Identification of the cytochrome P450 involved in the degradation of neonicotinoid insecticide acetamiprid in Phanerochaete chrysosporium

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Research paper

Identification of the cytochrome P450 involved in the degradation of neonicotinoid insecticide acetamiprid in *Phanerochaete chrysosporium*

Jianqiao Wang[a], Haruka Ohno[b], Yuuri Ide[b], Hirofumi Ichinose[c], Toshio Mori[b], Hirokazu Kawagishi[b, d, e], Hirofumi Hirai[b, e*]

[a] Key Laboratory for Water Quality and Conservation of the Pearl River Delta, Ministry of Education, School of Environmental Science and Engineering, Guangzhou University, Guangzhou 510006, China

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

[c] Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

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

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

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* Corresponding author:
Hirofumi Hirai
Mailing address: 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

Abstract

We previously reported that cytochrome P450s play critical roles in neonicotinoid insecticide biodegradation by white-rot fungi. Here, we investigated the biodegradation of acetamiprid (ACET) by *Phanerochaete chrysosporium* to identify the cytochrome P450 involved in this degradation process. During a 20-day incubation period, *P. chrysosporium* degraded 21% and 51% of ACET in ligninolytic and nonligninolytic media, respectively. The degradation rate of ACET was markedly decreased by the addition of cytochrome P450 inhibitors. Recombinant cytochrome P450s in *P. chrysosporium* (PcCYP) were heterologously expressed in *Saccharomyces cerevisiae* strain AH22, and the PcCYP involved in ACET degradation was identified. The results showed that CYP5147A3 can degrade ACET, and two ACET metabolites, *N'*-cyano-*N*-methyl acetamidine and 6-chloro-3-pyridinemethanol, were identified. To the best of our knowledge, this study provides the first characterization of the fungal cytochrome P450 that is responsible for the degradation and detoxification of ACET.

1. Introduction

Acetamiprid (ACET) is one of the first-generation neonicotinoid insecticides, and neonicotinoid insecticides have been used for the last two decades because they are less toxic than older insecticides. All neonicotinoid insecticides exert toxic effects on the nervous system of insects by binding to nicotinic acetylcholine receptors (nAChRs) [1, 2]. Neonicotinoid insecticides are extensively used for controlling pests in a wide range of croplands [3], and as a result, neonicotinoid insecticides can contaminate soil and water and accumulate in the food chain. Increasing lines of evidence show that these neonicotinoid insecticides cause harm to nontarget organisms, such as honeybees and wild bees, and these findings have prompted increasing concerns about these insecticides [4-7]. In addition, recent studies have revealed the impacts of these insecticides on aquatic invertebrates [8, 9] and vertebrates [10, 11], including humans [12, 13]. Thus, the elimination of neonicotinoid insecticides is urgently needed. ACET undergoes chemical degradation in the environment [14, 15], but chemical methods are expensive and environmentally harmful. Therefore, microbial degradation is preferable because it can avoid these shortcomings and potentially provide a strategy for the remediation of ACET contamination.

The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) summarized the main metabolic pathway of ACET. In rats, ACET is first demethylated to N^1-[(6-chloro-3-pyridyl) methyl]-N^2-cyanoacetamidine (IM 2-1), and IM 2-1 is then converted to 6-chloronicotinic acid (IC 0) and an *N*-cyanoacetamidine derivative that is released after cleavage from the side chains [16]. In plants, ACET is demethylated to IM 2-1 and further degraded to 6-chloro-3-pyridyl)methyl-β-D-glucopyranoside. The other significant metabolic pathway in plants metabolize ACET to N^2-carbamoyl-N^1-[(6-chloro-3-pyridyl)methyl]-N^1-methylacetamidine (IM 1-2), *N*-[(6-chloro-3-pyridyl)methyl]-*N*-methylacetamide (IM 1-3), *N*-[(6-chloro-3-pyridyl)methyl]acetamide (IM 2-3), *N*-methyl(6-chloro-3-pyridyl)methylamine (IM 1-4), and IC 0 [16]. Some microorganisms can degrade ACET, and *Rhodotorula mucilaginosa* IM-2, *Ensifer meliloti* CGMCC 7333, and *Stenotrophomonas maltophilia* CGMCC 1.1788 convert ACET to IM 1-3, IM 1-2, and IM 2-1, respectively [17-19]. Mammalian cytochrome P450s (CYPs) are associated with neonicotinoid insecticide degradation via pathways such as hydroxylation, nitroimine reduction of imidacloprid and the oxidation of thiamethoxam and clothianidin [20, 21]. Microbial CYPs might be involved in thiacloprid degradation by *S. maltophilia* CGMCC 1.1788 [22]. However, the type of microbial CYPs involved in the degradation of ACET needs to be further studied.

