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Article

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RESEARCH ARTICLE

Agricultural and Environmental Chemistry

Metabolism of an Insecticide Fenitrothion by *Cunninghamella elegans* ATCC36112

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Running title: Metabolism of fenitrothion by *Cunninghamella elegans*

ABSTRACT: In this study, the detailed metabolic pathways of fenitrothion (FNT), an organophosphorus insecticide by *Cunninghamella elegans* was investigated. Approximately 81% of FNT was degraded within 5 d after treatment with concomitant accumulation of four metabolites (M1-M4). The four metabolites were separated by HPLC and their structures were identified by MS and/or NMR. M3 is confirmed to be an initial precursor of others and identified as fenitrothion-oxon (FNTO). On the basis of their metabolic profiling, the possible metabolic pathways involved in phase I and II metabolism of FNT by *C. elegans* was proposed. We also found *C. elegans* was able to efficiently and rapidly degrade other organophosphorus pesticides (OPs). Thus, these results will provide an insight into understanding of the fungal degradation of FNT and the potential application for bioremediation of OPs. Furthermore, the ability of *C. elegans* to mimic mammalian metabolism would help us elucidate the metabolic fates of organic compounds occurring in mammalian liver cells and evaluate their toxicity and potential adverse effects.

KEYWORDS: Bioremediation; *Cunninghamella elegans*; fenitrothion; fenitrothion-oxon; fungal metabolism; organophosphorus insecticide;

Abbreviations used: FNT, fenitrothion; FNTO, fenitrothion-oxon; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MNP, 3-methyl-4-nitrophenol; NMR, nuclear magnetic resonance spectroscopy; OPs, organophosphorus pesticides.

INTRODUCTION

Organophosphorus pesticides (OPs) are widely used as insecticides for controlling a wide range of pests. These pesticides act as acetylcholinesterase (AchE) inhibitor causing disruption of nervous impulse transmission and can thus be hazardous as a result of runoff from areas of application.[1] OPs have been listed as highly toxic to mammal, and exhibited chemical stability and resistance to biodegradation.[2-4] Although recently developed pesticides are considered to be much safer than those with a long history (e.g., organochlorine), unexpected health risks and potential environmental hazard are still inevitable (e.g., carcinogenesis, pesticide residual, environmental pollution).[5]

Fenitrothion (FNT), *O,O*-dimethyl *O*-4-nitro-m-tolyl phosphorothioate, an organophosphorus insecticide, has been extensively used in agriculture production to control a broad range of insects. FNT is categorized as a moderately hazardous compound and listed as an alternative insecticide for DDT, dieldrin, and endrin.[6] As a consequence of increasing use of FNT, serious environmental problems such as soil and water contamination have occurred as well as its acute poisoning from accidental or suicidal ingestion.[7] In some developing countries, aging and large quantities of stored FNT has become a potential threat to human health and environmental contamination.[6] Additionally, in terms of food safety, FNT has been reported the most common insecticide residue in Australian food products.[8] Therefore, it is necessary to study the metabolic fate of FNT and find a possible solution for this problem. Studies on biological and nonbiological degradation of FNT have been conducted.[9-10] Among those methods, microbial degradation proves to be an effective and environmentally friendly.[10]

Several bacterial species such as *Pseudomonas* sp., *Bacillus* sp. have been reported to degrade a wide range of organophosphorus insecticides including FNT.[11-14] However, there have been only a few studies of fungal-associated degradation of FNT.[9] Unlike prokaryotes, eukaryotic fungi have demonstrated diverse metabolic potential resulting in metabolites similar to those produced in the mammalian enzyme system,[15-20] which would help us reveal the metabolic fates of organic compounds occurring in mammalian liver cells. *C. elegans*, a filamentous fungus, has been extensively used as a microbial model system of mammalian metabolism for studying biotransformation of drugs, pesticides, and environmental cleanup of pollutants.[17,20-26] FNT has been documented to be hydrolyzed and cometabolized by the fungus *Trichoderma viride*,[9] however, little is known about the biodegradation fate of FNT by fungi. In this study, *C. elegans* was used as a model of mammalian metabolism for studying metabolic fate of FNT and its degradative potential for other OPs with the aim of using this application for bioremediation.

MATERIALS AND METHODS

Chemicals and reagents. FNT and the other OPs standards involved in this study were purchased from Chem Service (West Chester, PA), Dr. Ehrenstorfer (Augsburg, Germany), and Fluka (Buchs, Germany), with the highest available purity. HPLC-grade acetone, acetonitrile, and methanol were obtained from Honeywell (Morristown, NJ). LC-MS-grade formic acid and ammonium formate were from Fluka (Buchs, Germany). CD_3OD and $CDCl_3$ were from Merk (Darmstadt, Germany), and anhydrous Na_2SO_4 and NaCl were from Junsei (Japan). Meta-choroperbenzoic acid (MCPBA) (<77%) was from Sigma-Aldrich (Shanghai, China). All other

chemicals and regents used in this study were of analytical grade. Potato dextrose broth (PDB) and potato dextrose agar (PDA) were purchased from BD Korea (Seoul, Korea).

Microorganism. *Cunninghamella elegans* ATCC36122 was obtained from American Type Culture Collection (Manassas, VA). Fungal cultures were maintained on PDA at 27 □ on a rotary shaker operating at 170 rpm, while the liquid cultures were maintained on PDB at 27 □ (170 rpm). In this study, all culture media were sterilized at 121 □ for 15 min prior to use. Seed cultures were prepared on PDB at 27 □ (170 rpm) for 48 h prior to inoculation.

Metabolism of FNT by *C. elegans.*

Determination of standard curve for FNT. A stock standard solution of FNT (5000 mg/L) was prepared with acetonitrile and stored in dark at -18 □. A range of concentrations (0.1-100.0 mg/L) were prepared by diluting a standard solution with acetonitrile for determining a standard curve using an Agilent 1100 HPLC series (Agilent, Santa Clara, USA) equipped with a Halo C_{18} column (150 × 2.1 mm i.d., 2.7 μm; USA), using a mobile phase of water (A) and acetonitrile (B) containing 0.1% formic acid at a flow rate of 1 mL/min which was programed as follows: 0-1 min, 10%B; 1-40 min, 10-90%B; 40-45 min, 90%B; 45-47 min, 90-10%B; 47-55 min, 10%B. The ultraviolet (UV) detection was performed at 280 nm.

Metabolic reactions of FNT by C. elegans. Precultured mycelia (approximately 2 g) was added in fresh PDB (250 mL), supplemented with 0.5 mL (2.5 mg) of FNT standard solution and cultured at 27 □ (170 rpm). Aliquots of culture medium (10 mL) were sampled periodically and transferred into a 50 mL test tube with 5 g NaCl and extracted with acetonitrile (twice with 20 mL). The organic layer was combined and dried over anhydrous Na_2SO_4. After evaporating the solvent, the extract was dissolved with 2 mL of acetonitrile, and filtered using a 0.2 μm

polytetrafluoroethylene (PTFE) filter for HPLC analysis (analytical conditions as aforementioned). Controls consisted of culture broth with microorganism (blank control) and sterile medium with FNT (negative control). For sterilized control, cultures were sterilized at 121 □ for 15 min. All experiments were conducted in triplicate. The percent degradation of FNT at each sampling time was calculated using equation [1]:

$$\text{Percent degradation} = \frac{(C_0 - C_t)}{C_0} \times 100\% \quad [1]$$

To describe the degradation kinetics of FNT, a first-order kinetic equation [2] was used to fit the C_t data as a function of sampling time. $T_{1/2}$ (half-life time) was determined using equation [3]:

$$C_t = C_0 \times e^{-kt} \quad [2]$$

$$T_{1/2} = \frac{\ln 2}{k} \quad [3]$$

where C_0 is defined as initial FNT concentration at 2 h after treatment, and C_t represents the concentration at time t (day), respectively, *k* is degradation rate constant (d^{-1}), and $T_{1/2}$ corresponds to the time at which half of FNT has been degraded. HPLC analysis showed that 4 metabolites (M1-M4) were produced by *C. elegans*.

Isolation and identification of FNT metabolites by large scale culture. The identification of the 4 metabolites was carried out through large scale culture. Eight of the 1000 mL cultures were extracted at 15 d after treatment (maximum metabolite formation), combined, and concentrated as described previously. The 4 metabolites for a Varian 500-MS^n (Walnut creek, CA, USA) analysis were fractionated from the extract through an Aglient 1100 HPLC equipped with a semi-prep C18 (250 × 4.6 mm i.d., 10 μm, Phenomenex, USA) column. The same mobile phase as aforementioned was used at a flow rate of 1 mL/min and programed as follows: 0-1 min,

52%B; 1-15 min, 52-58% B; 15-16 min, 58-95%B; 16-22 min, 95%B; 22-23 min, 95-52%B; 23-30 min, 52%B. Parameters set for MS analysis were as follows: scan type, positive and negative ion modes (*m/z* 50–500); nebulizer gas pressure, 40 psi; drying gas pressure, 30 psi; drying temperature, 350 □; capillary voltage, 46.9 V; needle voltage, 4000 (+) and 4000 (-) V; collision induced dissociation voltage, 2.0 V. The Turbo Data Dependent Scanning (TurboDDS^{TM}, Varian, USA) mode was used to obtain MS^2 spectra of those metabolites. The proposed structures for the fragmentation ions were determined using Mass Frontier^{TM} software (Version 6.0, HighChem, Ltd., Slovakia). As sufficient amount of M1 was collected, ^{1}H-NMR and ^{13}C-NMR measurements were further performed with a NMR spectrometer (Advance 400, Bruker) at 400 MHz.

