RSC Advances

PAPER

Cite this: *RSC Adv.*, 2020, **10**, 19659

Metabolism of insecticide diazinon by *Cunninghamella elegans* ATCC36112

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Received 10th March 2020
Accepted 23rd April 2020

DOI: 10.1039/d0ra02253e

rsc.li/rsc-advances

The fungal metabolism of diazinon was investigated and the microbial model (*Cunninghamella elegans* ATCC36112) could effectively degrade the organophosphorus pesticide (diazinon) mediated by cytochrome P450, which was mainly involved in oxidation and hydrolysis of phase I metabolism. Approximately 89% of diazinon was removed within 7 days and was not observed after 13 days with concomitant accumulation of eight metabolites. Structures of the metabolites were fully or tentatively identified with GC-MS and ^{1}H, ^{13}C NMR. The major metabolites of diazinon were diethyl (2-isopropyl-6-methylpyrimidin-4-yl) phosphate (diazoxon) and 2-isopropyl-6-methyl-4-pyrimidinol (pyrimidinol), and formation of minor metabolites was primarily the result of hydroxylation. To determine the responsible enzymes in diazinon metabolism, piperonyl butoxide and methimazole were treated, and the kinetic responses of diazinon and its metabolites by *Cunninghamella elegans* were measured. Results indirectly demonstrated that cytochrome P450 and flavin monooxygenase were involved in the metabolism of diazinon, but methimazole inhibited the metabolism less effectively. Based on the metabolic profiling, a possible metabolic pathway involved in phase I metabolism of diazinon was proposed, which would contribute to providing insight into understanding the toxicological effects of diazinon and the potential application of fungi on organophosphorus pesticides.

1. Introduction

Organophosphorus pesticides (OPs) have largely replaced the organochlorines from the mid-1960s,[1] which constitutes an important aspect of modern agriculture. OPs are used to ensure better yield and quality of fruits and vegetables, but at the same time the contamination of food by OPs may increase their danger to humans.[2] Due to the widespread use of OPs, residual OPs continue to accumulate in animal tissues and pass from one trophic level to another within food chains.[3] So degradation of OPs with a more economical and pro-environmental strategy such as microorganisms is very important and urgent.

Diazinon [*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate] is an phosphorothioate insecticide and acaricide with contact, stomach, and respiratory action.[4] It has been used in animal houses and households to control the ingestion and chewing of insects and mites on various crops, lawns, fruits and vegetables.[5,6] Diazinon presents a water solubility of 60 mg L^{-1} and the partition coefficient (K_{oc}) is 1000 mL g^{-1} in soil.[5,7] Besides the solubility and adsorption of pesticides, the toxicity of pesticides to non-target species should also be considered.[8] Based on the concern about non-targeted biotoxicity of diazinon, more and more studies indicated that diazinon caused oxidative damage through the generation of free radicals and induced lipid peroxidation and DNA fragmentation,[2,9,10] and diazinon could damage the liver and kidneys, causing severe histopathological damage.[2,13] Furthermore, diazinon will be oxidatively denatured to diazoxon when it enters the human body.[11] This metabolite is more toxic than the parent compound, mainly in inhibiting acetylcholinesterase,[12] leading to a cholinergic syndrome and associated neurotoxicity.[2]

Metabolism studies are very important for the understanding of pesticide toxicity and safety.[14] At present, the research on diazinon metabolism mainly focuses on the metabolites, pathways and related metabolic toxicology of diazinon in mice, dogs and other mammals.[15–17] In addition, the metabolism and toxicity of the pesticide *in vitro* metabolic model (human colon carcinoma cells and human liver microsomes, *etc.*)[2,18] and bacteria (*Acinetobacter* and *Pseudomonas* sp,

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etc.)[19] have also been reported. However, there are a few reports on fungal metabolism of diazinon.[20]

Cunninghamella elegans (*C. elegans*), a filamentous fungus, is widely used as a microbial model of the mammalian metabolism of different xenobiotics, including pesticides, drugs and other pollutants.[21–24] Previous studies have shown that the fungal biotransformation of many pesticides is mainly mediated by the cytochrome P450 (CYP450s),[24–28] which has similar metabolic systems and processes as human metabolism. Diverse chemical reaction are catalyzed by P450 monooxygenases including classical reaction of hydroxylation, C=C double bond epoxidation reaction, and aromatic ring hydroxylation reaction, even the cleavage of the C–C bond subjected to multiple substrate oxidations.[29] So, the application of this microbial model and enzyme inhibitor can be speculated or assisted to confirm the pesticide metabolism pathway in mammals, therefore greatly reducing the cost of experimental animals and experiments. The main purpose of this study is to use *C. elegans* to biotransform diazinon and identify the metabolites with gas chromatography mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). Under the action of enzyme inhibitors, oxidases involved in metabolism have also been studied.

2. Materials and methods

2.1 Chemicals and reagents

Diazinon, diazinon-O-analog (diazoxon) and 2-isopropyl-6-methyl-4-pyrimidinol (pyrimidinol) standards (purity > 98%) purchased from Chem service (West Chester, PA, U.S.A.). Methimazole (MZ) and piperonyl butoxide (PB) were from Macklin® (Shanghai, China). Ethyl acetate and methanol were of HPLC grade and from Burdick and Jackson® (Seoul, Korea). Anhydrous sodium sulfate and sodium chloride were obtained from Junsei (Tokyo, Japan). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were supplied by BD Korea (Seoul, Korea). All other chemicals were of reagent grade and of the highest purity available.

