

Biotransformation of 3-Methyl-4-nitrophenol, a Main Product of the Insecticide Fenitrothion, by *Aspergillus niger*

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Biotransformation of the environmental pollutant 3-methyl-4-nitrophenol (MNP), a newly characterized estrogenic chemical, and the primary breakdown product of the heavily used insecticide fenitrothion was investigated using a common soil fungus. In 96 h, daily culture sacrifice, extraction, and analysis showed that the filamentous fungus, *Aspergillus niger* VKM F-1119, removed more than 85% of the MNP present in solution (original concentration = 25 mg/L), mostly through biodegradation. Additionally, in 16-day time-course studies, *A. niger* was capable of biotransformation of MNP at concentrations as high as 70 mg/L. Gas chromatography mass spectroscopy (MS) analyses of culture fluid extracts indicated the formation of four metabolites: 2-methyl-1,4-benzenediol, 4-amino-3-methylphenol, and two singly hydroxylated derivatives of MNP. Culture scale up and metabolite analysis by liquid chromatography MS resulted in the confirmation of the original metabolites plus the detection of an azo derivative metabolite that has not been previously reported before during MNP biodegradation by any micro-organisms.

KEYWORDS: fenitrothion; 3-methyl-4-nitrophenol; *Aspergillus niger*; biodegradation; endocrine disruptor

INTRODUCTION

In 2004, Furuta et al. (1) showed for the first time that the compound 3-methyl-4-nitrophenol (MNP) exhibited estrogenic activity after isolating MNP and other nitrophenol derivative compounds from diesel exhaust particles. They evaluated the estrogenic activity of MNP by recombinant yeast strain assay, myometrial contractility assay, and in vivo uterotrophic assays and found that MNP was positive in all. They had previously isolated MNP from diesel exhaust particles and explained that, generally, environmental levels and distribution of MNP are relatively unknown (2). In the agricultural fields, it has been known for approximately 20 years that MNP is a main product of the broad-spectrum organophosphorus insecticide fenitrothion (*O,O*-dimethyl-*O*-4-nitro-*m*-tolyl phosphorothioate), first introduced by Sumitomo Chemical in 1959 as Sumithion and now in use worldwide. Under aerobic environmental conditions, fenitrothion breakdown to MNP may proceed relatively rapidly,

in days to weeks (3–6); however, the environmental burden of MNP is indeed currently relatively unknown.

As mentioned, fenitrothion is still used throughout the world, to protect fruit, vegetable, and grain crops, to control household insects, as a vector control agent for malaria, and it is the most common insecticide residue in Australian food, for example (7). In addition to its extensive use, fenitrothion is listed as an alternative insecticide for chlordane, DDT, dieldrin, and endrin in the United Nations Environment Program (UNEP) Persistent Organic Pollutants (POPs) Alternatives Database (<http://dbserver.irptc.unep.ch:8887/irptc/owa/ini.init>). As those insecticides are phased out under the UNEP POPs initiative, an increase in the use of alternative chemicals may occur and concomitant with such an increase will be a renewed interest in the environmental fates of these chemicals and their biodegradation products. Indeed, groups are now focusing on nonbiological processes for the catalytic and photocatalytic degradation of fenitrothion (8–10). Aging fenitrothion stocks also pose a serious environmental threat in developing countries, and a 1997 UN Food and Agriculture Organization (FAO) (11) report indicated that large quantities of stored fenitrothion in Africa and the Middle East are a major hazard because these unused stockpiles are stored under poor conditions and are contributing to soil and water contamination. Rather than costly transport and incineration of such stocks, the FAO considers bioremediation to be a possible solution to this problem.

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In insects, plants, and animals, fenitrothion is oxidized by monooxygenases to the metabolite fenitrothion-oxon; however the major excretion products are MNP and sugar esters of MNP (12, 13). Indeed, MNP is now directly detectable in human urine in fenitrothion-exposed individuals by liquid chromatography tandem mass spectrometry (LC-MS/MS) (14, 15). Reports of micro-organisms known to biotransform fenitrothion include a *Burkholderia* sp. strain that was shown to grow on fenitrothion by utilizing genes coded on 2 plasmids (16) and the fungus *Trichoderma viride*, which hydrolyzed fenitrothion and co-metabolized the hydrolyzed product, MNP (17). MNP itself has recently been shown to be biotransformed by the bacterial isolate *Ralstonia* sp. SJ98 (18); however little is known about the biodegradation fate of MNP.

