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Isolation and Characterization of a Novel Atrazine Metabolite Produced by the Fungus *Pleurotus pulmonarius*, 2-Chloro-4-Ethylamino-6-(1-Hydroxyisopropyl)Amino-1,3,5-Triazine

SEGULA MASAPHY,[1]* DAN LEVANON,[2] JACOB VAYA,[1] AND YIGAL HENIS[3]

Migal Technological Center, Kiryat Shmona 10200,[1] Institute of Soil and Water, Volcani Center, Bet Dagan,[2] and Otto Warburg Center for Biotechnology in Agriculture, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100,[3] Israel

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The white rot fungus *Pleurotus pulmonarius* exhibited metabolism of atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) in liquid culture, producing the dealkylated metabolites desethylatrazine, desisopropylatrazine, and desethyl-desisopropylatrazine. A fourth, unknown metabolite was also produced. It was isolated and was identified as 2-chloro-4-ethylamino-6-(1-hydroxyisopropyl)amino-1,3,5-triazine by gas chromatography-mass spectrometry, Fourier transformed infrared spectroscopy, and ^{1}H nuclear magnetic resonance analysis. The structure of this metabolite was confirmed by chemical synthesis of the compound and comparison with the fungally produced metabolite.

The chlorinated triazine atrazine is widely used as an herbicide all over the world. However, it causes ecological and public health problems and is considered recalcitrant (2, 7, 11). There is a need for its removal from nontarget environments, and much work is being done to isolate microorganisms (mainly from enriched environments) capable of degrading triazines (7, 9, 12). Atrazine is only slowly degraded in the soil, yielding metabolites such as desethylatrazine (CEAT; 2-chloro-4-ethylamino-6-amino-1,3,5-triazine), desisopropylatrazine (CAIT; 2-chloro-4-amino-6-isopropylamino-1,3,5-triazine), desethyl-desisopropylatrazine (CAAT; 2-chloro-4-amino-6-amino-1,3,5-triazine), and hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-1,3,5-triazine) (15, 24), which can be degraded further (3). The nomenclature proposed by Cook (7) for atrazine metabolites is used here and includes the following abbreviations: A, amino; C, chloro; E, ethylamino; I, isopropylamino; O, hydroxy; and T, triazine ring. In some studies, soil fungi were found to be dominant in causing the dealkylation of atrazine, while bacteria were responsible for its further degradation and mineralization (8, 14, 17). Bacterial transformation and clay mineral surfaces could oxygenate atrazine to hydroxyatrazine (13). Other than hydroxyatrazine, no oxygenated or hydroxylated biologically transformed atrazine has been isolated from either fungal or bacterial cultures.

In recent years, white rot fungi have been shown to be capable of biodegrading environmental pollutants (in particular, halogenated aromatic compounds) because of their oxidative degradation capacity, which enables them to decompose lignin (6). In our laboratory, we studied the white rot fungus *Pleurotus pulmonarius*, which was found to be capable of lignocellulose degradation via induceable enzymes (18). The same strain (*P. pulmonarius* 3014; Somycel Co., Langeais, France) was used to study atrazine degradation in order to find out more about the degradation and biotransformation abilities of this fungus. The present report concerns the kinetics of the process, the disappearance of atrazine, and the accumulation of metabolites in the culture supernatant, as well as identification of the metabolites, focusing on a novel metabolite produced by this fungus.

Atrazine degradation and metabolite production studies. *P. pulmonarius* was cultured in 100 ml of potato dextrose broth (15 g/liter; Difco), autoclaved at 121°C for 20 min in 250-ml flasks, and incubated in an orbital shaker (New Brunswick Scientific, Edison, N.J.) at 120 rpm and 28°C (18). Atrazine (98%; Riedel-De-Haen, Hanover, Germany) in methanol at a 20-mg/liter final concentration was added to the culture after 4 days of growth (three replicates). Atrazine was also added to a 4-day-old culture, which was heat killed by autoclaving. The disappearance of atrazine and the accumulation of metabolites were monitored by gas chromatography (GC) analysis in a GC (model 6000; Varian, Palo Alto, Calif.) equipped with a nitrogen-phosphorus detector. At each sampling time, a 1-ml aliquot of the culture supernatant was extracted in 2 ml of ethyl acetate. Prometryne (98%; Riedel-De-Haen) was used as an internal standard. The extract was further concentrated to 0.1 ml by air flow, and 2 μl of the extract was injected into a 30-m DB17 capillary column (J and W Scientific, Folsom, Calif.) with a split (1:20) injection system. The column oven was programmed from 200°C (2-min hold) to 250°C, with a gradient of 3°C/min. The temperature of the injector was 230°C, and that of the detector was 300°C. The carrier gas was He.