Synthesis of FNTO and its degradation by C. elegans. As FNTO was confirmed to be an initial precursor of other metabolites, to reveal the metabolic relationship among those metabolites, FNTO was synthesized by a previous described method[27] and subjected to degrade by *C. elegans*. A solution of 400 mmol MCPBA dissolved in 10 mL methylene chloride was prepared and then added dropwise to a round-bottled flask containing 200 mmol FNT in an ice bath and then stirred for 20 mins. The reaction solution was transferred to a separatory funnel, washed with Na_2CO_3 and saturated NaCl solution (three times). The organic phases were combined and dried with N_2. The extract was analyzed by HPLC/MS and ^{1}H and ^{13}C NMR accordingly. The study of degradation of FNTO by *C. elegans* was performed as aforementioned for FNT.

Biodegradation of other OPs by *C. elegans*. The degradative potential of *C. elegans* to other OPs was also investigated. Individual stock standard solutions of the twenty nine OPs (1000 mg/L) were prepared with acetonitrile and diluted to a range of concentrations (0.5-100.0 mg/L) for each individual to determine a standard curve using an Agilent HPLC 1100 series equipped

with a Kinetex C_{18} column (100 × 2.1 mm i.d., 2.6 μm; Phenomenex, USA), using the same mobile phase as aforementioned at a flow rate of 0.2 mL/min and programmed as follows: 0-1 min, 5%B; 1-40 min, 5-95% B; 40-45 min, 95%B; 45-47 min, 95-5%B; 47-55 min, 5%B. UV detection wavelengths, retention times, and standard curve regression equations of 29 OPs were shown in Table S1. Linear relationships (R^2> 0.99) between peak area and pesticide concentrations were found within the concentration range of 0.1-50.0 mg/L with a limit detection (LOD; S/N> 3) of 0.5 mg/L. Recovery experiments performed at two spike levels (0.2 and 1.0 mg) demonstrated that recoveries of those OPs ranged between 76.7 and 112.3% with relative standard deviation (RSD) values lower than 10.2% (Table S2). Procedures for biodegradation of 29 OPs by *C. elegans* were same as that of FNT. Cultures extraction were conducted at 2 h, 4 d, and 7 d after treatment and the residues were determined by HPLC. The percent degradation was calculated based on equation [1]. All experiments were conducted in triplicate.

RESULTS AND DISCUSSION

Metabolism of FNT by *C. elegans*. Standard regression equation (y= 35.9x-5.3) of FNT demonstrated a strong linear relationship (R^2= 1) between peak area and concentrations within the concentration range of 0.1-100.0 mg/L. LOD (S/N> 3) was calculated 0.01 mg/L.

Regarding metabolism of FNT by *C. elegans*, HPLC analysis of the culture extracts revealed that four metabolites M1, M2, M3, and M4 were detected at 17.7, 19.3, 21.6, and 25.5 minutes, respectively, whereas blank and negative controls had no peaks corresponding to any of those metabolites (Figure 1). Accumulation of metabolites was accompanied by concomitant decrease of FNT (Figure 2A and 2B). Among those metabolites, M2 and M3 were detected at 1 d after

treatment and gradually increased after that, however, the accumulation of M3 reached a maximum at 9 d after treatment and decreased gradually afterward, indicating that M3 is an initial precursor of other metabolites (Figure 2B). M1 and M4 were detected at 4 d after treatment. The accumulation of M1 continuously increased and became the most abundant metabolite until the end of experiment, whereas M4 increased slowly and was detected at low concentrations (Figure 2B). FNT was detected approximately 81% degraded in the culture at 5 d after treatment and 91.5% at 7 d after treatment, whereas only 3% of FNT was naturally degraded in the negative control at 7 d. At 15 d after treatment, it was almost undetectable and the proportions for those metabolites (M1-M4) in culture were 58.7%, 15.1%, 20.8%, and 4.8%, respectively. The kinetic model determined for degradation of FNT is $C_t = 3220.3 \times e^{-0.37t}$ with $R^2 = 0.997$, and the $T_{1/2}$ is 1.88 d. The identification of the four metabolites (M1-M4) was performed through large scale culture.

Mass spectrum of those metabolites showed FNT and M3 were detected in a positive ion mode and M1, M2, and M4 were detected in a negative ion mode (Figure 3), respectively. MS^2 fragmentation ions of those metabolites are summarized in Figure 4. M3 showed its molecular ion at *m/z* 262 ($[M + H]^+$) (100), which was 16 Da lower than that of FNT, indicating that an S atom in FNT may be substituted by an O atom (P=S → P=O) (Figure 3A and 4). To verify this hypothesis, FNTO was synthesized with 96.4% purity and identified by NMR and HPLC/MS. The ^1H and ^{13}C NMR spectrum of synthesized FNTO matched with its structure (Table 1). HPLC/MS analysis showed FNTO was detected at 22.1 minute and its molecular ion was at m/z 262 ($[M + H]^+$) (100), which are consistent with the results obtained for M3 (Figure 5), demonstrating that M3 is FNTO (*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphate).

In the case of M1, it displayed a molecular ion at *m/z* 232 ([M - H]$^-$) (100), which was 29 Da lower than that of M3, indicating that two *O*-methyl groups in M3 may be hydroxylated to produce M1 (-OCH_3 → -OH) (Figure 3B and 4**)**. Additionally, the fragment ion at *m/z* 152 ([M - H]$^-$) (40) corresponds to 3-methyl-4-nitrophenol (Figure 3B and 4).[6-7,10,28-29] The ^{1}H NMR and ^{13}C NMR spectra of M1 were obtained with purified samples to further determine its chemical structure. The ^{1}H NMR spectrum of M1 showed the presence of 3 aromatic ring protons (7.42, 7.46, and 7.18 ppm) and 3 carbohydrate protons with one methyl group (2.63 ppm). No signal was detected for hydroxy group proton, which may be due to hydrogen–deuterium exchange (CD_3OD as solvent). The ^{13}C NMR spectrum indicated the presence of 6 aromatic carbons (154.2, 149.3, 144.8, 126.9, 121.7, and 119.8 ppm) and one methyl group (20.9 ppm) (Table 1). On the basis of these results, M1 was identified 3-methyl-4-nitrophenyl dihydrogen phosphate.

With regard to M2, it gave a molecular ion value of *m/z* 262 [M - H]$^-$ (100) in mass spectrum (Figure 3C). A decrease in molecular weight of 14 in comparison with FNT which probably corresponds to a -CH_2. The fragment ion at *m/z* 152 ([M - H]$^-$) (23) corresponding to 3-methyl-4-nitrophenol was observed (Figure 3C and 4). [6-7,10,28-29] These results indicated that one of two *O*-methyl groups in FNT was hydroxylated (-OCH_3 → -OH), thus yielding M2. It was tentatively identified *O*-methyl *O*-(3-methyl-4-nitrophenyl) hydrogen phosphorothioate.

As for M4, its structural identification by NMR was infeasible due to the samll amout isolated. The mass spectrum of M4 exhibited a molecular ion at *m/z* 372 (100), which was significantly greater than that of FNT, indicating that a conjunction reaction may have occurred (Figure 3D). MS^2 showed that M4 gave fragment ion m/z at 168 ([M - H]$^-$) (45), which probably corresponds to hydroxylated 3-methyl-4-nitrophenol. On the basis of these results and literature data,[29] M4

was tentatively identified as glucuronide-3-(hydroxymethyl)-4-nitrophenol (Fig. 4). The molecular ion at *m/z* 372 was speculated as $[M + HCN]^-$.[30-31]

As M3 (FNTO) was found to be an initial precursor of other metabolites, FNTO was synthesized and its metabolism by *C. elegans* was further studied to reveal the metabolic relationship among those metabolites.

Metabolism of FNTO by *C. elegans.* HPLC analysis of the culture extracts showed FNTO was rapidly degraded to two metabolites O1 and O2 within 3 d after treatment by *C. elegans* (Figure 6), whereas no significant degradation was observed in negative control (data not shown). As a small amount of FNT (approximately 4%) was contained in synthesized FNTO, a small increase in FNTO was observed at 1 d after treatment, indicating that FNT was transformed to FNTO within 1 d (Figure 2C). This is consistent with the time of appearance of M3 in FNT treated culture (Figure 2B). At 3 d after treatment, O1 and O2 were detected at 17.1 and 19.0 minutes by HPLC, respectively, and gradually increased afterward (Figure 2D and 6). At 7 d after treatment, approximately 50% FNTO was degraded. Approximately 22% incomplete degraded FNTO persisted in the culture until the end of the experiment and the proportions for O1 and O2 were 53.1% and 27.4%, respectively. The kinetic model determined for degradation of FNTO is $C_t = 2491.5 \times e^{-0.116t}$ with $R^2 = 0.994$. The $T_{1/2}$ of FNTO is 5.97 d which is longer than that of FNT (1.88 d), which may be due to its high toxicity compared with FNT.[2]

O1 and O2 were detected in a positive and negative ion mode, respectively (Figure 6). O1 showed its molecular ion at *m/z* 278 ($[M + H]^+$) (34), which was 17 Da higher than that of FNTO, indicating that a methyl group in FNT may be alcoholized to produce O1 ($-CH_3 \rightarrow -CH_2OH$). Moreover, O1 also gave a fragment ion m/z at 260 ($[M - H]^+$) (100). On the basis of

these results, O1 was tentatively identified *O,O*-dimethyl *O*-[3-(Hydroxymethyl)-4-nitrophenyl] phosphate (Figure 7).

