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Title:

White-rot fungus *Phanerochaete chrysosporium* metabolizes chloropyridinyl-type neonicotinoid insecticides by an *N*-dealkylation reaction catalyzed by two cytochrome P450s

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Abstract

We previously identified a cytochrome P450 (CYP) derived from the white-rot fungus *Phanerochaete chrysosporium* as involved in degradation of acetamiprid, a neonicotinoid (NEO) insecticide. In the present study, we investigated biodegradation of other NEOs by *P. chrysosporium*, and attempted to identify the CYP enzyme responsible for NEO degradation. *P. chrysosporium* was able to degrade some NEOs (acetamiprid, clothianidin, imidacloprid, and thiacloprid) in nutrient-rich medium. Degradation was inhibited by the addition of a CYP inhibitor. Two CYPs in *P. chrysosporium* (PcCYPs), CYP5037B3 and CYP5147A3, were identified as major isozymes involved in metabolism of three neonicotinoids that have in common a chloropyridinyl moiety (acetamiprid, imidacloprid, and thiacloprid) by screening yeast that heterologously express PcCYPs. Both PcCYPs catalyzed cleavage of the chloropyridinyl moiety and side chain of the three NEOs by *N*-dealkylation, resulting in 6-chloro-3-pyridinemethanol and respective side chain fragments. In a culture of *P. chrysosporium*, 97% and 74% of imidacloprid and thiacloprid were modified to form degradation products, and one of these, 6-chloro-3-pyridinemethanol, was further degraded. These two PcCYPs catalyzed almost the same reaction but their substrate specificity and expression pattern are slightly different. Altogether, we found that *P. chrysosporium* degrades NEOs via the activity of at least two different CYP isozymes.

Keywords

bioremediation, neonicotinoids, *Phanerochaete chrysosporium*, cytochrome P450

Highlights

Phanerochaete chrysosporium is able to degrade four neonicotinoid insecticides.

Two cytochrome P450 isozymes are involved in degradation of neonicotinoids.

These enzymes react with all chloropyridinyl neonicotinoids tested except nitenpyram.

The degradation reactions are probably carried out via *N*-dealkylation.

1 **1. Introduction**

2 Neonicotinoids (NEOs) are insecticides that have in common a nicotine-related
3 molecular structure. NEOs are very commonly used insecticides in various countries, and are
4 very effective because of their transferability to plants, water solubilities, and residual
5 effectiveness. The first NEO, imidacloprid (IMI), appeared in the 1980s and was followed by
6 the development of several IMI analogues. NEOs act as exogenous agonists of nicotinic
7 acetylcholine receptors (nAChRs) and show high affinity to insect nAChRs (Tomizawa and
8 Casida, 2003). Indeed, NEOs exhibit activity against a broad spectrum of invertebrates by
9 continuously stimulating neural transmission in the central nervous system. NEOs usually show
10 a lower affinity for mammalian nAChRs than for insect nAChRs (e.g. (Tomizawa et al., 2000)),
11 such that these compounds show higher selective toxicity to invertebrates. NEOs are used for a
12 wide range of pest control applications, including for the control of important crop, soil, timber,
13 and animal pests.

14 Contrary, such a broad spectrum of insecticidal activity can also be a disadvantage
15 because NEOs also affect non-target insects. Whitehorn et al. have suggested that for bumble
16 bees, NEOs have a considerable negative impact on colony growth rates and production of new
17 queens (Whitehorn et al., 2012). Moreover, NEOs have impacts on nontarget invertebrates such
18 as butterflies, moths, wasps, beetles, earthworms, and aquatic invertebrates, as reviewed by Pisa
19 et al. (Pisa et al., 2015). As described above, NEOs display lower toxicity against mammals as
20 compared with insects; however, these compounds are known to nonetheless exert direct and

21 indirect negative effects on a wide range of vertebrates (Gibbons et al., 2015). For example,
22 thiacloprid (THI) and thiamethoxam (THX) are considered likely to be human carcinogens and
23 acetamiprid (ACE), and IMI are toxic to birds and fish (Tomizawa and Casida, 2005).
24 Moreover, these metabolites clearly show toxic effects in mammals. The metabolites (a desnitro
25 metabolite of IMI, descyano and olefin analogue type metabolites of THI) showing high affinity
26 against human nAChR showed lethal toxicity to mice, similar to nicotines (epibatidine and
27 nicotine) (Tomizawa et al., 2000). Therefore, some of NEOs metabolites possibly show
28 nicotine-like effect to mammals including human.

29 Therefore, the use of NEOs has been limited in several countries. In 2013, the
30 European Union partially banned the use of three NEOs, IMI, clothianidin (CLO), and THX, in
31 an effort to protect honey bees and other pollinators, then totally banned outdoor use in 2018.
32 Invalidation of THI was decided in 2019, and dinotefuran (DIN) and nitenpyram (NIT) have
33 never been approved until now. At this time, only ACE has avoided imposition of legal controls.
34 In France, use of five NEOs (ACE, CLO, IMI, THI and THX) has already been disallowed in
35 2018. However, NEOs are still used in a variety of countries. As a result of these issues,
36 studying the environmental fate of NEOs has been attracting interest as a research topic. NEOs
37 spread via runoff or dust, and contaminate soil and water in agricultural and nearby areas after
38 use (Bonmatin et al., 2015). Moreover, in some environments (sandy and clay loam soils) NEOs
39 are absorbed into soil and their half-lives can reach 1,000 days (Goulson, 2013). Although
40 NEOs can persist and accumulate in natural environments, soil or water ecosystems are surely

41 capable of degrading some parts of contaminating NEOs. THX in particular shows low nAChR
42 binding activity and is known as a ‘CLO precursor,’ as THX in soil is mainly converted to CLO,
43 along with other metabolites (Nauen et al., 2003). Several bacteria able to degrade NEOs have
44 already been isolated from nature, and the corresponding mechanisms and degradation
45 pathways have also been elucidated in part (Hussain et al., 2016). However, information about
46 biodegradation of NEOs by bacteria remains limited, except for IMI. Furthermore, studies of
47 fungal degradation of NEOs are even more limited. Altogether, this indicates the importance of
48 developing microorganisms capable of degrading NEOs, and in particular ACE, CLO, IMI and
49 THI, in order to establish bioremediation applications for removal of NEOs from contaminated
50 environments.