In consideration of the potentially damaging health effects of MNP to humans and animals as an endocrine-disrupting chemical or other means, combined with its rapid production from fenitrothion, we examined its susceptibility to and mode of biodegradation by common fungi with the aim of potentially applying fungi to bioremediate contaminated soils or decontaminate aging pesticide stocks in stirred-tank reactors.

MATERIALS AND METHODS

Chemicals. MNP (98% purity) and dibenzofuran were purchased from Aldrich Chemical (Milwaukee, WI). Ethyl acetate, acetonitrile, and methanol (all high-performance liquid chromatography (HPLC) grade) were purchased from Fisher (Fairlawn, NJ), and anhydrous sodium sulfate was from Sigma (St. Louis, MO).

Fungal Culture Maintenance. Fungal strains, including *Aspergillus niger* VKM F-1119 and *Cunninghamella elegans* 36112, were stored at 4 °C on potato dextrose agar (PDA; Difco, Becton Dickinson, MD) plates in the dark and transferred every 3–4 weeks to fresh solid media.

Fungal Screening Against MNP. Initially, eight fungal strains representing six different genera (*Aspergillus*, *Cunninghamella*, *Fusarium*, *Mucor*, *Phanerochaete*, and *Penicillium*) were screened for biotransformation activity against MNP. Fungi were grown on PDA plates for 5–7 days at 28 °C, after which a 0.25-cm² agar plug was removed from each plate with a steel scalpel under sterile conditions and transferred in duplicate to each of eight pairs of 250-mL Erlenmeyer flasks that contained 50 mL of potato dextrose broth (PDB; Difco, Becton Dickinson, MD). This method of inoculation was used in all experiments. After 24 h of growth at 28 °C and 150 rpm on a rotary shaker in the dark, 50 μ L of a solution of MNP dissolved in dimethyl sulfoxide (DMSO; 99.9% purity, Aldrich Chemical) were added to each flask resulting in a final concentration of 25 mg/L MNP. Microcosms were returned to the shaker under the same conditions as indicated above and incubated for 14 days. After 14 days, all cultures were extracted with an equal volume of ethyl acetate by shaking overnight, followed by organic and aqueous phase separation by further extraction in 500-mL separatory funnels. The organic phases were filtered through oven-dried anhydrous sodium sulfate, concentrated to dryness in vacuo at 33 °C with an Eyela Rotary Evaporator (Tokyo, Japan), and resuspended in 2 mL of HPLC-grade methanol plus a dibenzofuran reference standard for a final volume of 2 mL. Analyses of culture extracts by gas chromatography (GC) were performed using a Hewlett-Packard instrument fitted with a fused silica capillary column (DB-5; 30 m \times 0.25 mm \times 0.25 μ m) by manual injection with a flame-ionization detector. The temperature program was as follows: 150 °C for 2 min, followed by 10 °C/min to 280 °C, final hold at 280 °C of 5 min. The injection volume was 1 μ L, and the carrier gas was helium (flow rate, 1 mL/min).

Time-Course Biodegradation Assays with *A. niger*. MNP biodegradation was examined first in 96-hour time-course biodegradation assays. *A. niger* was grown on PDA plates for 7 days at 28 °C, after which fungal inoculum was added to 250-mL Erlenmeyer flasks containing PDB and 25 mg/L MNP and incubated under identical conditions as described above. Replicate cultures were incubated for

96 h with culture sacrifice every 24 h by addition of ethyl acetate followed by extraction and analysis of extracts by GC also as described above. Additionally, killed fungal cultures were analyzed to determine if there were MNP losses via adsorption to fungal biomass after 24 and 96 h. In such killed cultures, *A. niger* was inoculated into 50 mL of PDB and incubated for 96 h, after which the cultures were subjected to sterilization twice by steam autoclaving at 121 °C for 40 min each. After sterilization, MNP, final concentration of 25 mg/L, was added to each culture and the cultures were incubated further for 24 and 96 h, at which times they were extracted and analyzed by GC as described above.

