Biodegradation

Removal of diclofenac and mefenamic acid by the white rot fungus *Phanerochaete sordida* YK-624 and identification of their metabolites after fungal transformation

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Abstract The non-steroidal anti-inflammatory drugs diclofenac (DCF) and mefenamic acid (MFA) were treated with the white rot fungus *Phanerochaete sordida* YK-624. DCF completely disappeared and MFA decreased by about 90% after 6 days of treatment. It was also confirmed that the fungus almost completely removed the acute lethal toxicity of DCF and MFA towards the freshwater crustacean *Thamnocephalus platyurus* after 6 days of treatment. Mass spectrometric and ^{1}H nuclear magnetic resonance analyses demonstrated that two mono-hydroxylated DCFs (4'-hydroxydiclofenac and 5-hydroxydiclofenac) and one di-hydroxylated DCF (4',5-dihydroxydiclofenac) were formed via fungal transformation. The four metabolites of MFA were identified as 3'-hydroxymethylmefenamic acid (mono-hydroxylated MFA), 3'-hydroxymethyl-5-hydroxymefenamic acid (di-hydroxylated MFA), 3'-hydroxymethyl-6'-hydroxymefenamic acid (di-hydroxylated MFA) and 3'-carboxymefenamic acid. These results suggest that hydroxylation catalyzed by cytochrome P450 (CYP) in *P. sordida* YK-624 may be involved in the elimination and detoxification of DCF and MFA. This notion was further supported by the fact that smaller decreases in DCF and MFA were observed in cultures of *P. sordida* YK-624 incubated with 1-aminobenzotriazole, a known inhibitor of CYP.

Keywords Diclofenac · Mefenamic acid · Hydroxylation · Cytochrome P450 · White rot fungus

Introduction

The removal of many pharmaceuticals during municipal wastewater treatment has been found to be incomplete. As a result, residual amounts of these compounds have been detected ubiquitously in wastewater treatment plant effluents at the μg/l level, and they also frequently occur at the ng/l level in surface water, ground water and even drinking water (Rodríguez et al. 2003; Tixier et al. 2003; Ashton et al. 2004; Carballa et al. 2004; Wiegel et al. 2004). Moreover, environmental risk assessment studies have shown that their concentrations in aquatic environments may exceed the predicted no effect concentration (Stuer-Lauridsen et al. 2000; Santos et al. 2007). Consequently, the presence of pharmaceuticals in the environment as potential water pollutants has been attracting an increasing attention (Jones et al. 2007).

Non-steroidal anti-inflammatory agents, such as diclofenac (DCF) and mefenamic acid (MFA), are used extensively as non-prescription drugs, and residues of these compounds have been detected ubiquitously in the aquatic environment. Several laboratory-scale studies on the removal of DCF in a fixed-bed bioreactor (FBBR) and a membrane bioreactor (MBR) have been carried out. It was demonstrated that a FBBR filled with river sediment under aerobic conditions exhibits rapid removal of DCF (Gröning et al. 2007). In contrast, some reports have showed no significant removal of DCF using a laboratory-scale FBBR (González et al. 2006) or MBR (Quintana et al. 2005). These contradictory results for DCF were also observed at several conventional wastewater treatment plants (WWTPs) (Ternes 1998; Heberer 2002; Zwiener and Frimmel 2003; Clara et al. 2005). In the case of 3 WWTPs in Austria, although one WWTP had removal rates of up to 70%, no removal was observed at the other two (Clara et al. 2005). Furthermore, it has been also reported that the removal of MFA at 3 WWTPs in Switzerland varies from 2% to 50%, and that MFA apparently presents a risk to the aquatic environment, with a ratio of the predicted environmental concentration

(PEC) to the predicted no effect concentration (PNEC) (PEC/PNEC ratio) of higher than one (Tauxe-Wuersch et al. 2005). These widely varying removal rates for DCF and MFA suggest that the currently applied biological wastewater treatment techniques are not sufficiently effective for the removal of these pharmaceuticals, and that enhanced wastewater treatment technologies may be crucial for the future.