2.2 Microorganism

Cunninghamella elegans ATCC36112 was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). Stock cultures of *C. elegans* ATCC36112 were maintained on PDA plates at 28 °C. Spores and mycelia from several plates were used to inoculate on PDA medium. At 28 °C, *C. elegans* ATCC36112 grew for 48 h.

2.3 Metabolic reaction

Approximately 2 g mycelia was transferred into fresh PDB medium (500 mL), followed by the addition of diazinon (5 mg in 1 mL acetonitrile), which were incubated at 28 °C with shaking at 170 rpm for 13 days. The final pH value was adjusted to 7.0 with 0.1 M phosphate buffer.[30] Control experiments were conducted in the absence of either diazinon (blank control) or fungi (negative control). According to the sterilization standard of PDA and PDB, all the media were sterilized at 121 °C for 15 min. The natural pH value of the medium was 5.1 ± 0.2. The pH was measured by Orion Star™ A211 pH Benchtop Meter.

2.4 Enzyme inhibitor reaction

Additionally, the effects of enzyme inhibitors (PB and MZ) on the biodegradation of *C. elegans* were studied. Different concentrations (2, 10, 50 mg L^{-1}) of enzyme inhibitor stock solutions were added to the inoculated PDB medium (500 mL, pH 7.0), followed by adding diazinon (5 mg in 1 mL acetonitrile) and pre-incubated for 12 hours. Sample was collected at 2 hours, 1, 3, 5, 7, 10, and 13 days, respectively.

2.5 Extraction of metabolites

To detect parent pesticide and its biotransformation products, the culture medium was extracted. At each metabolic reaction time (2 hours, 1, 3, 5, 7, 10, and 13 days), 50 mL of the culture medium was transferred to a 1000 mL separatory funnel with 20 g of NaCl, and afterward extracted twice with ethyl acetate (100 and 50 mL). The combined ethyl acetate phases were passed through anhydrous Na_2SO_4 and evaporated at 35 °C. Subsequently, the obtained residue was dissolved in methanol (2 mL) and filtered through a 0.22 μm polytetrafluoroethylene (PTFE) filter for HPLC and GC-MS analysis. Before GC-MS analysis, the extract was dried by nitrogen and dissolved in dry pyridine, derivatized with BSTFA + TMCS (99 : 1) at 70 °C for 37 min.

2.6 Enrichment and isolation of metabolites by large scale culture

To collect enough metabolites for subsequent analysis, 9 bottles (800 mL per each) culture medium were set up. The culture medium was extracted in multiple portions in a separatory funnel, then concentrated and dissolved in methanol. Isolation of the metabolites was carried out using a previously reported method.[25] Separation of metabolites was through silica gel (20 g) column chromatography (1.5 cm i.d. × 40 cm) with 50 mL of hexane/ethyl acetate by stepwise gradient elution (100 : 0, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90 and 0 : 100, v/v). Each eluted component was detected by HPLC. **M1** was eluted in a fraction of 30 : 70 (hexane/ethyl acetate), and **M2** was collected in a fraction of 0 : 100 (hexane/ethyl acetate). After drying, **M1** and **M2** were then dissolved in $CDCl_3$ (99.8%, Merck) for NMR.

2.7 Instrumental analysis condition

Quantitative analysis of diazinon was conducted using an Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, U.S.A.) equipped with Luna C18 column (250 mm × 4.6 mm, 5 μm, Phenomenex®, Torrance, CA, U.S.A.) at 40 °C. The mobile phase consisted water (A) and methanol (B), while the flow rate was 1 mL min^{-1} and the volume of injection was 10 μL. The gradient condition was as follows: 5% B at 0 to 1 min, 90% B at 20–25 min, 95% B at 30 min, 5% B at 32–40 min. Samples were detected by UV absorption at 245 nm.

GC-MS analysis was performed with a Bruker SCION TQ equipped with an CP-8400 autosampler. A fused-silica capillary column (Rxi-5Sil MS, 30 m × 0.25 mm i.d., 0.25 μm film thickness) was used for GC separation. The oven program was 1 min at 80 °C, 7 °C min^{-1} to 240 °C, and 5 °C min^{-1} to 295 °C (30 min). In the full-scan mode, electron ionization (EI) mass spectra in the range of 50–600 (m/z) were recorded at 70 eV electron energy. Helium was used as carrier gas at 1.0 mL min^{-1}. Splitless injections of 2 μL sample were carried out. The injector temperature was 260 °C, the interface temperature was set at 280 °C, and the solvent delay time was set to 5 min. Data analysis was performed with Bruker MS workstation software (version 8.0, Germany) and ACD/MS Fragmenter 2017 software (ACD Labs, Canada).

NMR spectroscopy was used to confirm the structure of metabolites. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz NMR spectrometer (Jeol JNM-LA400, JEOL Ltd., Tokyo, Japan) in $CDCl_3$ (99.8%, Merck) at 298 K.

