Metabolism of 2,6-Dinitro-4-(trifluoromethyl)benzenamine by a *Streptomyces* Isolated from Soil

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2,6-Dinitro-4-(trifluoromethyl[*ring*-^{14}C]benzenamine (I) was metabolized to several products by a *Streptomyces* sp. isolated from soil. Metabolites were separated by dry column chromatography, purified by high-pressure liquid chromatography, and identified by chemical ionization and electron impact mass spectral analysis and infrared spectroscopy. Products identified were I, 3-nitro-5-(trifluoromethyl)-1,2-benzenediamine (II), *N*-[2-amino-5-(trifluoromethyl)phenyl]acetamide (III), *N*-[2-amino-3-nitro-5-trifluoromethyl)phenyl]methanesulfinamide (V), a diphenyldiazene (VI), and a diphenyldiazene oxide (VII).

The dinitroanalines constitute a rapidly expanding and extensively used class of herbicides. Several members of this class are *N*,*N*-dialkyl derivatives of 2,6-dinitro-4-(trifluoromethyl)benzenamine (I) (Figure 1) and individual herbicides differ by variations in the structure of the alkyl group. Numerous metabolic studies, primarily on 2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)benzenamine (trifluralin), indicate that the alkyl group can be altered, with dealkylation being a major reaction (Probst et al., 1975). Relatively little attention has been directed toward studies on the fate of the resultant benzenamine. From an environmental standpoint, I represents an important residue from several structurally related dinitroanaline herbicides used widely in agriculture.

This paper describes the isolation, from aerobic soil, of a microbiological culture capable of metabolizing I, and the identification of several metabolites produced by those microorganisms.

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. 2,6-Dinitro-4-(trifluoromethyl)[U-*ring*-^{14}C]benzenamine (I; 0.02 μCi/mg) (Figure 1) was supplied by the Lilly Research Laboratories, Greenfield, IN. The labeled material was 99.62% pure by thin-layer chromatography (TLC).

Synthesis of Authentic Compounds. 3-Nitro-5-(trifluoromethyl)-1,2-benzenediamine (II) was synthesized as described by Soper (1969). *N*-[2-Amino-3-nitro-5-(trifluoromethyl)phenyl]acetamide (III) was prepared by reacting II (104 mg) and acetic anhydride (200 μL) in dichloromethane (4 mL) at reflux for 2 h. After cooling, pentane was added and III was collected by filtration as a yellow solid, mp 185–186 °C (98 mg, 85%).

The preparation of *N*-(2-amino-3-nitro-5-(trifluoromethyl)phenyl)methanesulfinamide (V) is described elsewhere (Oliver and Lusby, 1979).

Bis[2-amino-3-nitro-5-(trifluoromethyl)phenyl]diazene (or isomer) (VI) was prepared by oxidation of II. A mixture of nickel peroxide [NiO_x, Nakagawa et al. (1962); 3 g, activity 2.1×10^{-3} g-atom of oxygen/g of NiO_x], II (0.91 g, 4.1×10^{-3} mol), and benzene (70 mL) was stirred at room temperature. Aliquots for TLC analysis were removed periodically and three additional 1-g portions of NiO_x were added over 2 h. After 3 h, the mixture was filtered and concentrated, and portions of the residue were recrystallized from various solvents. One sample was recrystallized twice from chlorobenzene, once from acetic acid plus water, and again from chlorobenzene to give VI as a bright red powder, mp 277–279 °C (rapid sublimation above 265 °C).