White rot fungi are the best-known and most effective lignin-degrading microorganisms. There is currently great interest in these fungi and their ligninolytic enzymes due to their potential for degrading and detoxifying recalcitrant environmental pollutants, such as polychlorinated dioxins (Kamei et al. 2005), chlorophenols (Ehlers and Rose 2005), polycyclic aromatic hydrocarbons (Cambria et al. 2008) and dyes (Asgher et al. 2008). We recently demonstrated that ligninolytic enzymes such as manganese peroxidase (MnP) and laccase, which are produced extracellularly by white rot fungi, are effective in removing the estrogenic activities of bisphenol A, nonylphenol, 4-*tert*-octylphenol, 17β-estradiol, ethinylestradiol, genistein, iso-butylparaben and *n*-butylparaben (Tsutsumi et al. 2001; Suzuki et al. 2003; Tamagawa et al. 2005, 2007; Mizuno et al. 2009). Furthermore, recent studies showed the capability of white rot fungi to degrade the pharmaceuticals such as ibuprofen, ketoprofen, clofibric acid, carbamazepine, atenolol and propranolol (Marco-Urrea et al. 2009a, 2009b, 2009c). These studies have prompted investigation into the biodegradation of DCF and MFA using white rot fungi.

In this study, we examined the disappearance of DCF and MFA and the removal of their acute lethal toxicity towards the freshwater crustacean *Thamnocephalus platyurus* following treatment with the white rot fungus *Phanerochaete sordida* YK-624. We also identified the metabolites of fungal transformation.

Materials and methods

Treatment of DCF and MFA with white rot fungus

P. sordida YK-624 (ATCC 90872) was used in this study. The fungus was maintained on potato dextrose agar (PDA; Difco Laboratories) slants. A new PDA plate was inoculated with the fungus, and was then precultured for 3 days at 30ºC. Five disks punched from the growing edge of the precultured mycelium were blended with an homogenizer for 30 s with 50 ml of PMY medium (3.0% glucose, 1.0% peptone, 1.0% malt extract, 0.4% yeast extract), and this was added to a 500-ml Erlenmeyer flask containing 150 ml of PMY medium. This flask was then shaken at 150 rpm and 30ºC to give a mycelium suspension culture. After 3 days, 2.2 ml of this culture was again blended with an homogenizer for 30 s with 17.8 ml of Kirk medium (Tien and Kirk 1988), and was added to a 100-ml Erlenmeyer flask containing 200 μl of 10^{-2} M DCF or MFA from a stock solution in dimethyl sulfoxide (final concentration, 10^{-4} M DCF or MFA; Wako, Osaka, Japan). The flask was then shaken at 150 rpm and 30ºC. For some experiments in fungal treatment of MFA, the cytochrome P-450 inhibitor 1-aminobenzotriazole (1-ABT) (0.1 and 1.0 mM) was added to Kirk medium, and the flask was shaken for 5 days.

Analyses of DCF and MFA by liquid chromatography

Residual DCF or MFA concentrations in the fungal cultures were determined by high-performance liquid chromatography (HPLC). Each of the whole fungal cultures (10 ml) containing mycelia, in which 3,4-dimethoxyacetophenone was added as an internal standard (I.S.) after incubation, was blended with an homogenizer for 30 s, followed by extraction with ethyl acetate (EtOAc) (10 ml × 3) after acidification (pH 2-3) with 1 N-HCl. The EtOAc layer

was dried over anhydrous Na_2SO_4 and was evaporated under reduced pressure. The residue was dissolved in methanol, which was analyzed by HPLC. HPLC analytical conditions were as follows: Cadenza 5CD-C18 column (75 mm × 4.6 mm i.d.; 5 μm; Imtakt, Kyoto, Japan); mobile phase of 20 mM phosphate (X) and methanol (Y); elution with a linear gradient of 30% to 100% Y in 30 min after isocratic elution with 30%Y for 5 min; flow rate of 1.0 ml/min; and detection at 254 nm.

Acute lethal toxicity test using freshwater crustacean

Toxicity of DCF and MFA before and after fungal treatments was evaluated by acute lethal toxicity test using the freshwater crustacean *T. platyurus*. Cysts of *T. platyurus* in EPA water (2.29 mM $NaHCO_3$, 0.70 mM $CaSO_4$, 0.49 mM $MgSO_4$, 0.11 mM KCl) were placed in an incubator at 25ºC under a fluorescent lamp, and the neonates that hatched within 24 h were used in toxicity tests. Tests were performed using a 24-well microplate with ten individuals per 1 ml of test sample in each well. Test samples were prepared as follows; each of the whole fungal cultures (10 ml) containing mycelia was blended with an homogenizer and then extracted with EtOAc after acidification. EtOAc extracts were dissolved in EPA water (10 ml), thus ensuring that the concentration of DCF or MFA before fungal treatment corresponded to 10^{-4} M in the test solution. A total of thirty neonates in three wells were used for each test solution. The neonates were exposed to the test sample for a period of 24 h at 25ºC in the dark. Lethality was calculated from the number of living crustaceans counted using a binocular microscope. Relative acute lethal toxicity (%) was defined as the percentage of lethality of fungal-treated DCF or MFA, as compared to that of untreated DCF or MFA.