Sixteen-day time-course biodegradation assays were performed with the intention of examining biodegradation of MNP at a higher concentration than previously investigated and to examine metabolite production. In triplicate, cultures were incubated with 70 mg/L MNP in 50 mL of PDB and sacrificed after 7, 12, and 16 days by the addition of ethyl acetate followed by extraction and extract preparation as described above. Extract analyses were performed by GC-MS using an Agilent Technologies 6890NGC (Palo Alto, CA) with 5973N Mass Selective Detector fitted with a Hewlett-Packard HP-5 fused silica capillary column (5% phenyl methyl siloxane, 30 m \times 0.25 mm \times 0.25 μ m). The GC temperature program was as follows: 150 °C for 2 min, followed by 10 °C/min to 280 °C, final hold at 280 °C of 5 min. The injection volume was 1 μ L, and the carrier gas was helium (flow rate, 1 mL/min).

Biomass Determinations. *A. niger* fungal biomass was determined by filtering post-extracted fungal biomass through cotton wool. Recovered wet biomass samples were placed in preweighed aluminum dishes and dried at approximately 50 °C to constant weight, and the final dry mass was recorded.

Scaled-Up Cultures for Metabolite Analysis by LC. Scaled-up cultures were prepared and incubated as described for 50-mL cultures except that the culture volume was increased to 1.5 L. After 2 weeks, cultures were extracted also as described above and filtered through 0.2- μ m cellulose acetate syringe filters (MFS-25, Advantec MFS, Inc., Pleasanton, CA) before being analyzed by using a Varian ProStar HPLC (Walnut Creek, CA) that was equipped with a Spherisorb ODS-2 C18 column (5 μ m particle size, 4.6 mm \times 25 cm, Waters Corp. Milford, MI) and a photodiode array detector. The mobile phase, which was composed of water–acetonitrile containing 1% formic acid, was ramped linearly as follows: 10% acetonitrile at 0 min, 20% acetonitrile at 20 min, 40% acetonitrile at 40 min, 70% acetonitrile at 50 min, and finally 90% acetonitrile by 60 min, and the flow rate was 1 mL/min. After metabolite detection by HPLC, further analysis by LC/MS (The National Center for Inter-University Research Facilities, Seoul National University, Seoul, Korea) was undertaken using identical conditions but performed on a Capcell PAK C18 reverse-phase column (5- μ m particle size, 4.6 mm \times 25 cm, Shiseido Fine Chemicals, Japan). As before, the flow rate was 1 mL/min, and in this case, one-third of the effluent was delivered via electrospray probe. Mass spectrometry was carried out by coupling an HP1100 system to a Quattro LC triple quadrupole tandem mass spectrometer (Micromass, Manchester, UK) in electrospray ionization (ESI⁺) mode. Full scans were acquired in positive-ion mode. The source temperature, desolvation temperature, cone voltage, and capillary voltage were kept at 110 °C, 180 °C, 28 V, and 3.88 kV, respectively. An electron multiplier voltage of 640 V was used. The nebulizer gas and desolvation gas were ultrapure nitrogen set at 81 and 300 L/h.

RESULTS AND DISCUSSION

After a 14-day screening of eight strains of fungi against MNP, it was found that at least two of the eight strains were capable of MNP biodegradation: the zygomycete *C. elegans* 36112 and the deuteromycete *A. niger* VKM F-1119 (data not shown). We continued to examine both strains with emphasis on biodegradation by *A. niger*. As shown in **Figure 1**, when *A. niger* was administered 25 mg/L MNP and monitored during a 96-h incubation period, initial biodegradation was found to

