



HAL
open science

Cleavage of diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases

Christian Mougin, François-Didier Boyer, Eliane Caminade, Rachel Rama

► **To cite this version:**

Christian Mougin, François-Didier Boyer, Eliane Caminade, Rachel Rama. Cleavage of diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases. *Journal of Agricultural and Food Chemistry*, 2000, 48 (10), pp.4529-4534. 10.1021/jf000397q . hal-02697764

HAL Id: hal-02697764

<https://hal.inrae.fr/hal-02697764>

Submitted on 19 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Cleavage of the diketonitrile derivative of the herbicide isoxaflutole by
extracellular fungal oxidases

Christian Mougin*, François-Didier Boyer, Eliane Caminade and Rachel Rama^{\$}

INRA, Unité de Phytopharmacie et Médiateurs Chimiques, Route de saint-Cyr, 78026
Versailles Cedex, France

^{\$} Present address: Aventis CropScience, 14-20 Rue P. Baizet, BP 9163, 69263 Lyon Cedex
09, France

*corresponding author

Tel: +33-1-30-83-31-02

Fax: +33-1-30-83-31-19

Email: mougin@versailles.inra.fr

1 Abstract

2 Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the
3 active herbicide principle. Then, the diketonitrile derivative undergoes cleavage to the
4 inactive benzoic acid analog. In this paper, we establish that an oxidative mechanism
5 implicating two successive reactions in the presence of dimethyldioxirane can chemically
6 initiate the cleavage of the diketonitrile. We also show that two white rot strains,
7 *Phanerochaete chrysosporium* and *Trametes versicolor*, are able to convert the diketonitrile to
8 the acid, when cultured in liquid media. This main metabolite amounts to 24.6 and 15.1% of
9 initial herbicide content after 12-15 days of culture. Another polar metabolite represents less
10 than 3.7% of parent compound amount during the same period. Oxidative enzymes produced
11 by the fungi show a similar time course to diketonitrile degradation. Purified laccase (EC
12 1.10.3.2), in the presence of 2 mM 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) acting
13 as a redox mediator at pH3, supports the reaction with rates of 0.3-0.4 nmol h⁻¹ U⁻¹.

14

15

16

17 Keywords: herbicide, metabolism, dimethyldioxirane, white-rot fungi, *Phanerochaete*
18 *chrysosporium*, *Trametes versicolor*, laccases

19

1 INTRODUCTION

2

3 Isoxaflutole (IUPAC name: 5-cyclopropyl-1,2-oxazol-4-yl α,α,α -trifluoro-2-mesyl-*p*-tolyl
4 ketone, compound **1** Figure 1) is a recent herbicide for pre- and postemergence control of a
5 wide range of important broadleaf and grass weeds in corn and sugarcane (Luscombe *et al.*,
6 1995). After herbicide application, susceptible weed species show a bleaching symptomology
7 of newly developed leaves, followed by growth suppression and necrosis prior to plant death,
8 similar to that seen with herbicidal inhibitors of carotenoid biosynthesis. Furthermore,
9 reduction of carotenoid and chlorophyll content is associated with an indirect inhibition of the
10 phytoene desaturase due to the depletion of the cofactor plastoquinone. That depletion is
11 caused by the inhibition of the enzyme 4-hydroxy-phenylpyruvate dioxygenase (Pallett *et al.*,
12 1998; Viviani *et al.*, 1998). The dioxygenase catalyses the oxidative decarboxylation of 4-
13 hydroxyphenylpyruvate forming homogentisate.

14 In plants and soils, isoxaflutole is rapidly converted to a diketonitrile derivative (DKN,
15 compound **2** Figure 1) by opening of the isoxazole ring (Viviani *et al.*, 1998). DKN is the
16 active herbicide principle and is a potent inhibitor of the dioxygenase (Pallett *et al.*, 1998).
17 DKN undergoes degradation to the inactive benzoic acid analog (BZA, compound **3** Figure 1)
18 in treated plants. The extent of this degradation is a basis for herbicidal selectivity, being most
19 rapid in tolerant plants than in the susceptible species.

20 Because of their implication in herbicide selectivity (agronomic impact) and breakdown
21 (environmental impact), it is of great importance to identify enzymatic systems involved in
22 the conversion of DKN to BZA. Enzymes responsible for the cleavage of diketone bonds
23 have been poorly characterized to date. Sakai *et al.* (1986) reported a bacterial β -diketone
24 hydrolase (EC 3.7.1.7) involved in the degradation mechanism of poly(vinyl alcohol). The
25 enzyme was also active on aliphatic β -diketones, and on aromatic β -diketones such as 1-

1 phenyl-1,3-butanedione, presenting a framework close to this of DKN. Nevertheless, such an
2 hydrolytic mechanism producing a methyl ketone is not relevant to the formation of BZA.
3 White-rot fungi are known for many years for their ability to transform various xenobiotics,
4 by using their rich enzymatic equipment (Barr and Aust, 1994). These organisms are
5 commonly leaving in woody plants, but they can also found in soils. For that reason, their
6 ability to cleave the diketone bond of the herbicide was investigated. We propose in this paper
7 an hypothetical pathway for the oxidative transformation of DKN. Then, we report herbicide
8 transformation by two strains of white-rot fungi cultured in liquid media, and by purified
9 oxidases.