51 White-rot fungi are well known due to their unique ability to degrade lignin which is
52 one of major wood components. lignin is a recalcitrant aromatic polymer with bearing on wood
53 strength and generally tolerant of biological attack. White-rot fungi have not only
54 lignin-degradation activity but also are able to degrade various recalcitrant organic pollutants
55 (Gao et al., 2010; Pointing, 2001). Our research group has been evaluating the possibility of
56 using white-rot fungi for bioremediation. We have already reported on biodegradation of NEOs
57 (ACE, CLO, NIT and DIN) by the white-rot fungus *Phanerochaete sordida*, including data on
58 the metabolic products and their toxicity (Mori et al., 2017; Wang et al., 2012, 2019b). Overall,
59 we have found that cytochrome P450 enzymes play important roles in NEO degradation
60 reactions in white-rot fungi. Additionally, we have reported the identification of fungal CYP

61 isozymes involved in ACE degradation using the typical white rot fungus *Phanerochaete*
62 *chrysosporium* (Wang et al., 2019a). Although a dealkylation reaction catalyzed by CYPs might
63 have been carried out as the main reaction bringing about ACE degradation by both fungi, the
64 structures of the metabolic products differed. *N*-demethylation of ACE occurred in the case of
65 metabolism by *P. sordida*, converting ACE to
66 (*E*)-*N*¹-[(6-chloro-3-pyridyl)-methyl]-*N*²-cyanoacetamide (Wang et al., 2012), whereas
67 6-chloropyridine methanol was obtained as a metabolite from culture of *P. chrysosporium* in the
68 presence of ACE (Wang et al., 2019a). This suggests the possibility that the reaction
69 mechanisms for NEO metabolism (other than ACE) and the specific CYP isozymes involved in
70 NEO metabolism might also be different between these fungi. In the present study, we
71 investigated the reactivity of *P. chrysosporium* to NEOs and identified the metabolites resulting
72 from degradation by this fungal species. We also identified the CYP isozymes involved in the
73 NEO-degrading metabolic reactions, shedding light on the mechanisms of degradation of NEOs
74 by white-rot fungi.

75

76 **2. Materials and methods**

77 *2.1. Strains and chemicals*

78 *P. chrysosporium* ME446 (ATCC 34541) was kept on potato dextrose agar (PDA)
79 medium at 4°C. The *P. chrysosporium* CYP (PcCYP) expression library used in the screen to
80 select CYPs responsible for NEOs metabolism was constructed previously (Hirosue et al., 2011).

81 *Saccharomyces cerevisiae* AH22 transformants heterologously expressing PcCYPs were stored
82 at -80°C until use. ACE was obtained from KANTO CHEMICAL Co., Inc., and other NEOs
83 (CLO, DIN, IMI, NIT and THI) were purchased from FUJIFILM Wako Pure Chemical Co. All
84 NEOs used were pesticide residue analysis grade (> 98% purity). 6-Chloro-3-pyridylmethanol
85 (I), 2-nitroamino-2-imidazoline (III) and 6-chloro-3-pyridinemethanol (IV) were obtained from
86 Tokyo Chemical Industry Co., Ltd.

87

88 2.2. Biodegradation of NEOs by *P. chrysosporium*

89 Two mycelium pellets (1 cm diameter) of *P. chrysosporium* grown on a PDA plate at
90 30°C were inoculated into a 100-ml Erlenmeyer flask containing 10 ml potato dextrose broth
91 (PDB, Difco). After 5 days of preincubation, 100 µl of NEOs or their metabolic products in
92 solution (10 mM in dimethyl sulfoxide) were added to each culture flask (final concentration:
93 0.1 mM). After the prescribed incubation period (1 to 4 weeks), the reaction was stopped by
94 addition of an equal amount of methanol, followed by homogenization. For quantification of
95 residual NEOs concentration, the homogenate was centrifuged ($10,000 \times g$ for 10 min, at 4°C),
96 then filtered (0.2 µm membrane filter) for quantification analysis using high-performance liquid
97 chromatography (HPLC), to quantify NEO remaining. HPLC analysis was performed using a
98 JASCO PU-2089 pump with an MD-2018 photodiode array detector (HPLC-PDA), fitted with
99 an Inertsil ODS-3 column (4.6 × 250 mm, GL Science). The eluent used was 30% methanol aq.
100 at 1 ml/min flow rate. Autoclaved fungal cultures were used as controls.

101 To evaluate the effects of a CYP inhibitor, we used 1-aminobenzotriazole (1-ABT).
102 A solution of 1-ABT (0 to 100 mM in DMSO) was added to the culture at the time of NEOs
103 addition to adjust the concentration to 0, 0.01, or 0.1 mM. After 2 weeks (for IMI) or 4 weeks of
104 incubation, recovery of NEOs was determined as described above.