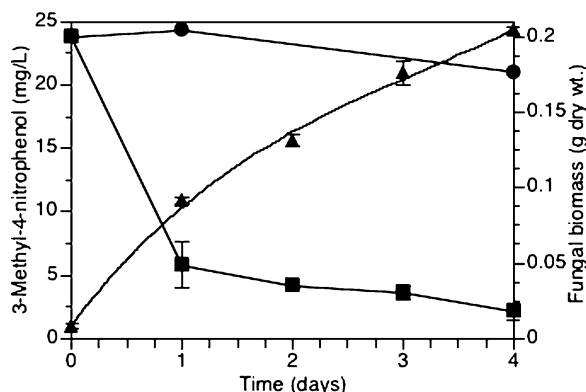


Figure 1. Elimination of MNP (25 mg/L) when added to growing (■) or pre-grown and killed (●) cultures of *A. niger* and increase in *A. niger* biomass (▲) during elimination. Averages of duplicates; error bars represent the coefficient of variation and are omitted when smaller than the symbol.

proceed rapidly in the first 24 h, declined rapidly over then next 72 h, and overall resulted in greater than 85% elimination of MNP in 4 days in liquid culture. Biomass analyses of *A. niger* at each time point indicated that growth continued through day 4 with the highest rate of growth occurring in the first 24 h. Amounts of biomass recovered for each replicate microcosm (g dry weight \pm coefficient of variation) were: 0.091 ± 0.002 , 0.131 ± 0.004 , 0.176 ± 0.008 , and 0.204 ± 0.002 at 24, 48, 72, and 96 hours, respectively. Also as shown in **Figure 1** there were negligible losses of MNP due to binding to *A. niger* biomass in the first 24 h. However, after 96 h of contact time between fungus and target chemical, approximately 15% of the starting MNP concentration was eliminated due to biosorption or abiotic transformation. On the basis of these data and calculations by difference, *A. niger* was capable of biotransforming at least 70% of the original MNP. Additionally, when *A. niger* was incubated and monitored for 10 tens days under identical conditions with 25 mg/L MNP in mineral medium (Staniers basal medium; 19) growth did not occur.

When *A. niger* cultures were administered MNP at an increased concentration of 70 mg/L and incubated over a 16-day time period, 4 metabolites were detected and some inhibition of fungal growth occurred. After 3 days, fungal biomass recovery was 0.106 ± 0.002 g (dry weight), and this value represents a 40% decrease in biomass recovery during the same

time period when compared to *A. niger* exposed to 25 mg/L MNP. By consideration, any treatment process design in the future will require some optimization of the MNP levels. The results of GC-MS analysis are given in **Figure 2**. Triplicate culture analysis at each time point were compared to positive controls of *A. niger* growing on only PDB without MNP. Those peaks that appeared in cultures that contained MNP but which did not appear in the PDB-only positive controls were identified as potential metabolite peaks from the biodegradation of MNP. GC analyses were continued for 20 min; however all peaks that eluted after 9 min from the samples (PDB plus MNP) were identical in retention time and mass spectrum to peaks found in the positive controls (PDB only).

The retention time of the internal reference standard dibenzofuran was 5.0 min, and the retention time of the parent compound, MNP, was 5.3 min under these conditions (**Figure 2**.) Four metabolites of MNP biodegradation were detected by GC-MS analysis when compared to positive controls during the 16-day time period. By 7 days, two metabolites (III, IV) were detected with retention times of 7.5 and 8.0 min, and their concentrations in the media increased slightly through day 16. Two early-eluting metabolites (I, II) were detected by day 16 at retention times of 3.0 and 3.2 min. The mass spectra of these metabolites are presented in **Figure 3**. Metabolite I was identified as 2-methyl-1,4-benzenediol with a molecular ion peak of m/z 124 plus 107, 95, 77, and 67 mass fragments, and metabolite II was identified as 4-amino-3-methylphenol with a molecular ion peak at m/z 123 plus 106, 94, 77, and 67 as the main mass fragments. Both were exact matches to the spectral library. Metabolites III and IV exhibited different retention times but produced almost identical mass spectra, yielding base peak ions at m/z 152, with 169, 124, 77, and 65 as the main mass fragment ions in both cases. The m/z 152 ion resulted from a loss of water from the molecular ion m/z 169, and we therefore identified metabolites III and IV as singly hydroxylated derivatives of MNP, with hydroxylation occurring either on the benzene ring or on the methyl group.