3. Results and discussion

3.1 Metabolism of diazinon by *C. elegans*

HPLC analysis of the culture extract indicated that diazinon was transformed to two metabolites (**M1** and **M2**). In the range of 0.1 to 100.0 mg L^{-1}, there is a linear relationship ($Y = 6.2191x - 1.3314$, $R^2 = 0.9999$) between the concentrations of diazinon standard and the peak area. LOD (S/N > 3) was calculated as 0.1 mg L^{-1}. The structure of metabolites was shown in Fig. 5. For example, diazinon was detected at approximately 50% degraded in the culture at 2 days after treatment and was undetectable after 13 days (Fig. 1A), **M1** and **M2** were observed as major metabolites. With the production of metabolites, the accumulation of **M1** reached a maximum at 5 days and gradually decreased to trace level after 13 days, while **M2** showed a trend of continuous growth. Then the identification of those was carried out through large scale culture. Approximately, eight metabolites of diazinon were observed in culture supernatants including two major metabolites (**M1** and **M2**) by GC-MS (Fig. 3). Minor metabolites (**M3–M8**) were hardly located on the chromatogram because of the trace levels, so their formation pattern could not be established. In a sterilized control experiments, no appreciable degradation was observed.

In GC-MS, mass spectral details of those metabolites were described in Table 1. Molecular ion of **M1** was observed at m/z 288, which was 16 Da lower than that of diazinon (M^+, 304), suggesting that metabolic oxidative desulfuration took place in diazinon (P═S → P═O). The molecular ion and the fragment ions of **M1** could be speculated as diazoxon. In the case of TMS-derivatized **M2**, gave a molecular ion of m/z 224 from GC-MS analysis, it was 72 Da higher than pyrimidinol (M^+, 152), which would be fragmented from diazinon (Fig. 4). **M2** could be speculated as pyrimidinol. To verify this hypothesis, diazoxon and pyrimidinol were purchased and analyzed by GC-MS and NMR. GC-MS analysis showed the retention times of two standards at 30.9 and 18.2 min, and their molecular ion peaks at m/z 288 (16) and m/z 224 (17), respectively. ^{1}H and ^{13}C NMR spectra of metabolites matched with the structures of standards (Table

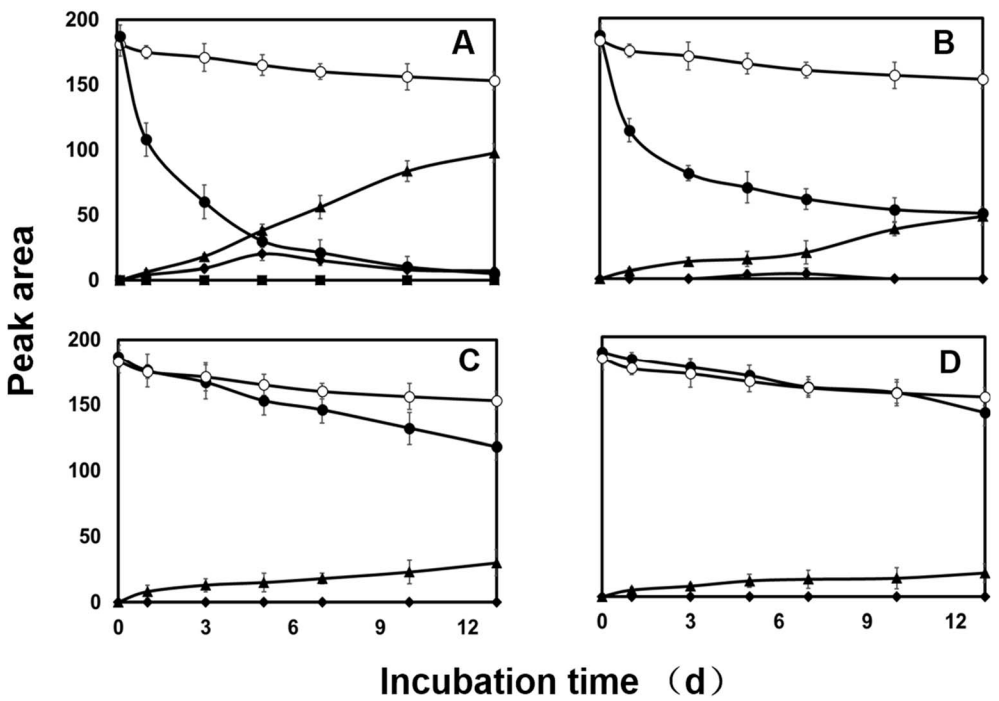


Fig. 1 Degradation and accumulation pattern of diazinon and metabolites by *C. elegans* with and without PB treatment (2, 10, and 50 mg L^{-1} PB for (B), (C), and (D); no PB for (A)). Controls include culture medium with fungi (blank control, ■) and sterile medium with diazinon (negative control, ○). Filled circles represent degradation of diazinon (●). The formation of metabolites is represented by filled rhombuses (M1, ◆) and triangles (M2, ▲), respectively. Error bars represent standard errors.