Under these conditions, four metabolites were detected in the culture supernatant, and their concentrations increased with time (Fig. 1) in the live culture. Three of the metabolites were identified as CAIT, CEAT, and CAAT by comparison with authentic compounds (obtained from Riedel-De-Haen). The comparison was performed by GC (conditions as mentioned above) and by thin-layer chromatography (TLC) on silica gel (kieselgel 60 F_{254}; Merck, Frankfurt, Germany), with ethyl acetate as the mobile phase. In this comparison, R_f values were as follows: atrazine, 0.86; CAIT, 0.75; CEAT, 0.69; and CAAT, 0.5. The fourth metabolite was unknown and had an R_f value of 0.6. None of the four metabolites was detected in the heat-killed culture, and only 5% of the atrazine disappeared from this culture after 18 days of incubation.

Identification of the unknown metabolite. The unknown

* Corresponding author.

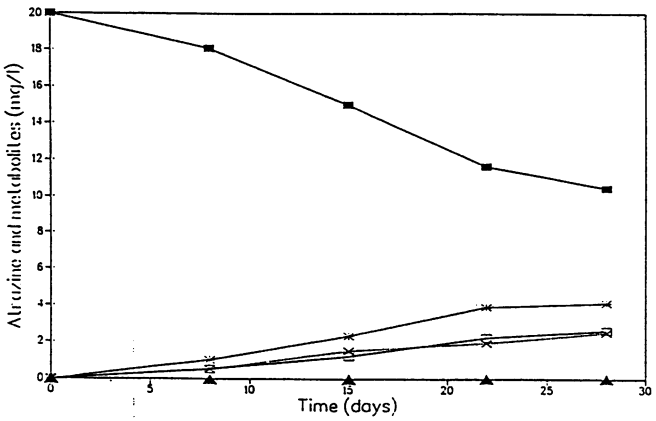

FIG. 1. Atrazine disappearance and metabolite accumulation in *P. pulmonarius* culture as monitored by GC analysis. Atrazine was added in a 20-mg/liter final concentration on day 4 of growth. The concentration (milligrams per liter) was calculated after identification of all compounds. ■, atrazine; □, CEAT; *, CAIT; ▲, CAAT; ×, CEIOT.

metabolite was found to have a lower R_f value then CAIT, CEAT, and atrazine, but a higher R_f value than CAAT, in the TLC system. This indicated that the unknown metabolite was more polar than atrazine, CAIT, and CEAT but less polar than CAAT. The crude ethyl acetate extract (containing atrazine and metabolites from the culture supernatant) was then subjected to mass spectrometry (MS) (Finnigan 4020 GC-MS low-resolution quadrapole; Finnigan Instruments, San Jose, Calif.). Two modes of GC-MS analysis were used; electron impact and chemical impact (CI). One microliter of the extract was injected into a 30-m DB5 capillary column. Operational conditions were as follows: oven temperature, programmed from 180 to 250°C at 3°C/min; injector temperature, 230°C; detector temperature, 300°C; carrier gas, He, with a split ratio of 1:20. Methane was used as the reagent gas in the CI mode. Direct CI-mode detection was also performed for the isolated unknown metabolite (as described below), and in this case the source temperature was 210°C. The GC-MS profiles of characteristic ion fragmentation of the unknown metabolite were first obtained in the electron impact mode. The unknown metabolite had a profile similar to that of the parent molecule, atrazine (atrazine profile not shown); i.e., the chlorine and alkyl groups were present in the product MS profile (Fig. 2A). However, whereas for atrazine the molecular ion could be detected (*m/z* 215, M^+), no molecular ion peak could be identified for the unknown metabolite in this mode; i.e., the molecule had been easily fragmented. To analyze the unknown metabolite, CI-mode GC-MS was used, with methane as the reagent gas, injecting the crude ethyl acetate extract. The molecular ion peak was identified as *m/z* 232 ($M^+ + 1$); i.e., $M^+ = 231$ (Fig. 2B), concluded from peaks 272 ($M^+ - C_2H_5^+$) and 260 ($M^+ - C_2H_5^+$). This is 16 mass units higher than the peak for atrazine itself, which could indicate the incorporation of a single oxygen atom into the molecule as a hydroxyl group, but not as a ketone, aldehyde, or amide group, which would cause the release of two protons. A fragment of 18 mass units (H_2O) was eliminated ($M^+ + 1 - H_2O = 214$). This could indicate, again, the presence of a hydroxyl group in the molecule. The same fragmentation profile was obtained when the pure isolated unknown metabolite was subjected to direct MS analysis.

