Biotransformation and detoxification of the neonicotinoid insecticides nitenpyram and dinotefuran by Phanerochaete sordida YK-624

Research paper

Biotransformation and detoxification of the neonicotinoid insecticides nitenpyram and dinotefuran by *Phanerochaete sordida* YK-624

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Abstract

Neonicotinoid insecticides have been widely used throughout the world over the last two decades. In the present study, we investigated the degradation of neonicotinoid insecticides nitenpyram (NIT) and dinotefuran (DIN) by the white-rot fungus *Phanerochaete sordida* YK-624. While NIT was completely degraded by *P. sordida* YK-624 under ligninolytic conditions, only a 20% decrease was observed under nonligninolytic conditions. On the other hand, *P. sordida* YK-624 degraded 31% of DIN under ligninolytic conditions after a 20-day incubation, while it did not degrade DIN under nonligninolytic conditions. We found that cytochromes P450 played a key role in the biotransformation of NIT and DIN by *P. sordida* YK-624. A novel NIT metabolite (*E*)-*N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-*N'*-hydroxy acetimidamide (CPMHA) and a novel DIN metabolite *N*-((4a*S*,7a*S*,*E*)-1-methylhexahydrofuro[2,3-*d*]pyrimidin-2(1*H*)-ylidene)nitramide (PHPF) were identified in this study. In addition, to evaluate neurotoxicity, the effects of NIT, DIN and their metabolites on the viability of human neuroblastoma cells SH-SY5Y were determined. PHPF showed higher neurological toxicity than DIN, whereas the metabolite of NIT, CPMHA, showed no toxic effect. Our results indicated that the neurological toxicity of NIT could be effectively removed by *P. sordida* YK-624.

1. Introduction

Neonicotinoid insecticides are nicotine-based neurotoxicants, and nicotinic acetylcholine receptors (nAChRs) are their main target (Matsuda et al., 2001; Tomizawa et al., 2003). Neonicotinoid insecticides have been widely used over the past two decades because of their lower binding efficiencies to mammalian nAChRs (Tomizawa et al., 2003; Tomizawa et al., 2005). However, it has been reported that neonicotinoid insecticides had harmful effects to honeybees (Henry et al., 2012), aquatic invertebrates, vertebrates, and even humans (Van Dijk et al., 2013; Morrissey et al., 2015; Wang et al., 2015; Harada et al., 2016). Based on the European Food Safety Authority (EFSA) report, three neonicotinoid insecticides (clothianidin, imidacloprid and thiamethoxam) were banned starting from April 2018 (EFSA, 2018). Nitenpyram (NIT), [(*E*)-*N*-(6-chloro-3-pyridylmethyl)-*N′*-ethy-*N′*-methyl-2-nitrovinylidenediamine], was discovered by the Sumitomo Chemical Company. Dinotefuran (DIN), *N*-methyl-*N′*-nitro-*N″*-[(tetrahydro-3-furanyl) methyl]guanidine, was developed in 2002 by Mitsui Chemicals (Bass et al., 2015). These pesticides have been broadly used in rice, fruit, and other agricultural products (Simon-Delso et al., 2015) and contaminated the environment. There are no reports on the degradation of NIT and DIN by microorganisms. Degradation of NIT by a low-temperature plasma treatment has been reported (Li et al. 2013). However, the cost of physical or chemical methods is high, and they may also produce secondary pollution.

There are limited reports on the metabolic mechanism of NIT and DIN. The mechanism of NIT metabolism was only reported in mice and spinach. The metabolic routes of NIT involve *N*-demethylation and further oxidation of the nitromethylene carbon, as well as chloropyridinyl and nitromethylene moiety cleavage (Ford and Casida 2006b, 2008). The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) summarized the metabolism of DIN in animals and plants (FAO, 2012). The major metabolites of DIN are 1-methyl-3-(tetrahydro-3-furylmethyl)urea (UF) and (1-methyl-3-(tetrahydro-3-furylmethyl)guanidine (DN) (FAO, 2012). Approximately twenty metabolites of DIN were identified by LC/MS selective detection in rats (Ford and Casida, 2006a). Other metabolic routes include *N*-demethylation, tetrahydrofuran moiety hydroxylation, and *N*-methylene hydroxylation (Ford and Casida, 2006a; FAO, 2012). The metabolism of DIN in plants has been studied in rice, apples, potato, lettuce and spinach. Most of the metabolites of DIN in rats are also observed in plants, including methylguanidine, nitroguanidine, and urea metabolites (Ford and Casida, 2008; FAO, 2012). However, microbial biodegradation mechanism of NIT and DIN have not yet been reported.