Enzyme assay

Activities of laccase, MnP and lignin peroxidase (LiP) were determined according to our previous report (Mizuno et al. 2009).

Isolation and characterization of fungal metabolites of DCF and MFA

After 3 days of treatment of DCF or MFA with *P. sordida* YK-624, each of the whole fungal cultures (200 ml) was blended with an homogenizer and then extracted with EtOAc after acidification. EtOAc extracts were dissolved in chloroform ($CHCl_3$), and metabolites of DCF and MFA in the residue were then purified by preparative thin-layer chromatography (PTLC) (Merck Kieselgel 60 F_{254}, solvent: DCF metabolites, methanol/$CHCl_3$ = 5/95; MFA metabolites, EtOAc/*n*-hexane = 1/2). The DCF and MFA metabolites were analyzed by direct inlet-mass spectrometry (DI-MS, electron impact), and by gas chromatograph-mass spectrometry (GC-MS) after trimethylsilylation with TMSI-H (GL Science, Tokyo, Japan). DI- and GC-MS analyses were carried out at 70 eV on a Shimadzu GCMS-QP 5050 equipped with a capillary column (TC-1; 0.25 μm × 0.25 mm × 30 m; GL Sciences). Oven temperature was programmed to increase from 100ºC to 270ºC at a rate of 5ºC/min. Metabolites of DCF and MFA were also analyzed by ^{1}H nuclear magnetic resonance (^{1}H NMR). ^{1}H NMR spectra were recorded with JEOL JMM EX-270 FT-NMR spectrometer.

Results and discussion

Treatment of DCF and MFA with white rot fungus

Figure 1 shows the decrease in DCF and MFA observed during treatment with *P. sordida* YK-624. The concentration of DCF decreased by about 90% after 3 days of treatment and

DCF completely disappeared after 6 days of treatment (Fig. 1A). On the other hand, MFA decreased by about 60 and 90% after 3 and 6 days of treatment, respectively (Fig. 1B). These results indicate that fungal treatment effectively eliminates DCF and MFA. Furthermore, it was confirmed that *P. sordida* YK-624 almost completely removed the acute lethal toxicity of DCF and MFA towards the freshwater crustacean *T. platyurus* after 6 days of treatment (Fig. 1A and 1B).

It has been suggested that the MnP, LiP and laccase produced extracellularly by white rot fungi are involved in the oxidative breakdown of lignin (Martínez et al. 2005). Therefore, these enzyme activities were determined every day during the treatment of DCF and MFA with *P. sordida* YK-624. No appreciable levels of laccase, MnP or LiP activity were detected throughout the treatment period; maximum activity of each of these ligninolytic enzymes was up to 0.005 nkat/ml during treatment of DCF or MFA. On the other hand, for example, this fungus produced about 4 nkat/ml MnP and 0.5 nkat/ml laccase on the fourth day of treatment of endocrine-disrupting genistein, and the profiles of MnP and laccase productions and genistein decrease during treatment were very similar (Tamagawa et al. 2005). These raised the question whether ligninolytic enzymes may be involved in the decrease in DCF and MFA concentrations or in the removal of the toxicity of these compounds by *P. sordida* YK-624. However, our preliminary experiments showed that both 10 nkat/ml MnP and laccase effectively eliminate DCF and MFA. Further investigation is needed to verify whether or not the combinations of MnP and laccase with some other enzymes may be involved in the degradation of DCF and MFA by *P. sordida* YK-624.

Metabolites of fungal DCF transformation

During HPLC analyses of residual DCF in the fungal cultures, the occurrence of additional

chromatographic peaks, which were suspected to be DCF metabolites, with shortened retention when compared to DCF became apparent (Fig. 2). Thus, the isolation of DCF metabolites (peaks D1-D3 in Fig. 2) was achieved by PTLC and the isolated metabolites were analyzed by MS and ^{1}H NMR for structural elucidation. Spectral data of DI-MS and ^{1}H NMR are shown in Table 1.