O2 displayed a molecular ion at *m/z* 232 ($[M - H]^-$) (100) and fragment ion at *m/z* 152 ($[M - H]^-$) (45), which coincided with M1 (Figure 4 and 6). HPLC analysis showed retention time of O2 was 19.0 minutes, which was comparable to that of 17.7 minutes for M1. These results demonstrated that O2 is identical to M1.

In synthesized FNTO treated cultures, O2 was detected at 3 d after treatment, while M1 was detected at 4 d after treatment in FNT treated cultures. Considering that unsynthesized FNT in FNTO treated cultures were transformed to FNTO at 1 d after treatment, it could be concluded that M1 was directly derived from M3 in FNT treated cultures (Fig. 2B and 2D). At 15 d after treatment, the residue of FNTO in synthesized FNTO treated cultures was 462 mAU*min (peak area), which was slightly lower than that of 567 mAU*min (peak area) for M3 in FNT treated cultures. There results demonstrated that FNT was continuously transformed to FNTO (M3) within 15 d, and FNTO (M3) was further transformed to O2 (M1). According to comparative analysis of the metabolites derived from those two pesticides, no trace amount of O1 was detected in FNT treated cultures. We speculated that the presence and accumulation of M2 in culture may inhibit the formation of O1, and this may also explain why there is no available data published on the metabolite O1. Additionally, M2 was found at the initial step in FNT treated cultures, in contrast, FNTO treated cultures had no trace amount of M2 detected due to the presence of low amount (4%) of FNT. If M2 did form, however, it did so in amounts too small to be detected under our analytical conditions.

On the basis of these results, the metabolic pathway was proposed (Figure 7). Two major degradation pathways are proposed: the first pathway occurs with the substitution of an S atom

by an O atom, and the second pathway involves the hydroxylation of the organophosphate band. The reaction of oxidation to form the oxon derivatives is quite common in phosphorothioate pesticides such as parathion,[32] cyanox,[33] and bromophos.[34] FNTO (M3) has been the most common metabolite found in plants, animals, and microorganisms during the degradation of FNT.[2,9,29] In the presence of *C. elegans*, FNT was first biotransformed to FNTO (M3) by oxidation of the P=S moiety, accumulated and became the initial precursor of other metabolites, whereas M2 was formed from the hydrolysis of FNT (*O*-methyl group) (Figure 7). M2 has been found in soil, microbial metabolism, and photoreaction in previous studies.[9,29] Due to the presence of thiomethyl, the rearrangement of the methoxy (thiono-thiolo) could result in formation of an isomer of M2.[29,35] Furthermore, our findings demonstrated that M1 (O2) and O1 were directly formed via M3 (FNTO) by the hydroxylation reaction. This provided supplementary evidence to further elucidate the detailed degradation process of FNT by *C. elegans* (Figure 7). To the best of our knowledge, M1 is rarely reported, however, it is the most abundant metabolite of FNT by *C. elegans* in the present study. O1 resulted from the oxidation of methyl group to hydroxymethyl. Formation of hydroxymethyl via oxidation of methyl by *C. elegans* has been reported by several authors.[23,36] The identified O1 in this study has not been reported in any other studies to date. Moreover, although 3-methyl-4-nitrophenol (MNP) was reported as a major metabolite of FNT in previous studies,[6-7, 9-10,28,37] it was not detected in the present study. Instead, the corresponding sugar conjugate M4 was detected. Conjugation of phenol with sugar is a common process during metabolic degradation steps.[38] Several glucosides have been reported for several pesticides such as clomazone,[39] oxamy,[40] and diphenamide.[41] In the present study, according to the identification and formation process of M4, it can be concluded that 3-hydroxymethyl-4-nitrophenol which conjugates glucuronic acid was derived

279 from 3-methyl-4-nitrophenol (MNP) (Figure 3D, 4 and 7). In terms of structure, MNP may be
280 derived from all metabolites by hydroxylation reaction in a certain period of time (Figure 7, as
281 dash lines indicated).

282 The metabolism of FNT in *C. elegans* was different from those of soil and plants. For
283 example, in soil, fenitrothion was degraded primarily by reduction of the nitro group to amino-
284 fenitrothion with lower redox, which was further degraded to demethyl amino.[42] Malhat et al.[28]
285 reported soil extracts of FNT showed MNP was the major degrade of FNT in soil. Regarding the
286 metabolic pathways of FNT in and on plants, although oxidative desulfuration and conjugation
287 were involved as those confirmed in the current study, the oxidation of methyl group and
288 demethylation of the *O*-methyl group were not found.[29] The primary degradation of FNT was
289 MNP with a trace amount of demethyl fenitrooxon, 3-methyl-4-nitroanisole, and 1,2-dihydroxy-
290 4-methyl-5-nitro-benzene.[43] In contrast, 3-methyl-4-nitrophenyl dihydrogen was the most
291 abundant metabolite of FNT by *C. elegans*. In FNT treated cultures of *Burkholderia sp.*, the
292 oxidative nitrite group-removing pathway was found, resulting in the formation of
293 methylhydroquinone,[44] however, this pathway was not found in *C. elegans* in the current study.
294 In addition, an azo derivative was detected during MNP biodegradation by *Aspergillus niger*,[6]
295 however, this has not been reported in *C. elegans*. Other studies have shown that *C. elegans* can
296 metabolize a wide variety of xenobiotics as those reported in human and animals studies through
297 *N*-oxidation and *N*-deethylation,[17,20] demethylation,[25] hydroxylation.[22-23,36] Cytochrome P450
298 was considered to be responsible for those metabolisms (phase I metabolism).[21,45] The two major
299 degradation pathways of FNT by *C. elegans* in the current study were also found in other
300 animals such as rats,[46] female goats,[47] and birds.[48] Furthermore, aromatic hydroxylation during
301 the metabolism of FNT in birds,[48] which has not reported in plant and soil, was also detected in

C. elegans. Recently, flavin-dependent monooxygenase was also found to be involved in certain parts of metabolism in *C. elegans*.[22] In addition to phase I metabolism, *C. elegans* was also involved in almost all of phase II metabolisms, such as sulfation, glucosylation, and glucuronide formation.[26,49-50] The ability of *C. elegans* to mimic mammalian metabolism and to perform novel biotransformations would help us reveal the metabolic fates of organic compounds occurring in mammalian liver cells instead of using live organisms. This microbial system also provides an alternative method for the production of metabolites in large quantities.

Biodegradation of other OPs by *C. elegans.* *C. elegans* was also capable of biodegradation of other common used OPs including insecticide, herbicide, and fungicide (Table S2). At 4 d after treatment, the percent degradation for those pesticides ranged between 23.1-94.5%, with 21 pesticides exhibiting degradation greater than 50%. At 7 d after treatment, the percent degradation was greater than 70%, with 27 pesticides exhibiting the degradation greater than 80%. Five pesticides including azinphos-methyl, phosmet, pirimiphos-methyl, pirimiphos-ethyl, and pyrazophos were not detectable due to the response value of these pesticides lower than LODs. *C. elegans* exhibited a wider degradation spectrum and stronger degradation ability to OPs compared with several bacterial degradations reported in previous studies.[11,13,51-52] The results indicated the potential of this fungus to be used in OPs pollution remediation.

In conclusion, this study reported a detailed metabolism of FNT by *C. elegans* by phase I (oxidation and hydrolysis) and II (conjugation) metabolisms through several different pathways, including those common in soil, plants, and animals. In addition to FNT, *C. elegans* was also capable of biodegradation of 29 common used OPs. The findings of this study will provide detailed insights regarding the fungal metabolism of FNT and complement the fungal degradation of OPs. Its potential for bioremediation of OPs residual contamination may be useful

for future remediation efforts. Additionally, this microbial system would serve as a model of mammalian metabolism to elucidate the metabolic fates of organic compounds, evaluate their toxicity and potential adverse effects. *Burkholderia* sp. strain NF100 has been found to carry a plasmid encoding a FNT-hydrolyzing enzyme in FNT metabolism.[45] Whether the metabolism of FNT by *C. elegans* in the present study also involves plasmid encoding relevant enzymes needs to be investigated in future studies.

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Notes

The authors declare no competing financial interest.

Yong-Zhe Zhu and Min Fu contributed equally to this work.

REFERENCES

(1) Carvalho, F. P. Pesticides, environment, and food safety. *Food Energy Secur*. **2017**, *6*, 48–60.