105

106 2.3. Degradation of NEOs by recombinant *PcCYP*-expressing yeasts

107 NEO degradation experiments using *PcCYP*-expressing yeast (*Saccharomyces*
108 *cerevisiae* strain SH22) were performed as described in previous reports (Hirosue et al., 2011;
109 Wang et al., 2019a). For screening, the yeast strains were separately cultured in 96-deep well
110 plates containing 0.5 ml synthetic dextrose liquid medium with 0.5 mM substrate in each well.
111 Next, the reaction was stopped by adding methanol/acetone and centrifuged, and then the
112 supernatant was filtered for HPLC analysis. To characterize selected *PcCYPs*, a recombinant
113 yeast suspension prepared from SDL pre-culture was inoculated into 10 ml of newly SDL,
114 along with 100 μ l of 10 mM NEOs/DMSO solution (final concentration: 0.1 mM). After
115 incubation for 3 days at 30°C, 200 rpm, the reaction was stopped by the addition of 10 ml
116 methanol. The supernatant was collected following centrifugation (10,000 \times *g* for 10 min, at
117 4°C) and filtrated for HPLC analysis using a membrane filter (0.2 μ m). HPLC analysis was
118 performed as described above to quantify the amount of NEO and metabolites. If necessary, the
119 supernatant was concentrated prior to HPLC analysis. Yeast transformed with a version of the
120 pGYR vector containing a GFP gene instead of *PcCYPs* was used as the control strain.

121

122 *2.4. Identification of degradation products*

123 Supernatant obtained from yeast culture reacted with NEOs was extracted with ethyl
124 acetate. The organic layer was evaporated and dissolved in methanol. The metabolite contained
125 in the extract was isolated by preparative HPLC using a Develosil C30 UG-5 column (20 × 250
126 mm, Nomura Chemical Co., Ltd.). The eluent used was 15% or 30% methanol aq. at 5 ml/min
127 flow rate. Newly isolated metabolites were provided for HPLC-PDA, GC-MS, ESI-MS and/or
128 ¹H-NMR analyses. GC-MS was performed using a Shimadzu gas chromatograph-mass
129 spectrometer (Shimadzu Co.) equipped with a Rtx[®]-5MS capillary column (3.0 m × 0.25 mm ×
130 0.25 μm, GL sciences, Tokyo, Japan). ESI-MS and NMR analyses were done using a
131 JMS-T100LC mass spectrometer (JEOL Ltd.) or JEOL Lambda-500 spectrometer (JEOL Ltd.),
132 respectively. Metabolites of IMI and THI obtained from fungal cultures in flasks were identified
133 by comparison with the retention time and UV-spectrum of authentic standards by HPLC
134 analysis.

135

136 *2.5. Analysis of expression of PcCYPs responsible for NEOs metabolism*

137 *P. chrysosporium* was cultivated in PDB medium as described above. After 5 days of
138 cultivation and every additional week, mycelium was recovered from each flask. Total RNA
139 extraction from the mycelium, purification, and on-column DNA digestion was performed using
140 an Rneasy Mini Kit (Qiagen) and RNase-free DNase set (Qiagen), then cDNA was synthesized

141 from 200 ng of total RNA using a PrimeScript RT-PCR kit (TaKaRa Bio Inc.). TaKaRa ExTaq
142 DNA polymerase was used for reverse transcription (RT)-PCR with specific primers for actin
143 (5'-aaggactcttacgtcggtgatg-3' and 5'-atcttctcacggttagccttgg-3', amplicon size; 209 bp),
144 CYP5147A3 (5'-agcttggtttaccgtctagc-3' and 5'-ttactaaacaagaaagagtcgccg-3', amplicon size;
145 1581 bp) and CYP5037B3 (5'-tctgccatgctgtgtttgc-3' and 5'-tgcacatcgcccagacatt-3', amplicon
146 size; 1178 bp). The reaction was begun with denaturation at 95°C for 30 sec, and 26 (for actin)
147 or 28 (for PcCYPs) cycle reactions were performed. Reaction cycles were repeated as follows:
148 95°C for 10 sec, 65°C (actin) or 60°C (PcCYPs) for 10 sec, 72°C for 20 sec (actin) or 80 sec
149 (PcCYPs). Subsequently, a final extension was done at 72°C for 2 min. Unless otherwise noted,
150 all experimental procedures were done following the product manufacturer's protocols. Since it
151 was difficult to design primers for quantitative RT-PCR of PcCYPs, RT-PCR products were
152 then run on a 0.7% agarose gel and the intensity of the bands were semi-quantified using
153 ImageJ software (Abràmoff et al., 2004). The relative expression levels of both CYP genes were
154 expressed using actin as reference.

155 Mycelium of *P. chrysosporium* were obtained from PDB cultures by filtration after
156 preincubation and every additional week. Obtained mycelium was dried at 105 °C, then
157 weighted. Residual concentration of reducing sugars in filtrate was measured by
158 Somogyi-Nelson method (Somogyi, 1952).

159

160 **3. Results and Discussion**

161 3.1. Biodegradation of NEOs by *P. chrysosporium*

162 Previously, we reported the biodegradation of ACE by *P. chrysosporium* and
163 identified CYP5147A3 as an enzyme responsible for ACE metabolism, based on activity
164 screening of PcCYPs heterologously expressed in *S. cerevisiae* (Wang et al., 2019a). In the
165 study, we also identified 6-chloro-3-pyridylmethanol and *N'*-cyano-*N*-methyl acetamidine as
166 ACE metabolites from both *P. chrysosporium* and recombinant yeast cultures. In the present
167 study, we investigated biodegradation by *P. chrysosporium* of a panel of NEOs, namely CLO,
168 DIN, IMI, NIT, THI and ACE. No remarkable degradation of DIN or NIT by *P. chrysosporium*
169 was observed. The results of a time-course analysis of degradation of ACE, CLO, IMI and THI
170 are shown in Fig. 1. Control experiments using autoclaved *P. chrysosporium* did not show any
171 NEOs degradation during 4-week incubation. ACE degradation was only observed at a later
172 cultivation stage, with just a slight amount (less than 5%) degraded during the 2 weeks
173 incubation period, but 27.5% degraded at 4 weeks of incubation. The time course of CLO
174 degradation was almost the same as that observed for ACE, i.e. 28% of CLO was degraded at 4
175 weeks of incubation. Degradation of IMI began in the first 7-day cultivation period and the
176 degradation rate for IMI increased over time. During the first week, 13.7% of IMI disappeared
177 from the culture, 31.8% was degraded in next a week, and 49.8% was degraded in 3rd week. At
178 4 weeks, IMI was completely degraded. THI degradation was much slower than what was
179 observed for IMI, and the time course of THI degradation was between that of ACE and
180 IMI/CLO. Thus, the time courses of degradation of each NEO showed a different pattern,

181 suggesting that degradation of each NEO by *P. chrysosporium* is caused by different enzymes.
182 Therefore, we presumed that there are other PcCYPs involved in degradation of NEOs, i.e.
183 additional to CYP5147A3, which is responsible for ACE degradation (Wang et al., 2019a).

