Identification of Biotransformation Products from 2,4-Dinitrotoluene†

NEIL G. MCCORMICK,* JOHN H. CORNELL, AND ARTHUR M. KAPLAN

Environmental Protection Group, U.S. Army Natick Research and Development Command, Natick, Massachusetts 01760

Received for publication 5 July 1977

The products of microbial transformation of 2,4-dinitrotoluene by Mucrosporium sp. were identified by thin-layer chromatography and by gas chromatography/mass spectrometry as 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2,2'-dinitro-4,4'-azoxytoluene, 4,4'-dinitro-2,2'-azoxytoluene, and 4-acetamido-2-nitrotoluene. A third azoxy compound, believed to be a "mixed" type (i.e., 2,4'-azoxy or 4,2'-azoxy), was isolated but not yet identified.

Water discharges and effluents from ammunition plants, loading facilities, and demilitarization operations contain low levels of a variety of nitro aromatic compounds, including 2,4-dinitrotoluene (DNT) and 2,4,6-trinitrotoluene (TNT). Low levels of these compounds are toxic to various forms of life, including mammals (6). The metabolic fate of TNT has been documented (4, 5, 7, 10, 13, 15), and it does not involve cleavage of the aromatic ring with subsequent degradation of the molecule; instead, TNT is transformed into a variety of products. Although both nitro groups of DNT are capable of undergoing enzymatic reduction to amino groups (7), knowledge of the metabolic fate of DNT and of the nature of the resulting biotransformation products is lacking. In this paper we describe the isolation, identification, and synthesis of these products and propose a pathway for their formation.

MATERIALS AND METHODS

Growth of cultures. A synthetic medium (8) containing 1% glucose was inoculated with spores of Mucrosporium sp. (strain QM9651, NLABS Culture Collection of Fungi) and incubated with shaking for 4 days at 30°C. An equal volume of identical medium, lacking the carbon source but containing DNT (Eastman, 200 mg/liter), was sterilized by autoclaving and added aseptically to the 4-day-old culture. If DNT crystals formed on cooling, the solution was heated to dissolve the DNT before addition to the culture flask. Although the mycelial yield was diluted twofold, this procedure maintained the salt concentration and provided a means of adding the DNT to a final concentration of 100 mg/liter without risking precipitation. Culture flasks were removed from the shaker at 0, 12, 24, 48, 96, and 168 h after the addition of DNT. The mycelia were recovered by filtration through glass wool and membrane filters (45 μm, Millipore Corp., Bedford, Mass.). Inoculated control flasks with no DNT and uninoculated control flasks with DNT were also incubated and sampled at the various times.

Chromatography. The supernatant from the filtered culture medium was extracted three times with 0.25 volume of dichloromethane (DCM) (Burdick and Jackson Laboratories). The extract was evaporated to a small volume, chromatographed by thin-layer chromatography (TLC) on 250-μm silica gel plates with fluorescent indicator (Kontes/Quantum Preadsorbent TLC-LQDF), and developed with benzene-hexane (50:50). Ultraviolet (UV) quenching areas were scraped off and extracted with DCM. A number of solvent systems were investigated, but no resolution was achieved between certain isomers in any of these solvents. To resolve these compounds, the TLC plate was developed, removed from the solvent, dried, and developed again in the same solvent system. This process of multiple development (12) was repeated as many times as necessary to achieve separation between areas of almost equal Rf values.

Gas chromatography/mass spectrometry (GC/MS). The mass spectra of both the metabolically produced and reference compounds were obtained on a model 21-491 DuPont mass spectrometer, coupled to a model 3920 Perkin-Elmer gas chromatograph with a glass column packed with 3% OV-17 on Gas-Chrom Q. All samples were injected as DCM solutions.