It is well known that white-rot fungi can be used to degrade recalcitrant environmental pollutants, such as persistent organic pollutants and endocrine-disrupting compounds, due to their unique ability to degrade lignin (Bumpus et al., 1985; Kirk and Farrell, 1987; Xiao et al., 2011; Wang et al., 2012b, 2014). White-rot fungi secrete ligninolytic enzymes for lignin degradation, which include lignin peroxidase, manganese peroxidase, laccase and versatile peroxidase. In addition to the extracellular enzymatic system, white-rot fungi have approximately 150 kinds of cytochromes P450, which are involved in the biodegradation of recalcitrant organic pollutants. We previously reported biodegradation of acetamiprid and clothianidin by the white-rot fungus *Phanerochaete soridida* YK-624. An *N*-demethylated acetamiprid metabolite IM 2-1 and a less neurologically toxic metabolite of clothianidin, TZMU, were detected (Wang et al., 2012a; Mori et al., 2017).

In the present study, the degradation of NIT and DIN by *P. sordida* YK-624 is reported for the first time. In addition, a novel NIT metabolite (*E*)-*N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-*N'*-hydroxy acetimidamide (CPMHA) and a novel DIN metabolite *N*-((4a*S*,7a*S*,*E*)-1-methylhexahydrofuro[2,3-*d*]pyrimidin-2(1*H*)-ylidene)nitramide (PHPF) were identified. The results of neurological toxicity experiments showed that CPMHA had a less toxic effect than NIT; however, PHPF showed higher toxicity than DIN. This is the first report that white-rot fungus can effectively remove the neurological toxicity of NIT.

2. Materials and methods

2.1. Strain and chemicals

The white-rot fungus *P. sordida* YK-624 (ATCC 90872), isolated from rotted wood (Hirai et al., 1994), was maintained on potato dextrose agar (PDA) slants at 4 °C. NIT and DIN standards (99% purity) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 1-Aminobenzotriazole (ABT) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. NIT and DIN degradation by *P. sordida* YK-624

P. sordida YK-624 was incubated on PDA plates at 30 °C for 3 days. The mycelium was inoculated into nitrogen-limited (LN, Tien and Kirk, 1988) and potato dextrose broth (PDB) media for degradation experiments. NIT or DIN (final concentration, 0.1 mM) was added to the cultures after 5 days of incubation. The cultures for NIT degradation were further incubated for 1-5 days, while those for DIN degradation were incubated for 5, 10, 15, and 20 days. The internal standard material (acetamiprid) was added to the culture, and then homogenized with

20 mL of acetone. The homogenate was evaporated to dryness and then dissolved in methanol (MeOH) for analysis by high-performance liquid chromatography (HPLC). The standard curve was obtained with the ratio of four different concentrations of NIT or DIN and the internal standard material. HPLC analysis for the quantification of NIT or DIN was carried out under the following conditions: column, Inertsil ODS-3 (4.6 x 250 mm, 5 μm; GL Sciences, Tokyo, Japan); mobile phase, 20% aqueous MeOH for NIT and 15% MeOH for DIN; flow rate, 1 mL/min; and monitored at 270 nm using a JASCO MD-2018 PDA detector. Every measurement was performed in triplicate.

2.3. Biomass and enzyme activities

NIT and DIN cultures were obtained as described in Section 2.2. Mycelia were separated from the culture fluid by filtration (0.2 μm) and dried at 105 °C for over 12 h following PDA disc removal and then weighed. The resulting filtrate was used for enzymatic assay of lignin peroxidase (LiP) and manganese peroxidase (MnP) activity according to a method described previously (Sugiura et al., 2003; Wang et al., 2011). Protein concentration in the filtrate was determined using a Bradford protein assay kit (Takara Bio Inc., Shiga, Japan).

2.4. Effect of ABT on NIT and DIN degradation

After preculturing *P. sordida* YK-624 in LN medium as described in Section 2.2., NIT or DIN was added at a final concentration of 0.1 mM, and final concentrations of 0.01, 0.1, and 1 mM cytochrome P450 inhibitor ABT were added. NIT cultures were then incubated for 1-3 days, while DIN cultures were incubated for 5, 10, 15, and 20 days. The samples treatment and analysis methods were described as Section 2.2.

2.5. Identification of metabolites of NIT and DIN

P. sordida YK-624 was inoculated into LN medium for large-scale cultivation (total 5 L). After precultivation for 5 days of incubation, NIT (final concentration, 148 μM) was added, and the culture was incubated at 30 °C for an additional 3 days, and the culture with DIN (final concentration, 197 μM) was incubated for 20 days. The cultures were evaporated to approximately 250 mL and extracted with ethyl acetate (EtOAc). The EtOAc extracts containing NIT or DIN metabolites were separated by silica gel flash column chromatography (silica gel 60 N). The elution sequence for NIT metabolites (*n*-hexane/acetone/MeOH=10/0/0, 8/2/0, 7/3/0, 6/4/0, 5/5/0, 4/6/0, 3/7/0, 2/8/0, 0/10/0, 0/5/5, and 0/0/10; v/v/v) and that for DIN