(2) Miyamoto, J.; Mikami, N.; Mihara, K.; Takimoto, Y.; Kohda, H.; Suzuki, H. Biological activity of fentriothion and its degradation products. *J. Pesticide Sci.* **1978**, *3*, 35–41.

(3) Harada, K.; Hisanaga, T.; Tanaka, K. Photocatalytic degradation of organophosphorus insecticides in aqueous semiconductor suspensions. *Water Res*. **1990**, 24, 1415–1417.

(4) Barceló, D. Environmental protection agency and other methods for the degermination of priority pesticides and their transformation products in water. *J. Chromatogr*. **1993**, *643*, 117–143.

(5) Ohi, M.; Dalsenter, P. R.; Andrade, A. J. M.; Nascimento, A. J. Reproductive adverse effects of fipronil in Wistar rats. *Toxicol. Lett*. **2004**, *146*, 121–127.

(6) Kanaly, R. A.; Kim, I. S.; Hur, H. G. Biotransformation of 3-methyl-4-nitrophenol, a main product of the insecticide fenitrothion by *Aspergillus niger*. *J. Agric. Food Chem*. **2005**, *53*, 6426–6431.

(7) Inoue, S.; Saito, T.; Miyazawa, T.; Mase, H.; Inokuchi, S. A simple method for detecting fenitrothion, its metabolite 3-methyl-4-nitrophenol, and other organophosphorus pesticides in human urine by LC-MS. *Forensic Toxicol*. **2009**, *27*, 32–36.

(8) Meaklim, J.; Yang, J.; Drummer, O. H.; Killalea, S.; Staikos, V.; Horomidis, S.; Rutherford, D.; Ioannides-Demos, L. L.; Lim, S.; Mclean, A. J.; McNeil, J. J. Fenitrothion: toxicokinetics and toxicologic evaluation in human volunteer. *Environ. Health Perspect*. **2003**, *111*, 305–308.

(9) Baarschers, W.; Heitland, H. S. Biodegradation of fenitrothion and fenitrooxon by the fungus *Trichoderma viride*. *J. Agric. Food Chem*. **1986**, *34*, 707–709.

(10) Katsumata, H.; Okada, T.; Kaneco, S.; Suzuki, T.; Ohta, K. Degradation of fenitrothion by ultrasound/ferrioxalate/UV system. *Ultrason. Sonochem*. **2010**, *17*, 200–206.

(11) Singh, B. K.; Walker, A. Microbial degradation of organophosphorus compounds. *EMS Microbiol. Rev*. **2006**, *30*, 428–471.

(12) Malik, D. K.; Bhatia, D.; Rathi, M. Bacterial Degradation of Some Organophosphate Compounds. *Microbial Divers. Biotechnol. Food Secur*. **2014**, *1*, 531–541.

(13) Deng, S.; Chen, Y.; Wang, D.; Shi, T.; Wu, X.; Ma, X.; Li, X.; Hua, R.; Tang, X.; Li, Q. X. Rapid biodegradation of organophosphorus pesticides by Stenotrophomonas sp. G1. *J Hazard Mater*. **2015**, *297*, 17–24.

(14) Singh, B.; Kaur, J.; Singh. 2014. Microbial degradation of an organophosphate pesticide, malathion. *Crit. Rev. Microbiol*. **2014**, *40*: 146–154.

(15) Cha, C. J.; Doerge, D.R.; Cerniglia, C. E. Biotransformation of malachite green by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol*. **2001**, *67*, 4358–4360.

(16) Zhang, D.; Hansen, E. B. Jr.; Deck, J.; Heinze, T. M.; Sutherland, J. B.; Cerniglia, C. E. Fungal biotransformation of the antihistamine azatadine by *Cunninghamella elegans*. *Appl. Environ. Microbiol*. **1996**, *62*, 3477–3479.

(17) Seo, J.; Lee, Y. G.; Kim, S. D; Cha, C. J.; Ahn, J. H.; Hur, H. G. Biodegradation of the insecticide *N,N*-diethyl-m-toluamide by fungi: identification and toxicity of metabolites. *Arch. Environ. Contam. Toxicol*. **2005**, *48*, 323–328.

385 (18) Park, M. K.; Liu, K. H.; Lim, Y.; Lee, Y. H.; Hur, H. G.; Kim, J. H. Biotransformation of a
386 fungicide ethaboxam by soil fungus *Cunninghamella elegans*. *J. Microbiol. Biotechnol*. **2003**,
387 *13*, 43–49.

388 (19) Moody, J. D.; Zhang, D.; Heinze, T. M.; Cerniglia, C. E. Transformation of amoxapine by
389 *Cunninghamella elegans*. *Appl. Environ. Microbiol*. **2000**, *66*, 3646–3649.

390 (20) Moody, J. D.; Freeman, J. P.; Fu, P. P.; Cerniglia, C. Biotransformation of mirtazapine by
391 *Cunninghamella elegans*. *Drug Metab. Dispos*. **2002**, *30*, 1274–1279.

392 (21) Zhang, D.; Yang, Y.; Leakey, J. E.; Cerniglia, C. E. Phase I and phase II enzymes produced
393 by *Cunninghamella elegans* for the metabolism of xenobiotics. *FEMS Microbiol. Lett*. **1996**,
394 *138*, 221–226.

395 (22) Zhu, Y. Z.; Keum, Y. S.; Yang, L.; Lee, H.; Park, H.; Kim, J. H. Metabolism of a fungicide
396 mepanipyrim by soil fungus *Cunninghamella elegans* ATCC36112. *J. Agric. Food Chem*. **2010**,
397 *58*, 12379–12384.

398 (23) Kang, S. I.; Kang, S. Y.; Kanaly, R. A.; Lee, E.; Lim, Y.; Hur, H. G. Rapid oxidation of ring
399 methyl groups is the primary mechanism of biotransformation of gemfibrozil by fungus
400 *Cunninghamella elegans*. *Arch. Microbiol*. **2009**, *191*, 509–517.

401 (24) Williams, A.; Deck, J.; Freeman, J. P.; Chiarelli, M. P.; Adjei, M. D.; Heinze, T. M.;
402 Sutherland, J. B. Biotransformation of flumequine by the fungus *Cunninghamella elegans*.
403 *Chemosphere*. **2007**, *67*, 240–243.

404 (25) Keum, Y. S.; Lee, Y. H.; Kim, J. H. Metabolism of methoxychlor by *Cunninghamella*
405 *elegans* ATCC36112. *J. Agric. Food Chem*. **2009**, *57*, 7931–7937.

406 (26) Keum, Y. S.; Lee, H. R.; Park, H. W.; Kim, J. H. Biodegradation of bisphenol A and its
407 halogenated analogues by *Cunninghamella elegans* ATCC36112. *Biodegradation*. **2010**, *21*,
408 989–997.

409 (27) Sánchez-Baeza F.; Durand G.; Barceló D.; Messeguer, A. Dimethyldioxirane conversion of
410 phosphine sulfides and phosphorothioates into their corresponding oxygen analogues.
411 *Tetrahedron Lett*. **1990**, *31*, 3359–3362.

412 (28) Malhat, F. M. Residues and dissipation of fenitrothion in green bean (*Phaseolus vulgaris*)
413 and soil. *Soil Sci*. **2012**, *3*, 1–4.

414 (29) Fukushima, M.; Fujisawa, T.; Katagi, T.; Takimoto, Y. Metabolism of fenitrothion and
415 conjugation of 3-methyl-4-nitrophenol in tomato plant (*Lycopersicon esculentum*). *J. Agric.*
416 *Food Chem*. **2003**, *51*, 5016–5023.

417 (30) Tong, H.; Bell, D.; Tabei, K.; Siegel, M. M. Automated data massaging, interpretation, and
418 emailing modules for high throughput open access mass spectrometry. *J. Am. Soc. Mass.*
419 *Spectrom.* **1999**, *10*, 1174–1187.

420 (31) Keller, B. O.; Sui, J.; Young, A. B.; Whittal, R. M. Interferences and contaminants
421 encountered in modern mass spectrometry. *Anal. Chim. Acta.* **2008**, *627*, 71–81.(30) Ali, E, R.;
422 Hopkins, T. L. Parathion absorption, translocation, and conversion to paraoxon in bean plants. *J.*
423 *Agric. Food Chem*. **1966**, *14*, 588–592.

424 (32) Ali, E, R.; Hopkins, T. L. Parathion absorption, translocation, and conversion to paraoxon in
425 bean plants. *J. Agric. Food Chem*. **1966**, *14*, 588–592.

426

427 (33) Chiba, M.; Kato, S.; Yamamoto, I. Metabolism of Cyanox and Surecide in bean plants and
428 degradation in soil. *J. Pestic. Sci*. **1976**, *1*, 179–191.

429 (34) Stiasni, M.; Deckers, W.; Schmidt, K.; Simon, H. Translocation, penetration, and
430 metabolism of *O*-(4-bromo-2,5-dichlorophenyl)-*O,O*-dimethylphosphorothioate (Bromophos) in
431 tomato plants. *J. Agric. Food Chem*. **1969**, *17*, 1017–1020.