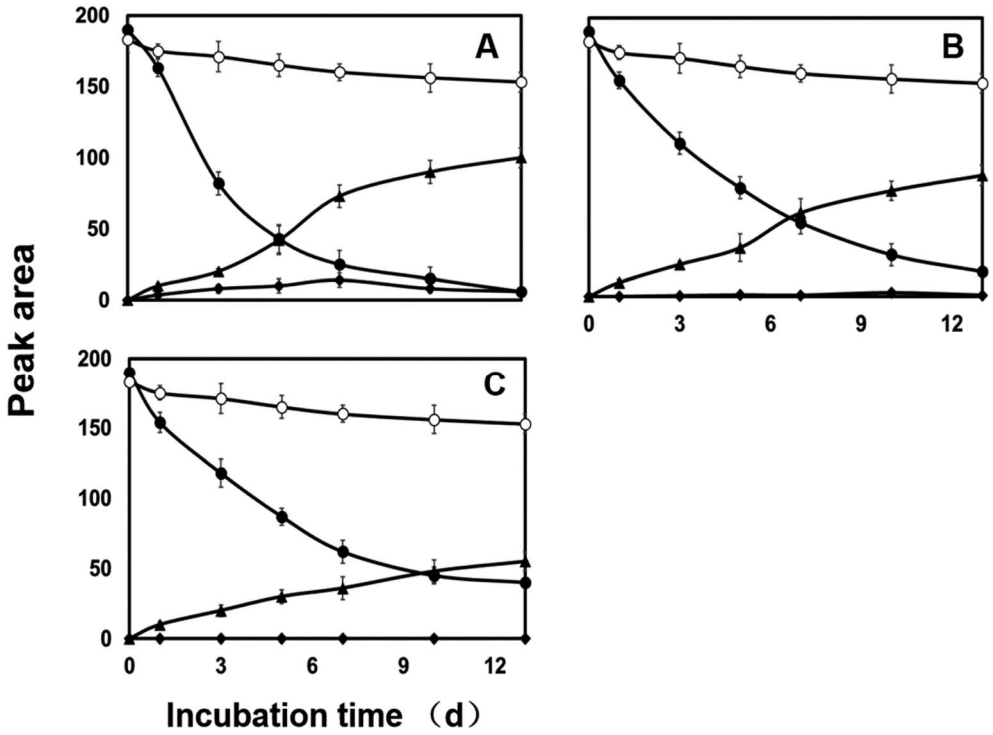


Fig. 2 Degradation and accumulation pattern of diazinon and metabolites by *C. elegans* in MZ treatment (2, 10, and 50 mg L^{-1} MZ for (A), (B), and (C), respectively). Controls include culture medium with fungi (blank control, ■) and sterile medium with diazinon (negative control, ○). Filled circles represent degradation of diazinon (●). The formation of metabolites is represented by filled rhombuses (**M1**, ◆) and triangles (**M2**, ▲), respectively. Error bars represent standard errors.

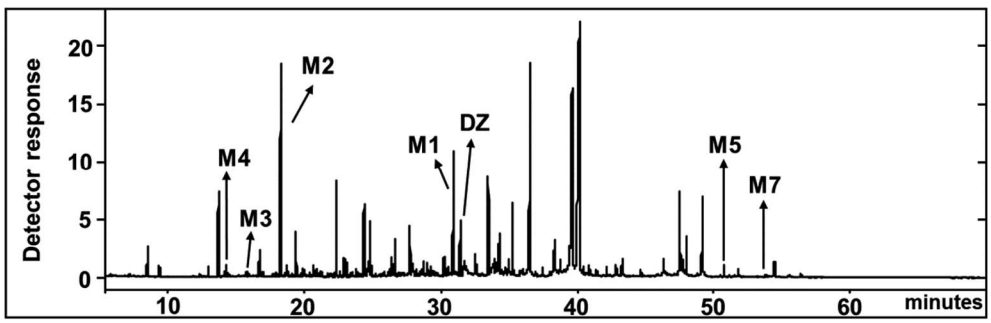


Fig. 3 Total ion chromatograms (TIC) of diazinon (DZ) and its TMS-derivatized metabolites by *C. elegans* at 3rd day.

2). On the basis of these results, **M1** and **M2** were identified as diazoxon and pyrimidinol, respectively.

To obtain useful information for explaining the ion spectrum of unknown degradation products, we continued to study the MS^2 fragment ions of diazinon, diazoxon and pyrimidinol (Fig. 4, Table 1). Diazinon showed its molecular ion at *m*/*z* 137 ($[M + H]^+$) (100), which was the most abundant fragment ion. This may be associated with the cleavage of the P–O-pyrimidine group. Similar reactions have been observed in diazoxon at *m*/*z* 137. The ion peaks at *m*/*z* 248 and 216 from parent represent consecutive eliminations of ethylene molecules, which may be formed by a classical elimination reaction involving a four-member transition state.[31] The ion at *m*/*z* 179 represents the cleavage of the P–O bond, accompanied by ethyl rearrangement or migration to an aromatic bonded oxygen atom.[32] As for pyrimidinol derivatized, it has a stable structure and fewer fragment ions are obtained under the condition of electron ionization, its fragment ion *m*/*z* 209 as the base peak may be formed by losing a methyl from isopropyl.