10

11 MATERIALS AND METHODS

12

13 **Chemicals and reagents.** The unlabelled DKN derivative of isoxaflutole, [Ring-UL-
14 ¹⁴C]DKN (909 MBq/mmol, radiochemical purity 96%) and standard of BZA were gifts from
15 Rhône-Poulenc Agro. NAT 89 was a commercial phospholipid source supplied by Natterman
16 Phospholipid GmbH (Cologne, Germany). All other chemicals and reagents were obtained
17 from Sigma-Aldrich (St-Quentin Fallavier, France), and solvents came from Carlo Erba (Val
18 de Reuil, France).

19

20 **Synthesis of putative transformation products of DKN.** NMR data (¹H: 300 MHz; ¹³C:
21 75.5 MHz) are recorded on a Varian Gemini 300 instrument (Les Ulis, France). All NMR
22 spectra are recorded in deuteriochloroform (CDCl₃). Chemical shifts are reported in δ ppm
23 relative to CHCl₃ (CDCl₃) as internal reference: 7.27 ppm for ¹H (77.14 ppm for ¹³C).
24 Coupling constants (*J*) are given in Hertz (Hz). Multiplicities are recorded as s (singlet), d
25 (doublet), t (triplet), q (quadruplet) and m (multiplet) and br (broad). Mass spectra (MS) were

1 obtained on a Nermag (Poissy, France) R10–10C by direct insertion probe or linked to a
2 Varian 3300 GC. Ionization was obtained either by electronic impact (EI) or chemical
3 ionization with ammonia (CI, NH₃). Infrared spectra (IR) were obtained on a Nicolet Avatar
4 320 FT-IR (Trappes, France) and are reported in terms of frequency of absorption (ν , cm⁻¹).

5 To a 0°C solution of diketone **2** (69 mg, 0.19 mmol) in CH₂Cl₂ (3 mL) was added an acetone
6 solution (0.01 M) of dimethyldioxirane (3 mL, 0.30 mmol) prepared by the well-formulated
7 protocol of Adam *et al.* (1991). The resultant solution was stirred for 5 minutes at 0°C, dried
8 (MgSO₄) filtered and evaporated under reduced pressure. The crude product (69 mg) was
9 immediately dissolved in CDCl₃ for NMR analysis. We observed the presence of epoxide **4**
10 and lactone **5** (33/66 the ratio was determined by NMR integration of signals of methyl
11 groups). After NMR analysis, CDCl₃ was removed under reduced pressure and the residue
12 kept at room temperature overnight and NMR analysis of the residue showed only the
13 presence of lactone **5**.

14 The solution of diketone **2** in CH₂Cl₂ and dimethyldioxirane was stirred overnight at room
15 temperature and treated as above for direct oxidation to the aromatic acid BZA **3** and
16 cyclopropanic acid **6**. These compounds **3** and **6** were characterized by esterification with
17 diazomethane to give ester **7** (35 mg) and **8** (2 mg) separated by chromatography on silica gel
18 (30 AcOEt / 70 cyclohexane).

19 **4**: – ¹H NMR: δ = 8.25 (s, 1 H), 8.00 (d, 1 H, J = 7.5 Hz), 7.55 (d, 1 H, J = 7.5 Hz), 3.20 (s, 3
20 H), 2.85 (m, 1 H), 1.50-1.20 (m, 4 H). – ¹³C NMR (partial): δ = 197.3 (s), 81.3 (s), 45.5 (q),
21 17.3 (d), 16.4 (t), 15.7 (t).

22 **5**: – ¹H NMR: δ = 8.40 (s, 1 H), 8.05 (s, 2 H), 6.25 (s, 1 H), 3.35 (s, 3 H), 2.30 (m, 1 H), 1.40-
23 1.20 (m, 4 H). – ¹³C NMR (partial no aromatic carbons): δ = 194.7 (s), 163.8 (s), 134.8 (m),
24 112.3 (s), 67.0 (d), 45.0 (q), 17.9 (d), 14.3 (t), 14.0 (t). – DEI MS m/z (%) 376 (MH⁺) (0.5),
25 356 (2), 280 (1), 251 (30), 69 (100). – DCI (NH₃) MS m/z (%) 393 (M + NH₄⁺) (100), 377 (M

1 + 2 H⁺) (10), 286 (8). – IR (CDCl₃, cm⁻¹): 3170, 3014, 2929, 2250, 1760, 1735, 1672, 1324,
2 1157.