184 To evaluate the effects of a CYP inhibitor on degradation of NEOs, we added 1-ABT
185 and NEOs (ACE, CLO, IMI and THI) to the fungal culture at the same time. After incubation
186 for 2 weeks (for IMI) or 4 weeks (for the other NEOs: ACE, THI, and CLO), we observed a
187 gradual inhibition of NEO degradation as indicated by changes in the 1-ABT concentration.
188 These results suggest that PcCYPs are involved in IMI, CLO and THI degradation by *P.*
189 *chrysosporium*, as with ACE degradation. Although 1-ABT effectively inhibited metabolism of
190 ACE, IMI and THI by *P. chrysosporium*, the inhibition by 1-ABT of CLO degradation was less
191 than for the other 3 NEOs. Additionally, degradation of ACE and THI was sensitive to 1-ABT
192 addition, 0.01 mM 1-ABT inhibited about 60% of ACE and THI degradation. IMI degradation
193 was moderately inhibited by 1-ABT addition. Because different inhibitory effects of 1-ABT for
194 IMI, CLO and ACE/THI degradation by *P. chrysosporium* was observed, it is suggested that
195 different PcCYPs isozyme involves in degradation of each NEOs.

196

197 3.2. IMI and THI degradation mechanism of *P. chrysosporium*

198 We next conducted functional screens for degradation activity using a library of
199 recombinant PcCYPs heterologously expressed in *S. cerevisiae*. All NEOs other than ACE were
200 used for screening. CYP5147A3 was identified as one of the PcCYPs responsible for

201 degradation of ACE (Wang et al., 2019a). In the present study, CYP5037B3 showed clear IMI
202 degradation, and moderate THI degradation was observed in both CYP5037B3 and
203 CYP5147A3-expressing yeast cultures. For other NEOs, a clear reaction was not observed.
204 Because this screening system using 96-well plates does not have high sensitivity, subsequent
205 functional characterization of selected two PcCYPs was performed in flask-scale. After 3 days
206 of cultivation, ACE, IMI and THI were clearly degraded in both recombinant yeasts expressing
207 CYP5037B3 and CYP5147A3 (Table 1). CYP5037B3 expressing yeast showed higher
208 IMI-degradation activity than CYP5147A3 expressing yeast. Contrary, ACE and THI was more
209 degraded in CYP5147A3 expression yeast culture than CYP5037B3 expressing yeast culture.
210 Although CLO degradation by *P. chrysosporium* was inhibited by the addition of a CYP
211 inhibitor, neither yeast strain showed metabolic activities against CLO. This result suggests that
212 PcCYPs other than CYP5037B3 and CYP5147A3 are responsible for degradation of CLO in *P.*
213 *chrysosporium*. And the activities of PcCYPs responsible to CLO degradation might not be
214 successfully expressed in the presented yeast expression system. In addition, we found that
215 neither DIN nor NIT was degraded by either yeast strain expressing PcCYPs. This result is
216 consistent with our finding that *P. chrysosporium* was not able to degrade DIN or NIT.

217 HPLC analyses revealed that ACE, CLO, IMI and THI were clearly degraded in both
218 yeast cultures. For ACE, we observed that 51.0 and 92.5 μ M of ACE was degraded in
219 CYP5037B3 and CYP5147A3 expressing yeast cultures, respectively. In addition, the same two
220 metabolites (**I** and **II**, retention time (RT) = 4.7 min and 9.7 min, respectively) were detected

221 from both recombinant yeast cultures containing ACE following HPLC analysis of these
222 extracts. Metabolites of ACE that react with CYP5147A3 have already been identified
223 previously, and including 6-chloro-3-pyridylmethanol and 2-nitroamino-2-imidazoline (Wang et
224 al., 2019a). Thus, it was clear that in addition to CYP5147A3, CYP5037B3 also catalyzes
225 *N*-dealkylation of ACE. While only 14.1 μ M of IMI was degraded by CYP5147A3-expressing
226 yeast during a 3-day incubation, CYP5037B3 expression yeast exhibited higher IMI-degrading
227 activity (67.5 μ M). We detected 6-chloro-3-pyridylmethanol (**I**) and metabolite **III** (RT=3.7
228 min) in both culture extracts of CYP5037B3 and CYP5147A3-expressing yeasts. Metabolite **III**
229 showed mass spectra at m/z 129 (M^+ -H) on negative ion mode, and 131 (M^+ +H) and 153 (M^+ -H,
230 +Na) on positive ion mode on ESI-MS, and the molecular mass of metabolite **III** was
231 determined to be 130. The 1 H-NMR spectrum of metabolite **III** had only one doublet peak at
232 3.71 ppm in CD₃OD. These spectra indicated that metabolite **III** is a fragment of the side chain
233 region of IMI. Based on these results, as well as a comparison of the retention time and
234 UV-spectrum on HPLC-PDA analysis with the authentic standard, we identified metabolite **III**
235 as 2-nitroamino-2-imidazoline. Three metabolites of THI (metabolites **I**, **IV** (RT=4.7 min) and
236 **V** (RT=15.0 min)) were obtained from the culture extract of CYP5147A3-expressing yeast.
237 Metabolite **V** was only detectable when a concentrated extract was analyzed by HPLC. By
238 contrast, only metabolites **I** and **IV** were detected in a culture of CYP5037B3-expressing yeast.
239 On ESI-MS analysis, metabolite **IV** showed molecular ions m/z 128 (M^+ +H) and 150 (M^+ +Na)
240 on positive ion mode. By comparing these findings with the retention time and UV-spectrum of