432 (35) Eto, M. Chemical reactions. *Organophosphorus Pesticides: Organic and Biological*
433 *Chemistry*; CRC Press: Cleveland, 1974; Chapter 3, pp 57–124.

434 (36) Lee, H.; Kim, E.; Shin, Y.; Lee, J. H.; Hur, H. G.; Kim, J. H. Identification and formation
435 pattern of metabolites of cyazofamid by soil fungus *Cunninghamella elegans*. *J. Korean Soc.*
436 *Appl. Biol. Chem*. **2016**, *59*, 9–14.

437 (37) Chanda, A.; Khetan, S. K.; Banerjee, D.; Ghosh, A.; Collins, T. J. Total degradation of
438 fenitrothion and other organophosphorus pesticides by catalytic oxidation employing Fe-TAML
439 peroxide activators. *J. Agric. Food Chem*. **2006**, *128*, 12058–12059.

440 (38) Katagi, T.; Molecular orbital approaches to the photolysis of organophosphorus insecticide
441 fenitrothion. *J. Agric. Food Chem*. **1989**, *37*, 1124–1130.

442 (39) Weston, L. A.; Barrett, M. Tolerance of tomato and bell pepper to clomazone. *Weed Sci*.
443 **1989**, *37*, 285–289.

444 (40) Harvey, J.; Jr.; Han, J. C. Y.; Reiser, R. W. Metabolism of oxamyl in plants. *J. Agric. Food*
445 *Chem*. **1978**, *26*, 529–536.

446 (41) Hodgson, R. H.; Frear, D. S.; Swanson, H. R.; Regan, L. A. Alteration of diphenamide
447 metabolism in tomato by ozone. *Weed Sci.* **1973**, *21*, 542–549.

448 (42) Adhya, T. K.; Barik, S.; Sethunathan, N. Stability of commercial formulations of
449 fenitrothion, methyl parathion, and parathion in anaerobic soils. *J. agric. food Chem.* **1981**, *29*,
450 90–93.

451 (43) Takimoto, Y.; Ohshima, M.; Miyamoto, J. Degradation and fate of the fenitrothion applied
452 to harvested rice grains. *J. pestic. Sci.* **1978**, *3*, 277–290.

453 (44) Hayatsu, M.; Hirano, M.; Tokuda, S. Involvement of two plasmids in fenitrothion
454 degradation by Burkholderia sp. strain NF100. *Appl. Environ. Microbiol*. **2000**, *66*, 1737–1740.

455 (45) Asha, S.; Vidyavathi, M. Cunninghamella: A microbial model for drug metabolism studies:
456 A review. *Biotechnol. Adv*. **2009**, *27*, 16–29.

457 (46) Roy, S.; Roy, S.; Kumar, R.; Sharma, C. B.; Pereira, B. J. Biodegradation and
458 bioaccumulation of fenitrothion in rat liver. *Biomed. Chromatogr.* **2004**, *18*, 132–136.

459 (47) Mihara, K.; Okunno, Y.; Misaki, Y.; Miyamoto, J. Metabolism of fenitrothion in goats. *J.*
460 *Pesticide Sci.* **1978**, *3*, 233–242.

461 (48) Mihara, K.; Okunno, Y.; Misaki, Y.; Miyamoto, J. Metabolism of fenitrothion in birds. *J.*
462 *Pesticide Sci.* **1979**, *3*, 175–185.

463 (49) Amadio, J.; Murphy, C. D. Biotransformation of fluorobiphenyl by *Cunninghamella elegans*.
464 *Appl. Microbiol. Biotechnol*. **2010**, *86*, 345–351.

465 (50) Lin, L.; Huang, H.; Zhang, P.; Qi, X.; Zhong, D. Microbial transformation of
466 dextromethorphan by *Cunninghamella blakesleeana* AS 3.153. *Chem. Pharm. Bull*. **2007**, *55*,
467 658–661.

(51) Ohshiro, K.; Kakuta, T.; Sakai, T.; Hirota, H.; Hoshino, T.; Uchiyama, T. Biodegradation of organophosphorus insecticides by bacteria isolated from turf green soil. *J. Biosci. Bioeng*. **1996**, *82*, 299–305.

(52) Karpouzas, D.G.; Fotopoulou, A.; Menkissoglu-Spiroudi, U.; Singh, B. K. Non-specific biodegradation of the organophosphorus pesticides, cadusafos and ethoprophos by two bacterial isolates. *FEMS Microbiol. Ecol*. **2005**, *53*, 369–378.

Figure captions

Fig. 1. HPLC profiles of FNT and its metabolites produced by *C. elegans*. (A) blank control; (B) negative control; (C) at day 0; (D) at 7 days.

Fig. 2. Degradation and accumulation patterns of FNT and its metabolites (A, B) and FNTO (C, D).

Fig. 3. Mass spectra of four metabolites M3 (A), M1 (B), M2 (C), and M4+HCN (D) of FNT by *C. elegans*. M3 was detected under ESI (+) mode and other three metabolites were detected under ESI (-) mode, respectively.

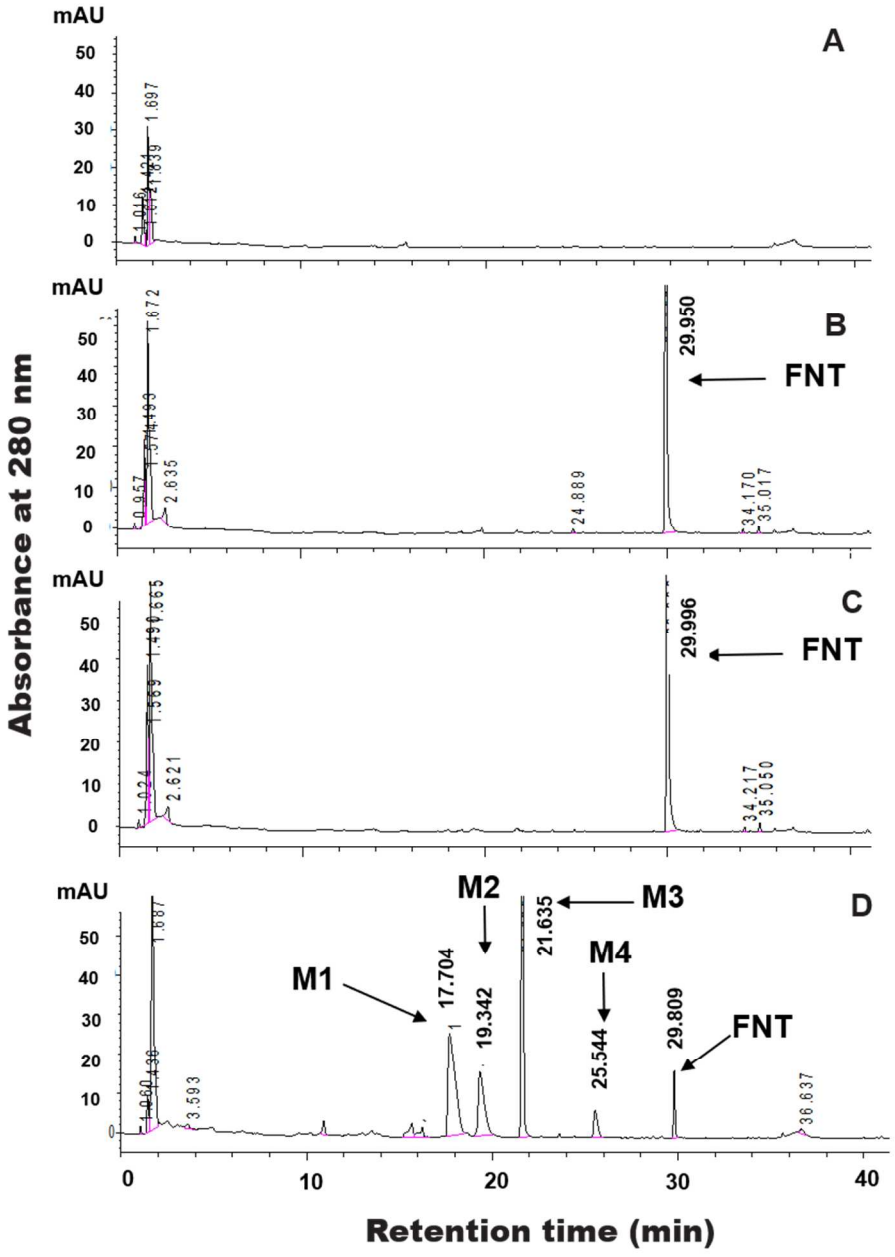
Fig. 4. MS^2 fragmentation scheme of FNT and its metabolites (M1-M4).

Fig. 5. HPLC/MS information comparison between metabolite M3 (A) and synthesized FNTO (B).

Fig. 6. HPLC elution profiling of two metabolites (O1 and O2) of FNTO by *C. elegans* at 7 days after treatment and their mass spectra. O1 and O2 were detected under ESI (+) and ESI (-) mode, respectively.