metabolites (*n*-hexane/acetone/MeOH=10/0/0, 8/2/0, 7/3/0, 6/4/0, 5/5/0, 4/6/0, 3/7/0, 2/8/0, 0/10/0, and 0/0/10; v/v/v) were used to obtain 54 and 45 fractions, respectively. The fraction purity was determined by thin-layer chromatography (Merck F254; Merck, Darmstadt, Germany) and HPLC on a C30-UG-5 column (4.6 x 250 mm; Nomura Chemical, Seto, Japan). The fractions 12-18 of DIN were subjected to silica gel flash column chromatography for a second time (*n*-hexane/acetone/MeOH=10/0/0, 8/2/0, 7/3/0, 5/5/0, 0/10/0, and 0/0/10; v/v/v), and 15 fractions were obtained. The NIT fractions were used without a second separation by silica gel flash column chromatography. The metabolites were further separated by HPLC on a C30-UG-5 column (20 x 250 mm). The pure NIT and DIN metabolites were analyzed by HR-ESI-MS and NMR spectroscopy. The HR-ESI-MS data were acquired by a JMS-T100LC mass spectrometer (JEOL, Tokyo, Japan). The NMR spectra were recorded on a Jeol Lambda-500 spectrometer (JEOL, Tokyo, Japan) operated at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The IR spectrum was recorded on a Jasco grating infrared spectrophotometer, and the specific rotation values were measured using a Jasco DIP1000 polarimeter.

2.6. Neurotoxicity of NIT and DIN and the metabolites

NIT, DIN or their metabolites were dissolved in dimethyl sulfoxide at a concentration of 300 mM and diluted to 0.01, 0.03, 0.1, or 0.3 mM with Dulbecco's modified Eagle's medium (10% fetal bovine serum and 1% penicillin-streptomycin solution) before use. SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) were seeded in a 96-well plate at a density of 6000 cells/well. Cells were cultured for 24 h and then incubated with NIT, DIN or the metabolites at various concentrations for an additional 72 h. Cells treated with culture medium or culture medium containing 0.1% dimethyl sulfoxide were used as the negative control or vehicle control, respectively. The cell viability in each well was determined by CCK-8 assay (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocol.

3. Results and discussion

3.1. NIT and DIN degradation by *P. sordida* YK-624

A low-temperature plasma treatment method has been reported to degrade NIT (Li et al. 2013). Recently, it has been reported that NIT was degraded in finished drinking water through oxidation or hydrolysis by Cl_2 (Noestheden et al., 2016). However, there are no reports on the degradation of NIT and DIN by microorganisms. There are a few publications on the microbiological degradation of neonicotinoid insecticides. Some pure bacteria have demonstrated the ability to degrade imidacloprid, acetamiprid, thiacloprid and thiamethoxam

(Zhao et al., 2009; Dai, et al., 2010; Tang et al., 2012; Zhou et al., 2013). Moreover, in our previous studies, we have reported that *P. sordida* YK-624 can degrade acetamiprid and clothianidin (Wang et al., 2012a; Mori et al., 2017). In the present study, we determined the degradation of NIT and DIN by *P. sordida* YK-624 in LN and PDB media. *P. sordida* YK-624 completely degraded NIT after 5 days of incubation in LN medium, while only 20% of NIT was degraded in PDB medium (Fig. 1A). We further found that DIN was degraded by 31% in LN medium by *P. sordida* YK-624 after 20 days of incubation. However, no degradation of DIN was observed in the PDB medium (Fig. 1B). The degradation ability of NIT and DIN by a white-rot fungus was confirmed for the first time in this study.

Besides the degradation ability of NIT and DIN by *P. sordida* YK-624, we also determined the growth responses of *P. sordida* YK-624 in the degradation experiments. We found that the biomass of *P. sordida* YK-624 for NIT and DIN degradation in the PDB medium were significantly higher than that in the LN medium (Fig. 2A&C). However, they both increased within the incubation time. In consist with that of biomass, the protein concentrations of *P. sordida* YK-624 in PDB medium were also significantly higher than that in LN medium (Fig. 2B&D). Different from that of biomass, the protein concentrations did not change over the incubation period. Therefore, our results suggested that the degradation of NIT and DIN by *P. sordida* YK-624 was not dependent on growth factors.

In addition, we further measured the the ligninoytic enzymes activities of *P. sordida* YK-624 during the degradation experiments. However, we only detected the activities of ligninoytic enzymes in LN medium. In this medium, LiP activity were very low during the incubation period, which were ~0.03 nkat mL^{-1} (data not shown). In the NIT degradation experiment, the MnP acticity significantly increased and reached a maximum of 2 nkat mL^{-1} at day 3 and then sharply decreased (Fig. 3A). In the DIN degradation experiment, the MnP activity decreased toward the end of incubation time (Fig. 3B). Since the production behavior of MnP did not correspond to the degradation behavior of NIT and DIN, we concluded that the involvement of ligninolytic enzymes including MnP in the degradation of NIT and DIN was very low, and that another enzyme system would be involved in the degradation.