241 an authentication standard by HPLC-PDA analysis, metabolite **IV** was identified as
242 2-cyanoimino-1,3-thiazolidine. Metabolite **V** is a minor metabolite that required concentration
243 for detection by HPLC. The ESI-MS spectrum of isolated **V** showed a molecular ion at m/z 271
244 on positive ion mode, and an ion peak at m/z 293 ($M^+ - H$, $+Na$) was also observed. Both ions
245 were accompanied by $+2$ mass chlorine isotope peaks. From the mass spectrum and 1H - and
246 ^{13}C -NMR spectra in CD_3OD (shown in Table 2), metabolite **V** was identified as thiacloprid
247 amide (THI-amide) (Dai et al., 2010).

248 As shown in Table 1, the yields of metabolite **I** were over 80% of degraded ACE,
249 IMI and THI in cultures of yeast expressing CYP5037B3. Although recovery of metabolite **II**
250 could not be determined because there is no authentic standard, yeast expressing CYP5037B3
251 also exhibited a high rate of conversion of IMI and THI to side-chain fragments **III** and **IV**
252 (around 93% or more). The conversion rates of ACE and IMI by yeast expressing CYP5147A3
253 were nearly the same as that observed for CYP5037B3. However, the rates of conversion of
254 THI to **I** and **IV** by CYP5147A3 were slightly lower than others. The reason seems to be that
255 CYP5147A3 is able to catalyze two reactions to form two major metabolites **I**, **IV** or a minor
256 metabolite **V**. In Fig. 3, we present a summary of degradation of NEOs and metabolite
257 production. Both CYP5037B3 and CYP5147A3 are likely to have almost the same catalytic
258 mechanism. All metabolites, except metabolite **V** (THI-amide), were produced via
259 *N*-dealkylation.

260 Some functions of these PcCYPs have been reported previously. For example,

261 CYP5037B3 catalyzes *O*-dealkylation of 7-ethoxycoumarin, and CYP5147A3 catalyzes
262 *S*-oxidation of dibenzothiophene, 11 β -hydroxylation of testosterone, as well as *O*-dealkylation
263 of 7-ethoxycoumarin (Ichinose, 2013). Although CYP5147A3 shows a broad range of substrate
264 specificity, both of the PcCYPs might share a catalytic mechanism with regards to the
265 *O*-dealkylation reaction. In the present study, both PcCYPs catalyzed the reaction with identical
266 NEOs as substrates and produced same *N*-dealkylation products. Accordingly, we propose that
267 both CYP5037B3 and CYP5147A3 catalyze the *N*-dealkylation reaction of ACE, IMI and THI
268 via the same mechanism, which might be either a hydrogen atom transfer or a single electron
269 transfer (Wang et al., 2019a). In addition, we suggest that the chloropyridinyl structure might be
270 important for showing the activity of these PcCYPs, as CYP5037B3 and CYP5147A3 degraded
271 ACE, IMI and THI but not CLO (Fig. 2). However, neither selected PcCYP showed activity
272 against NIT, which has a chloropyridinyl structure. This could be due to the bulky nature and/or
273 electronegativity of the nitrovinyl structure. Additionally, *N*-nitro and *N*-cyanoimine moieties
274 that are critical for binding to nAChR, along with structures of the heterocyclic or acyclic spacer,
275 do not seem to be important for the activity of these PcCYPs (Casida, 2011). THI-amide
276 production was only observed in a culture of CYP5147A3-expressing yeast; this might be
277 attributable to broad substrate specificity of CYP5147A3. Because it has been reported
278 CYP5147A3 can catalyze reaction to broad range substrate by comparison of CYP5037B3, as
279 described above (Ichinose, 2013). However, information of strict substrate specificity of these
280 PcCYPs must be provided by the experiments using purified PcCYPs.

281 To confirm whether the mechanism of degradation by *P. chrysosporium* is the same
282 as that of recombinant yeasts, we next analyzed metabolites formed from ACE, IMI and THI.
283 Although we note that only a tiny amount of metabolite **I** was obtained from a vast amount of
284 culture extract of *P. chrysosporium* with ACE in a previously study, no accumulation of
285 metabolite **I** from ACE was observed in the present study, as was also true for IMI and THI.
286 When compound **I** was added to the *P. chrysosporium* culture, only $10.0 \pm 2.4\%$ of added **I** was
287 recovered after one week of incubation. The result indicates that metabolite **I** is more easily
288 metabolized than the parent compound ACE, IMI or THI. Furthermore, this compound does not
289 accumulate in the culture. Metabolite **II** was clearly produced in a culture of *P. chrysosporium*
290 with ACE, as previously reported (Wang et al., 2019a). Metabolites **III** and **IV** were found by
291 HPLC analysis from fungal culture with IMI and THI, respectively. Although **I** was not
292 detectable in extracts of either culture, 97.0% of degraded IMI and 74.2% of degraded THI in
293 the fungal cultures had been converted to metabolites **III** and **IV**, respectively. **V**, a minor
294 metabolic product of THI in CYP5147A3 expressing yeast, was not observed by HPLC analysis,
295 even though the extract of the fungus culture with THI had been highly concentrated. Therefore,
296 the majority of IMI degradation is probably catalyzed by both PcCYPs to form major metabolite
297 **I** and side-chain fragments (**II**, **III** and **IV**) via *N*-dealkylation, then **I** is further metabolized. On
298 the other hand, we considered two possibilities for the cause of lower recovery of **IV** from THI
299 degradation. The first is further metabolism of **IV** and the other is the involvement of other
300 PcCYPs giving different metabolites. We confirmed degradability of **III** and **IV** in a culture of

301 *P. chrysosporium*. In this further degradation reaction, 63% of the initial **IV** (100 μ M) was
302 degraded during a 2-week incubation; however, only 4% of **III** was degraded. This indicates
303 that *P. chrysosporium* metabolizes THI by a reaction involving CYP5037B3 and CYP5147A3
304 to form mainly **I** and **IV**, **I** is quickly metabolized, and **IV** is slowly metabolized.

