almost entirely of benzimidazole **9**.

5-Amino-4-[(1-ethylpropyl)amino]-2-methyl-3-nitrobenzenemethanol (**14**). A solution of **13** (200 mg) in EtOH (10 mL) was stirred at room temperature and treated with four 1-mL portions of 52–60% aqueous $(NH_4)_2S$ over 2 h. After another hour the mixture was partitioned between water and CH_2Cl_2. The dried organic phase was concentrated to give 157 mg of material that was recrystallized from hexane to give 127 mg (71%) of a red solid that was predominately **14** but that contained a small amount of what was presumably the isomer resulting from reduction of the more hindered nitro group [see Smith et al. (1979)]; TLC, C, R_f 0.16 (major) and 0.21 (minor). The mass spectrum was run on a sample of the major isomer obtained by preparative TLC: mass spectrum, electron impact, *m/e* 267 (35, $(M^{+\cdot})$, 238 (100, $M^{+\cdot} - C_2H_5$).

4-[(1-Ethylpropyl)amino]-2-methyl-3-nitro-5-[(pentafluorophenyl)methyleneamino]benzenemethanol (**18**) was prepared from **14** and pentafluorobenzaldehyde similarly to the preparation of **7**. The orange **18** was recrystallized from hexane, mp 127–135 °C, and gave a single spot on TLC (C, R_f 0.24): mass spectrum, electron impact, *m/e* 487 (56, $M^{+\cdot}$), 485 (16, $M^{+\cdot} - 2H$), 458 (70, $M^{+\cdot} - C_2H_5$), 168 (100, $C_6F_5H^{+\cdot}$).

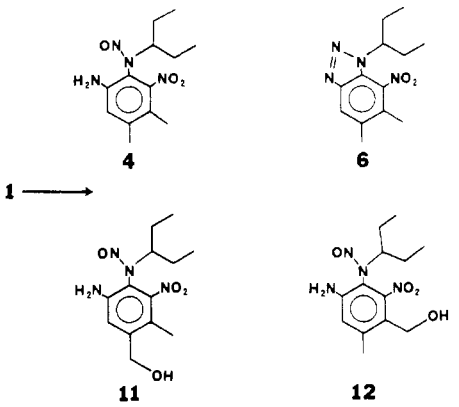
The acetate **19** was prepared with acetic anhydride–pyridine at room temperature and was isolated as an orange oil. The mass spectrum was run on a small sample obtained by preparative TLC (A, R_f 0.19; C, R_f 0.63): mass spectrum, electron impact, *m/e* 487 (56, $M^{+\cdot}$), 485 (16, $M^{+\cdot} - 2H$), 458 (70, $M^{+\cdot} - C_2H_5$).

4-[(1-Ethylpropyl)nitrosoamino]-2-methyl-3-nitro-5-[(pentafluorophenyl)methyleneamino]benzenemethanol Acetate (**20**). A solution of **19** (160 mg) in CH_2Cl_2 (10 mL) containing ~200 mg of pulverized NaOAc was cooled to −78 °C and treated with 0.7 mL of 0.75 M N_2O_4 in CH_2Cl_2. The solution was warmed to 0 °C, then the solvent was stripped to remove excess N_2O_4, then CH_2Cl_2 was added again, the mixture was filtered, and the solvent was again stripped. The yellow residue was triturated with cold MeOH to give **20** as a white solid, mp 143–144 °C (89 mg): mass spectrum, chemical ionization, isobutane, *m/e* 573 [6, $(M + C_4H_9)^+$], 559 [9, $(M + C_3H_7)^+$], 517 [7, $(M + H)^+$], 487 [100, $(M + H - NO)^+$].