When 2-week old 1.5-L scaled-up cultures of *A. niger* that were grown with 70 mg/L MNP were extracted and analyzed by LC-MS, molecular ion peaks corresponding to the molecular weights of the four metabolites previously identified by GC-MS analysis were confirmed. Scans were acquired in positive-ion mode and retention time, mass ion, and percent of metabolite recovery (based upon HPLC peak area integration) were observed as follows: (1) 2.7 min, $[M + H]^+ = 124.5$,

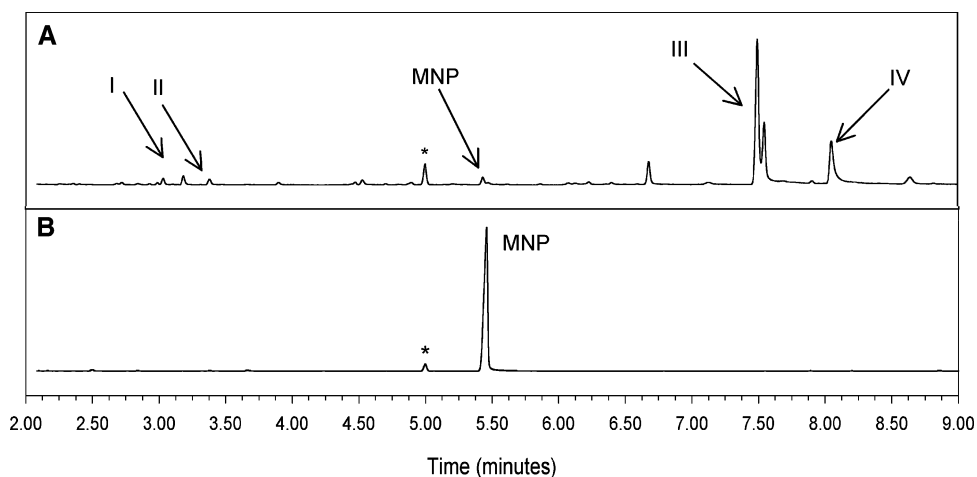


Figure 2. GC-MS total ion chromatograms showing the appearance of four metabolites (I–IV) during growth of *A. niger* on 70 mg/L MNP over a 16-day time period. (A) After 16 days; (B) at time 0. The position of the reference standard dibenzofuran is indicated by an asterisk (*).

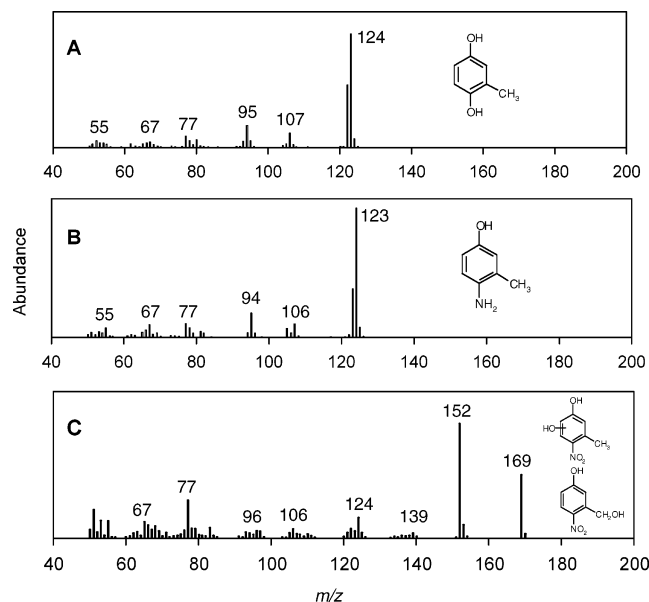


Figure 3. Mass spectra of the four metabolites formed during the biodegradation of MNP by *A. niger*. (A) Metabolite I, 2-methyl-1,4-benzenediol; (B) metabolite II, 4-amino-3-methylphenol; (C) metabolites III and IV (share the same mass spectra), hydroxylated derivatives of MNP.