305 A similar reaction to form 6-chloronicotinic acid (6-CNA) or
306 6-chloro-3-pyridylmethanol from NEOs with chloropyridinyl structures has been found in mice
307 and spinach, along with several other reactions (Ford and Casida, 2006)(Ford and Casida, 2008).
308 The reports of these activities describe that both products are produced as major metabolites by
309 oxidation (in mice or spinach) or by reduction (spinach) of 6-chloronicotinaldehyde formed
310 from *N*-dealkylation of ACE, IMI, NIT and THI. The metabolism of IMI in several organisms
311 including bacteria, plant and mammals has been particularly well investigated. Schulz-Jander
312 and Casida (Schulz-Jander and Casida, 2002) performed *in vivo* IMI-metabolic reaction with 8
313 recombinant human CYPs. These human CYPs catalyzed hydroxylation and desaturation of
314 imidazolidine moiety or reduction and elimination of *N*-nitro moiety, however, 6-CNA and
315 6-chloro-3-pyridylmethanol were not detected in these reactions. That is to say, the enzymes
316 catalyzing direct *N*-dealkylation of NEOs with a chloropyridinyl moiety have not been well
317 understood, such that the findings for fungal CYPs catalyzing direct *N*-dealkylation will be
318 useful information for understanding the mechanisms of metabolism of NEOs in various
319 organisms.

320

321 3.3. Expression analysis of CYP5037B3 and CYP5147A3

322 After a 5-day preincubation, total RNA was extracted from a culture of *P.*
323 *chrysosporium* subjected to additional 0- to 4-week incubation periods. The results of
324 expression analysis are shown in Fig. 4. Expression of the actin gene was clearly observed at all
325 incubation periods. Semi-quantitative expression levels of PcCYPs were provided by image
326 processing using ImageJ software. *CYP5037B3* was weakly expressed during 0-1 weeks
327 cultivation and higher expression levels were detected at 2- and 3-week incubation periods.
328 Expression levels then dropped at 4 weeks. *CYP5147A3* showed a much weaker expression at
329 0- and 1-week cultivation periods, expression levels were increased at the 2nd week, and then
330 levels decreased as the incubation period extended. IMI degradation and *CYP5037B3*
331 expression correlated well, as metabolic activity of IMI of *CYP5037B3* was much higher than
332 that of *CYP5147A3* (Table 1). As shown in Fig. 1, *P. chrysosporium* accelerated ACE and THI
333 degradation after 2 weeks of cultivation, such that ACE and THI degradation seem likely to
334 depend mainly on expression of *CYP5147A3*.

335 Dried mycelial weight after pre-incubation, 1- or 2-weeks incubation, were $54.5 \pm$
336 2.3 , 56.0 ± 0.1 and 42.8 ± 0.3 mg/flask, respectively. *P. chrysosporium* consumed almost half of
337 reducing sugar (47.6% remaining) during preincubation, and most of residual sugars was
338 consumed during additional 1-week incubation (2.6% remaining). From this result, it is
339 expected that *P. chrysosporium* got into nutrient starvation condition during 1-2 weeks
340 incubation period and started autolysis, in PDB culture. In the previous report, *P.*

341 *chryso sporium* increased the expression of some CYPs associating to secondary metabolism
342 under starvation condition (Doddapaneni and Yadav, 2005). From these facts, it is presumed
343 that CYP5037B3 and CYP5147A3 expressed under starvation condition.

344

345 **4. Conclusion**

346 In the present study, we investigated degradation of several NEOs by the white-rot
347 fungus *P. chryso sporium*, which is able to metabolize acetamiprid via the activity of CYP. We
348 found that this fungus can degrade CLO, IMI and THI in addition to ACE. Metabolic reactions
349 with ACE, IMI and THI, which have in common that they each contain a chloropyridinyl
350 structure, were catalyzed by two PcCYPs to form a common *N*-dealkylated product and
351 respective side-chain fragments. Although no metabolic product of CLO was yet identified,
352 these results show the applicative potential of *P. chryso sporium* for bioremediation of NEOs
353 because the fungus was able to degrade four NEOs that appear to be major pollutants in soil
354 environments. We demonstrated that both CYP5037B3 and CYP5147A3, which were identified
355 as major PcCYPs, are involved in ACE, IMI and THI metabolism, catalyzing direct
356 *N*-dealkylation of these NEOs. By contrast, these PcCYPs were not able to react with CLO,
357 indicating the presence of other PcCYPs involved in CLO degradation. Future work could be
358 focused on the identification of the PcCYPs that catalyze CLO degradation and identification of
359 its metabolites. Although CYPs also play important roles in degradation of NEOs by *P. sordida*,
360 the target NEOs and metabolic products formed by *P. sordida* are completely different from

361 those we have identified for *P. chrysosporium* (Mori et al., 2017; Wang et al., 2012, 2019a,
362 2019b). It is possible that the differences in the mechanisms of degradation of individual NEOs
363 by each fungal species result from diversity of CYPs in white-rot fungi (Floudas et al., 2012).