5-Amino-4-[(1-ethylpropyl)nitrosoamino]-2-methyl-3-nitrobenzenemethanol (**11**). Compound **20** (84 mg) was stirred in EtOH (4 mL) plus HOAc (0.5 mL) containing 2 drops of concentrated H_2SO_4. After 0.5 h, a mixture of H_2O (0.5 mL), HOAc (0.5 mL), and H_2SO_4 (1 drop) was added, and stirring was continued 10 more hours. The mixture was partitioned between H_2O and CH_2Cl_2; the CH_2Cl_2 was stripped and replaced with EtOH (3 mL) and H_2O (2 mL), and then 10 drops of 1 N NaOH was added. After 0.5 h the mixture was neutralized with HOAc and partitioned between CH_2Cl_2 and H_2O. Evaporation of the CH_2Cl_2 and crystallization of the residue from hexane plus a trace of EtOAc gave 33 mg of **11** as bright yellow crystals, mp 131–132 °C. Recrystallization from benzene–hexane raised the mp to 133–135 °C: mass spectrum, electron impact, *m/e* 278 (53, $M^{+\cdot} - H_2O$), 266 (100, $M^{+\cdot} - NO$), 249 (75); mass spectrum, chemical ionization, isobutane, *m/e* 297 (4, $M + H^+$), 279 (52, $M + H^+ - H_2O$), 268 (100, $M + H^+ - C_2H_5$), 267 (85, $M + H^+ - NO$).

4-[(1-Ethylpropyl)nitramino]-2-methyl-3,5-dinitrobenzenemethanol (**15**). A mixture of *N*-(1-ethylpropyl)-4-(hydroxymethyl)-3-methyl-2,6-dinitrobenzenamine (**13**; 20 mg), acetic anhydride (7 mg), and acetic acid (4 mg) was heated to 110–120 °C to form the 4-acetoxy derivative. Fuming 90% nitric acid (94 μL) freed from nitrogen oxides with urea and air was added to acetic anhydride (177 μL) at 0 °C, and the mixture was stirred at that temperature for 15 min. Then the crude acetate in 0.4 mL of HOAc was added dropwise, and stirring was continued an additional hour at 0 °C. Ice and water were added, and the mixture was neutralized with $NaHCO_3$ and extracted with CH_2Cl_2. The CH_2Cl_2 was stripped and replaced with MeOH (2 mL), and then 0.67 mL of 0.1 N NaOH was added. The solution was stirred at room temperature for 1.5 h, acidified with 1 drop of 6 N HCl, and extracted with CH_2Cl_2. The CH_2Cl_2 solution was purified by preparative TLC (D), and a rather broad, pale yellow band, R_f 0.21–0.37, was scraped. The silica gel was extracted with dichloromethane to provide pure **15**: mass spectrum, electron impact (18 eV), *m/e* 313 (14, $M^{+\cdot} - C_2H_5$), 296 (27, $M^{+\cdot} - NO_2$), 267 (100, $M^{+\cdot} - C_2H_5 - NO_2$). The synthetic material was determined by its mass spectrum and high-pressure LC retention volume to be identical with the later eluting of the isomeric hydroxylated *N*-nitropendimethalins isolated from the *Streptomyces* fermentation.

Scheme I. Structures of Isolated Metabolites



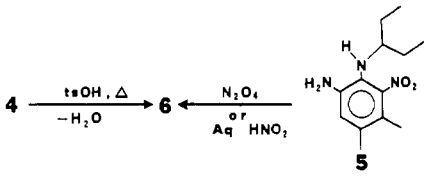
3-[(1-Ethylpropyl)nitramino]-6-methyl-2,4-dinitrobenzenemethanol (**16**). The procedure described above was repeated with 3-[(1-ethylpropyl)amino]-6-methyl-2,4-dinitrobenzenemethanol (**17**) to provide **16**: mass spectrum, electron impact, *m/e* 313 (14, $M^{+\cdot} - C_2H_5$), 296 (100, $M^{+\cdot} - NO_2$), 267 (95, $M^{+\cdot} - C_2H_5 - NO_2$). This material was identical (mass spectrum and high-pressure LC retention volume) to the earlier eluting material of the isomeric hydroxylated *N*-nitropendimethalins isolated from the *Streptomyces* fermentation.

