Soil Persistence and Metabolism of *N*-*sec*-Butyl-4-*tert*-butyl-2,6-dinitroaniline

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The persistence of the herbicide *N*-*sec*-butyl-4-*tert*-butyl-2,6-dinitroaniline (I) in soil was essentially the same as that of its enantiomorphs. Transformation products were isolated from extracts of Chillum silt loam and from culture solutions of a soil fungus, *Paecilomyces* sp. The major product isolated from soil was the dealkylated derivative of I, 4-*tert*-butyl-2,6-dinitroaniline. The major metabolite isolated from the fungal extracts was an oxygenated analog of I (mol wt 311), and was identified as 3-(4-*tert*-butyl-2,6-dinitroanilino)-2-butanol from its nmr spectrum using shift reagents. The *Paecilomyces* sp, however, did not oxidize I to a dealkylated product.

N-*sec*-Butyl-4-*tert*-butyl-2,6-dinitroaniline (I) is one of the newer members of the dinitroaniline class of herbicides. Metabolic reactions of several dinitroaniline herbicides have been reviewed by Probst and Tepe (1969). In soils, trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine) is dealkylated to *N*-monopropyl- and unsubstituted α,α,α-trifluoro-2,6-dinitro-*p*-toluidine and is reduced to form the 2-amino and 2,6-diamino analogs of trifluralin. Four species of fungi, *Sclerotium rolfsii, Aspergillus niger, Fusarium* sp, and *Trichoderma* sp, were limited in their ability to metabolize trifluralin, although the monopropyl derivative was isolated and identified in extracts of the *A. niger* solutions.

Recently, Laanio *et al.* (1973) undertook microbial studies with dinitramine (N^3,N^3-diethyl-2,4-dinitro-6-(trifluoromethyl)-*m*-phenylenediamine) using *Aspergillus fumigatus* Fres, *Fusarium oxysporum* Schlecht, and *Paecilomyces* sp. They obtained a monoalkyl derivative of dinitramine [N^3-ethyl-2,4-dinitro-6-(trifluoromethyl)-*m*-phenylenediamine] and the corresponding completely dealkylated diamine [2,4-dinitro-6-(trifluoromethyl)-*m*-phenylenediamine]. In addition, two previously unreported products, 6-amino-1-ethyl-2-methyl-7-nitro-5-(trifluoromethyl)-benzimidazole and 6-amino-2-methyl-7-nitro-5-(trifluoromethyl)benzimidazole, were isolated and identified. Simultaneously, Smith *et al.* (1973) reported that dinitramine gave the same two products in Anaheim silt loam. The present investigation examines the persistence of I and its enantiomorphs in soil, and describes the identification of the metabolites isolated from soil and from culture solutions of a soil fungus, *Paecilomyces* sp.

METHODS AND MATERIALS

Persistence. Compound I has an asymmetric carbon at the 2 position in the *sec*-butyl moiety of the molecule. Optically active compound I was prepared by the reaction of the corresponding enantiomorph of *sec*-butylamine (resolved with *d*-tartaric acid), $[\alpha]^{22}D$ +7.65 and −7.40° (neat) with the tosylate of 4-*tert*-butyl-2,6-dinitrophenol. The enantiomorphs of compound I have $[\alpha]^{22}D$ +1.56° (*c* 0.4 g/cm^3 of acetone), mp 59–61°, and $[\alpha]^{22}D$ −1.63 (*c* 0.4 g/cm^3 of acetone), mp 59–61°, respectively. The racemic mixture of I and the + and − isomers were examined separately to determine whether differential metabolism might result in a residue from predominantly one isomer. Purified I (racemate) and the two isomers (+ and −) were established in two soils (Lakeland loamy sand and Matapeake silt loam) at rates of 0, 0.1, 0.5, 1, 2, and 8 ppm. Lakeland loamy sand has the following properties: pH 6.2, organic matter 0.9, and sand, silt, and clay contents of 71.6, 16.4, and 12.0%, respectively. Matapeake silt loam has the following properties: pH 5.3, organic matter 1.5, and sand, silt, and clay contents of 38.4, 49.4, and 12.2%, respectively. Since the Lakeland had been stored air dry for several months, it was supplemented with small amendments of sucrose and sewage sludge microorganisms. Soils were moistened and one crop of bioassay plants was grown and harvested prior to initiating the persistence study. Persistence was measured using plant bioassays [green foxtail (*Sertaria viridis*) and oats (*Avena sativa* L., var. Markton)] by growing approximately 10 to 30 plants in herbicide-treated soil, harvesting 3 weeks after planting, and measuring the fresh weight of the above ground portion of the plant. Identical numbers of seeds were planted in all pots for each planting. Plant tissue from herbicide-treated pots was expressed as a per cent of tissue obtained in the controls for each planting. Harvested tissue was returned to the pot and mixed with the soil. Soil was kept moist until the next bioassay, at which time the pots were reseeded. Pure (±)-I and its two optically active isomers and the ^{14}C-labeled I were generously supplied by Agricultural Chemicals Division, Amchem Products, Inc., Ambler, Pa.