Soil Microbiology. One milligram of I was added to a flask containing a soil–water slurry (10 g of Matapeake silt loam and 100 mL of nutrient solution) and maintained on a rotary shaker at 23 ± 1 °C for several months. The fresh Matapeake silt loam, representative of the soil subgroup Typic Hapludults, was taken from field plots at the Beltsville Agriculture Research Center. Microorganisms were isolated by streaking at 3-month intervals an inoculum from the soil–water slurry on agar plates containing I. The agar plates (purified agar, Baltimore Biological Laboratory, Baltimore, MD) were prepared by layering I (0.1 mg in ether) on the surface of the solidified agar and allowing the solvent to evaporate. After the agar plates were incubated at 30 °C for 10 days, selected colonies were transfered to test tubes containing sterilized nutrient solution plus 5 mg/L of I. Colonies were assayed for metabolic activity by incubation in 250-mL flasks containing 100 mL of sterilized nutrient solution, 0.1 g of yeast extract, and 5 mg/L of I. After 14–21 days the organisms were separated from the nutrient solution and extracted with 100 mL of methanol. The nutrient solution was extracted with 300 mL of ethyl acetate, and the extracts were combined and reduced in volume, and the extract was spotted onto TLC silica gel coated plates (Brinkman 60F-254 precoated). Products were separated by using benzene or benzene/ethyl acetate as solvent systems. On the basis of the appearance of metabolites, one organism was selected for further studies.

The isolated organism was Gram-positive and aerobic, producing slender (0.5–2.0-μm diameter), branched, vegetative hyphae, which when mature produced chains of nonmotile aerial spores. The mature colonies were small (1–4-mm diameter), leathery, and gray. On the basis of these characteristics, the organism was identified as a member of the genus *Streptomyces* (Buchanan et al., 1974). Identification to species was not attempted.

For mass culture studies, a 2.8-L Fernbach flask was charged with 1 L of mineral nutrient salt solution and 1 g of yeast extract. After autoclaving the flask, 5 mg (0.1 μCi) of [*ring*-^{14}C]I in about 1 mL of ethanol was added, and the flask was inoculated with the organism. Ten such flasks were agitated gently for 14 days at room temperature. During this period, spherical colonies 0.5–5 mm in diameter appeared in the flasks.

Isolation. The contents of the flasks were combined and filtered through a pad of Celite, and 1500-mL portions of the filtrate were extracted with ethyl acetate (3 × 200 mL). The combined extracts were concentrated on a rotary evaporator at 30 °C and made up to 10.0 mL, and an

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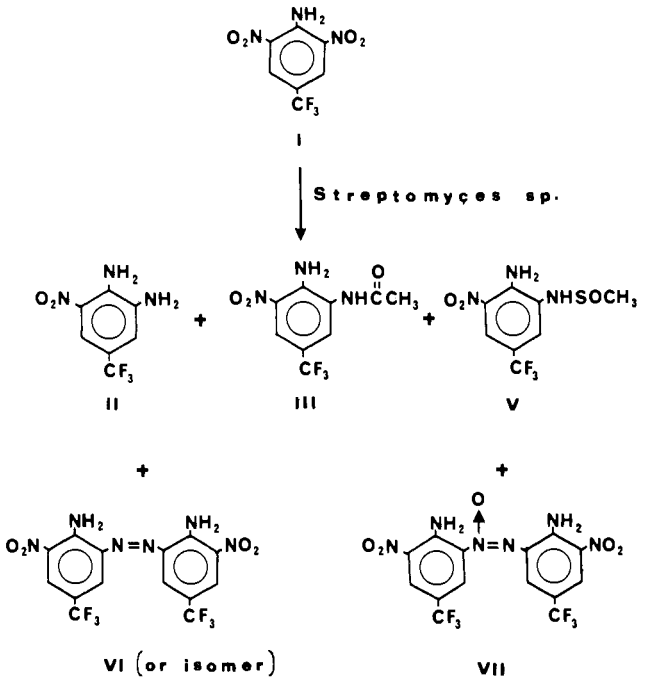


Figure 1. Metabolites from *Streptomyces* metabolism of 2,6-dinitro-4-(trifluoromethyl)benzenamine.

aliquot was counted for radioactivity. The Celite pads and residue were stirred for 4 h in 1500 mL of methanol. After filtering, the methanol extract was concentrated to 10.0 mL, and an aliquot was counted. The ethyl acetate extract was evaporated onto a few cubic centimeters of silica gel, which was then placed on top of a 90 × 2 cm i.d. silica gel (Silica Woelm TSC) dry column. The column was eluted with 20% ethyl acetate in toluene, then cut into 5-cm fractions, except for fractions 1, 2, and 16, which were, respectively, the origin band, the first 2.5 cm beyond the origin and the last 22.5 cm of the column. The silica gel of each fraction was extracted with diethyl ether. The ether extracts were concentrated to a volume of 3.0 mL, and aliquots were counted.