RESULTS AND DISCUSSION

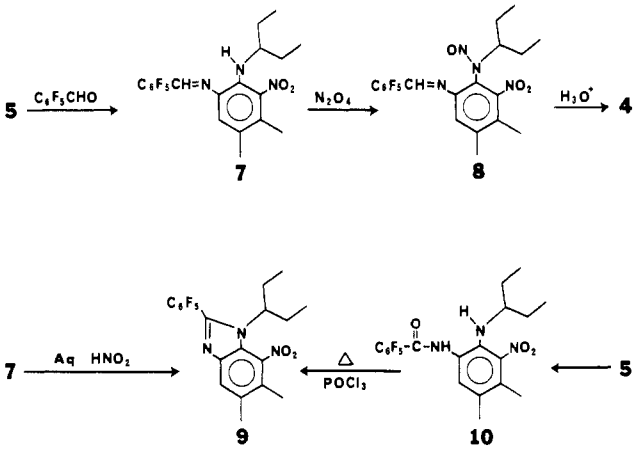
***N*-Nitrosopendimethalin** (**1**). Examination of the TLC autoradiograms of the 1-, 2-, 4-, 8-, and 14-day culture samples revealed a gradual loss of starting material accompanied by the progressive appearance of more polar metabolites. The ethyl acetate extract from the 14-day culture contained 54% of the added radioactivity. The aqueous phase and methanol extract each accounted for 8% of the radioactivity.

Six of the fifteen dry column fractions contained 91% of the recovered radioactivity. Dry column fraction 4 (R_f 0.094–0.16), which contained 20% of the recovered radioactivity, was further purified on TLC (solvent B, R_f 0.33) and yielded compound **4** (Scheme I). The isobutane CI mass spectrum of **4** indicated a molecular mass of 280 amu: *m/e* 281 [13, $(M + H)^+$], 337 [1.4, $(M + C_4H_9)^+$], 251 [100, $(M + H - NO)^+$]. In order to confirm the molecular mass and to ascertain the basicity of the compound, it was analyzed by using ammonia as the reagent gas. The base peak of the ammonia CI spectrum was *m/e* 298 which resulted from electrophilic attachment of an ammonium ion. The weak (0.3%) $(M + H)^+$ ion in the ammonia CI spectrum indicated **4** to be a weaker base than ammonia. The use of deuterium oxide as a reagent gas provided a quasi-molecular ion at 284 amu and demonstrated the presence of two exchangeable hydrogens. The facile loss of 30 amu from the molecular ion of 280 amu (0.2%) in the EI spectrum gave the base peak of 250 amu, suggesting the retention of the nitroso functional group in **4**. This facile loss of 30 amu was also observed in CI spectra and was highly dependent upon source block temperature. Bands at 3470 and 3388 cm^{-1} in the infrared spectrum indicated the presence of an amino group, and a band at 870 cm^{-1} was consistent with a penta-substituted benzene. The ^1H NMR spectrum was also consistent with retention of the *N*-nitroso group. Unlike that of **3**, wherein the chemically equivalent methyls of the 1-ethylpropyl side chain also showed magnetic equivalence at δ 0.86, the spectrum of **4** indicated magnetic nonequivalence of the methyl groups (chemical shift difference of 0.053 ppm) attributable to hindered rotation around the N–NO bond. The above suggested that one of the aromatic nitro groups

Scheme II. Formation of Benzotriazole 6



Scheme III. Synthesis of Metabolite 4 and Compound 9



of the starting material had been reduced to an amino group.

This structure was suspect because it is formally a nitrosation product of an *o*-phenylenediamine, and *o*-phenylenediamines, upon nitrosation, generally provide benzotriazoles directly. We know of no case in which intermediates have been isolated. For example, diamine **5** (Scheme II), upon treatment with either aqueous nitrous acid or with dinitrogen tetroxide in dichloromethane at −78 °C, instantly gave the benzotriazole **6**. In contrast, a sample of metabolite **4** was recovered unchanged after standing several hours in acetic acid containing a little hydrochloric acid.

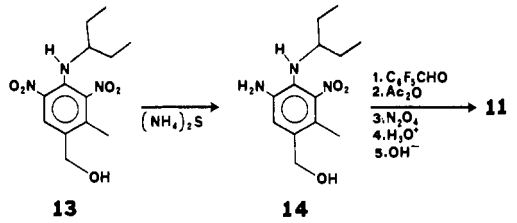
Our first confirmation that the proposed structure was correct was obtained by heating metabolite **4** in toluene containing a little *p*-toluenesulfonic acid. These conditions did effect the anticipated cyclodehydration, and the product was identical with benzotriazole **6** prepared earlier (Scheme II).