The mineralization of recalcitrant environmental pollutants, such as 1,1-bis(4-

chlorophenyl)-2,2,2-trichloroethane, 2,3,7,8-tetrachlorodibenzo-*p*-dioxine, lindane, and 3,3',4,4'-tetrachlorobiphenyl, by a white-rot fungus has been demonstrated [23], and thus, there is extensive interest in the use of white-rot fungi for bioremediation. The ligninolytic enzymes and CYPs of white-rot fungi have been the widely studied due to their significant roles in the degradation of recalcitrant environmental pollutants [24-28]. To date, a total of 154 CYPs in *Phanerochaete chrysosporium* (PcCYP) have been identified through bioinformatics surveys [29]. In our previous study, we showed that the white-rot fungus *P. sordida* YK-624 can degrade ACET and demonstrated that the CYP inhibitor piperonyl butoxide can inhibit the *N*-demethylation of ACET [30]. In the present study, we examined the biodegradation of ACET by the white-rot fungus *P. chrysosporium* to identify the PcCYP involved in the degradation of ACET. To that end, we used a functional screening system in which recombinant PcCYPs were heterologously expressed in *Saccharomyces cerevisiae* strain AH22 [29]. We also identified two lower-toxicity metabolites of ACET by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), gas chromatography mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). This study provides the first identification of the fungal CYP involved in ACET degradation and demonstrates the dealkylation of ACET by a pure fungal CYP.

2. Materials and methods

2.1. Equipment

GC-MS was performed using a Shimadzu gas chromatograph-mass spectrometer (Shimadzu Cooperation, Kyoto, Japan) equipped with a Rtx®-5MS capillary column (3.0 m × 0.25 mm × 0.25 μm, GL sciences, Tokyo, Japan). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The oven temperature was increased from 100°C to 250°C at a rate of 5°C/min and maintained at 250°C for 30 min. The ion source and interface temperatures were maintained at 250°C and 200°C, respectively. HR-ESI-MS data were obtained using a JMS-T100LC mass spectrometer, and NMR spectra were recorded using a Jeol Lambda-500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz). High-performance liquid chromatography (HPLC) analyses were conducted using a JASCO PU-2089 pump with an MD-2018 PDA detector.

2.2. Fungal degradation of ACET

P. chrysosporium ME-446 (ATCC 34541) was preserved on potato dextrose agar medium at 4°C. *P. chrysosporium* was inoculated into potato dextrose broth (PDB) and nitrogen-limited (LN) media, and ACET (Wako Pure Chemical Industries) was then added to a final concentration of 0.1 mM. The detailed fungal degradation procedures were previously

described [31]. ACET was quantified through an HPLC analysis using a Develosil column (5 μm C30-UG-5 4.6 x 250 mm, Nomura Chemical, Seto, Japan). Elution was performed at a flow rate of 1 mL/min with 30% aqueous methanol, and the eluate was monitored at 246 nm.

2.3. Inhibition of ACET degradation by 1-aminobenzotriazole (ABT)

The inhibition of ACET degradation by the CYP inhibitor ABT (Tokyo Chemical Industry） at final concentrations of 0.01, 0.1, and 1 mM was examined. The degradation experiment and analysis methods were previously described [31].

2.4. Identification of the *P. chrysosporium* PcCYP involved in ACET degradation using a functional screening system

The genome sequence of *P. chrysosporium* ME-446 was obtained from the US Department of Energy Joint Genome Initiative (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html), and the PcCYP was heterologously expressed in *S. cerevisiae* strain AH22 as described by Hirosue et al. [29]. The transformants were separately inoculated into 96-deep-well plates containing synthetic dextrose liquid (SDL) medium, and ACET was added to a final concentration of 0.5 mM for the rapid screening of ACET degradation. After 2 days of incubation, the reactions were stopped by the addition of methanol/acetone. After centrifugation and filtration, the removal of ACET and its metabolites was analyzed by HPLC.