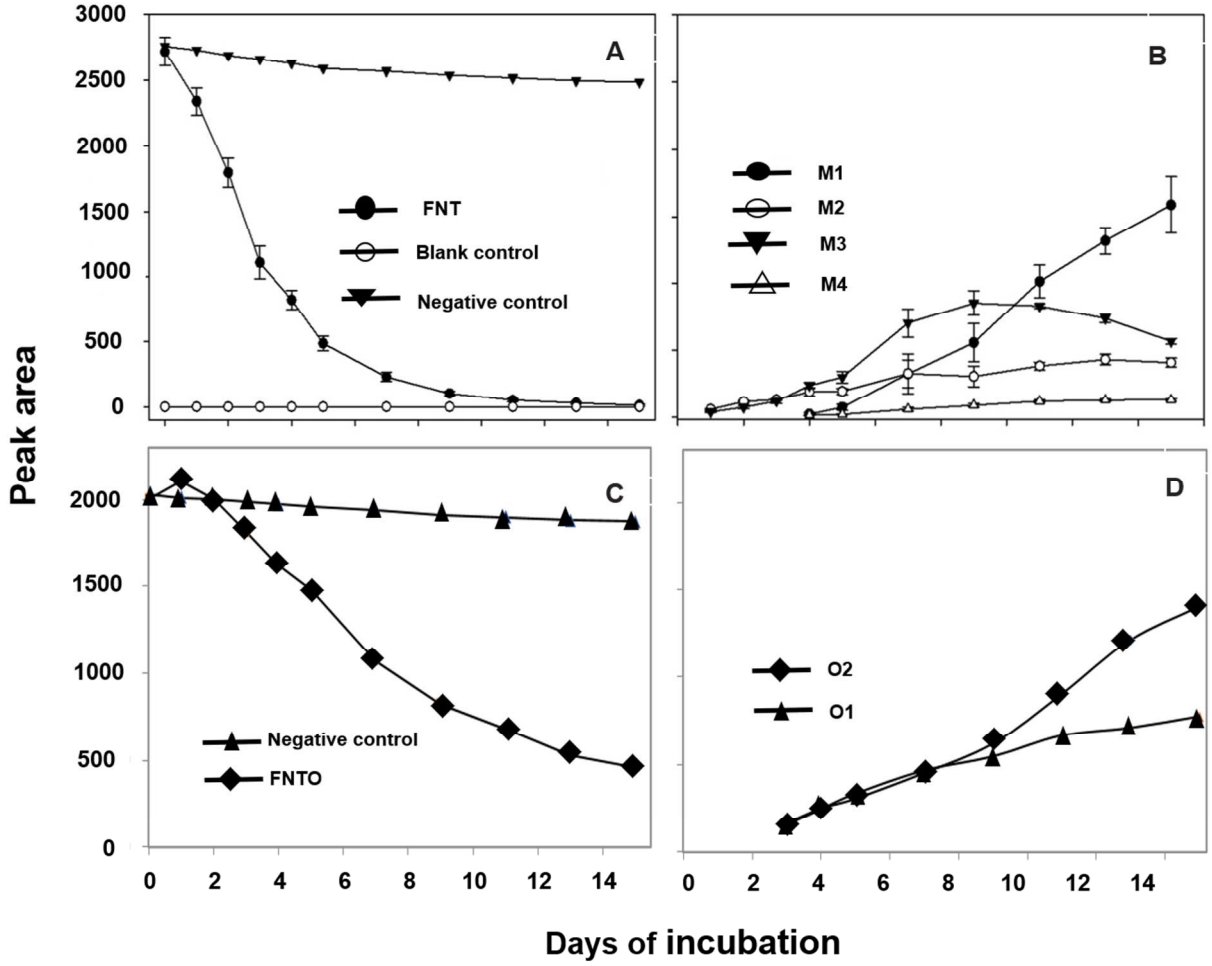
Fig. 7. Proposed metabolic pathway of fenitrothion by *C. elegans*. Abbreviations: s, soil; ph, photolysis; and mi, microorganism.

495 **Fig. 1.**


mAU
A
B
C
D
FNT
M1
M2
M3
M4
29.950
29.996
21.635
17.704
19.342
25.544
29.809
Absorbance at 280 nm
Retention time (min)

496

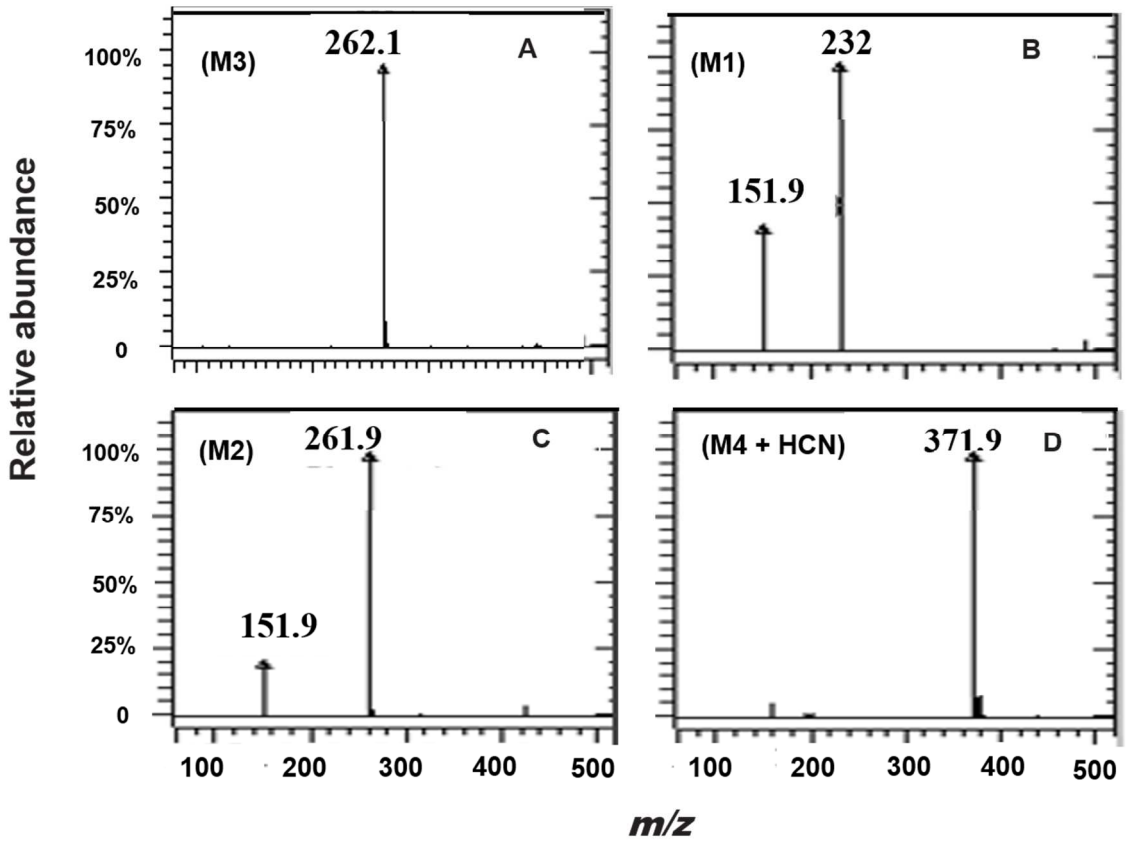
497 **Fig. 2.**


A
B
C
D
FNT
Blank control
Negative control
M1
M2
M3
M4
Negative control
FNTO
O2
O1
Peak area
Days of incubation
0
500
1000
1500
2000
2500
3000
0
2
4
6
8
10
12
14

