Bioremediation of the neonicotinoid insecticide clothianidin by the white-rot fungus Phanerochaete sordida

doi: 10.1016/j.jhazmat.2016.09.049

Research paper

Bioremediation of the neonicotinoid insecticide clothianidin by the white-rot fungus *Phanerochaete sordida*

Toshio Mori [a, †], Jianqiao Wang [a, †], Yusuke Tanaka [a], Kaoru Nagai [b], Hirokazu Kawagishi [a, c, d], Hirofumi Hirai [a, c*]

[a] Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[b] Department of Nutrition, Kohshien University, 10-1 Momijigaoka, Takarazuka 665-0006, Japan

[c] Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[d] Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

Keywords: *Phanerochaete sordida*; clothianidin; biodegradation

* Corresponding author: Hirofumi Hirai

Faculty of Agriculture, Shizuoka University

836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

Tel. & Fax: +81 54 238 4853

E-mail address: hirai.hirofumi@shizuoka.ac.jp

† Equal contributors

Contact Data: June 20th, 2016

Highlights

- CLO was transformed to TZMU by *Phanerochaete sordida*.
- Cytochrome P450 is involved in the degradation of CLO.
- The CLO metabolite TZMU exhibited no neurotoxicity.

Abstract

Clothianidin (CLO) is a member of the neonicotinoid pesticides, which have been widely used worldwide over the last two decades. However, its toxicity for bees and neurological toxicity for humans are urgent problems. Here, the degradation of CLO by the white-rot fungus *Phanerochaete sordida* was examined in nitrogen-limited liquid medium. After incubation for 20 days at 30 °C, 37% of CLO was degraded in the cultures. High-resolution ESI-MS and NMR analyses of the culture supernatant identified *N*-(2-chlorothiazol-5-yl-methyl)-*N*′-methylurea (TZMU) as a metabolite of CLO degradation. The addition of cytochrome P450 inhibitors to the culture medium markedly reduced the degradation of CLO by *P. sordida*. And manganese peroxidase, a major ligninolytic enzyme secreted by this fungus, were not carried out CLO degradation. The effects of CLO and TZMU on the viability of the neuronal cell line Neuro2a demonstrated that *P. sordida* effectively degrades CLO into a metabolite that lacks neurotoxicity.

1. Introduction

Clothianidin ((*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine, CLO) belongs to the family of neonicotinoid pesticides, which have been the most widely used group of pesticides worldwide over the last two decades. In addition to CLO, the neonicotinoid pesticides registered for use in Japan are acetamiprid (ACE), imidacloprid (IMI), dinotefuran, thiacloprid, thiamethoxam (THX), and nitenpyram. Neonicotinoid pesticides are agonists of nicotinic acetylcholine receptors (nAChRs) and exert their selective biological toxicity by disrupting the central nervous system of insects [1,2]. Recently, the impacts of CLO, IMI, and THX on bees and the neurological toxicity of ACE and IMI were reviewed by the European Food Safety Authority (EFSA). Because CLO and other neonicotinoids have a broad toxicity spectrum for crop pests, CLO has an undesirable environmental effect, and shows unwanted toxicity to non-target insects, such as honeybees. Therefore, EFSA and the United States Environmental Protection Agency are suspending CLO's registrations. Neonicotinoid pesticides are broadly used in agriculture, horticulture, tree nurseries, and forestry to control various pests [3]; however, the slow degradation of the pesticides may lead to residual amounts in the environment. For example, the accumulation of CLO in soil after multiple years of application has been reported [4]. For this reason, the elimination and detoxification of neonicotinoid pesticides are needed.

The metabolic pathways of CLO in livestock, crops, soil and water are summarized in FAO Plant Production and Protection Paper 206 [5]. In soil, two main pathways for CLO aerobic metabolism have been identified. The first pathway involves the *N*-demethylation of CLO to form *N*-(2-chlorothiazol-5-ylmethyl)-*N′*-nitroguanidine, and

N-methyl-*N′*-nitroguanidine is formed by the cleavage of the nitroguanidine moiety in another pathway. In addition, *N*-(2-chlorothiazol-5-yl-methyl)-*N′*-methylurea (TZMU) was obtained as a minor metabolic product resulting from transformation of the nitroguanidine. Although the metabolic pathways of CLO have been well studied, there has been no information about CLO-degrading microorganisms and enzymes.