Metabolism. A soil fungus, tentatively identified as a *Paecilomyces* sp, was isolated by the soil enrichment technique (Laanio *et al.*, 1973). A concentration of 100 ppm of I was established in 100 ml of water containing a few grams of Hagerstown silty clay loam. After several months, liquid extracts from these soil solutions were streaked on agar plates which contained 50 ppm of I as the primary source of carbon. Organisms were selected from agar plates and cultured in liquid solutions containing I as a sole source of carbon. Those cultures exhibiting growth were selected for metabolic studies.

Purified I, mp 59–61°, was homogeneous on tlc, gas chromatography, and high-pressure liquid chromatography. Initial microbial studies were conducted using flasks containing 50 ppm of I plus ring-1-^{14}C-labeled I (1 μCi) in 500 ml of nutrient medium. The specific activity of the ^{14}C-labeled I was 4.05 mCi/mmol and it gave a single spot on tlc plates in chloroform–carbon tetrachloride (1:1) (R_f 0.63 ± 0.04). The nutrient medium contained 0.2 g of K_2HPO_4, 0.3 g of NH_4NO_3, 0.2 g of $CaSO_4$, 0.1 g of $MgSO_4$, 0.01 g of $FeSO{\cdot}7H_2O$, 2 g of sucrose, and 1 g of yeast extract in 1 l. of distilled water. Large scale experiments were conducted using 30 l. of nutrient solution. After incubating for *ca.* 3 weeks at 22°, the nutrient solution and cells were separated by filtration through cheesecloth. The nutrient solution was reduced in volume in a rotary evaporator and extracted with three volumes of benzene and the cells were extracted with acetone and

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Table I. Persistence of *N-sec*-Butyl-4-*tert*-butyl-2,6-dinitroaniline and Its Enantiomorphs in Lakeland Loamy Sand and Matapeake Silt Loam[a]

Weeks	Isomer	Concentration in soil, ppm[b] 0	0.1	0.5	1.0	2.0	8.0
		Lakeland Loamy Sand					
3	±	100	77	32			
	+	100	87	34			
	−	100	101	26			
9	±	100	167	25	12		
	+	100	63	13			
	−	100	70	16	8		
25	±	100	67	62	19	10	7
	+	100	76	71	29	10	7
	−	100	95	57	19	10	10
37	±	100	89	64	43	18	13
	+	100	76	95	57	17	12
	−	100	84	70	35	16	9
		Matapeake Silt Loam					
3[c]	±	100	128	116	125	104	60
	+	100	132	116	114	97	68
	−	100	122	116	125	100	65
9	±	100	95	97	85	26	10
	+	100	95	97	85	31	10
	−	100	79	92	74	26	10
21	±	100	100	100	86	94	11
	+	100	109	105	97	113	11
	−	100	104	99	100		14

[a] Expressed as per cent of plant growth at zero soil concentration. [b] All results average of three replications. [c] Plant bioassay performed with oats on Matapeake soil at 3 weeks; all other bioassays performed with green foxtail.

methanol. The cellular extract contained about 94% of the recovered ^{14}C and was further investigated.