Because of the facile benzotriazole formation from *o*-phenylenediamines, a direct synthesis of **4** seemed impossible. Therefore, the Schiff base was chosen to protect the unsubstituted (and less deactivated) amino group. Initially benzaldehyde was used; a Schiff base formed, but upon nitrosation with dinitrogen tetroxide at −78 °C, a bright blue product formed (presumably nitrosation of the benzene ring had occurred) that was not characterized. We then prepared the pentafluorobenzaldimine **7** (Scheme III). When the nitrosation of **7** was attempted with aqueous nitrous acid, however, **7** was instantly oxidized to the benzimidazole **9**, and no **8** was detected.

The structure of **9** was deduced from its electron impact and chemical ionization mass spectra [stable molecular ion 2 amu lighter than precursor **7**, no exchangeable H's, loss of the fragment $C_5H_{11}O$ as observed with a related benzimidazole (Smith et al., 1979)] and was confirmed by an alternate synthesis from **5** as shown in Scheme III.

In contrast to the reaction with aqueous HNO_2, **7** reacted rapidly and smoothly with N_2O_4 at −78 °C under anhydrous conditions to give **8** as a white crystalline solid. Acid hydrolysis of **8** removed the protecting group and gave the desired *N*-nitroso-*o*-phenylenediamine **4** as a yellow solid. Not only did **4** not tend to cyclize under the acidic hy-

Scheme IV. Synthesis of Metabolite 11



drolysis conditions, but also, in fact, it has proved to be a surprisingly stable compound with a shelf life of >1 year at room temperature. The synthetic 4 was shown to be identical with the metabolite by comparison of their mass and infrared spectra and TLC R_f's. Compound 4 was also the major product formed from 1 in flooded anaerobic soil (Smith et al., 1979).

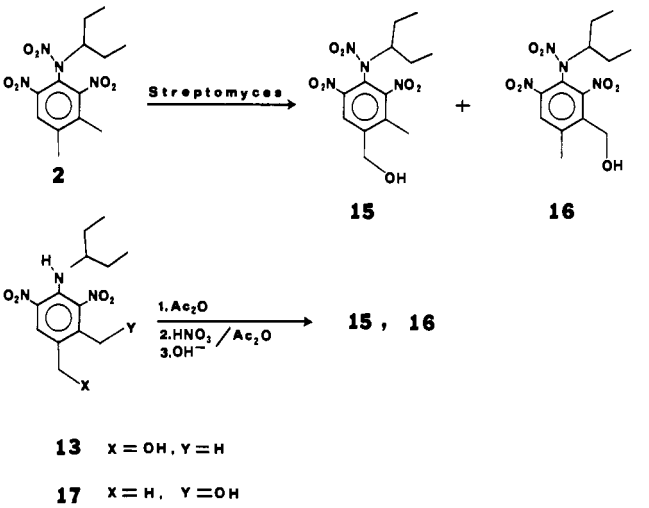
Dry column fraction 1 (origin band), which contained 24% of the recovered radioactivity, was further purified on TLC (system C) and yielded three bands which were radioactive. The first band (R_f 0.51) proved to be metabolite 4. The presence of metabolite 4 in a fraction well separated from the bulk of 4 contained in fraction 4 will be noted later. The major band (R_f 0.26) contained two compounds, both of which exhibited protonated molecular ions at m/e 297 under isobutane CI conditions. The base peaks of both electron impact spectra corresponded to losses of 30 amu from the molecular ions, indicating retention of the nitroso group. Low intensity ions at m/e 278 suggested the loss of water from the molecular ions. Deuterium-exchange chemical ionization indicated the presence of three exchangeable hydrogens. Proton nuclear magnetic resonance spectroscopy on a very small sample suggested the replacement of a ring methyl group by a functionalized methylene group. Structures 11 and 12 (Scheme I) were proposed for the minor and major isomers, respectively. An authentic sample of 11 was synthesized (Scheme IV) by essentially the same sequence used for 4, the only significant difference being the extra steps required by protection and deprotection of the benzylic alcohol as its acetate. The chromatographic and mass spectral properties of synthetic 11 matched those of the minor isomer in the isolated material. We have not yet been able to confirm the identity of metabolite 12.