White-rot fungi secrete ligninolytic enzymes and are able to mineralize lignin [6]. White-rot fungi secrete several oxidases as key ligninolytic enzymes, such as lignin peroxidase, manganese peroxidase (MnP), and laccase [7]. Generally, low nitrogen concentration in liquid medium induces ligninolytic enzymes. *Phanerochaete sordida* mainly produces MnP; however, the fungus produces hardly any lignin peroxidase or laccase in Mn-containing liquid medium [8,9]. These fungi also hold a large diversity of cytochrome P450 enzymes (CYPs) [10]. It is estimated that the diversity of the CYPs are the result of metabolic adaptation to the degradation of various lignin fragments, production of secondary metabolites, and detoxification of toxic compounds [10]. For these reasons, the use of white-rot fungi to degrade recalcitrant environmental pollutants, such as polyaromatic hydrocarbons, halogenated aromatics, nitro explosives [11], mycotoxins [12], and endocrine-disrupting compounds [13], has attracted attention as a cost-effective approach for the bioremediation of soils. We have also reported that the white-rot fungus *P. sordida* is able to degrade the neonicotinoid pesticide ACE and that the *N*-demethylated metabolite is detected in the culture [14].

In the present study, we investigated CLO degradation by *P. sordida* in liquid culture, and a metabolic product was identified by mass spectrometry and nuclear magnetic resonance

(NMR) analysis. The neurological toxicity of CLO and its metabolite were also examined using the mouse neuroblastoma cell line Neuro2a. This report demonstrates that a white-rot fungus is capable of degrading the neonicotinoid pesticide CLO into metabolites that lack neurological toxicity.

2. Materials and methods

2.1. Fungi and chemicals

P. sordida YK-624 (ATCC 90872) isolated from rotten wood [8] was maintained on potato dextrose agar slants at 4 °C.

All chemicals and solvents used in this study were of analytical grade and were used without further purification.

2.2. Degradation of CLO by *P. sordida*

Inoculation of *P. sordida* mycelium to nitrogen-limited (LN; ligninolytic conditions) and potato dextrose broth (PDB; nonligninolytic conditions) media (10 ml) was performed as previously [14]. After statically incubating the flasks at 30 °C for 5 days, 100 μl 10 mM CLO (DMSO solution) was added to the cultures, which were then further incubated for 5-20 days at 30 °C (n=3). After that, the cultures were homogenized with 20 mL acetone, then filtered. The filtrate was evaporated, and the residue, redissolved in methanol, was analyzed by high performance liquid chromatography (HPLC) on an Inertsil ODS-3 column (4.6 × 250 mm, 5 μm; GL Sciences, Tokyo, Japan) with 20% aqueous methanol (1 ml/min) at 270 nm. HPLC was performed using a JASCO PU-2089 pump with a JASCO MD-2018 PDA detector.

For determination of the effects of ligninolytic enzymes on CLO degradation, MnP was partially purified and reacted with CLO using the method described by Wang et al. [12]. Briefly, the culture fluid from LN culture was separated from mycelium, dialyzed and separated by anion-exchange chromatography (DEAE-Separose CL-6B; GE Healthcare). Then, partially purified MnP (10 nkat), 1 mM manganese sulfate, 0.1 % Tween 80, 5 nkat glucose oxidase, 2.5 mM glucose, and 10 μl of 10 mM CLO (in DMSO), 50 mM malonate (pH 4.5) were enzymatically reacted (1.0 ml) for 72 h at 30 ºC, 120 rpm. The same reaction mixture without MnP was used as a control.

2.3. Identification of CLO metabolite

The metabolite was obtained from LN medium (total 5 L) containing 200 mg CLO (in 5 ml DMSO) incubated with *P. sordida* for 20 days. An ethyl acetate extract of the culture was fractionated by chromatography on a silica gel 60 N column (φ40 × 600 mm) with successive elution by acetone/*n*-hexane (4/6, 6/4, 8/2, 10/0) and methanol. The metabolite was further purified by HPLC on a Develosil C30-UG-5 column (20 × 250 mm; Nomura Chemical, Japan) using 20% methanol. The purified metabolite was analyzed by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) using a JMS-T100LC mass spectrometer, and ^{1}H- and ^{13}C-NMR spectra were recorded using a JEOL Lambda-500 spectrometer.