3.2. Cytochrome P450 enzymes are involved in NIT and DIN degradation

The cytochromes P450 in white-rot fungi have been reported to be crucial in degrading

various recalcitrant aromatic compounds (Ichinose, 2013). Some reports have referenced the effect of cytochromes P450 in the degradation of neonicotinoid insecticides. CYP3A4, a kind of cytochrome P450 from the human liver, was able to hydroxylate imidacloprid and oxidize thiamethoxam and clothianidin (Schulz-Jander and Casida, 2002; Shi et al., 2009). Some insect cytochromes P450 have been reported to play a critical role in the detoxification of imidacloprid (Karunker et al., 2008; Puinean et al., 2010; Bass et al., 2011). A few reports verified that the common cytochrome P450 inhibitor ABT inhibited some bioconversion steps in many white-rot fungi (Marco-Urrea et al., 2009; Golan-Rozen et al., 2011). Accordingly, we investigated the inhibition of NIT and DIN degradation by *P. sordida* YK-624 using the P450 inhibitor ABT. As shown in Fig. 4A, the recovery of NIT was 30, 37, and 54 μM when 0.01, 0.1 and 1 mM ABT were added to *P. sordida* YK-624 cultures, respectively, compared to 6 μM in cultures without ABT. While 68 μM DIN was detected in *P. sordida* YK-624 after 20 days of incubation, the recovery of DIN after the addition of 0.01, 0.1 and 1 mM ABT was 79, 84, and 89 μM, respectively (Fig. 4B). Compared to the degradation rate of NIT and DIN without ABT, these results indicated that the degradation activity of NIT and DIN was markedly lower. Since ABT markedly inhibited the degradation of NIT and DIN, the result suggests that cytochromes P450 are involved in the degradation of NIT and DIN.

3.3. Identification of the metabolites from NIT and DIN

Large-scale cultures of *P. sordida* YK-624 were used to determine the metabolites produced from NIT and DIN after 5 and 20 days of incubation, respectively. A NIT metabolite and a DIN metabolite were purified and identified for further analysis. An *m/z* of 250.0700 $[M+Na]^+$ for the NIT metabolite and an *m/z* of 199.0200 $[M-H]^-$ for the DIN metabolite were observed by ESI-TOF-MS, and the molecular formulas of the purified metabolites were calculated to be $C_{10}H_{13}ClN_3O$ and $C_7H_{12}N_4O_3$, respectively. Moreover, ^{1}H-NMR, ^{13}C-NMR and distortionless enhanced polarization transfer (DEPT) experiments demonstrated the presence of three methylenes, two methines, two methyls and one quaternary carbon for the NIT metabolite, while two methylenes, three methines, one methyl and one quaternary carbon were identified for the DIN metabolite. The IR spectrum of the DIN metabolite showed that it contained imino and cyano groups (data not shown). Table 1 lists the assignment of the protons and carbon atoms in the metabolites of NIT and DIN. As a result, the metabolite of NIT was identified as CPMHA (Fig. 5A), and the metabolite of DIN was determined to be PHPF (Fig. 5B).

Because CPMHA and PHPF are novel compounds, new metabolic pathways of NIT and

DIN by the white-rot fungus *P. sordida* YK-624 were proposed. In NIT metabolization in mice and spinach, *N*-demethylation, *N*-deethylation, nitromethylene oxidation, and chloropyridinyl and nitromethylene moiety cleavage have occurred (Ford and Casida 2006b, 2008). The mechanism of NIT metabolism by *P. sordida* YK-624 was proposed to be that the nitro group of NIT was initially reduced by the action of cytochrome P450, and following denitrosation or deamination to NH-NIT, CPMHA was generated by a new metabolic pathway from NH-NIT (Casida, 2011). The metabolic pathways of DIN in plants and animals include *N*-demethylation, tetrahydrofuran moiety hydroxylation, and *N*-methylene hydroxylation (Ford and Casida, 2006a, 2008; FAO, 2012). In the present study, we proposed that DIN was hydroxylated by cytochrome P450, that the hydroxy aldehyde group opened the ring, and that the intermediate was then dehydrated to PHPF (Casida, 2011).