364

365 **Declaration of Competing Interest**

366 The authors declare no competing financial interest.

367

368 **CRedit authorship contribution statement**

369 **Toshio Mori:** Writing - original draft, Visualization. **Haruka Ohno:** Investigation,
370 Visualization. **Hirofumi Ichinose:** Investigation, Resources. **Hirokazu Kawagishi:** Writing -
371 review & editing. **Hirofumi Hirai:** Writing - review & editing, Supervision, Project
372 administration, Funding acquisition.

373

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377

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473

474 **Figure captions**

475 **Fig. 1. Time course of degradation of NEOs (ACE, CLO, IMI and THI) in a culture of *P.***
476 ***chryso sporium*.** Degradation rates of ACE (square), CLO (triangle), IMI (circle) and THI
477 (rhombus) in live fungal cultures were indicated as open symbols, and control experiment
478 using autoclave culture were indicate respective closed symbols. Values are means \pm
479 standard deviation of samples in triplicate.

480
481 **Fig. 2. Inhibitory effect of a cytochrome P450 inhibitor, 1-ABT, on degradation of ACE,**
482 **CLO, IMI and THI by *P. chryso sporium*.** 1-ABT and NEOs were added at the same time
483 to pre-incubated culture of *P. chryso sporium*. After 4 weeks (for ACE, CLO and THI) or 2
484 weeks (for IMI) of incubation, residual amounts of NEOs were measured on HPLC. Values
485 are means \pm standard deviation of samples in triplicate.

486
487 **Fig. 3. Schematic diagram of NEOs metabolic pathways in cultures of *P. chryso sporium*, or**
488 **of yeast expressing CYP5037B3 or CYP5147A3.** Values express the transformation rate
489 as calculated based on the amount of degraded NEOs and recovered metabolites. Asterisk
490 indicates that the reaction was confirmed in previous work (Wang et al., 2019a).

491
492 **Fig. 4. RT-PCR analysis of CYP5037B3, CYP5147A3, and actin expression in 0- to**
493 **4-week-old cultures of *P. chryso sporium*.** Values below images are relative expression

494 levels of PcCYPs semi-quantified using actin as reference gene by ImageJ software
495 (Abràmoff et al., 2004). Relative expression level is expressed by percentage of highest
496 expression (CYP5037B3 expression at week 3).
497

Table 1 Degradation of NEOs and metabolite production in cultures of recombinant yeast expressing CYP5037B3 or CYP5147A3

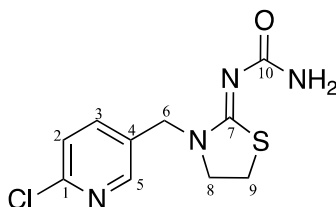
PcCYPs	NEOs	degradation (μM)	metabolites (μM)		other product
			I	II, III or IV	
CYP5037B3	ACE	56.7 ± 2.5	47.4 ± 0.2	accumulate ¹	
	CLO	< 5.0	n.d. ²	n.d. ²	
	IMI	59.6 ± 0.2	54.9 ± 0.9	57.4 ± 1.1	
	THI	53.2 ± 0.7	42.7 ± 2.8	49.4 ± 0.7	
CYP5147A3	ACE	97.3 ± 0.5	85.8 ± 1.2	accumulate ¹	
	CLO	< 5.0	n.d. ²	n.d. ²	
	IMI	14.6 ± 1.1	12.1 ± 0.3	13.8 ± 0.7	
	THI	73.9 ± 1.4	57.4 ± 0.4	65.6 ± 0.8	V (THI-amide)

The values are means \pm standard deviation of triplicated samples.

¹ Metabolic product II was clearly accumulated in both yeast cultures, however, the amount has not determined because no standard.

² n.d. means “not detectable”.

Table 2 ¹H- and ¹³C-NMR spectra for THI-amide (in CD₃OD)



Position	¹³ C δ_C	¹ H δ_H
1	151.5	-
2	125.7	7.44 (d), 1H, $J= 8.0$ Hz
3	141.0	7.82 (dd), 1H, $J_1= 8.6$ Hz, $J_2= 2.3$ Hz
4	133.4	-
5	150.5	8.36 (d), 1H, $J= 2.9$ Hz
6	48.0	4.79 (s)
7	171.5	-
8	27.6	3.60 (t), 2H, $J= 7.8$ Hz
9	50.5	3.12 (t), 2H, $J= 7.5$ Hz
10	166.9	-