2.4. Inhibition of CLO degradation by CYP inhibitors

After precultivation as described in Section 2.2, CLO (final concentration 100 μM) and piperonyl putoxide (PB) or 1-aminobenzotriazole (ABT) was added to the culture flasks (final

0.01, 0.1, and 1 mM, concentrations with low toxicity for white-rot fungus [15]). The flasks were incubated at 30 °C. HPLC was performed as described in Section 2.2.

2.5. Neurotoxicity of CLO and the metabolite TZMU

Neuro2a cell (Health Science Research Resources Bank, Japan) viability was assayed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Dojindo, Japan) as previously reported [16]. CLO or TZMU was applied to 24 h cultures at various concentrations, and the viability was measured by MTT assay after an additional 24 h incubation. Student's *t*-test was used to detect significant differences. Differences between means at a confidence level of 1% were considered to be statistically significant.

3. Results

3.1. CLO degradation by *P. sordida*

The time courses of CLO degradation in LN and PDB cultures of *P. sordida* are shown in Fig. 1. In LN medium, *P. sordida* degraded 37% of CLO after 20 d of culture. On the other hand, CLO was reduced by 8% after 20 d of cultivation in PDB medium. These results indicated that CLO can be degraded by *P. sordida*. On the other hand, CLO degradation was not observed in enzymatic reactions with MnP (data not shown).

3.2. Metabolite identification

To identify the metabolites produced during the degradation of CLO, *P. sordida* was cultured in LN medium with 160 μM CLO for 20 d. A metabolite was purified from the

culture supernatant. Based on HR-ESI-MS, the molecular formula of the purified metabolite was $C_6H_8ClN_3OS$, with an *m/z* 204.0003[M-H]$^-$ (calculated for $C_6H_7ClN_3OS$, 203.9998). This formula indicates that a nitroguanidine of CLO had been converted to urea. Additionally, the ^{1}H- and ^{13}C-NMR spectra of the metabolite were in accordance with those of CLO. Based on these findings and the chemical-shift assignments (Table 1), the metabolite was determined to be *N*-(2-chlorothiazol-5-yl-methyl)-*N′*-methylurea (TZMU; Fig. 2).

3.3. Inhibition of CYP

CLO degradation by *P. sordida* in LN medium was inhibited by the addition of CYP inhibitors (PB or ABT) into the culture (Fig. 3). After 20 days of incubation with PB, the remaining CLO concentration was 72, 71, and 90 μM in cultures containing 0.01, 0.1 and 1.0 mM PB, respectively, while CLO was reduced to 62 μM in cultures lacking PB (Fig. 3A). Similarly, after the addition of 0.01, 0.1, and 1 mM ABT, 64, 72, and 90 μM CLO, respectively, remained after the 20-d culture period (Fig. 3B). In contrast to the CLO degradation in cultures without CYP inhibitors, markedly lower degradation of CLO was observed in cultures with CYP inhibitors, particularly at inhibitor concentrations of 1 mM.

3.4. Neurotoxicity of CLO and its metabolite TZMU

To evaluate the neurotoxicity of CLO and TZMU, the effect of these compounds on the viability of the neuronal cell line Neuro2a was evaluated. Neuro2a cells were treated with 10, 30, 100, 300 μM CLO or TZMU for 24 h, and cell viability was quantified. CLO treatment significantly decreased the cell viability compared to the untreated control cells, whereas

TZMU treatment had no apparent effect on cell viability (Fig. 4). This result indicates that the metabolic modification of CLO by *P. sordida* converted the molecule to one that was no longer neurotoxic.