Other metabolites (**M3**–**M8**) were also observed but at trace levels. **M3** had shown its ion peak at *m*/*z* 242, suggesting that it would be a derivative of diethyl phosphorothioate. Other fragment ion observed at *m*/*z* 227, was corresponded to the loss of methyl groups from the precursor ion. The characteristic fragment at *m*/*z* 171 might be resulted from *m*/*z* 227 after losing two ethyl groups. Molecular ion of **M4** was observed at *m*/*z* 226, which was 16 Da lower than that of diethyl phosphorothioate (**M3**, *m*/*z* 242), indicating that P═S was oxidized to P═O. The

Table 1 Retention times and fragmentation patterns of diazinon and TMS-derivatized metabolites in GC-MS

Compound	Retention time (min)	Molecular weight and fragment ions[a] (*m*/*z*)
Diazinon (parent)	31.4	304 (M^+, 21), 276 (15), 248 (14), 216 (11), 199 (42), 179 (86), 152 (53), 137 (100), 93 (31)
Diazoxon (**M1**)	30.9	288 (M^+, 16), 273 (75), 260 (16), 217 (20), 151 (26), 137 (100)
Pyrimidinol (**M2**)	18.2	224 (M^+, 17), 209 (100), 196 (15), 181 (9), 126 (7)
Diethyl phosphorothioate (**M3**)	15.9	242 (M^+, 38), 227 (29), 199 (38), 171 (100), 165 (64), 153 (56), 137 (43), 121 (46)
Diethyl phosphate (**M4**)	14.3	226 (M^+, 4), 211 (9), 199 (14), 183 (12), 171 (4), 155 (100), 139 (7)
Hydroxydiazoxon (**M5**)	50.7	376 (M^+, 27), 361 (4), 303 (10), 251 (100), 236 (18), 197 (37), 155 (47), 105 (38)
Isopropenyl diazoxon (**M6**)	33.0	286 (M^+, 4), 271 (13), 192 (27), 179 (20), 149 (9), 147 (18), 137 (16), 73 (100)
2-Hydroxylated diazoxon (**M7**)	53.8	376 (M^+, 17), 361 (5), 343 (4), 296 (9), 251 (100), 223 (10), 209 (24), 195 (24), 181 (12), 169 (12), 155 (14)
Hydroxypyrimidinol (**M8**)	30.7	312 (M^+, 37), 297 (79), 282 (38), 267 (74), 253 (47), 223 (53), 193 (22), 126 (25), 73 (100)

[a] Values in parentheses are the relative abundance of specified fragment ions.

most abundant fragment ion *m*/*z* 155 might be resulted from *m*/*z* 211 after losing two ethyl groups. In addition, **M3** and **M4** can be matched by library search and they had also been confirmed in some animal experiments.[33,34] Therefore, **M3** and **M4** were tentatively identified as diethyl phosphorothioate and diethyl phosphate, respectively. Metabolites **M5** and **M7** gave similar fragment ions, and their molecular ions and base peaks were *m*/*z* 376 and *m*/*z* 251, respectively. The mass/charge *m*/*z* 251 might be formed by the cleavage of the aryl and alkyl group or ethyl rearrangement, and the fragment at *m*/*z* 223 was observed, indicating that an ethyl group was removed from the precursor ion *m*/*z* 251. Hydroxylation is an important way of pesticide metabolism by *Cunninghamella elegans*.[25,31,35] Similarly, hydroxylation may occur in **M5** and **M7**, both of them had been reported in the previous studies.[31,36,37] So **M5** and **M7** were tentatively identified as hydroxydiazoxon and 2-hydroxylated diazoxon, respectively. Molecular ion of **M6** was observed at *m*/*z* 286, which was 2 Da lower than that of metabolite **M1** (*m*/*z* 288). Fragment ion *m*/*z* 271 was corresponded to the loss of a methyl from the ion at *m*/*z* 286. The ion *m*/*z* 192 would be formed by the cleavage of the P–O-pyrimidine group and ethyl rearrangement. Furthermore, fragment ion *m*/*z* 149 and *m*/*z* 137 were obtained by the cleavage of the P–O-pyrimidine group. It was worth noting that *m*/*z* 149 was 2 Da lower than *m*/*z* 151 of diazoxon, indicating that two hydrogen atoms were removed from the isopropyl group of the nitrogen-containing heterocycle. Thus, metabolite **M6** was tentatively identified as isopropenyl diazoxon, which has also been reported before.[36] **M8** gave molecular ion at *m*/*z* 312, which was 88 Da (-OTMS) higher than that of metabolite **M2** (*m*/*z* 224). The characteristic fragment at *m*/*z* 297 may be obtained after losing a methyl, which has a similar fragmentation mechanism with pyrimidinol. It had been reported that Müecke *et al.* (1970) treated rats with diazinon ^{14}C-labeled in the pyrimidine ring and ethoxy groups, and hydroxypyrimidinol was identified in the urine.[38] So metabolite **M8** was tentatively identified as hydroxypyrimidinol (Fig. 5).