498

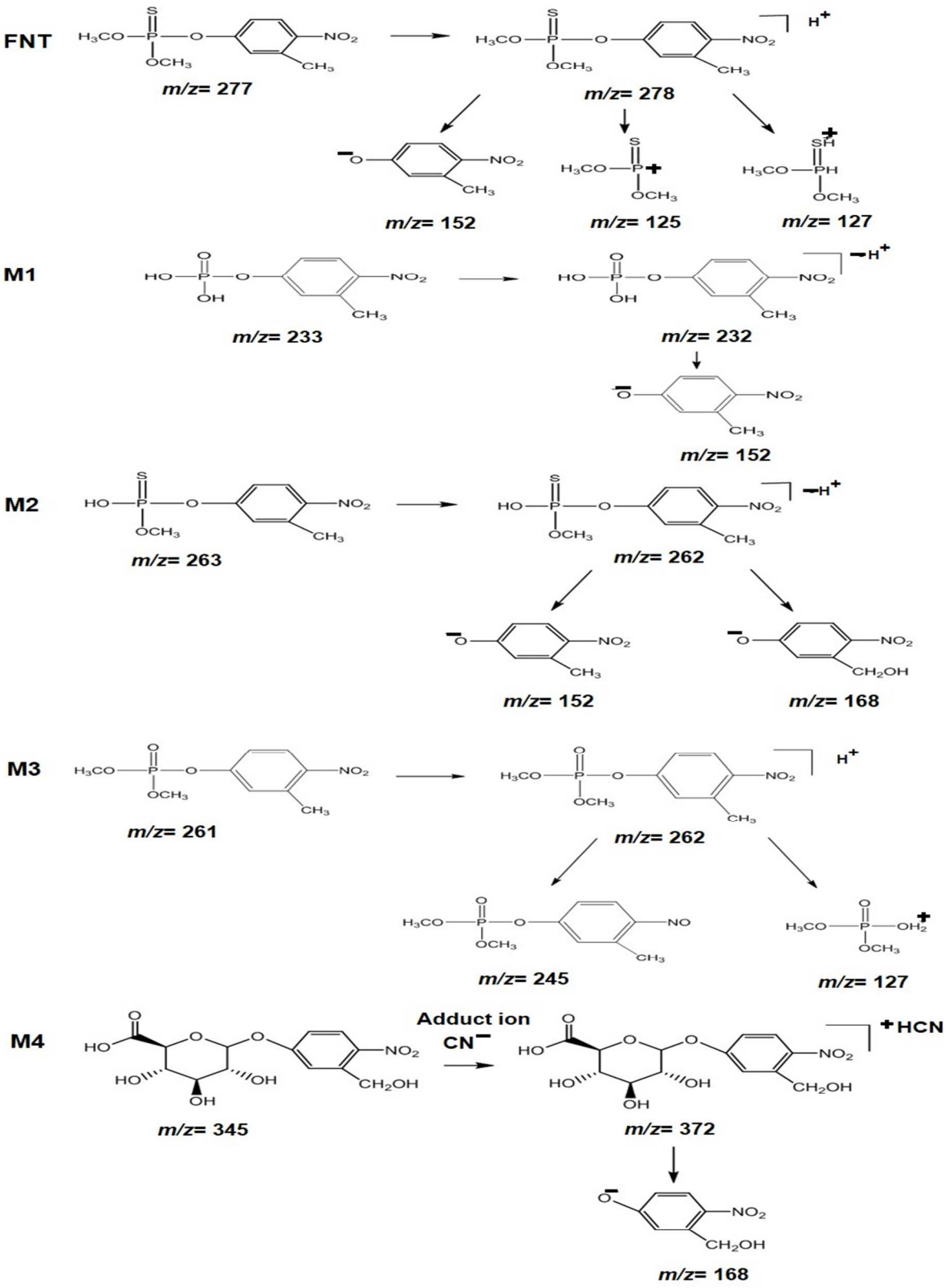
499 **Fig. 3.**

500



501

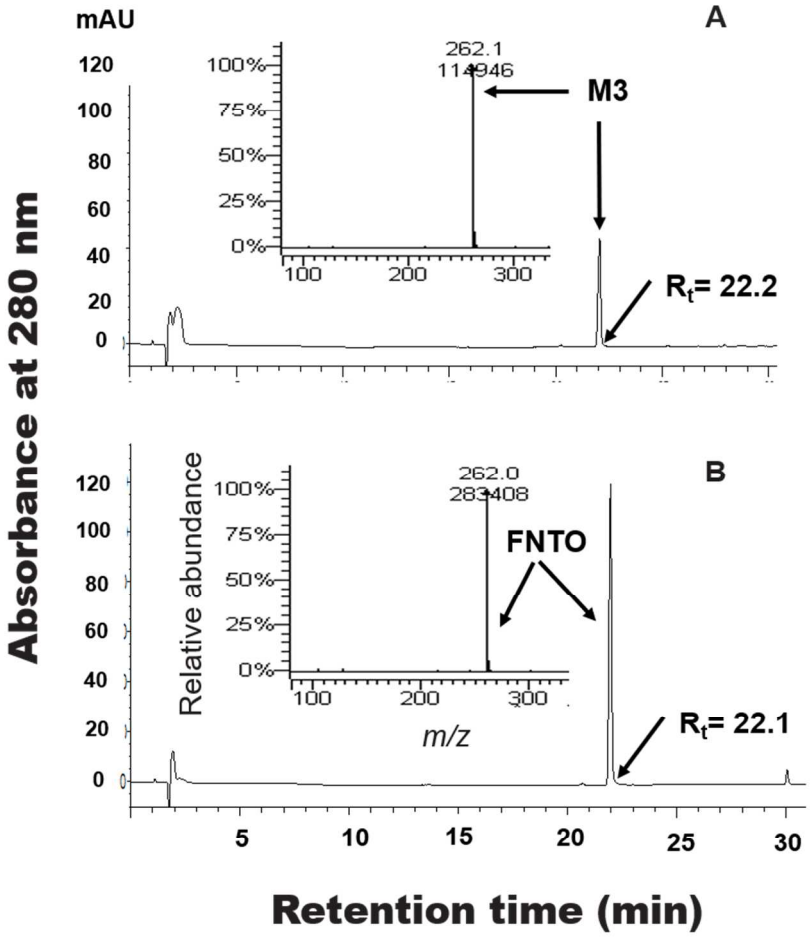
502 **Fig. 4.**


FNT
m/z= 277
m/z= 278
m/z= 152
m/z= 125
m/z= 127
M1
m/z= 233
m/z= 232
m/z= 152
M2
m/z= 263
m/z= 262
m/z= 152
m/z= 168
M3
m/z= 261
m/z= 262
m/z= 245
m/z= 127
M4
Adduct ion
CN−
+HCN
m/z= 345
m/z= 372
m/z= 168