4. Discussion

To our knowledge, this is the first report of the microbial biodegradation of CLO. We demonstrated that 37% of CLO was degraded by the white-rot fungus *P. sordida* in LN liquid medium during 20 days of cultivation (Fig. 1). Notably, TZMU was detected and identified as a major metabolite of CLO (Fig. 2). The metabolism of CLO has been studied in animals, plants, and soil [5]. In mice and spinach, the *N*-nitro group in CLO is reduced to generate *N*-nitrosoguanidine or *N*-aminoguanidine derivatives and cleavage to form guanidine and urea derivatives (like TZMU) [17]. Although TZMU was found in soil as a metabolite of CLO, metabolites having a methyl nitrosoguanidine group or methyl aminoguanidine group have not been detected in soil [5]. Pandey et al. [18] reported that the neonicotinoid pesticides IMI and THX are transformed to nitrosoguanidine, guanidine, and urea metabolites by the bacterium *Pseudomonas* strain 1G. Although microbial metabolism of CLO has not been described, the present results suggest that the metabolic pathway of CLO in *P. sordida* is similar to that observed in mice. Unfortunately, however, no intermediary metabolites were detected in this study. Although *P. sordida* is known as a good MnP producer, the fungus produced hardly any lignin peroxidase or laccase in Mn-containing liquid medium [9]. Therefore, we tried CLO degradation *in vitro* using partially purified MnP (containing several isozymes). However, 100% of the CLO was recovered, the same as the MnP-absent control,

after 24 h incubation, so we concluded that CLO metabolism was not carried out by MnP.

CYPs of white-rot fungi play an important role in the degradation of recalcitrant aromatic compounds. [10,19,20]. A number of reports have demonstrated that the CYP inhibitors PB and ABT inhibit the bioconversion steps in many white-rot fungal species [14,20-22]. In the present study, CLO degradation was markedly inhibited by PB and ABT (Fig. 3). Based on this finding, it is proposed that CYPs are involved in the degradation of CLO. A CYP isozyme in mammals is able to reduce nitro groups of the nitroguanidine moiety of THX and CLO to form the corresponding *N*-nitrosoguanidine and *N*-aminoguanidine derivatives; after that, urea derivatives (like TZMU) are formed [17]. This suggested that the reduction of the *N*-nitro group by CYP reductase is an initial step of CLO degradation by *P. sordida*. However, the enzymes involved in the formation of urea derivatives are unknown. The present study adds the knowledge that CLO degradation can be observed under ligninolytic conditions, and that CYP is involved in the degradation of CLO, although its degradation is not dependent on ligninolytic enzymes. In future studies, we plan to examine how CYPs are involved in CLO degradation by *P. sordida*.

Neonicotinoid pesticides are widely used worldwide and are effective against a wide range of insects, including nontarget insects and other organisms, particularly honeybees. Recent studies have provided evidence that colony collapse disorder, which has led to a serious decline in the honeybee population, is linked to the use of neonicotinoid pesticides [23,24]. Wang et al. [25] also reported that five neonicotinoid pesticides (ACE, IMI, CLO, thiacloprid, and nitenpyram) are highly toxic to earthworms. The insecticidal activity of neonicotinoid pesticides is based on their activity against nAChRs. IMI mediates toxic effects

in honeybees by binding to nAChRs and has an IC_{50} value of 2.9 nM; its urea metabolite has an IC_{50} value >100,000 nM [26]. Therefore, we speculate that the urea metabolite of CLO (TZMU) also has low toxicity in honeybees. Neonicotinoid pesticides are broadly used in agriculture because they have a much higher affinity for the nAChRs of insects compared to those in vertebrates [27]; however, these pesticides may be neurotoxic to humans [28]. In the biodegradation of environmental pollutants, toxicity reduction is the primary aim. Although acute and chronic toxicity tests of neonicotinoid pesticides often use insects, we evaluated the neurotoxicity of CLO and TZMU by analyzing the viability of the mouse neuronal cell line Neuro2a. CLO treatment at 30 to 300 μM significantly decreased neuronal cell viability, whereas no remarkable neuronal cell death was observed after TZMU treatment (Fig. 4). Thus, the metabolic modification of CLO by *P. sordida* is expected to abolish its neurological toxicity.

In summary, we demonstrated that the white-rot fungus *P. sordida* is able to degrade CLO into the nontoxic metabolite TZMU. The present findings also suggest that CYPs play an important role in the degradation of CLO by *P. sordida*. And CLO degradation is not dependent on manganese peroxidase.