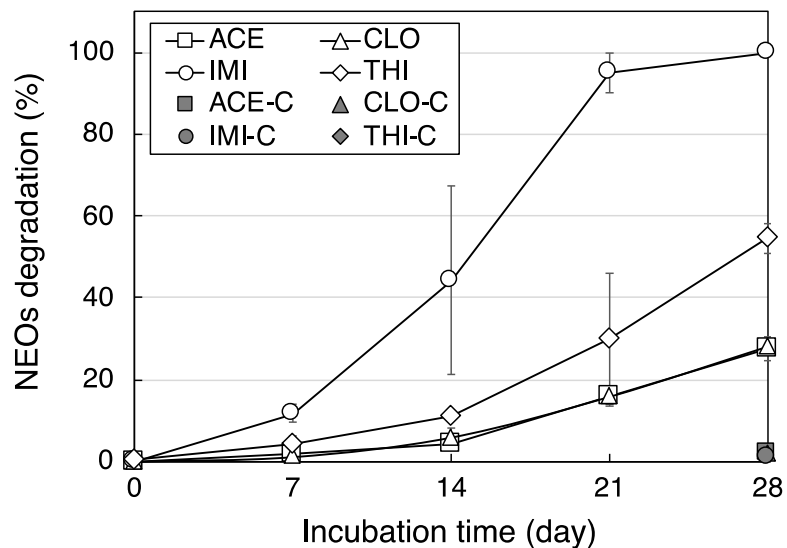


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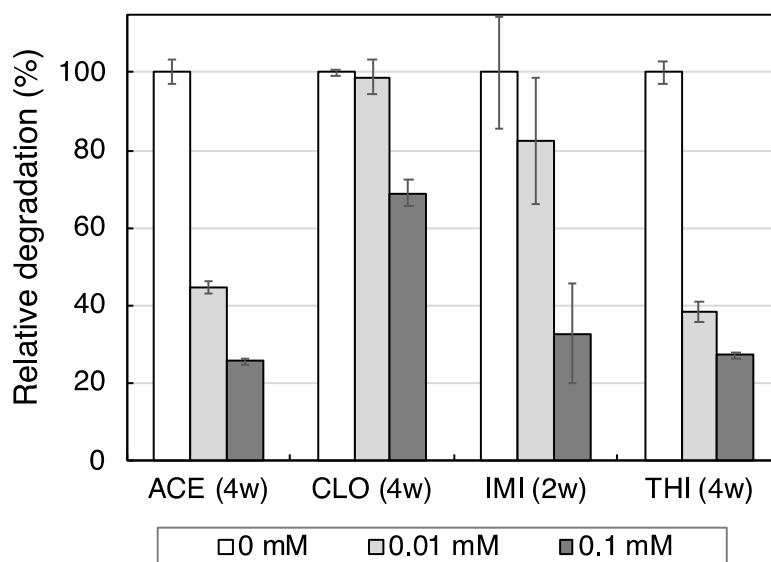


Fig. 2. Inhibitory effect of a cytochrome P450 inhibitor, 1-ABT, on degradation of ACE, CLO, IMI and THI by *P. chrysosporium*. 1-ABT and NEOs were added at the same time to pre-incubated culture of *P. chrysosporium*. After 4 weeks (for ACE, CLO and THI) or 2 weeks (for IMI) of incubation, residual amounts of NEOs were measured on HPLC. Values are means \pm standard deviation of samples in triplicate.

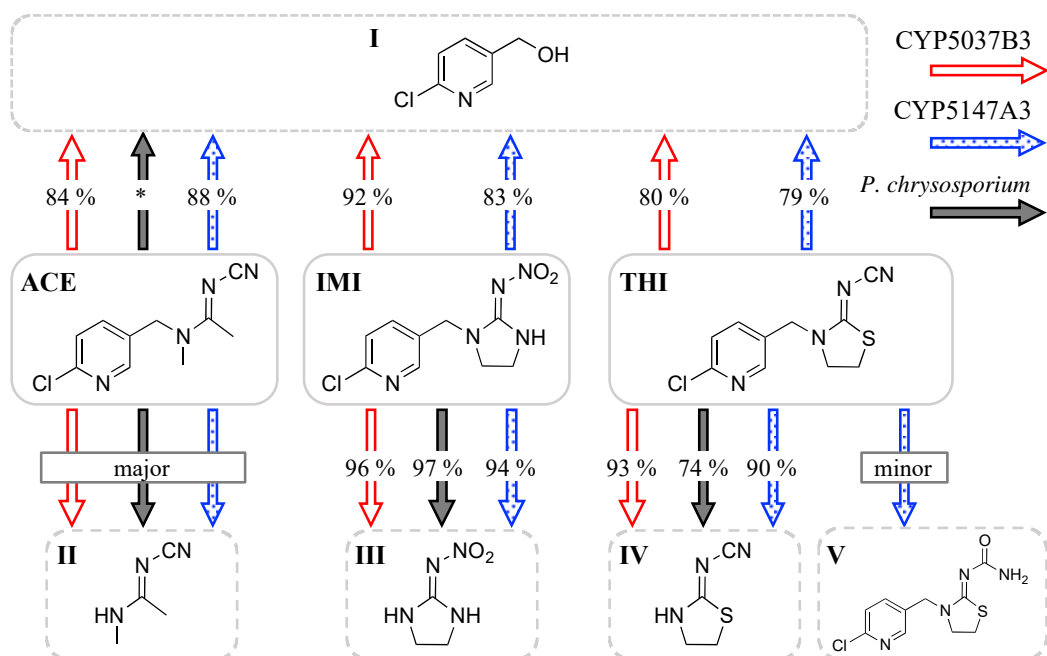


Fig. 3. Schematic diagram of NEOs metabolic pathways in cultures of *P. chrysosporium*, or of yeast expressing CYP5037B3 or CYP5147A3. Values express the transformation rate as calculated based on the amount of degraded NEOs and recovered metabolites. Asterisk indicates that the reaction was confirmed in previous work (Wang et al., 2019a).

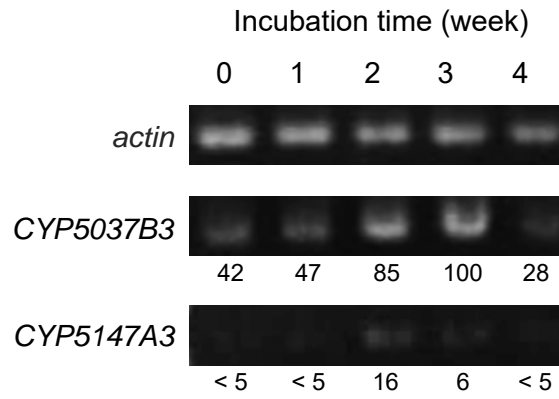


Fig. 4. RT-PCR analysis of CYP5037B3, CYP5147A3, and actin expression in 0- to 4-week-old cultures of *P. chrysosporium*. Values below images are relative expression levels of PcCYPs semi-quantified using actin as reference gene by ImageJ software (Abràmoff et al., 2004). Relative expression level is expressed by percentage of highest expression (CYP5037B3 expression at week 3).