Initial separations were achieved by column chromatography [1 × 20 cm of silica gel 60, particle size 0.063–0.200 mm (70–230 mesh ASTM) manufactured by E. Merck and distributed by Brinkman Instruments Inc., Westbury, N. Y.] using progressively more polar solvents to elute metabolites. The column was developed with 100 ml each of hexane, hexane–benzene (1:1), benzene, and benzene–ethyl acetate (95:5). Metabolite I appears as a yellow band and was eluted after 335 ml of solvent had passed through the column. Further purification was achieved by preparative thin-layer chromatography (tlc) on silica gel GF 254 coated plates (also purchased from Brinkman Instruments, Inc.) using as solvents chloroform–hexane–acetone (8:2:1), benzene–acetone (8:2), and carbon tetrachloride–chloroform (8:2). Metabolites were identified by cochromatography with authentic reference samples (when available) and by examination of their mass, ir, uv, and nmr spectra. Low-resolution mass spectral analyses were performed on a Perkin-Elmer Model 270 combination gas chromatograph–mass spectrometer, and high-resolution measurements were obtained on an AEI MS-9 mass spectrometer. Nmr spectra were taken at *ca.* 37° on a 60-MHz Varian A-60A spectrometer converted from the continuous wave mode with a Digilab FTS-3 pulsed, Fourier assembly for averaging 30 scans/min; this configuration neither permits spin decoupling experiments nor yields integrals. The parent compound and metabolite I were each analyzed in 0.4 ml of CCl_4 (stored over Na_2CO_3) with $(CH_3)_4Si$ as an internal standard. Spectra were resolved using deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium ($Eu(fod)_3$-d_{27}) nmr shift reagent. A DuPont 830 liquid chromatograph, fitted with a uv detector and a 1-m (2.1 mm i.d.) column packed with "Permaphase" ODS (octadecyltrimethoxysilane), was used to purify metabolite I. Metabolite I was purified by successive injections onto an analytical column (retention time 7.6 min) and eluted with a solvent of constant composition of 40% water and 60% methanol at 700 psi with a flow rate of 0.8 ml/min at 40°. Compound I was detected on the same instrument under the same column conditions using a solvent of constant composition of 60% water and 40% methanol which gave a peak after 8 min.

Table II. Distribution and R_f Values of ^{14}C among Metabolites of *N-sec*-Butyl-4-*tert*-butyl-2,6-dinitroaniline from Cellular Extracts of *Paecilomyces* sp

Compound	% ^{14}C	R_f in various solvent systems[a] 1	2	3	4
Parent	76.2	0.63	0.91	1.00	
Metabolite I	16.2		0.77	0.62	0.30
Metabolite II	2.6	0.56			0.40
Others	5.0				
Total recovery	100.0[b]				

[a] Solvent systems: (1) carbon tetrachloride–chloroform (1:1); (2) benzene–acetone (8:2); (3) chloroform–hexane–acetone (8:2:1); (4) carbon tetrachloride–chloroform (8:2). [b] This is based on the amount of ^{14}C in the acetone and methanol extracts of the cell fraction; 94% of the total recovered ^{14}C was found in the cell extract.

Soil metabolism studies were conducted in a Chillum silt loam from the Beltsville, Md. area. The Chillum soil has a moisture content (at 0.33 bar) of 26.6%, organic matter content of 3.1%, and pH 5.7, and sand, silt, and clay contents of 22.2, 51.5, and 26.3%, respectively. A concentration of 10 ppm of I plus 1 μCi of ^{14}C-labeled I per kg was established in soils by adding the herbicide in acetone, allowing the acetone to evaporate, and then thoroughly mixing the soils. The soil moisture content was maintained at about 70% field capacity. After 2, 4, and 6 months, *ca.* 1 kg of soil was sequentially extracted with 3 l. of benzene and 2 l. of methanol.