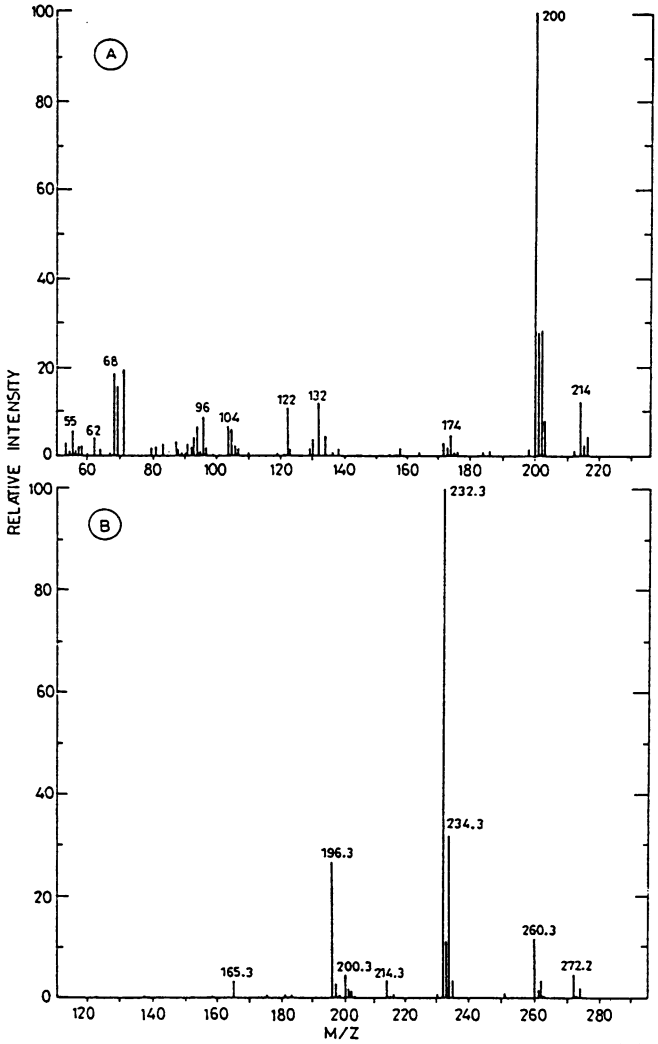

FIG. 2. Mass spectral data of the unknown metabolite: (A) electron impact mode; (B) CI mode. Methane was used as the reagent gas.