4,4’-Dinitro-2,2’-azoxytoluene (2,2’Az). The procedure for synthesis of the azoxy compounds was analogous to that used to prepare certain azoxy derivatives of TNT (11). To a solution of 1.52 g (10 mmol) of 2-amino-4-nitrotoluene (2A4NT) (Aldrich Chemical Co.) in 75 ml of DCM was added 4.1 g (20 mmol) of m-chloroperoxybenzoic acid (Aldrich Chemical Co.). The mixture was allowed to stand overnight at ambient temperature, and the precipitate of chlorobenzoic acid was removed by filtration. The filtrate was extracted with 5% sodium bicarbonate solution, and the DCM was allowed to evaporate from the solvent layer. The product was recrystallized from 95% ethanol and obtained as a slightly yellow solid, melting point (m.p.) 200 to 201°C. To our knowledge, this compound has

† Publication no. TP-1913 of the U.S. Army Natick Research and Development Command.
not been previously synthesized. Mass spectrum: m/e 316 (M+).

2,2'-Dinitro-4,4'-azoxytoluene (4,4'Az). The synthesis was carried out as for the 2,2'-azoxy derivative, except that the starting material was 4-amino-2-nitrotoluene (4A2NT) (Pfaltz and Bauer); m.p. 169 to 170°C; in literature (lit.) 164°C (3). Mass spectrum: m/e 316 (M+).

4HA. DNT was hydrogenated (2) and the product, 4-hydroxylamino-2-nitrotoluene (4HA), was recrystallized from DCM; m.p. 98 to 99°C; lit. 99 to 100°C (2). Solutions of 4HA exposed to the air were unstable, giving rise to a mixture of 4HA, 4A2NT, and 4,4'Az. Attempts to prepare 2-hydroxylamino-4-nitrotoluene (2HA) were not successful. The presence of 2HA could not be demonstrated by GS/MS analysis of the reaction mixture.

4-Acetamido-2-nitrotoluene (4Ac2NT). 4A2NT (1.5 g) was refluxed for 30 min with 3 ml of acetic anhydride, 5 ml of water was added, and the mixture was refluxed for an additional 5 min. Yellow crystals of 4Ac2NT separated on cooling. The product was recrystallized from an ethanol-water mixture; m.p. 144°C; lit. 144°C (3). Mass spectrum: m/e 194 (M+).

2-Acetamido-4-nitrotoluene (2Ac4NT). The synthesis was carried out as for 4Ac2NT, except that the starting material was 2A4NT. The yellowish-white crystals were recrystallized from aqueous ethanol; m.p. 150°C; lit. 150 to 151°C (9). Mass spectrum: m/e 194 (M+).

RESULTS AND DISCUSSION

The filtered supernatant solutions from the different samples were combined in order to provide ample material for purification and analysis. This operation was deemed appropriate because TLC analysis revealed no qualitative changes in the products during the period of incubation. There was a gradual decrease in the concentration of DNT and a corresponding increase in the concentrations of the products. No decrease in DNT concentration occurred in uninoculated control flasks, and no artifacts were introduced, as determined by analysis of inoculated control flasks lacking DNT. DCM extracts of the combined supernatants were concentrated and chromatographed on TLC plates as described in Materials and Methods. When the plates were visualized by UV quenching, the pattern shown in Fig. 1A was observed. Resolution on the first TLC separation was sufficient to allow initial separation of the products into four areas, which were consecutively numbered starting with the band nearest the origin.

Area 4 consisted of residual DNT as shown by co-chromatography with authentic samples of DNT. UV quenching areas 1, 2, and 3 were scraped off and extracted with DCM, and after evaporation to a small volume, each was chromatographed separately in the benzene-hexane solvent system. Even after reextraction with DCM and three passes in benzene-hexane, un-known 1 did not migrate appreciably from the origin. However, using chloroform as developing solvent, a major band with an Rf of about 0.2 did migrate. TLC migration patterns indicated that the compound was not 4HA. Unknown 1 was obtained in a pure state as a single migrating band by repeated development in chloroform. After reextraction and multiple development in benzene-hexane, band 2 was resolved into two bands (bands 2a and 2b, Fig. 1B). Area 3 was resolved into two bands (Fig. 1C) after two passes in benzene-hexane; the upper area (band 3b) separated quite cleanly from the rather diffuse lower area (band 3a). Area 3a was eluted and rechromatographed through four passes of multiple development and was resolved into bands 3a1 and 3a2 (Fig. 1D).