References

[1] K. Matsuda, S.D. Buckingham, D. Kleier, J.J. Rauh, M. Grauso, D.B. Sattelle, Neonicotinoids: Insecticides acting on insect nicotinic acetylcholine receptors, Trends Pharmacol. Sci. 22 (2001) 573–580. doi:10.1016/S0165-6147(00)01820-4.

[2] M. Tomizawa, J.E. Casida, Selective Toxicity of Neonicotinoids Attributable to Specificity of

Insect and Mammalian Nicotinic Receptors, Annu. Rev. Entomol. 48ne (2003) 339–364. doi:10.1146/annurev.ento.48.091801.112731.

[3] N. Simon-Delso, V. Amaral-Rogers, L.P. Belzunces, J.M. Bonmatin, M. Chagnon, C. Downs, L. Furlan, D.W. Gibbons, C. Giorio, V. Girolami, D. Goulson, D.P. Kreutzweiser, C.H. Krupke, M. Liess, E. Long, M. Mcfield, P. Mineau, E.A.D. Mitchell, C.A. Morrissey, D.A. Noome, L. Pisa, J. Settele, J.D. Stark, A. Tapparo, H. Van Dyck, J. Van Praagh, J.P. Van der Sluijs, P.R. Whitehorn, M. Wiemers, Systemic insecticides (Neonicotinoids and fipronil): Trends, uses, mode of action and metabolites, Environ. Sci. Pollut. Res. 22 (2015) 5–34. doi:10.1007/s11356-014-3470-y.

[4] D. Goulson, An overview of the environmental risks posed by neonicotinoid insecticides, J. Appl. Ecol. 50 (2013) 977–987. doi:10.1111/1365-2664.12111.

[5] T. van der Velde-Koerts, P.H. van Hoeven-Arentzen, C.M. Mahieu, Chlothianidin (238), Food and Agricultural Organization, Rome, Italy, 2010. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation10/Chlotiahinidin.pdf.

[6] T.K. Kirk, R.L. Farrell, Enzymatic “combustion”: the microbial degradation of lignin, Annu. Rev. Microbiol. 41 (1987) 465–505. doi:10.1146/annurev.mi.41.100187.002341.

[7] A. Hatakka, Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation, FEMS Microbiol. Rev. 13 (1994) 125–135. doi:10.1111/j.1574-6976.1994.tb00039.x.

[8] H. Hirai, R. Kondo, K. Sakai, Screening of lignin-degrading fungi and their ligninolytic enzyme activities during biological bleaching of kraft pulp, Mokuzai Gakkaishi. 40 (1994) 980–986.

[9] C. Ruttimann-Johnson, D. Cullen, R.T. Lamar, Manganese peroxidases of the white rot fungus

Phanerochaete sordida, Appl. Environ. Microbiol. 60 (1994) 599–605.

[10] H. Ichinose, Cytochrome P450 of wood-rotting basidiomycetes and biotechnological applications, Biotechnol. Appl. Biochem. 60 (2013) 71–81. doi:10.1002/bab.1061.

[11] D. Gao, L. Du, J. Yang, W.-M. Wu, H. Liang, A critical review of the application of white rot fungus to environmental pollution control, Crit. Rev. Biotechnol. 30 (2010) 70–77. doi:10.3109/07388550903427272.

[12] J. Wang, M. Ogata, H. Hirai, H. Kawagishi, Detoxification of aflatoxin B1 by manganese peroxidase from the white-rot fungus Phanerochaete sordida YK-624, FEMS Microbiol. Lett. 314 (2011) 164–169. doi:10.1111/j.1574-6968.2010.02158.x.

[13] J. Wang, N. Majima, H. Hirai, H. Kawagishi, Effective removal of endocrine-disrupting compounds by lignin peroxidase from the white-rot fungus phanerochaete sordida YK-624, Curr. Microbiol. 64 (2012) 300–303. doi:10.1007/s00284-011-0067-2.

[14] J. Wang, H. Hirai, H. Kawagishi, Biotransformation of acetamiprid by the white-rot fungus Phanerochaete sordida YK-624, Appl. Microbiol. Biotechnol. 93 (2012) 831–835. doi:10.1007/s00253-011-3435-8.

[15] T. Mori, R. Kondo, Oxidation of dibenzo-p-dioxin, dibenzofuran, biphenyl, and diphenyl ether by the white-rot fungus Phlebia lindtneri, Appl. Microbiol. Biotechnol. 60 (2003) 200–205. doi:10.1007/s00253-002-1090-9.