In order to confirm the chemical structure, an isolation procedure to obtain high concentrations of the pure compound was performed. The fungus was grown in high volumes (three 1-liter volumes of potato dextrose broth medium in 3-liter Erlenmeyer flasks, grown under the same conditions as given above). After 25 days, the biomass was separated from the culture supernatant by centrifugation for 10 min at 16,000 × *g*, and atrazine and the metabolites were extracted (three times) from the supernatant with 0.5 volume of ethyl acetate. This crude extract was concentrated in a rotary evaporator and mixed with a small amount of silica gel (230 to 400 mesh; Merck), and then the mixture was loaded onto a glass column packed with silica gel. The compounds were eluted with chloroform-methanol (95:5, vol/vol). Fractions of 1 to 2 ml were collected and screened on TLC silica gel plates for the unknown metabolite. The fractions containing the unknown metabolite were pooled, concentrated, and subjected to direct GC-MS, infrared (IR) spectroscopy (Nicolet Fourier transformed-IR instrument; Nicolet, Madison, Wis.), and ^1H nuclear magnetic resonance (NMR) spectroscopy (300 MHz, Bruker spectrometer; Bruker Instruments, Billerica, Mass.).

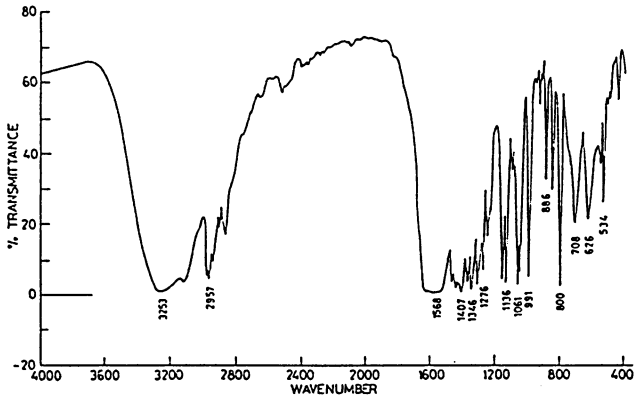

FIG. 3. IR spectrum of the unknown metabolite; a KBr pellet was used.

The presence of a hydroxyl group was confirmed by Fourier transformed-IR and NMR analyses. The IR spectrum of the unknown metabolite (Fig. 3) showed several significant peaks at 3,250 (NH, OH), 1,276 (OH bending) and 1,061 (C—O stretching) cm^{-1} (23). The two last peaks were found in the metabolite spectrum (Fig. 3) but were absent in the atrazine spectrum (data not shown). The ^1H NMR spectrometer was used at 300 MHz, with tetramethylsilane as the internal standard and $CDCl_3$ as the solvent. D_2O was added to detect exchangeable protons. Decoupling was performed at δ = 1.2 ppm to elucidate the structure further. The NMR spectrum showed the following signals of protons (Fig. 4A): peaks A (CH_3—g, t, CH_3—a, 6H), B (CH_2—e, f; m, 2H), C and D (CH_2—c, d; m, m, 2H), E (CH—b, br, 1H), F (OH, s, 1H), and G (NH, br, 2H) (a, b, c, d, e, f, and g are protons at the positions shown on the molecular structure in Fig. 4). When D_2O was added, peak F disappeared immediately and then peak G disappeared, indicating exchangeable protons (compare Fig. 4A and B). From decoupling studies (Fig. 4C), it seems that decoupling at 1.2 ppm (methyl groups) simplified peaks B and E (one proton), indicating that both peak groups are adjacent to the methyl groups in the molecule and that the NH group is bonded to the secondary carbon of the propyl group (^1H NMR showed one proton). Peak A (δ = 1.2 ppm) was composed of six protons, indicating the existence of two methyl groups in the molecule. The two methyl groups were not identical according to ^1H NMR (one clear triplet and the second unidentified splitting) (Fig. 4A), suggesting the presence of the methyl of the ethyl group (triplet H) and one methyl of the propyl group. It could therefore be concluded that the hydroxyl was bonded on a primary carbon of the propyl side chain of the molecule.

From the above data, the structure of the new metabolite was identified as 2-chloro-4-ethylamino-6-(1-hydroxyisopropyl)amino-1,3,5-triazine (CEIOT).