2.5. Identification of the metabolites of ACET

P. chrysosporium was inoculated into PDB medium for large-scale cultivation (4 L). After 5 days of incubation, ACET was added to a final concentration of 0.5 mM, and the culture was incubated for an additional 20 days. The cultures were evaporated and extracted with ethyl acetate.

SDL medium containing yeast transformants expressing PcCYP and 0.5 mmol/L ACET was incubated for 4 days. A 650-mL sample of solution was obtained after centrifugation and filtration, and the solution was evaporated and extracted using ethyl acetate. The residue was separated by flash column chromatography on silica gel (60 N, φ40 x 600 mm), and 41 fractions were obtained by eluting with *n*-hexane/ethyl acetate/methanol (10/0/0, 9/1/0, 8/2/0, 7/3/0, 6/4/0, 5/5/0, 4/6/0, 3/7/0, 2/8/0, 1/9/0, 0/10/0, 0/8/2, 0/5/5, 0/2/8, and 0/0/10; v/v).

The metabolites were further purified by preparative HPLC using a C30-UG-5 column (20 x 250 mm, Nomura Chemical, Seto, Japan) and then analyzed by GC-MS, HR-ESI-MS and NMR.

3. Results

3.1. Fungal degradation of ACET

LN and PDB media were used for the degradation of ACET by *P. chrysosporium*, and 21% and 51% of ACET was degraded after 20 days of incubation in LN and PDB medium, respectively (Fig. 1). These results showed that *P. chrysosporium* can effectively degrade ACET.

3.2. Inhibition of CYP and identification of the ACET-degrading PcCYP

The biodegradation of ACET by *P. chrysosporium* in PDB medium was inhibited by the CYP inhibitor ABT. Although 48 μM ACET remained in the cultures without ABT, ACET concentrations of 94, 98, and 99 μM were obtained in the presence of 0.01, 0.1 and 1 mM ABT, respectively (Fig. 2). Thus, the degradation rate of ACET in cultures containing ABT was significantly lower than that in the cultures without ABT.

To screen the ACET-degrading CYPs, 120 PcCYPs were heterologously expressed in the yeast *S. cerevisiae* strain AH22 for the degradation of ACET. The incubation of ACET with the transformants revealed that CYP5147A3 was able to degrade ACET. An HPLC analysis revealed a metabolite after both fungal degradation and CYP5147A3 treatment (data not shown).

3.3. Identification of the metabolites of ACET

Large-scale cultivations of ACET with *P. chrysosporium* and the transformant CYP5147A3 were performed for metabolite analysis. The metabolites were detected through comparisons of their GC-MS and HPLC retention times. The GC-MS analysis revealed that an ACET metabolite showed signals at m/z 143, 114 (143-CHO) and 78 (114-HCl) (Fig. S1), and this metabolite of ACET was further characterized by HR-ESI-MS and NMR analysis. The HR-ESI-MS data of the metabolite revealed a molecular ion $[M+H]^+$ at m/z 144.0189 (calcd for C_6H_7ClNO, 144.0217) (Fig. S2), which indicated a molecular formula of C_6H_6ClNO. Moreover, the distortionless enhanced polarization transfer (DEPT) experiment showed the presence of one methylene, three methines, and two quaternary carbons. Table 1 and Fig. S3 show the assignment of the protons and carbons in the metabolite, and the couplings in the heteronuclear multiple bond correlation experiment (HMBC, H-2/C-3, 4, 6; H-4/C-2, 6, 7; and H-7/C-2, 3, 4) are shown in Fig. 3. As a result, the metabolite was identified as 6-chloro-3-pyridinemethanol (IM 0, Fig. 3). Another metabolite with signals at m/z 97, m/z 82 (97-CH_3), m/z 67 (82-CH_3) and m/z 56 (97-HNCN) was detected by GC−MS (Fig. S4). The MS fragmentation pattern of this metabolite was the same as the reference data [32], which suggested that this metabolite was *N'*-cyano-*N*-methylacetamidine (ACET-c, Fig. 3).