Co-chromatography of the unknowns with the reference compounds (Fig. 1E–J) suggested the following identifications: (1) 4Ac2NT; (2) 4A2NT; (2b) 2A4NT; (3a) 2,2'Az; (3b) 4,4'Az; and (4) DNT. TLC and GC/MS analyses of a solution of the 4HA reference compound indicated the presence of a mixture of 4HA, 4A2NT, and 4,4'Az. This apparent instability of 4HA may explain the absence of hydroxylamino intermediates in the microbial transformations of DNT and the increased concentration of other metabolites (i.e., aminotoluidines and azoxy compounds). Under aerobic conditions it is probable that chemical species containing the hydroxylamino group persist long enough to undergo oxidative coupling to form the azoxy compounds we have observed. A similar nonenzymatic oxidation of N-phenylhydroxylamine to azoxybenzene in the presence of atmospheric oxygen has been reported (1). Under less aerobic conditions, nitro group reduction would proceed to the amino stage by enzymatic reduction of the intermediate hydroxylamino species.

Identities of the synthesized reference compounds and of the unknowns were confirmed by GC/MS. GC/MS analysis of unknown 1 showed a molecular ion at m/e 194, suggesting an acetyl or propyl group attached to a nitrotoluidine residue. Accordingly, the suspected compounds, 2Ac4NT and 4Ac2NT, were synthesized. In addition to the parent ion m/e 194, unknown 1 exhibited two major fragment ions at m/e 135 and 107, which were also exhibited by reference compound 4Ac2NT but not by 2Ac4NT. The mass spectrum of unknown 2a exhibited ions at m/e 152 (M+), 135, and 107, which agrees with the spectrum obtained from the reference compound, 4A2NT. Unknown 2b exhibited a parent ion at m/e 152 (M+), but ions at m/e 135 and 107 were absent, in agreement with the spectrum obtained from reference compound 2A4NT.

The mass spectrum of unknown 3a had ions...
BIOTRANSFORMATION OF 2,4-DINITROTOLUENE

FIG. 1. TLC separations of transformation products of DNT visualized by UV. (A) Concentrated DCM extract of spent growth medium; (B) concentrated eluate of area 2; (C) concentrated eluate of area 3; (D) concentrated eluate of area 3a; (E) 4Ac2NT; (F) 4A2NT; (G) 2A4NT; (H) 2,2'Az; (I) 4,4'Az; (J) DNT.

FIG. 2. Proposed pathways for the formation of transformation products from DNT. The hypothetical nitroso and hydroxylamino intermediates are enclosed in brackets. The potential formation of 2,4-diaminotoluene is indicated by dashed arrows.

We are currently attempting to synthesize a mixed azoxy compound of this type for use as a reference compound in the identification of unknown 3a2.

A scheme for the biotransformation of DNT is presented in Fig. 2. No compounds soluble in DCM, other than those reported, were detected in the concentrated extracts. No additional UV quenching areas corresponding to hydroxylamino or nitroso compounds were detected, but these intermediates are included in the scheme (enclosed in brackets) because the reduction of DNT to 2A4NT and 4A2NT proceeds through the nitroso and hydroxylamino compounds (14). Although no 2,4-diaminotoluene was detected in the present system, complete reduction of both nitro groups to amino groups has been reported in the biotransformation of DNT by anaerobic bacterial systems (7); hence, to generalize, this compound is included in Fig. 2.

ACKNOWLEDGMENTS

We wish to acknowledge the aid of W. G. Yeomans, who conducted the GC/MS analyses on the samples. Thanks are due to H. S. Levinson for valuable suggestions in reviewing the manuscript.

This paper reports research undertaken at the U.S. Army Natick Research and Development Command.

LITERATURE CITED

2. Brand, K., and J. Steiner. 1922. Catalytic reduction of aromatic nitro compounds and a new method of prep-