[16] K. Nagai, A. Chiba, T. Nishino, T. Kubota, H. Kawagishi, Dilinoleoyl-phosphatidylethanolamine from Hericium erinaceum protects against ER stress-dependent Neuro2a cell death via protein kinase C pathway, J. Nutr. Biochem. 17 (2006) 525–530. doi:10.1016/j.jnutbio.2005.09.007.

[17] J.E. Casida, Neonicotinoid metabolism: Compounds, substituents, pathways, enzymes, organisms, and relevance, J. Agric. Food Chem. 59 (2011) 2923–2931. doi:10.1021/jf102438c.

[18] G. Pandey, S.J. Dorrian, R.J. Russell, J.G. Oakeshott, Biotransformation of the neonicotinoid insecticides imidacloprid and thiamethoxam by Pseudomonas sp. 1G, Biochem. Biophys. Res. Commun. 380 (2009) 710–714. doi:10.1016/j.bbrc.2009.01.156.

[19] T. Hata, S. Kawai, H. Okamura, T. Nishida, Removal of diclofenac and mefenamic acid by the white rot fungus Phanerochaete sordida YK-624 and identification of their metabolites after fungal transformation, Biodegradation. 21 (2010) 681–689. doi:10.1007/s10532-010-9334-3.

[20] P. Xiao, T. Mori, I. Kamei, R. Kondo, A novel metabolic pathway for biodegradation of DDT by the white rot fungi, Phlebia lindtneri and Phlebia brevispora, Biodegradation. 22 (2011) 859–867. doi:10.1007/s10532-010-9443-z.

[21] E. Marco-Urrea, M. Pérez-Trujillo, G. Caminal, T. Vicent, Dechlorination of 1,2,3- and 1,2,4-trichlorobenzene by the white-rot fungus Trametes versicolor, J. Hazard. Mater. 166 (2009) 1141–1147. doi:10.1016/j.jhazmat.2008.12.076.

[22] N. Golan-Rozen, B. Chefetz, J. Ben-Ari, J. Geva, Y. Hadar, Transformation of the recalcitrant pharmaceutical compound carbamazepine by pleurotus ostreatus: Role of cytochrome P450 monooxygenase and manganese peroxidase, Environ. Sci. Technol. 45 (2011) 6800–6805. doi:10.1021/es200298t.

[23] R.J. Gill, O. Ramos-Rodriguez, N.E. Raine, Combined pesticide exposure severely affects individual- and colony-level traits in bees., Nature. 491 (2012) 105–108. doi:10.1038/nature11585.

[24] D. Cressey, Europe debates risk to bees., Nature. 496 (2013) 408. doi:10.1038/496408a.

[25] K. Wang, S. Pang, X. Mu, S. Qi, D. Li, F. Cui, C. Wang, Biological response of earthworm, Eisenia fetida, to five neonicotinoid insecticides, Chemosphere. 132 (2015) 120–126. doi:10.1016/j.chemosphere.2015.03.002.

[26] R. Nauen, U. Ebbinghaus-Kintscher, R. Schmuck, Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in Apis mellifera (Hymenoptera: Apidae), Pest Manag. Sci. 57 (2001) 577–586. doi:10.1002/ps.331.

[27] M. Tomizawa, D.L. Lee, J.E. Casida, Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors, J. Agric. Food Chem. 48 (2000) 6016–6024. doi:10.1021/jf000873c.

[28] European Food Safety Authority, Scientific Opinion on the developmental neurotoxicity potential of acetamiprid and imidacloprid, EFSA J. 11 (2013) 1–47. doi:10.2903/j.efsa.2013.3471.

Figure captions

Fig. 1 Time course of CLO degradation by *P. sordida*. *Closed circles*: PDB medium, and *open circles*: LN medium. Values are the means ± SD of triplicate samples.

Fig. 2 *Heteronuclear multiple bond correlation* (HMBC) for the identified CLO metabolite.

Fig. 3 Effects of the cytochrome P450 inhibitors PB (A) and ABT (B) on the degradation of CLO by *P. sordida* in LN medium. *Closed circles*: 0 mM, *closed squares*: 0.01 mM, *closed triangles*: 0.1 mM, and *closed diamonds*: 1 mM. Values are the means ± SD of triplicate samples.