3.2 Effects of enzyme inhibitor on the degradation of diazinon

Both CYP450s and flavin monooxygenases (FMO) are phase I metabolic enzymes that catalyze the oxidation of sulfur and phosphorus compounds of xenobiotic compounds.[39] The enzyme inhibitor piperonyl butoxide (PB) and methimazole (MZ) were introduced to explore the response in diazinon metabolism.[25]

In PB-treated cultures, a large portion of diazinon still persisted compared to control. For example, residual diazinon was approximately 25–70% of the initial dose (Fig. 1B–D) after 13 days. **M2,** as a major metabolite, its concentration gradually increased until the end of the experiment in all cultures. However, the concentrations of **M2** were far less than those of the control. **M1** was only at trace level (2 mg L^{-1}, Fig. 1B) or was not observed (10, 50 mg L^{-1}, Fig. 1C and D) in PB-treatment experiment. Compared with the control, the degradation of diazinon was inhibited in the presence of PB, indicating that CYP450s may be involved in the biotransformation of diazinon. Previously, inhibition of CYP450s by metyrapone or carbon monoxide resulting in the attenuation of biotransformation reaction has been reported.[40,41] In MZ-treated cultures, diazinon rapidly dissipated in the low level culture (2 mg L^{-1} of MZ) and was undetectable at 13th day, while significant amounts of diazinon (5–20% of initial dose) still persisted in the high level cultures (10, 50 mg L^{-1} of MZ) (Fig. 2). Overall profiles of diazinon and metabolites with the lowest concentration of MZ (2 mg L^{-1}) were not distinguishable from those of the control (Fig. 2A). However, a less amount but similar response has been observed at higher concentrations of inhibitor (10 and 50 mg L^{-1}) (Fig. 2B and C). For example, **M1** was almost not

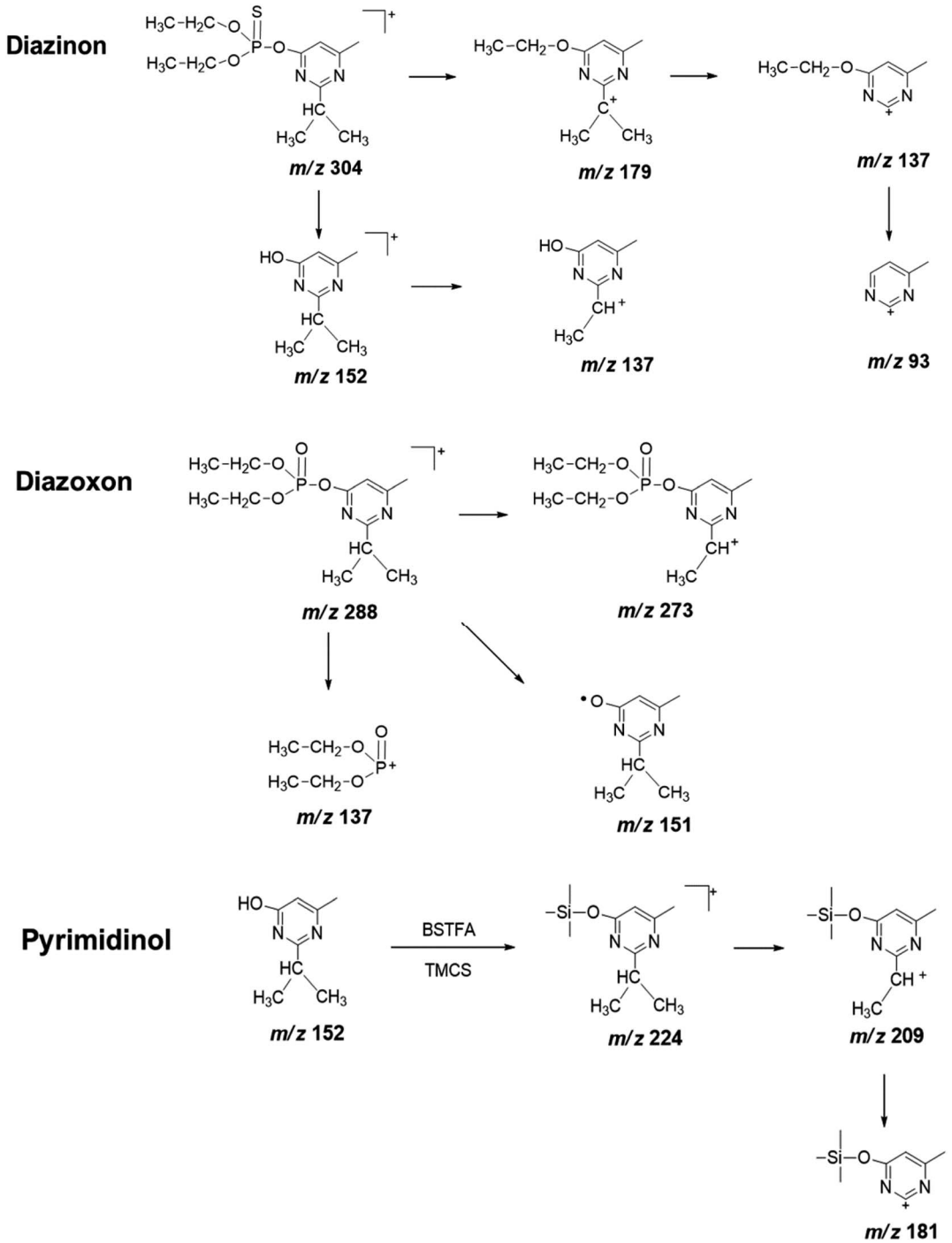
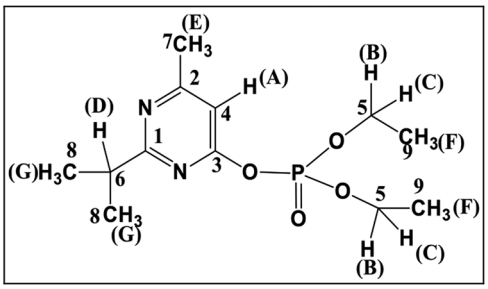
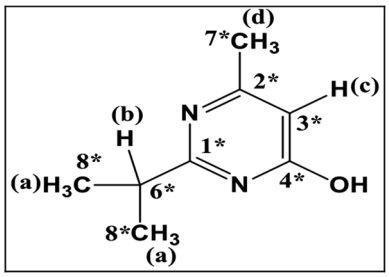