A final metabolite was isolated from dry column fraction 10 (R_f 0.49–0.57) by high-pressure LC. The electron impact mass spectrum, the high-pressure LC retention time, and the TLC R_f matched that of a sample of the authentic benzotriazole 6. Presumably 6 was derived from 4 by a simple cyclodehydration; whether this was a microbiological reaction or a simple chemical conversion is not known. Also isolated from fraction 10 by high-pressure LC was a small amount of the starting material 1.

What appeared to be anomalous dry column chromatographic behavior was noted. For example, the bulk of metabolite 4 was detected in dry column fraction 4; however, metabolite 4 was also found in fraction 1. Conjugates of some metabolites may have been present initially which subsequently decomposed upon handling; however, 1 was also detected in widely separated dry column fractions, and 1 does not appear to have any functional groups which would allow reversible conjugation.

N-Nitropendimethalin (2) was incubated 10 days under the conditions described for 1. Sixty-seven percent of the radioactivity was recovered in organic solvents (49% by EtOAc extraction and 18% by extraction of organisms with methanol). Of the recovered ^{14}C, 56% was associated with unmetabolized 2. Four additional ^{14}C-containing compounds were detected by high-pressure LC [two pairs

Scheme V. Metabolism of N-Nitropendimethalin (2) and Synthesis of Metabolites



of barely resolved "doublets" from two consecutive dry column fractions (R_f 0.29 and 0.45)]. Chemical ionization mass spectra of one pair of compounds, 15 and 16 (Scheme V), suggested that they were isomeric to each other, having been derived from addition of one oxygen to 2. The D_2O chemical ionization spectra indicated one exchangeable H per molecule. Their fragmentation under electron impact conditions resembled that of 2, suggesting that hydroxylation had probably occurred on ring methyls and not on alkylamino carbons. This conclusion was verified by synthesizing 15 and 16 as shown in Scheme V. Mass spectra and high-pressure LC retention volumes matched those of the metabolites.

The remaining two metabolites have not been identified. They may also constitute a pair of isomers; chemical ionization mass spectra of each contained peaks at m/e 297 (100%), suggesting molecular weights of 296. The electron impact mass spectra, however, both contained low intensity (6–15%) peaks at m/e 313, and fragmentation was extensive with groups or pairs of ions frequently encountered (m/e 297, 296; 268, 267, 266; 252, 251, 250). Interestingly, a peak at m/e 211 had a relative intensity of 100% in the spectrum of one "isomer" but only 12% in the spectrum of the other. A chemical ionization spectrum with deuterium oxide as the reagent gas was obtained on one compound but was also confusing; it had peaks at m/e 301, 300, and 299 with relative intensities 100, 16, and 80%, respectively. Insufficient material was available for further studies of these metabolites.

In summary, the predominant metabolic reaction of nitrosamine 1 was nitro group reduction to give the same metabolite 4 that had been isolated from anaerobic soil. Oxidation of ring methyls was also observed; hydroxylated metabolites 11 and 12 were the result of sequential reduction and oxidation of 1. Thus the N-nitroso group remained intact while both oxidative and reductive processes occurred elsewhere in the molecule. Benzotriazole 6, the only observed metabolite in which the N-nitroso group was not intact, presumably resulted from cyclodehydration of 4. Hydroxylation of the ring methyls of the nitramine 2 was the only metabolic process identified.

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Oligomers and Quinones from 2,4-Dichlorophenol

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Incubation of 2,4-dichlorophenol with a phenol oxidase from the fungus *Rhizoctonia praticola* caused the formation of several phenolic and quinonoid oligomers. Some of these products were highly reactive and several could be isolated and identified. Mass spectrometric analysis indicated the formation of oligomers up to the pentamer. Two dimeric quinones were identified by mass and NMR spectroscopy as 2-(2,4-dichlorophenoxy)-6-chloro-1,4-benzoquinone and 2-(2,4-dichlorophenoxy)-1,4-benzoquinone.