The dry column fractions which contained more than 5000 dpm were either analyzed directly or further purified by liquid chromatography.

An earlier metabolism study of [^{14}C]I was conducted similarly with the same organism, but in that case the metabolite isolation was achieved by column and preparative thin-layer chromatography.

Instrumentation. Gas chromatographic data were obtained from a Hewlett-Packard Model 5710A fitted with a 1.8 m × 2 mm (i.d.) 3% Dexsil 300 on 100/120 mesh Supelcoport column and flame ionization detector. The injection port temperature was 250 °C, the detector temperature was 300 °C, and the carrier (argon) flow was 46 mL/min. Liquid scintillation counting was measured on a Nuclear-Chicago Mark I liquid scintillation system. Liquiscint (National Diagnostics) was used as the scintillation cocktail. Samples for counting were taken to dryness before the scintillation counting solution was added, and corrections for background were made. Low-resolution mass spectrtal data were obtained from a Dupont 491-B mass spectrometer combined with a Dupont 094 data system. Sample entry was by solids probe. The spectrometer was operated at a resolution of 600 and a scan rate of 4 s/decade. The source block temperature was maintained at either 140 or 240 °C, depending upon the sample and the mode of ionization. An ionizing potential of 70 eV was used for electron impact spectra; isobutane, ammonia, water, and deuterium oxide were used as reagent gases for chemical ionization. High-resolution mass spectral data were obtained from an AEI-902 mass spectrometer at a resolving power of 10 000 and a block temperature of 200 °C by the Florida State University, High Resolution Mass Spectrometry Laboratory, Tallahassee, FL. A Perkin-Elmer 621 infrared spectrophotometer fitted with a beam condenser was used to acquire infrared spectra from 1.5-mm KBr micropellet samples. High-pressure liquid chromatography (LC) was performed on a Dupont 830 liquid chromatograph with either a Zorbax ODS or CN column.

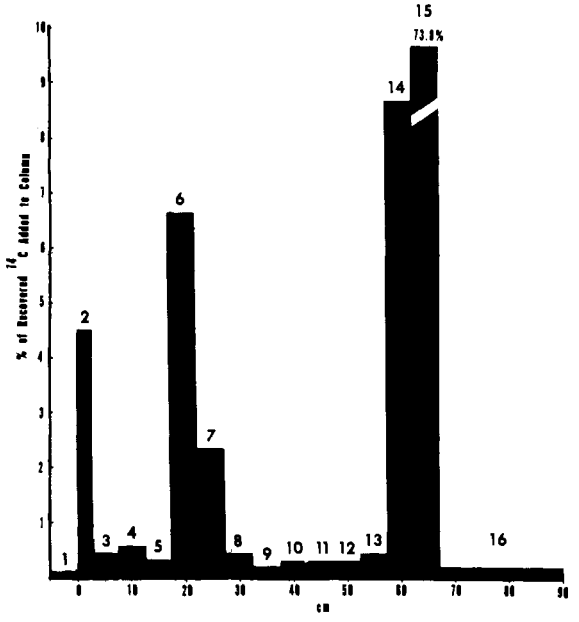


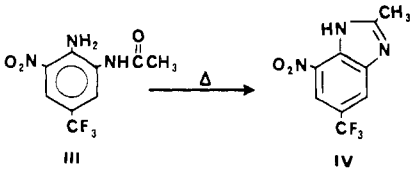
Figure 2. Distribution of radioactivity on silica gel dry column.

RESULTS AND DISCUSSION

The ethyl acetate phase from the extraction of the cell culture contained 74% of the added ^{14}C radioactivity. The methanol from the extraction of the Celite pad and organisms accounted for 2% of the radioactivity. The methanol extract was not examined further.