Fig. 4 Proposed fragmentation pathway of diazinon, diazoxon, and pyrimidinol standard under electron ionization conditions.

observed during the whole experiment at high concentrations, while **M2** gave the similar kinetic responses with those of the control or PB-treatment experiments. In this study, the residue of diazinon in MZ-treated culture was lower than that in PB-treated culture, which indicated that CYP450s were the major contributors to biotransformation. But the role of FMO in the oxidative metabolism was also not negligible,[25,36,42] which was mainly reflected in the inhibitory effect of MZ on biotransformation. Lim *et al.* (2017) have previously reported that CYP450s were the main catabolic enzymes in xenobiotic biotransformation and contribution of FMO was limited.[43] The change in **M1** was more pronounced only at the lowest concentration of inhibitor (2 mg L^{-1} of PB). This may be due to the inhibition of the activity of the responsible enzyme at higher dose, which affects the oxidative desulfurization process. In conclusion, CYP450s play a major role in the oxidative metabolism of diazinon.

Table 2 ^{1}H and ^{13}C NMR chemical shifts assignment of standards and metabolites

Analytes	^{1}H δ (ppm), assignment		^{13}C δ (ppm), assignment	
	1.31	H_G	16.08	C_9
	1.40	H_F	21.56	C_8
	2.49	H_E	24.23	C_7
	3.12	H_D	37.41	C_6
	4.34	H_B and H_C	65.03	C_5
	6.72	H_A	106.12	C_4
			164.40	C_3
			170.18	C_2
			175.81	C_1
M1, (metabolite)	1.31		16.05	
	1.40		21.50	
	2.49		24.11	
	3.12		37.26	
	4.34		65.13	
	6.72		106.12	
			164.35	
			170.15	
			175.76	
	1.33	H_a	20.51	C_{8*}
	2.30	H_d	24.24	C_{7*}
	2.92	H_b	34.57	C_{6*}
	6.17	H_c	110.15	C_{4*}
			165.42	C_{3*}
			165.99	C_{2*}
			166.67	C_{1*}
M2, (metabolite)	1.33		20.46	
	2.30		24.11	
	2.92		34.56	
	6.17		110.21	
			165.20	
			165.97	
			166.75	

On the basis of these results, the metabolic pathway of diazinon was proposed (Fig. 5). In the presence of *C. elegans*, two major pathways of biotransformation are proposed. The first pathway is oxidative desulfurization and hydrolysis reactions, and the second pathway is hydroxylation reaction. Two reactions in the first pathway were common in some animal experiments[44] and human liver microsome experiments.[11,45,46] Under the catalysis of CYP450s, diazinon was directly transformed into three different metabolites (**M1**, **M2** and **M3**). **M1** (diazoxon) was formed with the oxidative desulfurization pathway, **M2** and **M3** were formed with the hydrolysis reaction, which is consistent with the almost immediate onset of **M2** and **M3** at 2 h after treatment and fragment ion analysis. The low-toxic pyrimidinol (**M2**) was also obtained from diazinon *via* **M1** with oxidative desulfurization, hydrolysis pathways, which is the main degradation product in some soil,[47,48] plant[49] and animal[44] experiments, as well as documented in other pesticides.[50–52] For example, chlorpyrifos can form diethyl phosphate, diethyl phosphorothioate and 2,3,5-trichloro-4-pyridinyl alcohol through the above reactions.[53] In this study, **M1** is more toxic than diazinon, and there may be four ways to continue to decompose it. It will be degraded to **M2** and **M4** with the first pathway, and it will also be degraded to **M5** and **M7** with the second pathway. By observing the structure of minor metabolites (**M5**, **M7**, **M8**), we speculate on the occurrence of hydroxylation reactions, which may be related to CYP450s.[36]

One of the pathway for **M4** is the same as that of **M2**, it could be formed from diazinon *via* **M1** (diazinon → **M1** → **M4**). Here, we also detected diethyl phosphate (**M4**) and diethyl phosphorothioate (**M3**) except pyrimidinol, which indicated the occurrence of dearylation. We captured the trend that **M3** increased firstly and decreased gradually, while **M4** continued