Fig. 4 Neurotoxicity of CLO and its metabolite TZMU. Values are the means ± SD of 12 samples. Asterisks indicate values that were determined by Student's t-test to be significantly different from the untreated control (*$P < 0.01$).

Table legends

Table 1 ^{1}H- and ^{13}C-NMR data for TZMU (in CD_3OD).

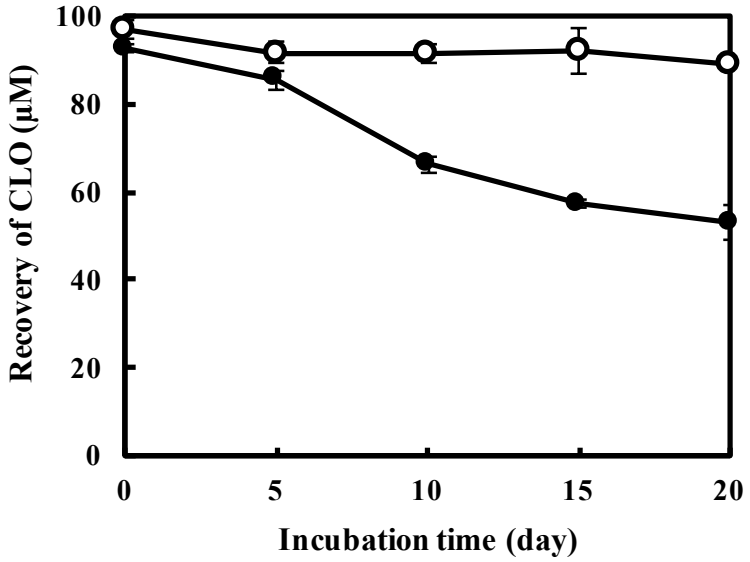
Recovery of CLO (μM)
100
80
60
40
20
0
0
5
10
15
20
Incubation time (day)