To fully identify the new metabolite found in this work, chemical synthesis was performed by the method of Palmer (20). Synthesis was carried out in two steps involving separate Cl substitution replacement of cyanuric chloride (Aldrich Chemical, Milwaukee, Wis.) in two different positions on the triazine ring. The two reactions took place in the same medium but under different conditions for each derivation. Ethylamine (in 70% water; Aldrich Chemical) was added to cyanuric chloride in acetone at 0°C in alkaline medium for the first (ethylamine) derivation. More drastic

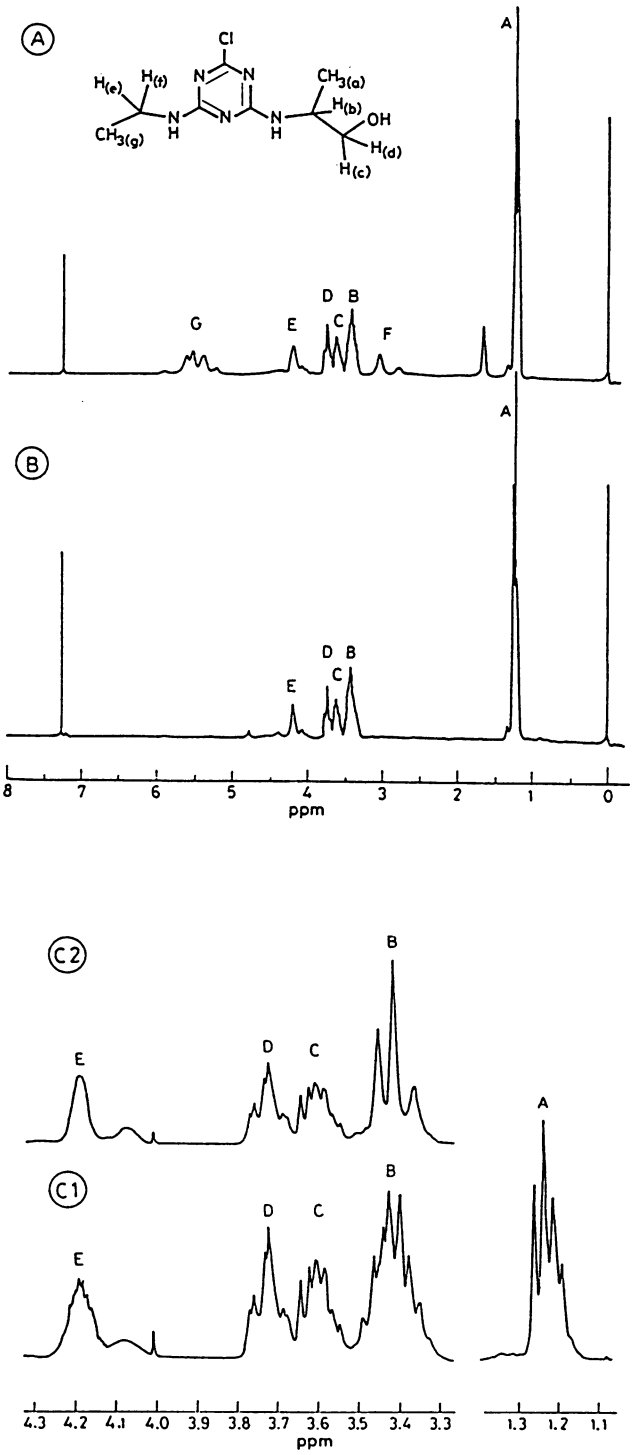

FIG. 4. ^1H NMR spectrum of the unknown metabolite. The isolated metabolite was dissolved in $CDCl_3$ and analyzed without (A) or with (B) the addition of D_2O and after methyl decoupling at δ = 1.2 ppm (C1 [before decoupling] and C2 [after decoupling]). Notice the disappearance of exchangeable H, indicating NH (5.7 to 5.2 ppm) and OH (3.1 to 2.7 ppm), after D_2O addition and the simplification of peaks B and E after decoupling.

conditions were used for the second (propanolamine) derivation (30°C and addition of two equivalents of NaOH), using 2-amino-1-propanol (Aldrich Chemical). Separation of the crude product on TLC yielded fluorescent bands which were subjected to GC to identify the compound by the similarity of its RT value to that of the unknown fungal metabolite. To purify the chemically synthesized compound for further analysis, flash chromatography on a silica gel column was used, as described above for the fungally produced chemical. The synthesis yielded two main compounds detectable by TLC, one of which was identical in its R_f on TLC and its retention time on GC analysis to the unknown metabolite produced by the fungus. After purification by flash chromatography, the yield of the chemically synthesized metabolite was found to be 50%. The compound was identical to CEIOT produced by the fungus on the basis of comparisons with data from GC, TLC, direct MS, IR spectroscopy, and NMR. The melting point of the new compound was determined with a Buchi 520 instrument: it was 135°C (ethyl acetate), compared with 174°C for atrazine.