4. Discussion

Few studies have investigated the biodegradation of the neonicotinoid insecticide ACET by microorganisms. In this study, we found that *P. chrysosporium* could degrade 21% and 51% of ACET in LN medium and PDB medium, respectively (Fig. 1). The present study showed that recombinantly expressed CYP5147A3 is capable of converting ACET to IM 0, which was detected as a product of ACET degradation by *P. chrysosporium*. In addition, this study provides the first demonstration that fungal CYPs can degrade ACET. The main metabolite of ACET in soil is IM 1-4, and the minor metabolites are IM 1-3 and IC 0 [17, 33, 34]. The main metabolic pathway for ACET degradation in mammals involves *N*-demethylation [16, 35], and in insects, ACET is metabolized to IM 1-3, IM 1-4, IM 2-1, IC 0, and IM 0 [36]. IM 0 and ACET-c have been detected in rats and plants as ACET metabolites [32, 35]. The microbial degradation of ACET produces IM 1-4, which was previously identified in *Pigmentiphaga* sp. AAP-1 cultures by GC-MS and NMR analysis [33]. *R. mucilaginosa* IM-2 and *E. meliloti* CGMCC 7333 can convert ACET into IM 1-3 and IM 1-2, respectively [17, 18]. The *N*-demethylated metabolite of ACET, IM 2-1, from *S. maltophilia* CGMCC 1.1788 was detected by liquid chromatography/tandem mass spectrometry and NMR analysis [19]. Similarly, the degradation of ACET to IM 2-1 by *P. sordida* YK-624 was previously reported [30]. Although some studies have investigated the metabolism of ACET by microorganisms, the present study reports the first detection of IM 0 and ACET-c as metabolites in microorganisms.

The degradation of lignin and a range of refractory organic contaminants using white-rot fungi has been highlighted in many studies. Extracellular ligninolytic enzymes, including lignin peroxidases, manganese peroxidases, laccase, and versatile peroxidase, are involved in lignin biodegradation [37, 38], and some metabolic systems, such as intracellular enzymes, including CYPs, and extracellular ligninolytic enzymes, are also involved in this process. CYPs have been shown to play a key role in the metabolism of organic contaminants by white-rot fungi [26, 28-31]. ABT is a well-known CYP inhibitor [39], and inhibition experiments revealed that ACET degradation was substantially inhibited by ABT (Fig. 2). Hence, we conjectured that CYPs are related to the degradation of ACET. The effect of CYPs on the degradation of neonicotinoid insecticides has been studied in other processes. Schulz-Jander and Casida have reported the hydroxylation of imidacloprid by CYP3A4 isolated from the human liver [20], and CYP3A4 can oxidize thiamethoxam and clothianidin [21]. In insects, CYP6CM1 (*Bemisia tabaci*), CYP6ER1 (*Nilaparvata lugens*), and CYP6CY3 (*Myzus persicae*) overexpression has

been associated with the detoxification of imidacloprid [40-42]. Recombinant CYP6CM1 is involved in the cross-metabolism of clothianidin and thiacloprid [43]. However, no CYPs responsible for ACET degradation have been identified in microorganisms. To screen for the active ACET-degrading CYP, 120 CYPs from *P. chrysosporium* were functionally expressed in *S. cerevisiae* strain AH22. We provide the first demonstration that a fungal CYP, CYP5147A3, can convert ACET into IM 0 and ACET-c. The putatively proposed *N*-dealkylation mechanism of ACET by CYP5147A3 of *P. chrysosporium* is shown in Fig. 4. Two mechanistic hypotheses regarding the CYP-mediated *N*-dealkylation process was been proposed [44]. The first hypothesis involves hydrogen atom transfer (HAT): the reaction starts through the transfer of a proton from the alpha-carbon of the tertiary amine to CYP (compound I, $[Fe(IV)=O]^{\bullet+}$). The second hypothesis involves single electron transfer (SET): an electron is first transferred to CYP (compound I) to form an ammonium cation radical, and internal electron transfer induces deprotonation at the alpha-carbon. Both reactions yield a carbon-centered radical. A hemiaminal structure is subsequently generated by oxygen rebound, and spontaneous degradation then produces an aldehyde and a corresponding *N*-dealkylamine (ACET-c). Although the HAT mechanism is considered the most acceptable based on computational and experimental results, the structure-reaction relationship is not yet well understood. Therefore, whether the *N*-dealkylation of ACET by CYP derived in *P. chrysosporium* proceeds through either mechanism remains unclear. The N-dealkylation of ACET through reaction with a carbonate radical appears to favor formation of the aminium cation radical, and an aldehyde and a *N*-dealkylated amine are generated, as was observed in the CYP reaction in this study. The aldehyde resulting from fungal ACET degradation was easily reduced to form IM-0, and this reaction was followed by further metabolism.