RESULTS AND DISCUSSION

Soil Persistence. The persistence of I and its enantiomorphs in two soil types is shown in Table I. Compound I was considerably more persistent in Lakeland loamy sand than in Matapeake silt loam. Approximately 30% more dissipation occurred between 9 and 21 weeks (2.0 ppm) in Matapeake than occurred between 9 and 37 weeks (0.5 ppm) in Lakeland. Degradation did occur, however, in the Lakeland loamy sand as evidenced by the lower residues measured after 37 weeks. A comparison of the residues of the enantiomorphs in a soil exhibiting relatively rapid degradation (Matapeake) or slow degradation (Lakeland) shows no consistent difference in persistence or plant response between either enantiomorph and the racemic mixture. A trend suggesting a more rapid disappearance of the + isomer in Lakeland at 0.5 and 1.0 ppm, particularly after 21 and 37 weeks, is indicated. However, differences between enantiomorphs were not significant. Consideration of these isomeric effects is important from a practical residue analysis standpoint, particularly to avoid the consequences that might arise if the nonphytotoxic enantiomorph should prove to be more persistent than the phytotoxic enantiomorph.

Microbial Studies. Approximately 94% of the radioactivity in the small flask experiments of the *Paecilomyces* sp incubated with I was recovered in the organic extracts of the cell and culture solution. The distribution of ^{14}C among metabolites separated by tlc and their R_f values in several solvent systems are shown in Table II. In addition to the large amount of unchanged parent compound, two major metabolites were detected which accounted for

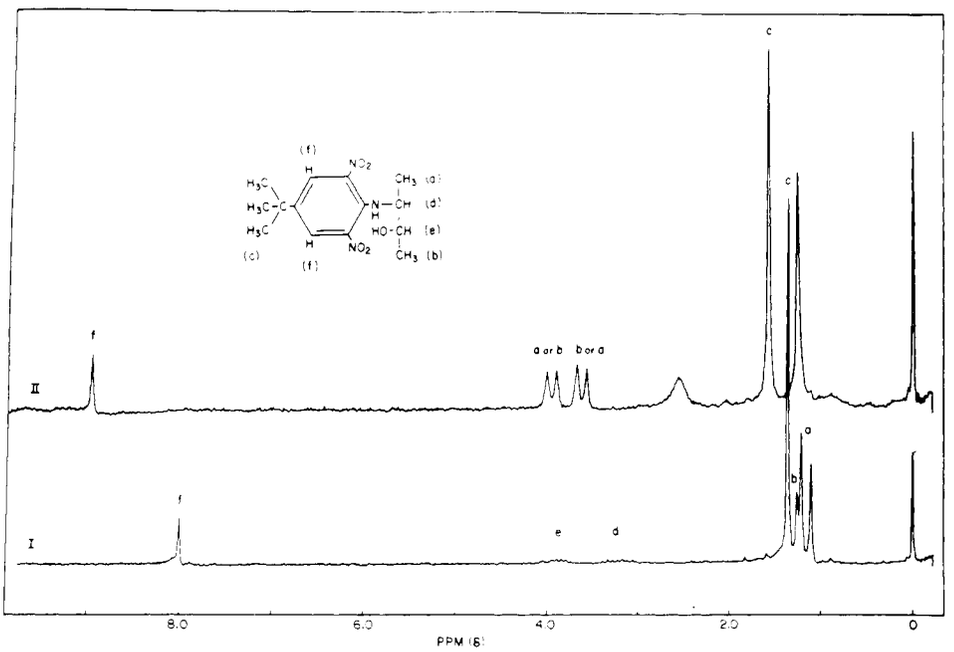


Figure 1. Nuclear magnetic resonance spectrum of 3-(4-*tert*-butylanilino-2,6-dinitro)-2-butanol (I in CCl_4; II with equimolar $Eu(fod)_3$-d_{27}).

Table III. High-Resolution Mass Spectrum of Metabolite I. Significant Fragments and Calculated Formulae

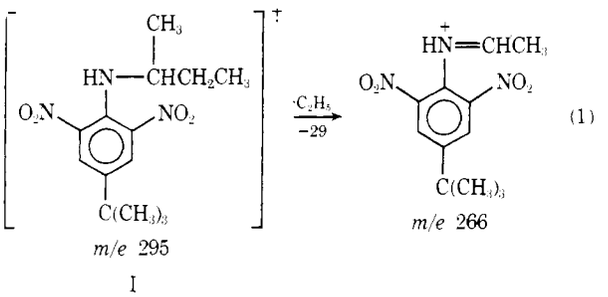
Rel intensity	Calcd mass	Obsd mass	Empirical formula
6.0	311.1480	311.1469	$C_{14}H_{21}N_3O_5$
0.4	296.1245	296.1266	$C_{13}H_{18}N_3O_5$
14.7	267.1173	267.1170	$^{12}C_{11}{}^{13}CH_{16}N_3O_4$
100.0	266.1139	266.1142	$C_{12}H_{16}N_3O_4$
3.4	250.1190	250.1220	$C_{12}H_{16}N_3O_3$
3.0	269.1113	249.1120	$C_{12}H_{13}N_3O_3$
4.2	220.1085	220.1103	$C_{11}H_{14}N_3O_2$
2.6	205.09760	205.0972	$C_{11}H_{13}N_2O_2$