3.4. Neurotoxicity of NIT, DIN and their metabolites

The insecticidal activity of neonicotinoid insecticides is based on their action on nAChRs. There are no reports on the toxicity of NIT and DIN metabolites because studies on the mechanisms of NIT and DIN metabolism are limited. The biological activity of imidacloprid and its metabolites by interference with imidacloprid binding sites in honey bee nAChRs indicate that IC_{50} values for imidacloprid is 2.9 nM, its hydroxylated metabolites show lower IC_{50} values, and urea metabolite show the lowest one (>100 000 nM) (Nauen et al., 2001). We estimate that CPMHA, the NIT metabolite, has low toxicity in honey bees since the nitromethylene unit ($CH\text{-}NO_2$) was reduced to form CPMHA. Although neonicotinoid insecticides are considered to be less toxic in humans, recent studies have reported that these compounds can affect human health (Harada et al., 2016; Han et al., 2017). Although insect toxicity tests are often used for neonicotinoid insecticides, a neurotoxicity test for human cells is also necessary. Therefore, in the present study, SH-SY5Y human neuroblastoma cells were used to evaluate the neurotoxicity of NIT and DIN and of their metabolites CPMHA and PHPF for the first time (Fig. 6). While DIN treatment caused minimal cytotoxicity (cell viability > 80%), even at a relatively high dose of 0.3 mM, treatment with PHPF, the metabolite of DIN, decreased cell viability (Fig. 6). This result suggested an enhanced neurotoxicity of DIN after its degradation. In contrast, the metabolic process of NIT by *P. sordida* YK-624 transformed the compound to a less neurotoxic metabolite, as indicated by the increased cell viability of SH-SY5Y cells treated with CPMHA compared to those treated with NIT (Fig. 6). It is expected that the degradation of NIT by *P. sordida* YK-624 can abolish the neurological toxicity of NIT. Additionally, higher toxicity has

been observed with DIN degradation products, and a further metabolic pathway for DIN by white-rot fungi is necessary.

4. Conclusion

To our knowledge, microbial biodegradation of NIT and DIN has not been reported. Here, we demonstrated the degradation of NIT and DIN by the white-rot fungus *P. sordida* YK-624. Subsequently, we identified a novel metabolite of NIT, CPMHA, and a novel metabolite of DIN, PHPF. We discovered that cytochrome P450 was essential for the degradation of NIT and DIN by *P. sordida* YK-624. Finally, we demonstrated for the first time that the white-rot fungus can effectively remove the neurological toxicity of NIT by converting it to CPMHA.

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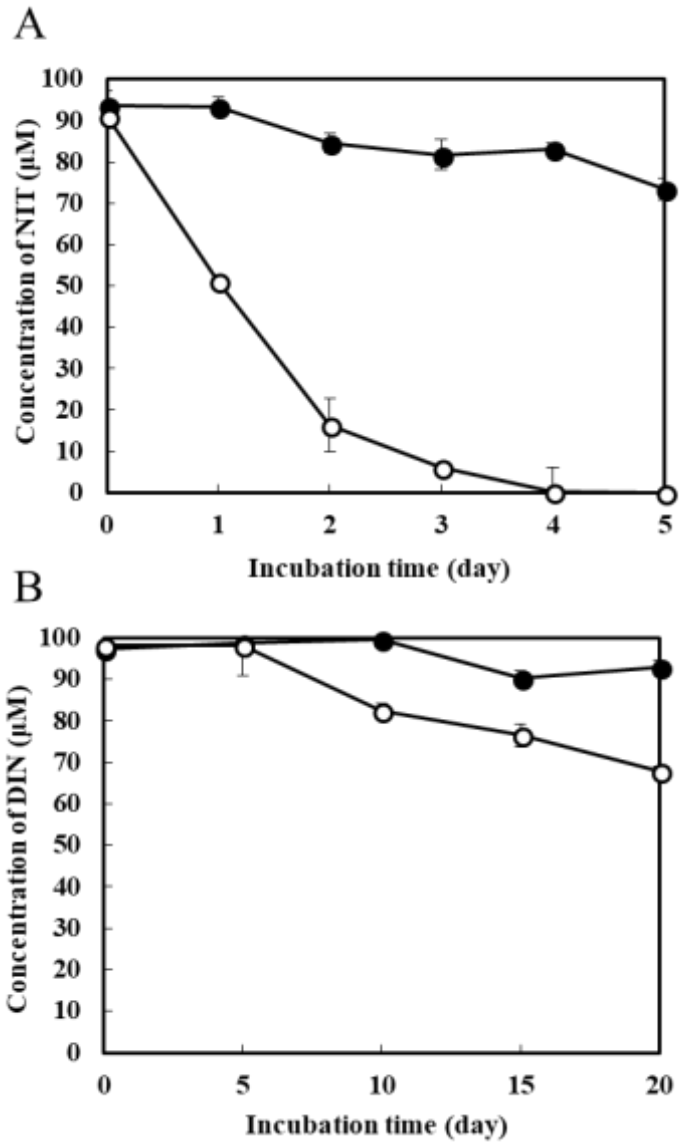

Fig. 1 Time-course of the degradation of NIT (A) and DIN (B) by *P. sordida* YK-624: (●) PDB medium, (○) LN medium. The values are presented as the mean ± standard deviation of triplicate samples.