Neonicotinoid insecticides are currently the most widely used insecticides worldwide, and thus, their persistence in environments and their potential risks to nontarget organisms must be explored. ACET is commonly detected in surface water and has a half-life of 450 days in soil [45, 9]. In addition, ACET is toxic to honeybees, with a lethal dose (LD_{50}) value of 7 μg/bee, whereas the metabolite IM 0 induces no honeybee mortality, with an LD_{50} value of 50 μg/bee [46]. Although neonicotinoid insecticides show better affinity for insect than vertebrate nAChRs [47], they were recently reported to be toxic to vertebrates [10-13]. A 13-

week study in rats showed that the no-observed-adverse-effect levels of ACET and the metabolite IM 0 were 7 mg/kg/day and 48.9 mg/kg/day, respectively [16]. These results showed that the toxicity of IM 0 was lower than that of ACET. A previous study identified a CYP enzyme that could detoxify thiacloprid and ACET to less-toxic metabolites [48]. We therefore predicted the detoxification of ACET via the CYP from *P. chrysosporium*.

In summary, our findings showed that *P. chrysosporium* and its CYP could transform the neonicotinoid insecticide ACET to IM 0 and ACET-c, which are less toxic than ACET. Our results provided the first identification of a fungal CYP, CYP5147A3, involved in the detoxification of ACET. This study will help further our understanding of the activity of detoxification enzymes for neonicotinoid insecticides.

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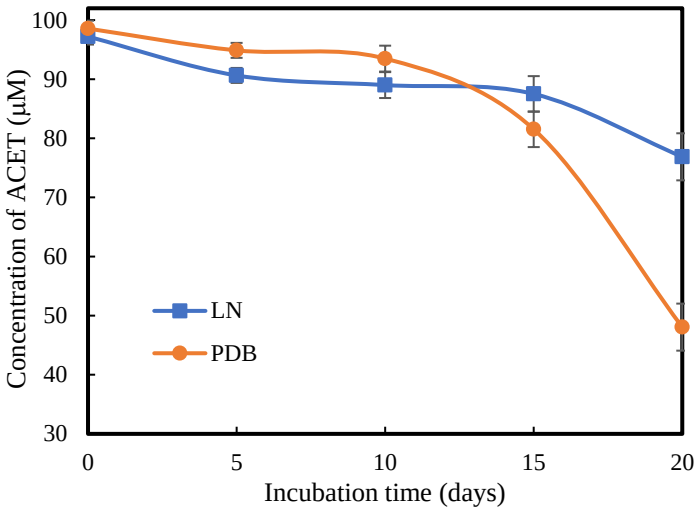

Fig. 1 Time-course of the degradation of ACET by *P. chrysosporium*. Blue squares: LN medium, and orange circles: PDB medium. The values are presented as the mean ± SD of triplicate samples.

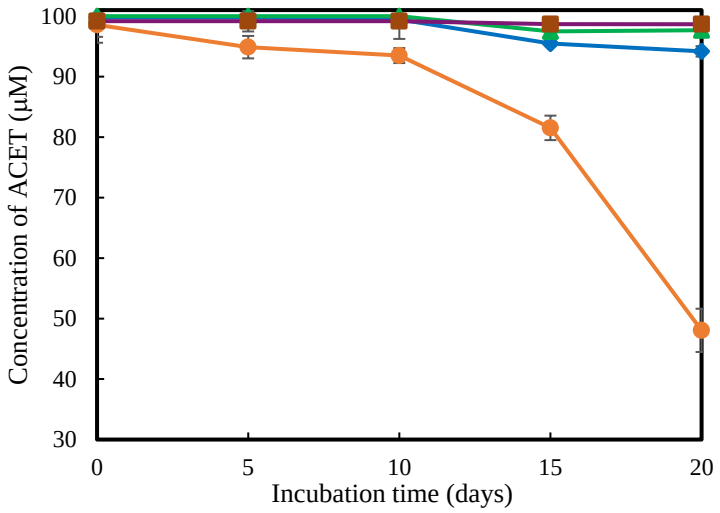

Fig. 2 Effect of ABT on the degradation of ACET by *P. chrysosporium* in PDB medium. Orange circles: 0 mM, blue diamonds: 0.01 mM, green triangles: 0.1 mM, and red squares: 1 mM. Values are the means ± SD of triplicate samples.