Five of the 16 dry column fractions accounted for 96% of the recovered radioactivity (Figure 2). Fraction 15, which was analyzed without further cleanup, contained 74% of this radioactivity. A yellow solid isolated from this fraction had chromatographic characteristics identical with those of the starting material I (TLC silica gel R_f = 0.55, CH_2Cl_2; LC retention time 8 min, Zorbax ODS, 65% methanol in water, 1340 PSI, 1.0 mL/min; single GLC peak when coinjected with authentic I). The electron impact mass spectrum of this compound also matched that of authentic I; m/e 251 [100%, $(M)^+$], 232 [16.1%, $(M - F)^+$], 221 [11.7%, $(M - NO)^+$].

Fraction 6 was also analyzed without further cleanup and provided compound II. Chemical ionization mass spectrometry (CI–MS) with isobutane as the reagent gas established a molecular weight of 221: m/e 222 (100%, protonated molecular ion), 262 (2.2%, alkyl adduct ion), 443 (2.6%, protonated molecular dimer). The molecular mass was confirmed by a base peak in the electron impact (EI) mass spectrum at m/e 221. The high abundance of the molecular ion suggested that labile functional groups or hydrocarbon side chains were not present. Deuterium exchange CI–MS with deuterium oxide gave a quasimolecular ion at m/e 227 and indicated the presence of four exchangeable hydrogens. These data suggested that one of the nitro groups of the starting material had been re-



III IV

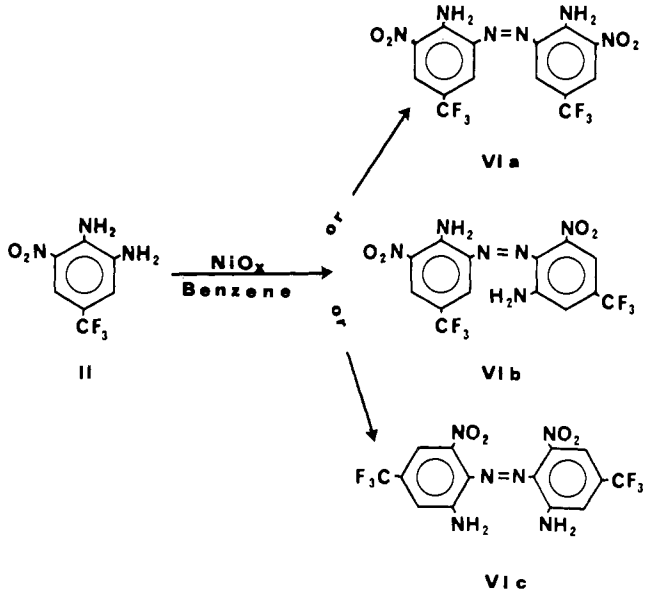
Figure 3. Formation of benzimidazole from metabolite III.

duced to provide an *o*-phenylenediamine. Compound II was chromatographically identical with authentic 3-nitro-5-(trifluoromethyl)-1,2-benzenediamine (TLC R_f = 0.23, CH_2Cl_2 single GLC peak when coinjected with authentic sample). The infrared spectrum and the EI mass spectrum of compound II also match those of the authentic sample. Diamine II has been reported to be an aerobic soil metabolite from both trifluralin and benefin (Probst et al., 1967; Golab et al., 1970).