The mass spectra of D2 and D3 gave molecular ion peaks 16 mass units higher than authentic DCF (*m/z* 295), and the molecular ion of D1 was at *m/z* 327, 32 mass units higher than authentic DCF (Table 1). These results suggest the introduction of one or two hydroxyl group into DCF to yield mono-hydroxylated DCF (D2 and D3) or di-hydroxylated DCF (D1). D2 and D3 were identified to be 4'-hydroxydiclofenac (4'-OH-DCF) and 5-hydroxydiclofenac (5-OH-DCF) by ^{1}H NMR analyses (Table 1), and the NMR data of D2 and D3 were consistent with those of 4'-OH-DCF and 5-OH-DCF described previously (Shen et al, 1999; Gröning et al. 2007). Furthermore, the MS and NMR data of D1 confirmed that hydroxylation occurred at the 4' and 5 positions of DCF. Thus, D1 was identified as 4',5-dihydroxydiclofenac (4',5-diOH-DCF) (Table 1). Since the authentic compounds of the identified metabolites were not available, the formation of metabolites was estimated from relative area (T_4/T_0) measured by HPLC peaks of the corresponding DCF metabolite at 4 days of treatment (T_4) and the starting compound DCF in the treatment at time zero (T_0). The T_4/T_0 value for 4',5-diOH-DCF (D1), 4'-OH-DCF (D2) and 5-OH-DCF (D3) was 0.11, 0.85 and 0.22, respectively (Fig. 2).

DCF is metabolized in the liver and follows two major pathways in both humans and experimental animals. First, DCF undergoes ring hydroxylation, catalyzed by cytochrome P450 (CYP) isoenzyme CYP2C9, resulting in the formation of the major oxidative metabolite, 4'-OH-DCF (Stierlin and Faigle, 1979). Other, quantitatively minor products of oxidative

metabolism include 5-OH-DCF, catalyzed by CYP3A4 (Shen et al., 1999; Tang et al., 1999), as well as a number of other mono- or di-hydroxylated metabolites (Blum et al., 1996; Bort et al., 1999). Both the 4'-OH-DCF and 5-OH-DCF can be further oxidized into quinone imines. The second important pathway is glucuronidation, resulting in DCF 1-*O*-β-acyl glucuronide (Stierlin and Faigle 1979; Krenz-Rommel and Boelsterli 1993).

In this study, 4'-OH-DCF (D2) was shown to be the predominant metabolite, and 5-OH-DCF (D3) and 4',5-diOH-DCF (D1) occurred as minor metabolites by treatment of DCF with the white rot fungus *P. sordida* YK-624 (Fig. 2). This suggested that *P. sordida* YK-624 possesses functions similar to those of mammalian CYP2C9 and CYP3A4, although no sequence of the model white rot fungus *P. chrysosporium* P450 genes showed a sequential similarity above 35% to either the CYP2 or CYP3 families (Matsuzaki and Wariishi 2004).

Studies on the metabolites of DCF transformation with pure cultures of filamentous fungi and yeasts have reported that 4'-OH-DCF is the predominant metabolite, while 5-OH-DCF and 3'-OH-DCF occur as minor by-products in few cases (e.g., with *Pestalotiopsis* sp. and *Epicoccum nigrum*) (Webster et al. 1998; Gröning et al. 2007). More recently, it was reported that the *p*-benzoquinone imine of 5-OH-DCF produced through autoxidation of 5-OH-DCF is a major metabolite of DCF treated with the indigenous microflora of river sediments but 4'-OH-DCF cannot be identified (Gröning et al. 2007). Thus, transformation of DCF in *P. sordida* YK-624 appeared to be similar to that in eukaryotic filamentous fungi and yeasts.

Metabolites of fungal MFA transformation

HPLC data on the transformation of MFA and formation of metabolites confirmed four chromatographic peaks (peaks M1-M4) that are suspected to be MFA metabolites (Fig. 3).

Thus, metabolites M1-M4 separated by PTLC were subjected to MS and ^{1}H NMR.

Table 2 shows the mass spectra of TMS derivatives of authentic MFA and the four MFA metabolites (M1-M4) by GC-MS analysis. The molecular ion of M3 was at *m/z* 401, which is 88 mass units (OTMS) higher than authentic MFA (*m/z* 313) and the molecular ions of M1 and M2 (*m/z* 489) were 176 mass units (2 × OTMS) higher than that of authentic MFA. These results suggest the transformation of MFA to mono-hydroxylated MFA (M3) and di-hydroxylated MFAs (M1 and M2). The molecular ion of M4 at *m/z* 415 was characterized by the conversion of a CH_3 group in the 2' or 3' position of MFA (*m/z* 313) to a COOH group.

The chemical structures of these metabolites (M1-M4) were determined by ^{1}H NMR analyses (Table 2). The mono-hydroxylated MFA (M3) was confirmed to be 3'-hydroxymethylmefenamic acid (3'-OH-MFA). The di-hydroxylated MFAs (M1 and M2) were identified as 3'-hydroxymethyl-5-hydroxymefenamic acid (3',5-diOH-MFA) and 3'-hydroxymethyl-6'-hydroxymefenamic acid (3',6'-diOH-MFA), respectively. Furthermore, metabolite M4 was identified as 3'-carboxymefenamic acid (3'-COOH-MFA). The value of relative area (T_4/T_0), which was measured by HPLC peaks of the corresponding MFA metabolite at 4 days of treatment (T_4) and the starting compound MFA in the treatment at time zero (T_0), was 0.17, 0.03, 0.67 and 0.13 for 3',5-diOH-MFA (M1), 3',6'-diOH-MFA (M2), 3'-OH-MFA (M3) and 3'-COOH-MFA (M4), respectively (Fig. 3).