corresponding to 4-amino-3-methylphenol, (<1.0%); (2) 5.0 min, $[M + H]^+ = 125.5$, corresponding to 2-methyl-1,4-benzenediol, (15.0%); (3) 27.7 min, $[M + H]^+ = 170.4$ corresponding to hydroxylated derivatives of MNP, (67.3%). In the case of LC analysis, one peak corresponding to a compound molecular weight of 169 (hydroxylated derivative(s) of MNP) was observed, which differed from the two peaks observed in the GC-MS analysis. Most likely the hydroxylated derivatives of MNP coeluted as one peak under the conditions that were employed in the LC analysis. In addition to confirmation of the previously identified metabolites from GC-MS analysis, the use of larger-scale cultures enabled the detection

of a new metabolite, metabolite V, by LC-MS analysis with a corresponding mass ion of $243.8 [M + H]^+$, peak retention time of 25.6 min (**Figure 4**), and represented 17.0% of the metabolite recovery. By consideration that the molecular weight of MNP is 153, the production of metabolite V would most likely have come about as a result of metabolite condensation. On the basis of the fact that the nitro group reduction product of MNP, the primary aromatic amine 4-amino-3-methylphenol, was also detected in the culture medium, a structure for metabolite V was proposed (**Figure 4**). This is the first report of the production of such an azo derivative during the biodegradation of MNP. Hydroxylated MNP derivatives were present in the largest concentration in the culture medium after incubation with *A. niger*, and by consideration that *A. niger* is known to possess cytochrome P-450 activity, such metabolites may likely be the result of this enzyme system. Hydroxylation of aromatic (20) and nonaromatic ring (21) structures by *A. niger* have indeed been shown previously. In addition to the oxidative metabolism of *A. niger*, the reduction product, 4-amino-3-methylphenol, was detected albeit at much lower levels than the oxidized products. Although *A. niger* has previously been shown to possess amine oxidase activity (22), it seems that this enzyme system may not act on aromatic amines. Instead, condensation of 4-amino-3-methylphenol to produce an azo derivative seems to have occurred. It has been shown very recently by Gorlewska-Roberts et al. (23) that aromatic amine condensation reactions occur when peroxidase and hydrogen peroxide are present with aromatic amines; in their case, aromatic amine condensation reactions which resulted in the production of azo and hydrazo derivatives occurred very rapidly in the presence of lactoperoxidase and hydrogen peroxide. In the case of *A. niger*, the organism has previously been shown to possess peroxidase activity (24), and it is also well known that certain oxidases in *A. niger*, such as D-amino acid oxidase or glucose oxidase may bring about the production of extracellular hydrogen peroxide during metabolism (25). Of course, direct oxidative coupling of aromatic amines via the production of laccase or peroxidase is another related possibility (26, 27). By consideration of these