Fig. 1

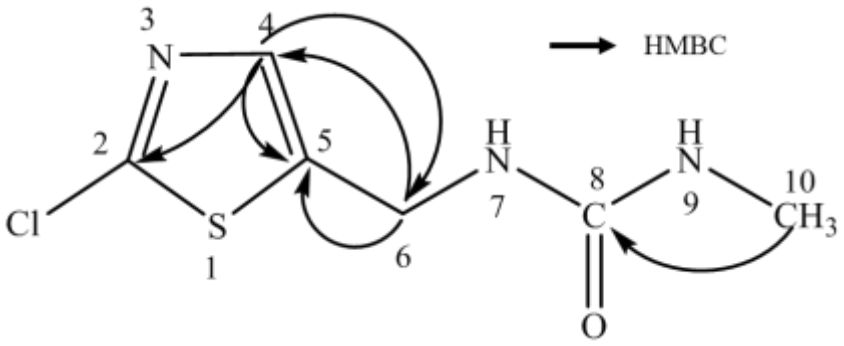

Fig. 2

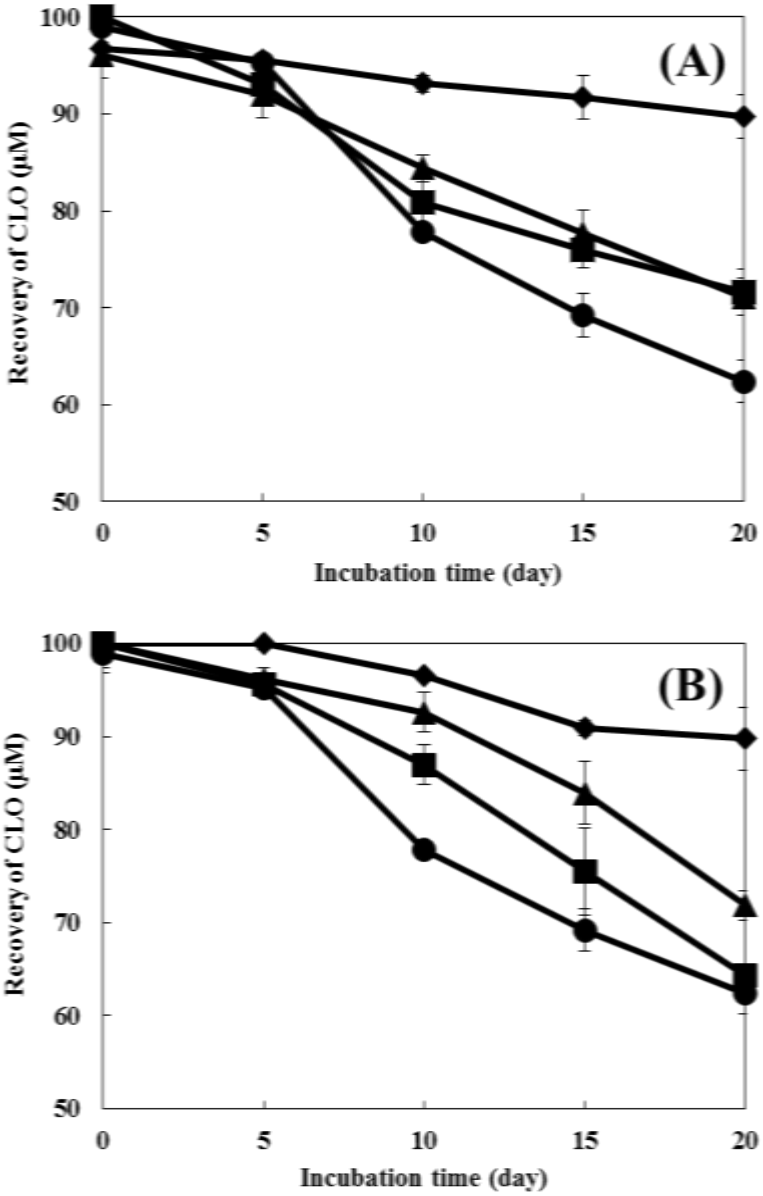
(A)
Recovery of CLO (μM)
100
90
80
70
60
50
0
5
10
15
20
Incubation time (day)
(B)
Recovery of CLO (μM)
100
90
80
70
60
50
0
5
10
15
20
Incubation time (day)

Fig. 3

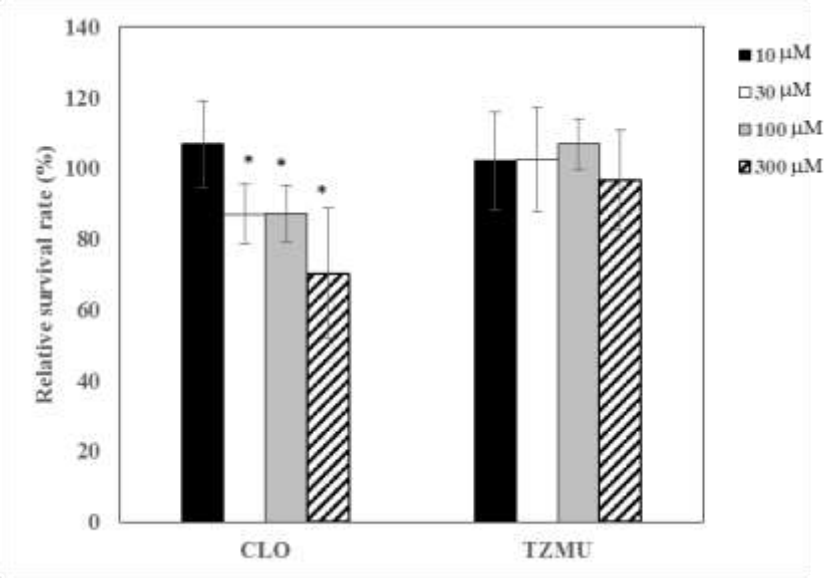
140
120
100
80
60
40
20
0
Relative survival rate (%)
*
*
*
CLO
TZMU
10 μM
30 μM
100 μM
300 μM

Fig. 4

Table 1 ^{1}H- and^{13}C-NMR data for TZMU (in CD_3OD).

Position	^{1}H	^{13}C
	δ_H	δ_c
2	-	152.7
4	7.40	139.5
5	-	142.8
6	4.40	37.4
8	-	161.3
10	2.69	27.0