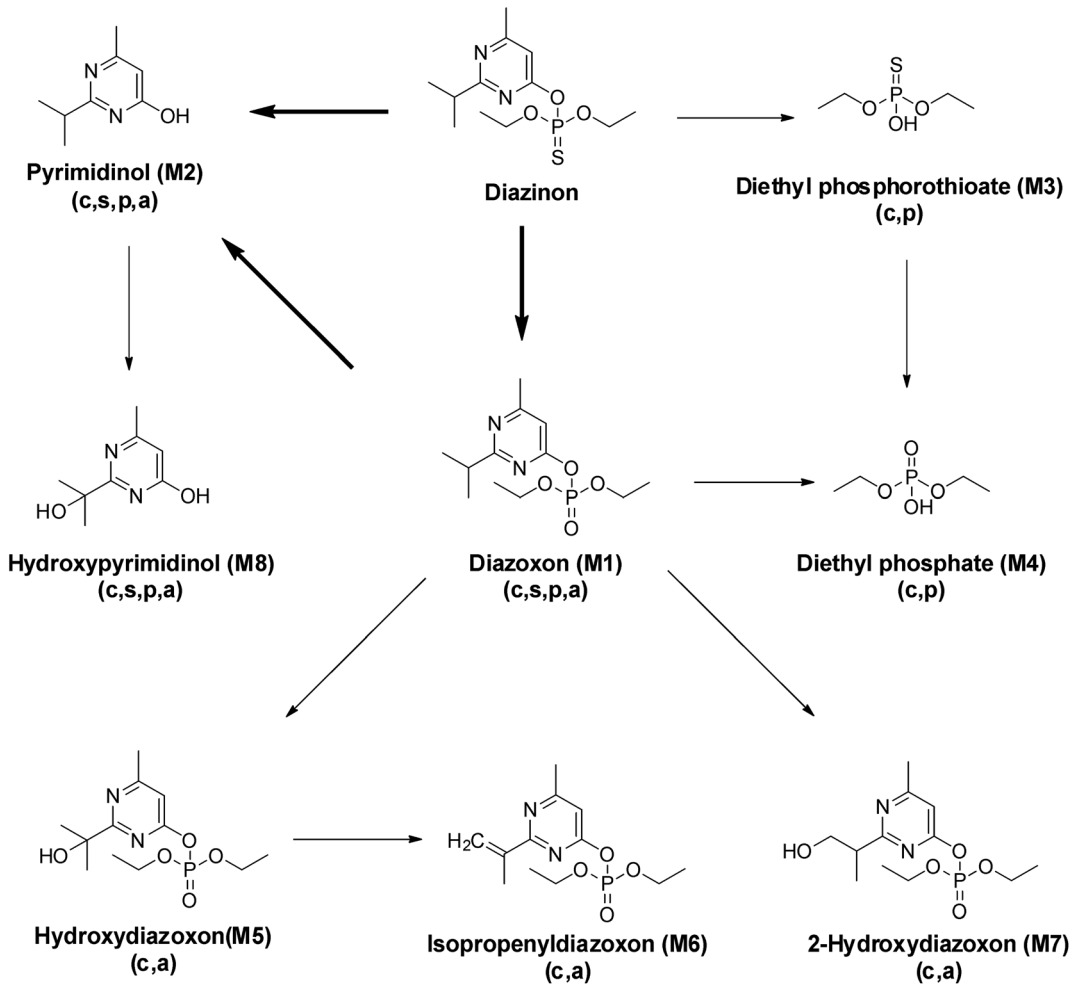


Fig. 5 Proposed metabolic pathways of diazinon by *C. elegans* and previously determined transformations in other environments. The main pathway is indicated by a thick arrow. Abbreviations: c, *C. elegans*; s, soil; p, plants; and a, animals.

to increase within 13 days. This may be the result of partial or complete conversion of **M3** to **M4**, so another way of **M4** is formed from diazinon *via* **M3** with hydrolysis and oxidative desulfurization pathways (diazinon → **M3** → **M4**). The rate of dearylation and desulfurization was related to the chemical structure of each pesticide and the CYP450s that catalyze the reactions.[11,38] Previous study using microsome from human liver showed that diazinon was more readily detoxified (dearylation) than bioactivated (desulphuration) under the catalysis of CYP450s,[45] which was necessary to fully understand the toxicological effects of pesticides on human body.

Furthermore, Casida (2011) had proved that CYP450s have *situ* selectivity in hydroxylation.[54] **M5** was firstly observed on the 5th day and **M7** on the 7th day. By comparing the structure of these two metabolites, we speculated that a hydroxylation reaction had occurred. **M5** was further metabolized through a dehydration reaction to give the respective isopropenyl substituted compounds **M6,** which was detected on the 7th day. **M8** was also found except **M1**, **M2**, **M3**, and **M4** on the first day. Based on the metabolic profiling, **M8** can only come from **M2**, which may be the result of hydroxylation reaction.

4. Conclusions

This study has demonstrated the biodegradation of diazinon in *C. elegans* and described its metabolic pathways. Eight metabolites were fully or tentatively identified with GC-MS and ^{1}H, ^{13}C NMR. CYP450s and FMO were involved in the phase I metabolism of diazinon. In addition, to fully understand the toxicological effects of pesticides on the human body, it is necessary to determine the relative importance of different pathways in future experiments.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This study was carried out with the support of the "Research Program for Agricultural Science & Technology Development (Project PJ0140182018)", National Institute of Agricultural Sciences, and Rural Development Administration, Republic of Korea.

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