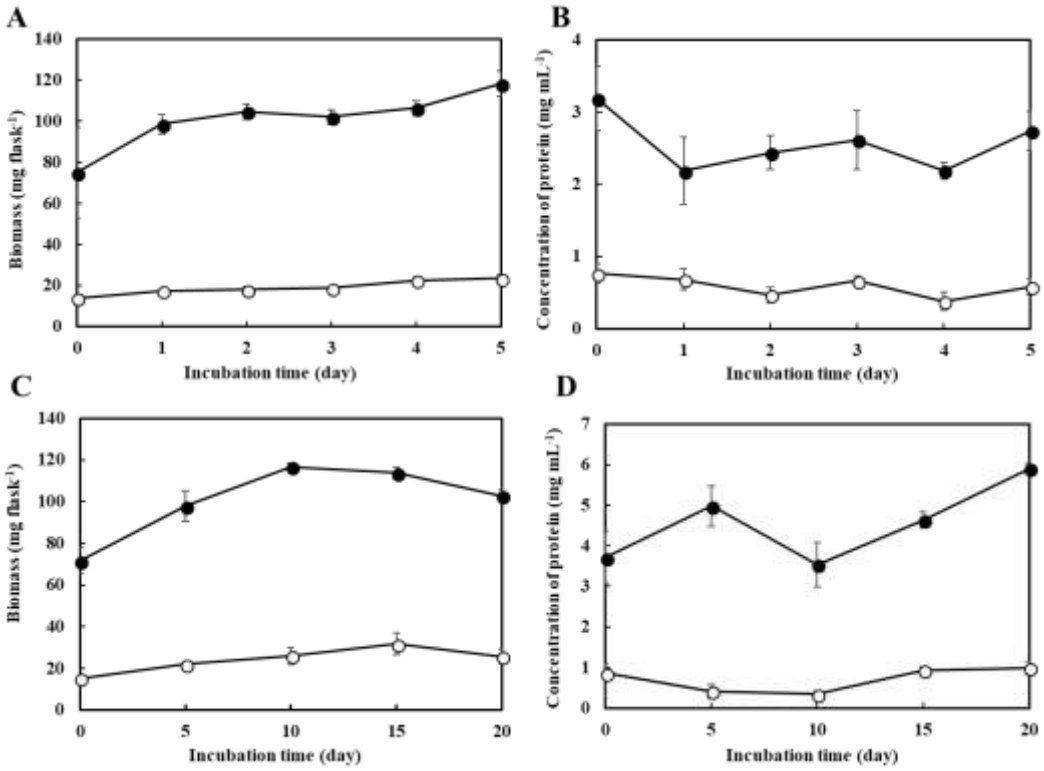

Fig. 2 Time-course of the biomass (A, C) and protein concentrations (B, D) of *P. sordida* YK-624 in the degradation of NIT (A, B) and DIN (C, D). The closed and open cycles represent that in PDB and LN media, respectively. The values are presented as the mean ± standard deviation of triplicate samples.