18.8% of the extracted ^{14}C. A number of other products absorbed 254-nm uv light when the extract was separated on fluorescent silica gel coated plates. Collectively, these accounted for about 5% of the recovered activity.

Metabolite I, the major metabolite, was less mobile than I on tlc plates. Metabolite I was also detected in the large scale experiment, and a sufficient quantity was isolated for uv, ir, mass, and nmr spectral analysis.

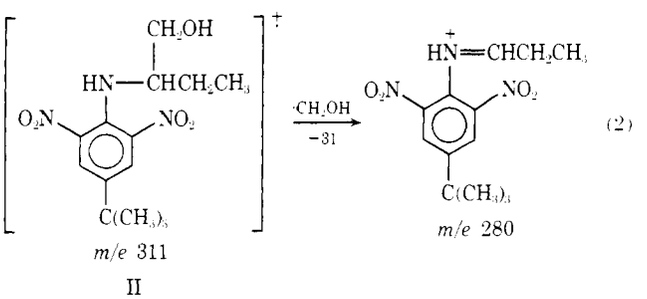
The general features of metabolite I and the location of the hydroxyl group were indicated by an examination of the mass spectrum. The parent compound I gives a fragment ion at *m/e* 266. This is the base peak of the spectrum and corresponds to the expulsion of a $[C_2H_5]$ radical from the molecular ion to form a fragment ion stabilized by localization of charge at the anilino-N atom. This fragmentation pattern is characteristic of a number of related N-alkylated dinitroanilines (Plimmer and Klingebiel, 1972).

The high-resolution mass spectrum of metabolite I (Table III) indicated a molecular formula $C_{14}H_{21}N_3O_5$, *i.e.*, the addition of an oxygen atom to I. The base peak of the spectrum was at *m/e* 266 and this fragment had composition $C_{12}H_{16}N_3O_4$ identical with that of the fragment obtained from I. The loss of C_2H_5O from the molecular ion was therefore identified with a fragmentation process occurring in the *N*-alkyl residue (eq 1). Since the ion at



m/e 266 was formed by loss of a C_2H_5 group in I, it was concluded that in metabolite I an oxidized form of this residue was present. A primary or secondary alcohol thus appeared most probable.

The possibility that the primary alcohol group might be located in the side-chain methyl substituent was eliminated by mass spectral study of the synthetic compound II, 2-(4-*tert*-butyl-2,6-dinitroanilino)-1-butanol. The base peak of II was at *m/e* 280 and corresponded to the loss of a fragment CH_2OH indicating that this alternative pathway was favored in the case of II (eq 2).



The uv absorption spectra of I and metabolite I are very similar with λ_{max} at 431 and 430 nm, respectively. The ir of I and metabolite I are very similar, showing prominent bands at 2690, 1630, 1530, 1340, 1260, 1205, 1150, 920, 900, 760, and 725 cm^{-1}. Metabolite I also exhibits a broad band in the 3300–3600-cm^{-1} region. From the uv, ir, and mass spectra of metabolite I, it is concluded that oxygen-

ation of the *sec*-butylamine moiety of I has occurred. The molecular ion of metabolite II was at *m/e* 251. Insufficient quantities of this metabolite were available for identification studies. At least six other metabolites were detected, but were present in quantities insufficient for further study.