MFA is metabolized in humans by CYP2C9 to 3'-OH-MFA, and further oxidation to a 3'-COOH-MFA may occur. Two oxidative metabolites, 3'-OH-MFA and 3'-COOH-MFA, are further converted to esterglucuronides (Glazko 1966; Sato et al. 1993). To our knowledge, almost no information on metabolites of MFA transformation by microorganisms is currently available. In this study, the four metabolites of MFA were identified as 3'-OH-MFA (M3), 3'-COOH-MFA (M4), 3',5-diOH-MFA (M1) and 3',6'-diOH-MFA (M2). These results

suggest that the transformation of MFA in *P. sordida* YK-624 is similar to that previously reported in humans, and that CYPs may be involved in the hydroxylation of MFA by the fungus.

Effect of cytochrome P450 inhibitor on degradation of DCF and MFA

The results on the identification of metabolites of DCF and MFA after fungal transformation suggested that hydroxylation catalyzed by CYPs in *P. sordida* YK-624 may be involved in the elimination and detoxification of DCF and MFA. Therefore, the CYP inhibitor 1-ABT was added to *P. sordida* YK-624 cultures containing DCF or MFA. After 5 days of incubation, the addition of 0.1 and 1.0 mM 1-ABT did not affect production of ligninolytic enzymes and mycelial dry weight on Kirk medium containing DCF or MFA (data not shown). On the other hand, the disappearance of DCF and MFA was inhibited in cultures with 1.0 mM 1-ABT, although no appreciable inhibition of DCF removal was observed by the addition of 0.1 mM 1-ABT (Fig. 4). These results support the notion that hydroxylation of DCF and MFA by *P. sordida* YK-624 is catalyzed by CYPs. However, it cannot be clearly concluded that the removal of DCF and MFA by the white rot fungi *P. sordid*a YK-624 is caused by only CYPs, not by ligninolytic enzymes, because our preliminary experiments showed that MnP and laccase eliminate DCF and MFA. The role of ligninolytic enzymes and CYPs on fungal elimination of DCF and MFA should be the subject of further studies.

Conclusions

In this study, we demonstrated that the white rot fungus *P. sordida* YK-624 eliminates DCF and MFA and removes the acute lethal toxicity of DCF and MFA towards the freshwater

crustacean *T. platyurus*. To our knowledge, this is the first report dealing with the fungal removal of the toxicity of DCF and MFA. Recently, it was reported that degradation of pharmaceuticals contained in real sewage sludges by white rot fungus *Trametes versicolor* in solid-phase and bioslurry systems is now underway in their research group and preliminary assays revealed promising results (Marco-Urrea et al. 2009b). These research approaches would be useful for the practical application of white rot fungi to the pharmaceutical degradation.

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Figure Legends

Fig. 1 Treatment of DCF (A) and MFA (B) with *P. sordida* YK-624. Indicated for each point are the mean and standard deviation of five experiments. (♦), Residual DCF or MFA; (■), Relative acute lethal toxicity.

Fig. 2 HPLC detection of DCF metabolites after fungal transformation.

Fig. 3 HPLC detection of MFA metabolites after fungal transformation.

Fig. 4 Effect of 1-ABT on decrease in DCF (A) and MFA (B) in cultures of *P. sordida* YK-624. Indicated for each point are the means and standard deviation of triplicate experiments.

Table 1 Chemical structures and analytical data of DCF and its fungal metabolites.