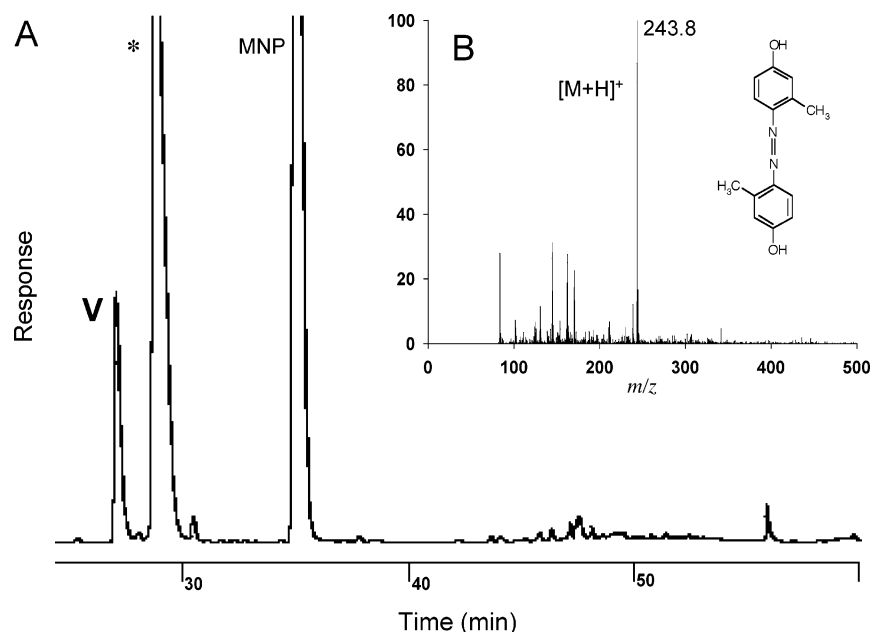


Figure 4. (A) HPLC chromatogram showing the appearance of metabolite V at 25.6 min after growth of *A. niger* in a 1.5-L volume of PDB with 70 mg/L MNP for 2 weeks. Also shown are peaks corresponding to (1) the parent compound (MNP), and (2) to the hydroxylated derivative(s) of MNP, indicated by an asterisk (*), which coeluted under these conditions, m/z of 170.2 $[M + H]^+$. (B) LC-MS spectrum of novel metabolite V, m/z of 243.8 $[M + H]^+$, and the proposed structure.

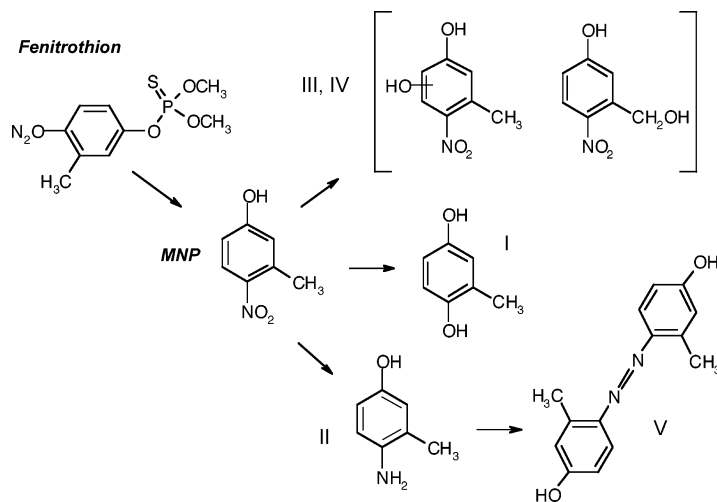


Figure 5. Proposed scheme for the biodegradation of MNP by *A. niger*. All metabolites indicated were tentatively identified in this study (I–V).

points, we postulate that these mechanisms likely play a role in the production of azo derivatives during the biodegradation of MNP by *A. niger*. The overall scheme of MNP biodegradation by *A. niger* to oxidized (metabolites II, III, IV), reduced (metabolite I), and dimerized products (metabolite V) is proposed in **Figure 5**. The major metabolism of MNP by *A. niger* appears to be hydroxylation of the benzene ring or the methyl group (metabolites III and IV).

In conclusion, biotransformation of MNP was shown to occur by the soil fungus *A. niger*, and five metabolites were identified, including an azo derivative that is most likely the result of aromatic amine condensation. Hydroxylated products of MNP were the predominant metabolites, and this may be beneficial in developing any waste-treatment process design by increasing the water solubility of the target compound and the potential for further biodegradation. Although low levels of a potentially carcinogenic arylamine, 4-amino-3-methylphenol, were detected, it seems that the predominant resultant product of MNP reduction was the azo derivative, and this may serve as a detoxification step of the arylamine. Cytochrome P-450 enzymes may be implicated in both the hydroxylation and reduction reactions of MNP; however a combination of peroxidase and oxidase activities may be responsible for the condensation reaction. Recently, the reduction product 4-amino-3-methylphenol was found to be a major metabolite of fenitrothion metabolism in rat liver in vivo; however condensation products were not reported (28).

Although the potential mutagenic effects are not clear (29, 30), MNP possesses estrogenic activity, has been implicated as a vasodilator (2), and may be present in food, on vegetation, and in soil, water, and air. By consideration of these points, an increase in our understanding of the biodegradation fate of MNP and its potential for its bioremediation may be useful for the future. Additionally, biological means to eliminating unwanted fenitrothion insecticide stocks may prove to be an alternative means to chemical elimination.

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