Nmr Studies. The nmr spectrum (2000 scans) of compound I yielded: δ 0.93 (triplet, J = 7 Hz, CH_2CH_3 group), 1.20 (doublet, J = 6 Hz, $NCHCH_3$ group), 1.38 (singlet, *tert*-butyl protons), *ca.* 1.5 (multiplet, $NCHCH_2CH_3$ group), *ca.* 3.2 (multiplet, CH_3NCHCH_2 group), and 8.03 ppm (singlet, aromatic protons). Although overlapping occurred in the high-field section, first-order signals of the C-1 and C-4 protons of the *sec*-butyl moiety were observed.

Figure 1(I) shows the spectrum of metabolite I (2415 scans). The proton absorptions [c, δ 1.36 ppm; f, 8.02] of the 2,6-dinitro-4-*tert*-butylanilino moiety were near those for compound I, as was the doublet at 1.16 (a, J = 7 Hz, $NCHCH_3$ group). The triplet of I at high-field (CH_2CH_3 group) was absent in the spectrum of I, and it appeared that another signal was present at *ca.* 1.2 ppm. Of the two multiplets at *ca.* 3.2 and 3.9 ppm, the former (proton d) was also observed for I.

Since the mass spectral data indicated loss of a C_2H_5O group, only two possible oxidation sites in the *N-sec*-butyl moiety remained for consideration. These can be represented by the partial structures $NHCHCH_2CH_2OH$ and $NHCHCHOHCH_3$. If a $NHCHCH_2CH_2OH$ group were present, a triplet would be expected near *ca.* 3.6 ppm (as with 1-propanol). A $NHCHCHOHCH$ group, however, would yield the observed multiplet (e) at *ca.* 3.9 (as with 2-propanol) while its methyl group (b), which gave a high-field triplet in I, would yield a lower field doublet.

Additions of solid $Eu(fod)_3$-d_{27} shift reagent to samples in CCl_4 in 5-mm o.d. nmr vials readily resolved the spectrum of metabolite I. With 1 mg of shift reagent (1744 scans), the *tert*-butyl singlet remained upfield near 1.50 ppm while the methyl doublets (J = 6 Hz) shifted downfield to 2.46 and 2.63, appearing almost as a quartet; the two C*H* multiplets were at *ca.* 6.4 and 6.9 and the aromatic singlet at 8.49. In Figure 1(II), 3 mg of $Eu(fod)_3$-d_{27} resolved (2339 scans) the two doublets (J = *ca.* 6 Hz); the Eu:metabolite molar ratio was *ca.* 1:1. The peaks at 1.25 and 2.56 ppm are from the incompletely deuterated shift reagent. Earlier experiments with 2-anilinoethanol showed that N,O-acetylation failed to yield a derivative in which the expected first-order methylene triplets could be observed; therefore N,O-acetylation was not attempted with metabolite I. The fact that two doublets are observed for the two methyl groups in the *N-sec*-butyl moiety of metabolite I, plus the evidence presented for the high-resolution mass spectral data, clearly confirm the structure to be 3-(4-*tert*-butyl-2,6-dinitroanilino)-2-butanol.

Soil Studies. The recovery of total ^{14}C after 2 and 4 months was 98 and 85%, respectively. The amount of ^{14}C removed from the soil by the combined benzene–methanol extract was 58 and 53%, respectively. A more rigorous extraction of the soil after 6 months [7 total extractions: benzene (3), methanol (3), and benzene–methanol (1)] removed only 43% of the calculated amount originally applied. Chromatography of the extracts from samples taken after 2 and 4 months yielded primarily unchanged I. After 6 months, a number of products were isolated.

The major product of I isolated from soil was identified as the 4-*tert*-butyl-2,6-dinitroaniline. This structure was confirmed by comparison with authentic 4-*tert*-butyl-2,6-dinitroaniline using uv, mass spectrometry, and tlc. Detection of the dealkylated product of I in soils is not unexpected, since similar products have been reported for trifluralin (Probst and Tepe, 1969) and dinitramine (Smith *et al.*, 1973). A second product (*m/e* 281) was not identical with two authentic samples of the same molecular weight: the *N*-acetyl derivative of 4-*tert*-butyl-2,6-dinitroaniline or the 4-*tert*-butyl-*N*-isopropyl-2,6-dinitroaniline. Efforts are continuing to characterize this compound and obtain sufficient quantities of other unknowns for further characterization.

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