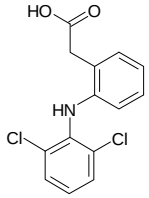
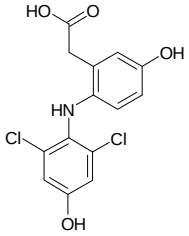
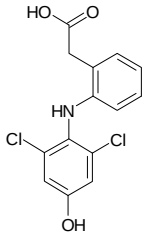
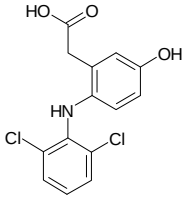
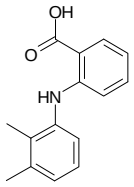
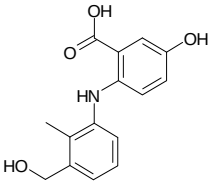
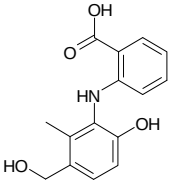
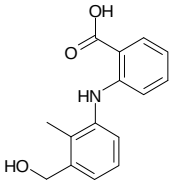
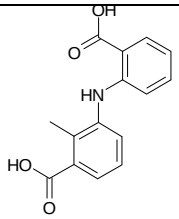
Metabolite	Chemical structure	Mass spectrum *m/z* (relative intensity, %)	^{1}H NMR spectrum δ (CD_3OD)
DCF (starting compound)		297 (22.6), 295 (M^+, 34.3), 279 (3.3), 277 (5.0), 244 (16.2), 242 (48.4), 216 (35.4), 214 (100), 179 (15.0), 151 (9.9), 107 (15.0)	3.62 (2H, s, -CH_2-), 6.35 (1H, dd, *J* = 1.2, 7.9, 3-H), 6.81 (1H, m, 5-H), 6.96 (1H, m, 4-H), 7.00 (1H, t, *J* = 7.9, 4'-H), 7.19 (1H, dd, *J* = 1.3, 7.4, 6-H), 7.35 (2H, d, *J* = 7.9, 3', 5'-H)
D1	 4',5-diOH-DCF	329 (26.5), 327 (M^+, 39.5), 311 (19.3), 309 (28.2), 285 (5.4), 283 (11.7), 276 (10.8), 274 (31.9), 248 (42.0), 246 (100), 211 (30.7), 203 (7.1), 183 (15.5), 123 (23.4)	3.55 (2H, s, -CH_2-), 6.08 (1H, d, *J* = 8.6, 3-H), 6.38 (1H, dd, *J* = 2.8, 8.6, 4-H), 6.59 (1H, d, *J* = 2.8, 6-H), 6.73 (2H, s, 3', 5'-H)
D2	 4'-OH-DCF	313 (25.9), 311 (M^+, 38.8), 295 (5.0), 293 (7.7), 260 (16.7), 258 (49.8), 232 (37.1), 230 (100), 195 (18.6), 167 (12.9), 166 (13.1), 139 (6.0), 115 (13.9)	3.70 (2H, s, -CH_2-), 6.26 (1H, dd, *J* = 1.2, 8.1, 3-H), 6.80 (1H, dt, *J* = 1.2, 7.4, 5-H), 6.87 (2H, s, 3', 5'-H), 7.02 (1H, m, 4-H), 7.16 (1H, dd, *J* = 1.5, 7.4, 6-H)
D3	 5-OH-DCF	313 (22.9), 311 (M^+, 34.9), 295 (11.7), 293 (18.2), 260 (12.5), 258 (33.8), 232 (37.0), 230 (100), 196 (8.6), 195 (21.4), 167 (19.2), 139 (6.7), 115 (16.5)	3.70 (2H, s, -CH_2-), 6.33 (1H, d, *J* = 8.6, 3-H), 6.50 (1H, dd, *J* = 2.8, 8.6, 4-H), 6.70 (1H, d, *J* = 2.8, 6-H), 6.93 (1H, t, *J* = 8.0, 4'-H), 7.32 (2H, d, *J* = 8.1, 3', 5'-H)

Table 2 Chemical structures and analytical data of MFA and its fungal metabolites.