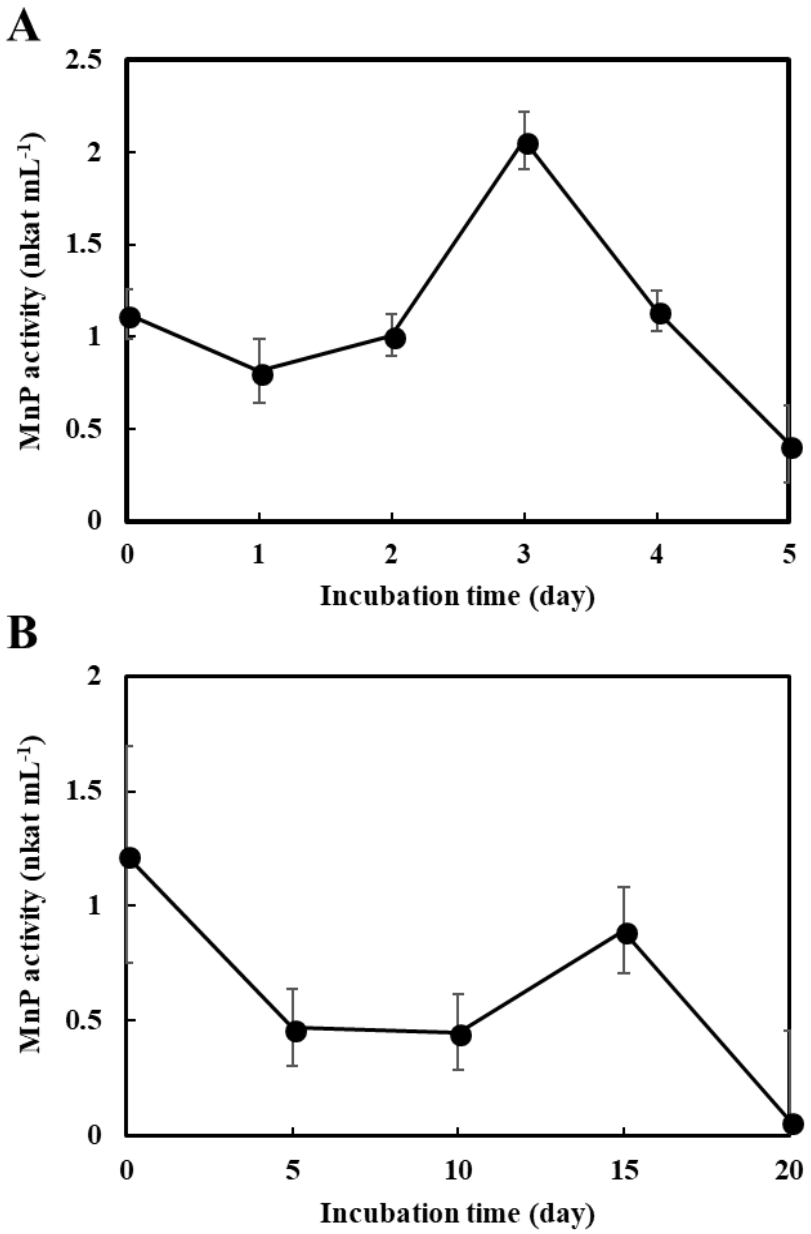

Fig. 3 MnP activity during the degradation of NIT (A) and DIN (B) by *P. sordida* YK-624 in LN medium. The values are presented as the mean ± standard deviation of triplicate samples.

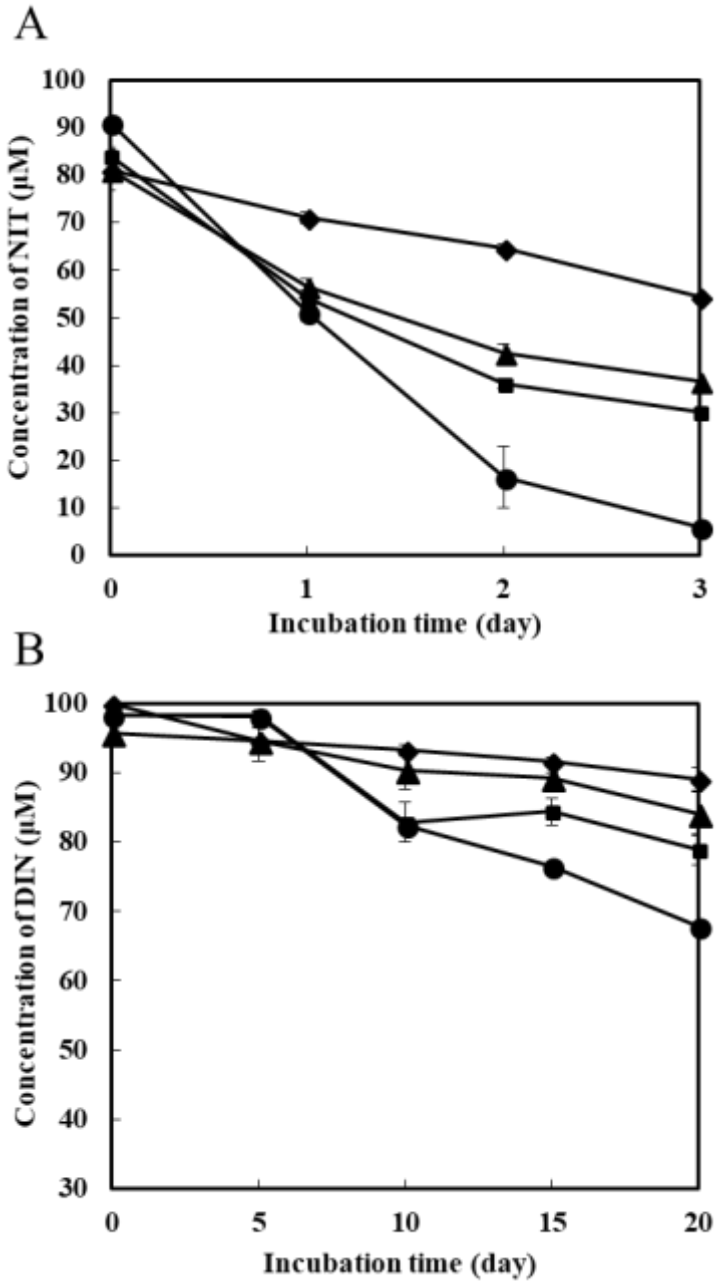

Fig. 4 Effect of the cytochrome P450 inhibitor ABT on the degradation of NIT (A) and DIN (B) by *P. sordida* YK-624 in LN medium: (●) without ABT, (■) 0.01 mM ABT, (▲) 0.1 mM ABT, and (◆) 1 mM ABT. Values are the means ± standard deviation of triplicate samples.