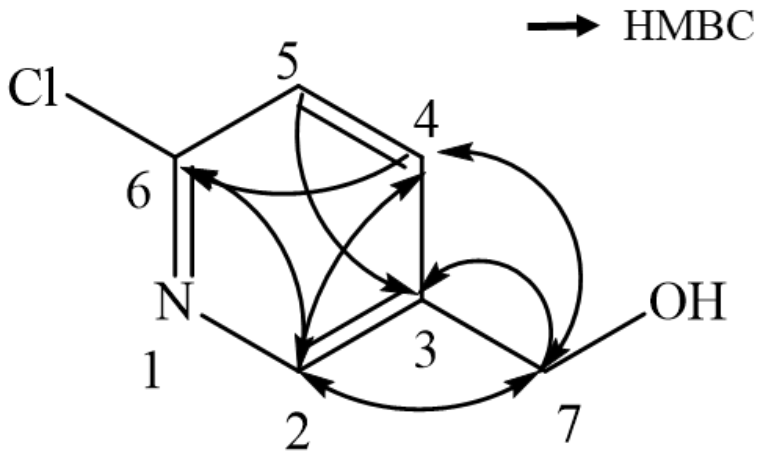

Fig. 3 Heteronuclear multiple bond correlation (HMBC) for the identified ACET metabolite.

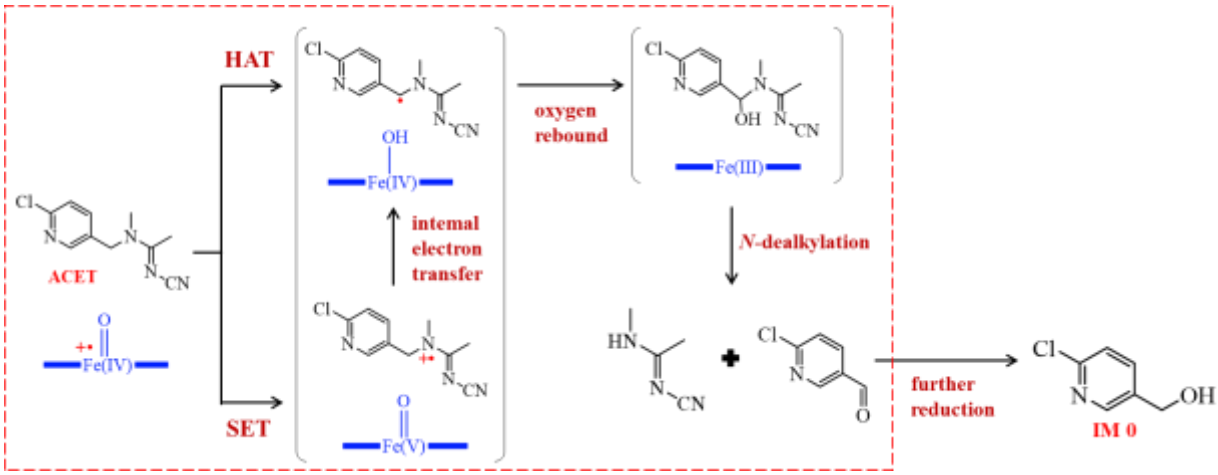

Fig. 4 Proposed reaction mechanism for the *N*-dealkylation of ACET with CYP5147A3 of *P. chrysosporium*, as well as further reduction to form IM 0 (outside of dashed line box). HAT: hydrogen atom transfer; SET: single electron transfer.

Table 1 ^{1}H- and ^{13}C-NMR data for IM 0 (in CD_3OD).

Position	^{1}H δ_H	^{13}C δ_C
2	8.33	149.1
3	-	138.1
4	7.80	139.7
5	7.43	125.4
6	-	151.0
7	4.63	61.8