Metabolite	Chemical structure	Mass spectrum [a] *m/z* (relative intensity, %)	^{1}H NMR spectrum δ (CD_3OD)
MFA (starting compound)		314 (13.9), 313 (M^+, 51.9), 299 (2.1), 298 (8.0), 224 (26.1), 223 (100), 208 (45.6), 194 (18.7), 180 (19.5), 75 (12.6), 73 (12.2)	2.14 (3H, s, 2'-CH_3), 2.31 (3H, s, 3'- CH_3), 6.60-6.67 (1H, m, 5-H), 6.63-6.68 (1H, m, 6'-H), 6.98-7.10 (3H, m, 3, 4', 5'-H), 7.22 (1H, ddd, *J* = 1.6, 7.3, 8.6, 4-H), 7.94 (1H, dd, *J* = 1.5, 8.1, 6-H)
M1	 3',5-diOH-MFA	490 (20.5), 489 (M^+, 46.2), 474 (2.5), 384 (2.5), 369 (5.9), 309 (20.3), 281 (9.7), 268 (3.8), 207 (4.0), 75 (24.0), 73 (100)	2.22 (3H, s, 2'-CH_3), 4.65 (2H, s, 3'-CH_2-), 6.72-6.78 (1H, m, 6'-H), 6.82 (1H, dd, *J* = 3.0, 9.1, 4-H), 7.10-7.20 (3H, m, 3, 4', 5'-H), 7.41 (1H, d, *J* = 2.8, 6-H)
M2	 3',6'-diOH-MFA	490 (26.0), 489 (M^+, 61.2), 475 (2.5), 474 (5.9), 384 (8.2), 369 (3.8), 327 (9.0), 310 (10.1), 309 (17.2), 296 (11.8), 282 (9.2), 266 (7.4), 237 (3.6), 222 (12.6), 207 (10.4), 180 (4.0), 73 (100)	2.13 (3H, s, 2'-CH_3), 4.78 (2H, s, 3'-CH_2-), 6.33 (1H, dd, *J* = 1.0, 8.6, 3-H), 6.47 (1H, ddd, *J* = 1.0, 6.9, 7.9, 5-H), 6.60 (1H, d, *J* = 8.4, 5'-H), 6.90 (1H, d, *J* = 8.6, 4'-H), 7.04 (1H, ddd, *J* = 1.6, 6.9, 8.6, 4-H), 7.81 (1H, dd, *J* = 1.5, 7.9, 6-H)
M3	 3'-OH-MFA	402 (17.6), 401 (M^+, 54.3), 386 (4.7), 311 (4.9), 296 (10.1), 266 (15.6), 222 (25.7), 221 (76.9), 194 (23.0), 193 (29.5), 180 (12.1), 75 (20.1), 73 (100)	2.22 (3H, s, 2'-CH_3), 4.66 (2H, s, 3'-CH_2-), 6.62-6.69 (1H, m, 5-H), 6.68-6.73 (1H, m, 6'-H), 7.16-7.26 (4H, m, 3, 4, 4', 5'-H), 7.95 (1H, dd, *J* = 1.5, 8.1, 6-H)
M4	 3'-COOH-MFA	416 (6.1), 415 (M^+, 30.1), 401 (3.0), 400 (6.2), 309 (4.2), 281 (21.8), 235 (19.4), 207 (19.2), 180 (13.4), 152 (5.5), 75 (40.7), 73 (100)	2.41 (3H, s, 2'-CH_3), 6.66-6.73 (1H, m, 5-H), 7.21-7.31 (2H, m, 4, 5'-H), 7.40-7.44 (1H, m, 3-H), 7.53-7.57 (1H, m, 4'-H), 7.96 (1H, dd, *J* = 1.5, 8.2, 6-H)

[a] TMS derivatives

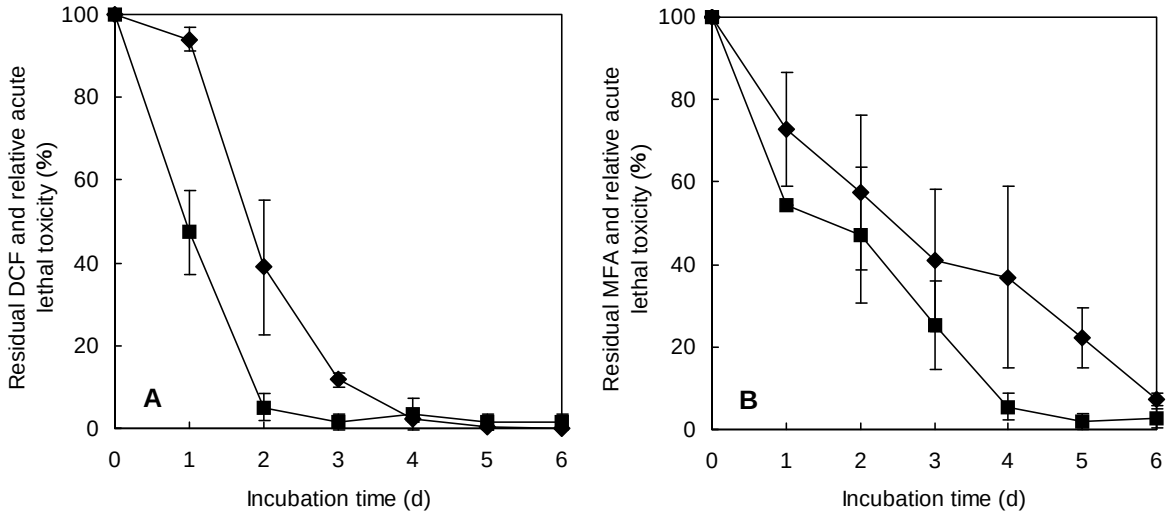


Fig. 1 Treatment of DCF (A) and MFA (B) with *P. sordida* YK-624. Indicated for each point are the mean and standard deviation of five experiments. (♦), Residual DCF or MFA; (■), Relative acute lethal toxicity.