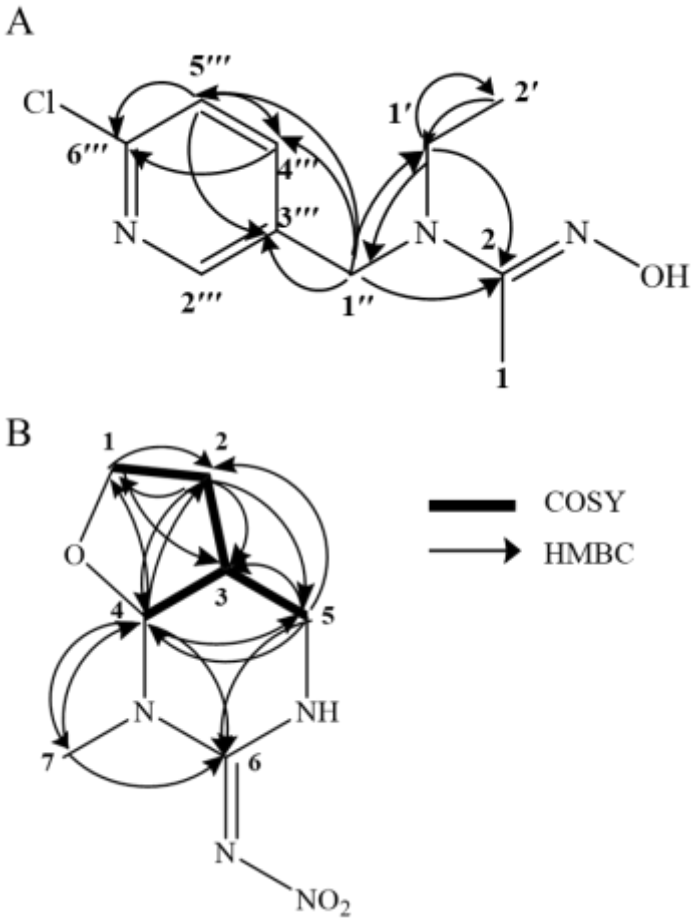

Fig. 5 Correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) for the identified of NIT metabolite CPHMA (A) and DIN metabolite PHPF (B).

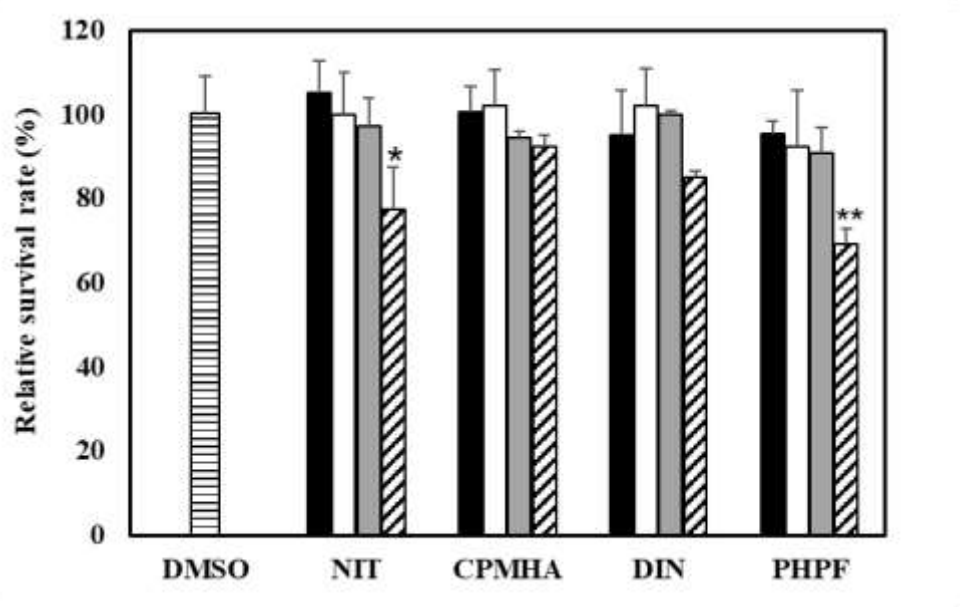

Fig. 6 SH-SY5Y human neuroblastoma cell viability by addition of NIT and DIN and their metabolites: (▤) DMSO, (■) 0.01 mM, (□) 0.03 mM, (■) 0.1 mM, (▨) 0.3 mM. Asterisks indicate values that were determined by Student's t-test to be significantly different from the untreated control (**P<0.01, *P<0.05).

Table 1 ^{1}H- and ^{13}C-NMR data for NIT and DIN metabolites (in CD_3OD)

	Position	^{1}H δ_H	^{13}C δ_C
NIT metabolite	1	3.90	67.0
	2	2.20	28.3
		1.91	
	3	2.72	37.2
	4	5.07	90.1
	5	3.51	39.3
		3.27	
	6	-	158.4
	7	2.99	34.2
DIN metabolite	1	2.74	27.8
	2	-	160.7
	1′	3.25	42.7
	2′	1.10	13.5
	1″	4.50	47.6
	2‴	7.42	125.5
	3‴	-	135.8
	4‴	7.71	140.1
	5‴	8.25	149.6
	6‴	-	150.9