Dry column fraction 2 was found to contain three radioactive components. These were purified by preparative LC. Metabolite III had a molecular weight of 263 (CI–MS, isobutane, m/e 264, 100%, $(M + H)^+$; 278, 1%, $(M + CH_3)^+$; 320, 1.6%, $(M + C_4H_9)^+$. The EI mass spectrum contained an abundant molecular ion m/e 263 (87%) and ions indicating a loss of water m/e 245 (25%) and loss of ketene m/e 221 (100%). The deuterium oxide CI–mass spectrum contained a quasimolecular ion at m/e 268 and therefore gave evidence for three exchangeable hydrogens. These data suggested compound III to be an acetylated derivative of II. This compound might be expected to lose water thermally to give corresponding benzimidazole IV (Figure 3). Indeed, upon electron impact GC–MS, a gas chromatograph oven temperature of 190 °C was sufficient to effect the dehydration, and the resulting GC–MS spectrum of compound III was consistent with the anticipated for IV (m/e 245, 100%). The monoacetylated diamine, III, was synthesized and analyzed via both probe–MS and GC–MS. The spectra matched those from the isolated material. Both the isolated metabolite and the authentic material had an R_f of 0.33 on silica gel TLC using ethyl acetate as the solvent. Although in principle two monoacetylation products are possible, the isomer shown represents reaction at the much more reactive of the two amino groups. The corresponding monotrifluoroacetylated diamine has been described as the product from the reaction of II and trifluoroacetic anhydride (Eli Lilly and Co., 1978).

Because ethyl acetate had been used for the extraction and cleanup of the metabolies, we felt it important to establish that III was a true metabolite and not an artifact that might have resulted from acetylation of II by ethyl acetate. Accordingly, a solution of II in ethyl acetate was prepared and divided into three portions. One portion was refluxed 24 h, then allowed to stand 24 h at room temperature. A second portion was allowed to stand 48 h at room temperature in contact with silica gel. The remaining portion simply stood at room temperature. All three solutions were assayed for III by LC; in all cases, the amount of III detected, if any, was <0.1% of the amount of II. In contrast, the amount of III isolated from the metabolite mixture was approximately 10% of the amount of II isolated. We are therefore confident that III was formed during the incubation and not during the workup.

The second radioactive component isolated from dry column fraction 2 had an LC elution time of 12.3 min and was designated compound V. Mass spectral data indicated a molecular mass 283, the presence of three exchangeable hydrogens and an unusual loss of 63 amu from the molecular ion in the EI mass spectrum. The identification of this compound as *N*-[2-amino-3-nitro-5-(trifluoromethyl)phenyl]methanesulfinamide will be described elsewhere (Oliver and Lusby, 1979). To the best of our knowledge, this is the first time any sulfinamide has been identified as a metabolite.



II VIa VIb VIc

Figure 4. Synthesis of metabolite VI.

The third radioactive component (LC elution time of 14.3 min) of dry column fraction 2 coeluted with an impurity; the material was, therefore, further purified by LC on a Zorbax CN column (7% 2-propanol in hexane, 3.2 mL/min, 9.5 min). The purified material proved to be another sample of diamine II, identical by mass spectroscopy and GLC with the authentic sample.

Compound VI, which was red in color, was isolated from the earlier metabolism study by column chromatography. Its EI mass spectrum contained an abundant (100%) ion at m/e 438. A fragment ion at m/e 419 (11%) suggested the loss of fluorine. The molecular mass of 438 amu was confirmed by a protonated molecular ion at m/e 439 (19%) under conditions of isobutane chemical ionization. The above data suggested a diphenyldiazene structure for compound VI.

Diphenyldiazenes are not uncommon metabolites of anilines (Kearney et al., 1970), and if VI was formed by oxidation of II, one might expect isomer VIa to be preferentially formed because that product, like metabolite III, would result from reaction of the more reactive amino group. It is also possible for diphenyldiazenes to be formed during the chemical reduction of nitrobenzenes. Since such a reduction is involved in the metabolic conversion of III, it is possible that the diphenyldiazene was formed during that conversion. In that case, VIa, or possibly VIb, but not VIc, would be expected. A further complication, possible because of the *o*-amino groups, would be the possible interconversion of the three isomers. However, we have no evidence of that process occurring. Nickel peroxide oxidation (Nakagawa et al., 1962) of II gave a diphenyldiazene identical by TLC and LC to metabolite VI. The fact that the synthetic product was the same isomer as the isolated metabolite would seem to support structure VIa (Figure 4).