Degradation of pesticides and other xenobiotics by biological or physicochemical factors often causes the formation of intermediary products which can react with themselves or with naturally occurring molecules. It is probable that in the soil environment the same agents which cause polymerization during the humification process oxidize or polymerize xenobiotics and consequently are responsible for their incorporation into soil organic matter.

Oxidation of phenols can yield quinones and radicals. Both molecular formulations are usually very reactive and form stable products by self-coupling or cross-coupling with available molecules. Although this reaction is well-known, it has received little attention as an alternate transformation reaction in determining the fate of xenobiotic products. The reason for this oversight can be explained by the difficulty in detecting and identifying compounds which are incorporated into complex, high molecular weight polymers.

In a previous investigation we selected 2,4-dichlorophenol as a representative phenolic intermediate from a pesticide (2,4-D, 2,4-dichlorophenoxyacetic acid), and we demonstrated the formation of oligomers and cross-coupling products with phenolic humus constituents in the presence of a laccase from the fungus *Rhizoctonia praticola* (Bollag et al., 1980). The purpose of this study was to investigate in more detail the lower molecular weight reactive intermediates formed initially by oxidation and by oxidative coupling of 2,4-dichlorophenol.

MATERIALS AND METHODS

An extracellular phenoloxidase was isolated from the growth medium of the fungus *R. praticola* and purified as previously described (Bollag et al., 1979). The enzyme (0.5 unit/mL) was incubated with 2,4-dichlorophenol at 100 μg/mL in 0.1 M phosphate buffer (pH 6.9) for 2 h at 30 °C. One unit of enzyme is defined as that amount which causes a change in optical density of 1.0 per minute at 468 nm in 3.5 mL of 0.1 M phosphate buffer solution (pH 6.9) containing 3.24 μmol of 2,6-dimethoxyphenol at 23 °C (Sjoblad and Bollag, 1977). The reaction mixture was extracted with an equal volume of methylene chloride, and the extract was evaporated to dryness after drying over anhydrous sodium sulfate. Enzyme boiled for 5 min was used as a control.

Analysis of the products was carried out by thin-layer chromatography (TLC) using silica gel F-254 plates with a layer thickness of 0.25 mm (Brinkman Instruments Inc., Westbury, NY) and ether–hexane (4:1 v/v) as a development solvent. For isolation of products, preparative silica gel F-254 plates (0.5-mm layer thickness) were used. Methylation was obtained by treating the products with diazomethane in ether.

Subsequently some of the products were extracted from the thin-layer plates and further analyzed and purified by high-performance liquid chromatography (LC) using a Waters Associates (Milford, MA) Model 6000 equipped with an U6K injector and a 440 UV detector operating at a wavelength of 254 nm. The column used was a 30 cm × 3.9 mm (i.d.), packed with normal phase μ-Porasil, particle size 10 μm (Waters Associates, Milford, MA). Separation of the products was achieved with a mobile phase consisting of 95% hexane and 5% dioxane at a flow rate of 1.2 mL/min. The samples were passed through a Florisil precolumn (Supelcosil-ATF-120, 100–200 mesh, Supelco Inc., Bellefonte, PA) equilibrated with methylene chloride, and the column was eluted with the same solvent unless otherwise stated.

For isolation of quinone products, the sample in methylene chloride was placed on a Florisil column equilibrated with hexane. Elution of the column with hexane removed the various polymers, but the quinones were retained on the column. Subsequently they were removed from the Florisil column with methylene chloride and separated by high-performance LC.

Mass spectrometry was based on electron impact analysis at 70 eV with sample introduction by direct insertion probe on an AEI MS-902 mass spectrometer or by a directly coupled gas chromatograph on a Finnigan 3200 mass spectrometer (1.5 m × 1.8 mm i.d. glass column, packed with 3% OV-1 or 3% OV-17 on 100–120-mesh Chromosorb W). The products were injected in a meth-

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