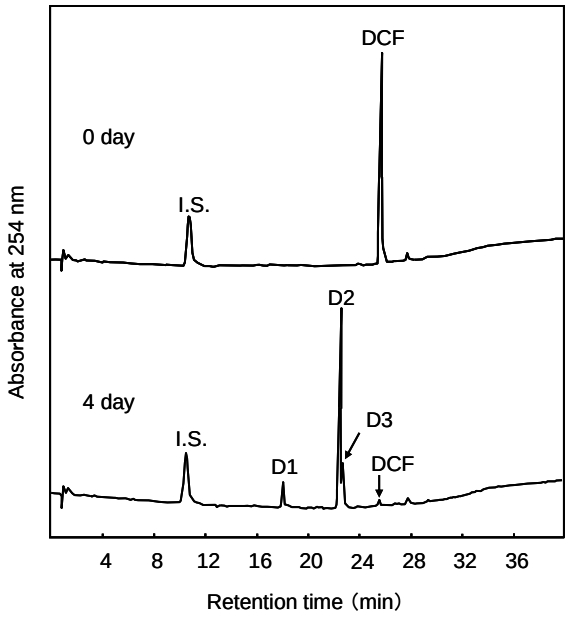


Fig. 2　HPLC detection of DCF metabolites after fungal transformation.

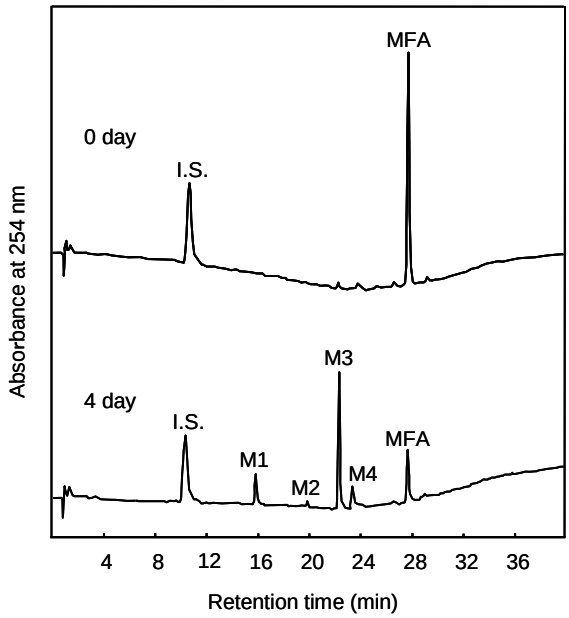


Fig. 3 HPLC detection of MFA metabolites after fungal transformation.

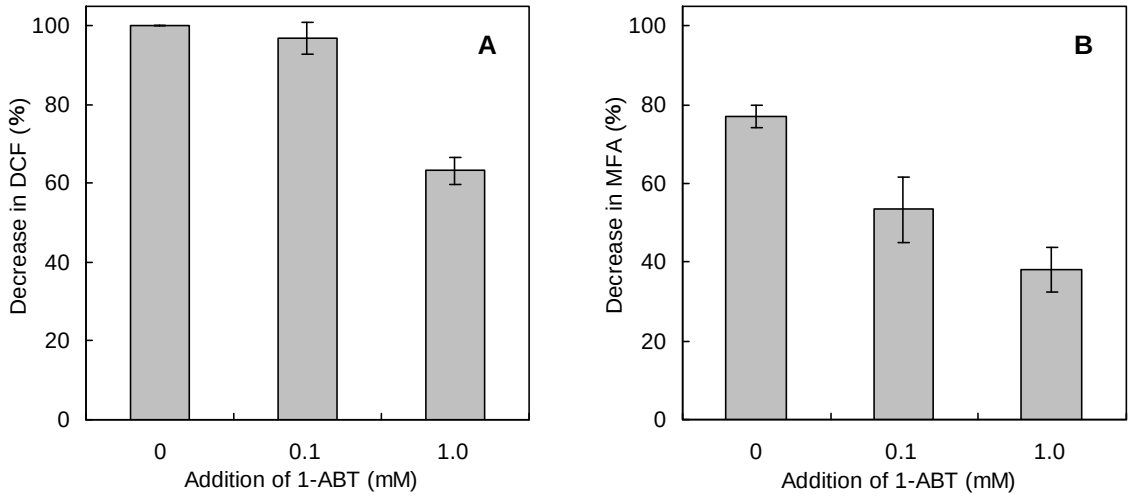


Fig. 4 Effect of 1-ABT on decrease in DCF (A) and MFA (B) in cultures of *P. sordida* YK-624. Indicated for each point are the mean and standard deviation of triplicate experiments.