Also isolated from the earlier metabolism study, compound VII had an abundant (57%) ion at m/e 454, which corresponds to the addition of oxygen to compound VI. We isolated too little of VII to permit a detailed investigation of its structure, but the most reasonable possibility would appear to be the diphenyldiazene oxide corre-

sponding to VI. Both diphenyldiazenes and diphenyldiazene oxides have recently been reported to be formed in the soil degradation of trifluralin (Golab et al., 1979).

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Growth Inhibitors from Spikerush

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A phytotoxic compound, dihydroactinidiolide (I), has been isolated from spikerush (*Eleocharis* spp.), synthesized, and shown to be a potent inhibitor of root length elongation and seed germination.

The management of aquatic vegetation in ponds, canals, and drainage ditches has been the subject of considerable investigation which has led to a number of mechanical, chemical, and biological methods of control. In some instances, animals or competitive plants (biological control agents) can be introduced into the aquatic environment to remove selectively or greatly diminish the unwanted vegetation while not becoming pests themselves.

One such plant, spikerush (*Eleocharis* spp.), has been observed to "displace" other unwanted aquatic weeds. Spikerush species are short, rapidly growing perennial sedges, which form a fairly thick mat from a profuse and dense system of rhizomes. Propagation takes place by the growth of the rhizome system as well as the spreading of seed and tubercules.

The competitive nature of spikerush was reported by Oborn et al. (1954), who showed that it can eliminate pondweeds (*Potamogenton* spp.). Likewise, Yeo and Fisher (1970), observing the movement of spikerush in several aquatic sites in California, found that it had eliminated or greatly reduced infestations of pondweed and elodea. The mechanism by which spikerush successfully competes with larger and more vigorous plants was not determined. The rather diminutive nature of spikerush and its bottom-dwelling habit suggest that it could not displace larger species of aquatic plants by mere competition for light, space, and nutrients. After several studies demonstrated that spikerush (*Eleocharis coloradoensis*) successfully inhibited the growth and spreading of American and Sago pondweed, Frank (1975) ascribed the activity to allelopathy.

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MATERIALS AND METHODS

Collection and Extraction of Spikerush. Spikerush which had been grown for seed production in Davis, CA, was mowed, dried, and threshed to remove seeds. The intact plant material (1.1 kg) was then steeped for 1 week at room temperature in 40% aqueous ethanol (6 gal) (Keen, 1978) to extract plant growth regulators. Removal of ethanol in vacuo at 50 °C and liquid–liquid extraction of the aqueous residue for 1 day with ethyl acetate afforded 1.77 g of dark-brown oil.

Bioassay. Fractions or purified samples (0.1–6 mg) were dissolved in 0.5 mL of acetone or methanol and diluted with 60 mL of hot 0.35% agar solution. After thorough mixing, the agar–sample solution was poured into 7-cm petri dishes, allowed to cool, and seeded with approximately 20 watercress seeds (*Nasturtium officinale*). After 7 days in a growth chamber fluctuating between 18 °C (6-h night) and 28 °C (18-h day), root lengths were measured to the nearest 0.5 mm and compared to those of the controls. In each case, replicates were prepared and fractions which showed root-length reduction of 20% or greater at 100 ppm or less were considered active.

Separation of Active Components. The ethyl acetate extract was chromatographed on silica gel (500 g) and eluted with chloroform (2 L) to give material showing phytotoxic activity. The fraction crystallized to give the flavone tricin, which shows no toxicity. Preparative TLC of the mother liquor on silica plates (2% acetic acid/ether) gave an active band at R_f 0.77–0.85. Rechromatography of the band on silica plates (10% methanol/chloroform) gave an active band at R_f 0.68–0.72, which crystallized from methanol to give "γ-sitosterol", i.e., a mixture of sterols. The sterols were shown to be inactive. The mother liquor was vacuum transferred at 1 mm pressure and 50 °C to give, by GC, a compound of about 73% purity. Mass spectral analysis suggested the butenolide I (Figure 1), i.e., dihydroactinidiolide (Chen et al., 1970, which was con-