The isopropyl-hydroxylated metabolite accumulated in the medium at rates similar to those of the monodealkylated products (Fig. 1). Although the incorporation of oxygen into the atrazine alkyl groups has been found in chemical photooxidation and ozonization, producing ketone, aldehydes, and amides (16, 22), and although bacteria can ring hydroxylate the molecule by dechlorination (7), no isolation of a propyl-hydroxylated product has been reported previously for any bacterially or fungally transformed atrazine. Alkyl hydroxylation of the ethyl group of the methylthiotriazine, terbutryne, by wheat and potato cell culture has been reported, as has ring hydroxylation of the same molecule (10). In the same study, atrazine was only either N dealkylated or glutathione conjugated. Hydroxylation (either aromatic or aliphatic) is an important initial detoxification step of xenobiotics in general, and pesticides in particular, by plants, mammals and microorganisms. Although *P. pulmonarius* could hydroxylate the isopropyl alkyl group, as reported here, no fungally produced hydroxyatrazine (aromatic hydroxylation) could be detected in the culture supernatant (using high-pressure liquid chromatography at a detection limit of as low as 100 μg/liter it was compared with authentic hydroxyatrazine). Other fungi have shown the ability to hydroxylate a wide range of pesticides, mainly by ring hydroxylation, including carbaryl, phenoxyacetic acid, and 2,4-dichlorophenoxyacetic acid (4). In recent years, the hydroxylation of polyaromatic hydrocarbons has also been demonstrated (21). Aliphatic hydroxylation has been less reported in fungal xenobiotic transformation, although hydroxylation of carbaryl by several soil fungi, producing a hydroxymethyl metabolite, has been demonstrated (5). It has been suggested that hydroxylation could occur in the oxygenation process of N dealkylation, although no hydroxylated compound has been isolated (7, 14, 19).

The fungus could also N dealkylate the atrazine in both of the alkyl groups. It has been suggested that N dealkylation is effected by several enzymatic systems, such as P-450 monooxygenases and chloroperoxidases, as well as by chemical ozonization and photooxidation (16, 19, 22). Several oxidative mechanisms are thought to cause N dealkylation, but the most widely accepted one is the incorporation of a single oxygen atom into the molecule in the alkyl side chain, producing an unstable intermediate, which is then readily degraded with cleavage of the oxygenated alkyl group. This is thought to be the case for N dealkylation of triazine in plant and mammalian tissues (1, 10), as well as in fungal and bacterial cultures from soil (7, 15).

Oxygenation of alkyl groups may occur before N dealkylation of triazine and other compounds, as suggested by many researchers referenced above. If this is true in N dealkylation of *P. pulmonarius*, then this fungus could be incorporating an oxygen atom separately into three different positions in the atrazine alkyl groups. The first would be incorporated into the C-2 position on the propyl side chain, and the second would be incorporated into the ethyl group. In both, an unstable intermediate would be produced; this would then be dealkylated and would therefore not be detectable in the culture. The third would be incorporated into the methyl group in the isopropyl side chain, producing a stable hydroxylated metabolite. Unlike *P. pulmonarius*, which exhibits isopropyl hydroxylation, another white rot fungus examined in our laboratory, *Phanerochaete chrysosporium*, could only N dealkylate the parent molecule, not hydroxylate it (unpublished data).

The fungus *P. pulmonarius* was not isolated from enriched culture, and its ability to dealkylate and hydroxylate atrazine was not dependent on previous incubation with the compound. This white rot fungus could therefore be demonstrating atrazine side chain dealkylation and hydroxylation via the constitutive metabolic activities of natural molecules.

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