503

504 **Fig. 5.**

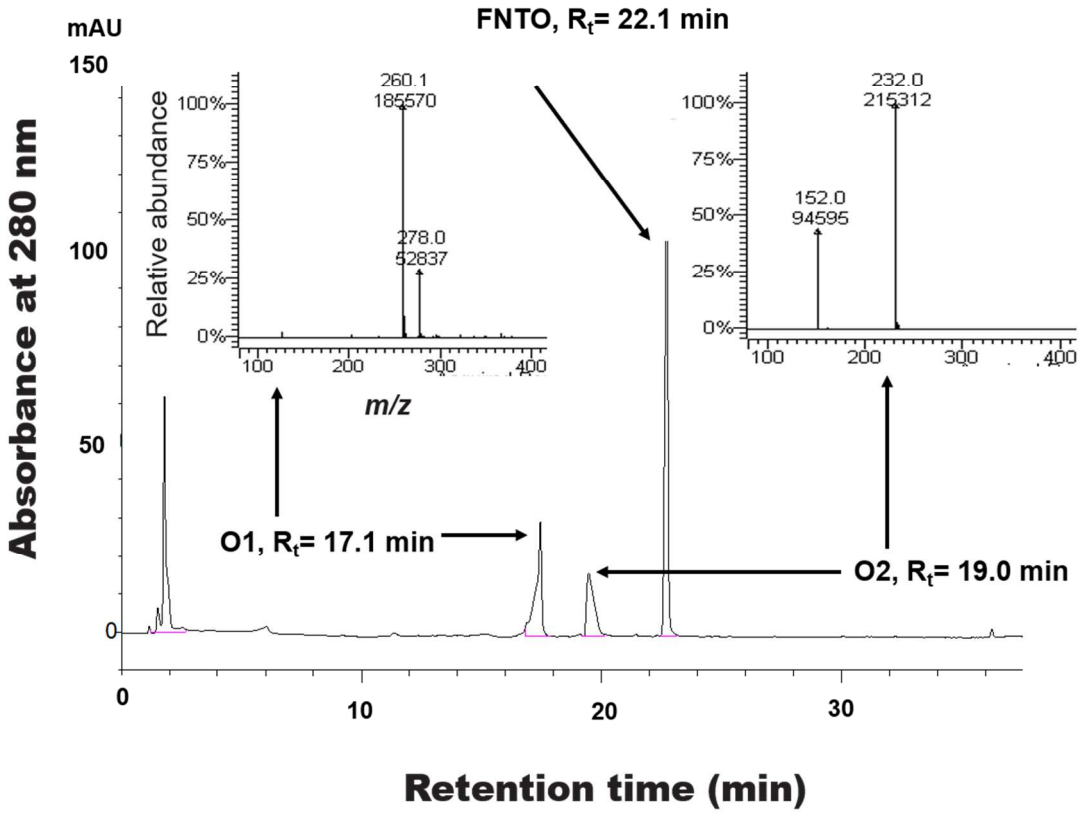
505



506

507 **Fig. 6.**

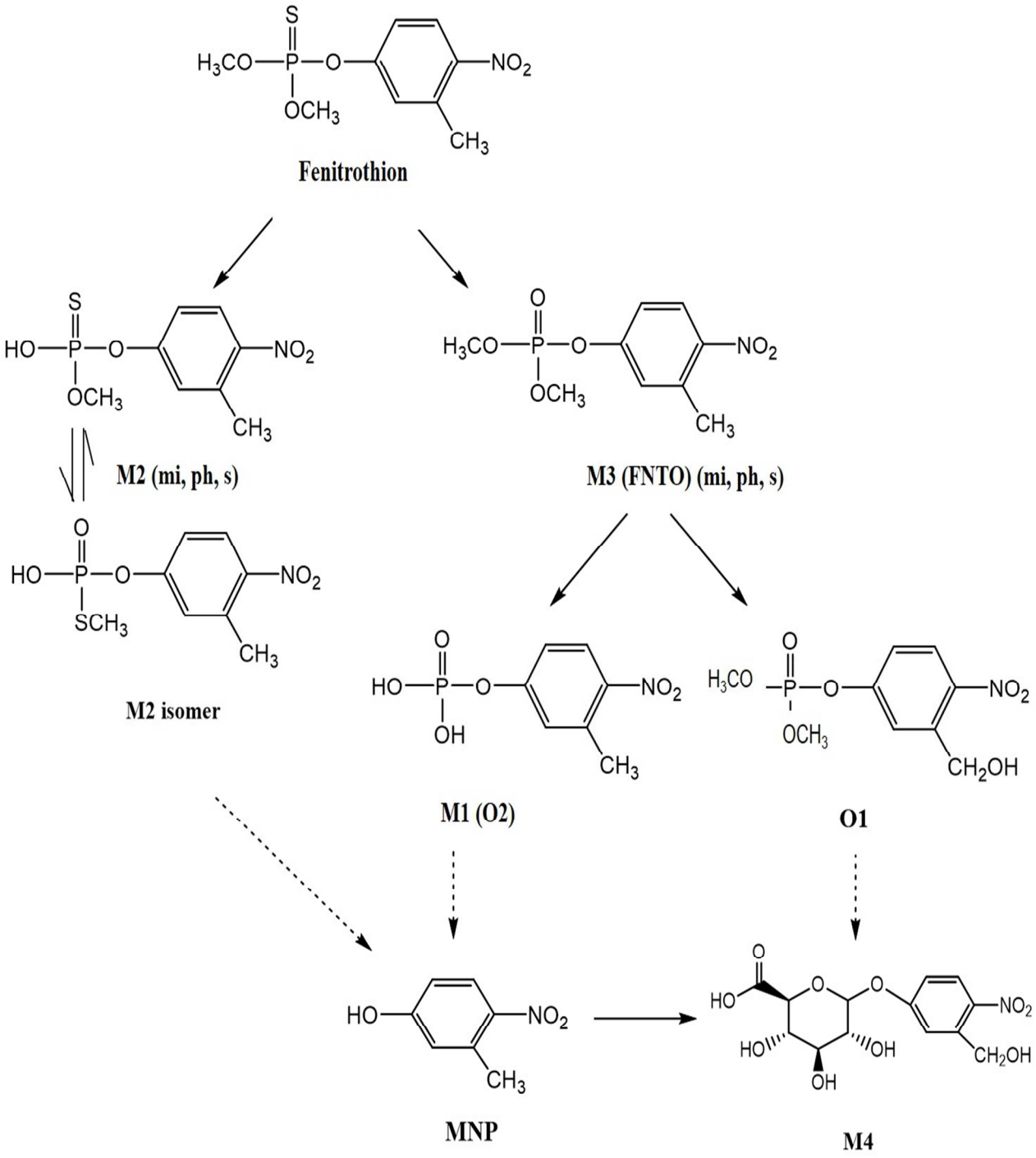
508



509

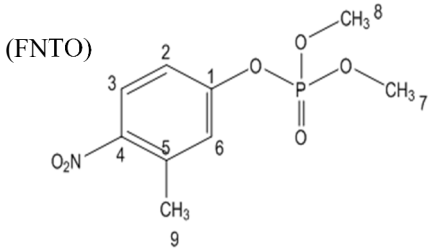
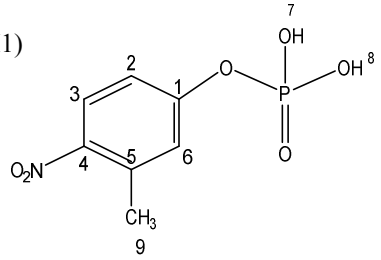
510 **Fig. 7.**

511



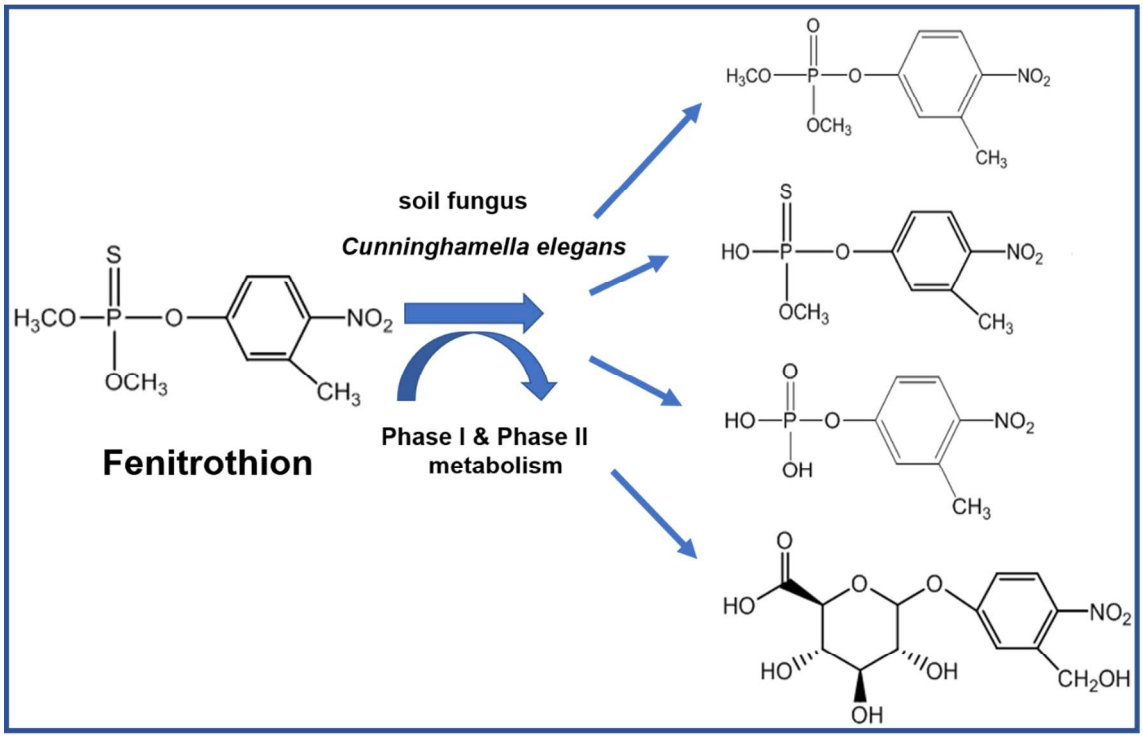
512

513 **Table 1.** ^{1}H and ^{13}C NMR assignments for synthesized FNTO and metabolite M1.

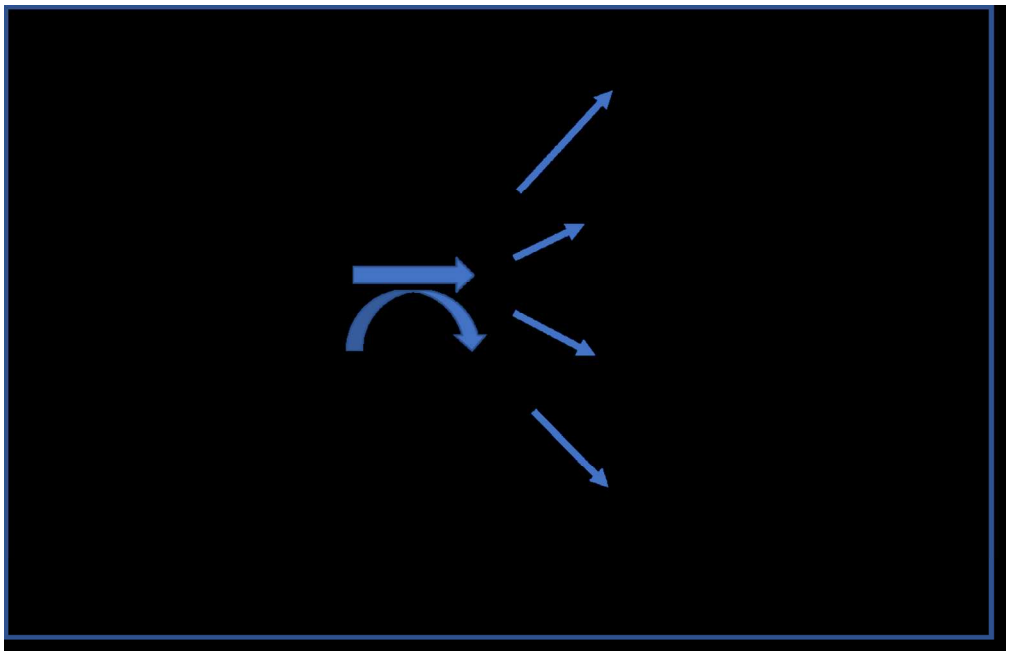
	(FNTO) 		(M1) 	
Position	^{1}H	^{13}C	^{1}H	^{13}C
1	-	153.7	-	154.2
2	7.12 (d, 1H)	119.3	7.42 (d, 1H)	119.8
3	8.05 (d, 1H)	124.8	7.46 (d, 1H)	126.9
4	-	136.6	-	149.3
5	-	127.0	-	144.8
6	7.19 (s, 1H)	124.8	7.18 (s, 1H)	121.7
7	3.90 (d, 3H)	55.6	-	-
8	3.87 (d, 3H)	55.6	-	-
9	2.62 (s, 3H)	21.1	2.63 (s, 3H)	20.9

514 **TOC graphic**

515



516



152x98mm (220 x 220 DPI)