3 **7**: – ¹H NMR: δ = 8.40 (s, 1 H), 7.95 (d, 1 H, *J* = 7.5 Hz), 7.85 (d, 1 H, *J* = 7.5 Hz), 4.00 (s, 3
4 H), 3.40 (s, 3 H). – ¹³C NMR (partial no aromatic carbons): δ = 166.5 (s), 134.0 (m), 53.7 (q),
5 45.0 (q). – GC analysis (AT-5, 0.32 mm ID x 30 m, 180-300 °C, 10 °C min⁻¹), retention time
6 2.12 min – EI MS *m/z* (%) 282 (M^{+o}) (2), 267 (20), 251 (100), 235 (2), 220 (10), 203 (12),
7 188 (10), 172 (10), 160 (15), 145 (20).

8 **8**: – ¹H NMR: δ = 3.90 (s, 3 H), 2.75 (m, 1 H), 1.30-1.10 (m, 4 H). – GC analysis (AT-5, 0.32
9 mm id. x 30 m, 50 °C (30 s)-300 °C, 10 °C min⁻¹), retention time 4.20 min – EI MS *m/z* (%)
10 128 (M^{+o}) (5), 69 (100), 59 (10), 41 (50).

11

12 **Fungal strains and culture conditions.** *Phanerochaete chrysosporium* (BKM-F-1767,
13 ATCC 24725) and *Trametes versicolor* were from the culture collections of the Unité de
14 Phytopharmacie et Médiateurs Chimiques (INRA, Versailles, France). The strains were
15 maintained at 37°C on solid medium of malt (20 g L⁻¹), agar (16 g L⁻¹) and yeast extract (1 g
16 L⁻¹).

17 We distributed 10-mL fractions of culture media in 150-mL Erlenmeyer flasks for *in vivo*
18 degradation studies (*P. chrysosporium* and *T. versicolor*), 200 mL in Roux flasks for biomass
19 production (*P. chrysosporium* and *T. versicolor*), and 5 L in bioreactor for laccase production
20 (*T. versicolor*).

21 We used for *P. chrysosporium* cultures a medium previously described (Mougin *et al.*, 1994).
22 The medium was inoculated with conidiospores (10⁸ spores/L medium). For *T. versicolor*
23 cultures, glycerol was replaced by maltose (20 g L⁻¹), bactopectone was added (2 g L⁻¹) and
24 NAT 89 was omitted (Lesage-Meesen *et al.*, 1996). Cultures were inoculated by 3-5 agar
25 plugs (5 mm) of mycelium taken from the solid medium plates.

1 The air-lift bioreactor used for laccase production was inoculated with pounded mats and
2 performed as previously described (Jolivalt *et al.*, 1999). It contained 5 liters mineral culture
3 medium (Collins *et al.*, 1996). We buffered the medium to pH 5 with 0.29 g L⁻¹ of dimethyl
4 succinic acid. The bioreactor was inoculated with a suspension of mycelium obtained by
5 pounding 2 mats of *T. versicolor* in 100 mL sterile water. Laccase production was stimulated
6 by supplementing the medium with 20 mM 2,5-xylydine.

7

8 **Incubation conditions for in vivo degradation studies.** For DKN degradation studies, the
9 medium (10 mL) was supplemented with 30 μM DKN consisting of a mixture of unlabelled
10 and labelled (2.5 kBq) pesticides dissolved in acetone (50 μL). The Erlenmeyer flasks were
11 enclosed in 1-L sealed flasks with two vials containing sodium hydroxide solution (1M, 10
12 mL, to trap carbon dioxide) and water (10 mL, to keep constant moisture). They were
13 incubated without shaking in the dark at 25°C. Every three days, the cultures were flushed
14 with ambient air and sodium hydroxide solutions were replaced. Uninoculated sterile controls
15 were submitted to a similar incubation design.

16

17 **Enzyme purification.** Purified lignin peroxidases (LiPs) and manganese-dependent
18 peroxidases (MnPs) were obtained from *P. chrysosporium* cultures. These enzymes were
19 generous gifts of Dr. M. Asther (INRA, Unité de Biotechnologie des Champignons
20 Filamenteux, Marseille-Luminy, France). Purified laccases (Lacs) were obtained from *T.*
21 *versicolor* cultures as previously described (Jolivalt *et al.*, 1999). The purified enzyme was
22 stored at -20°C in 25% w/v glycerol.

23

24 **Enzymatic Assays.** Lignin peroxidase (LiP, EC1.11.1.14) activity in the extracellular fluid
25 was determined by the rate of oxidation of veratryl alcohol ($\epsilon_{310} = 9\ 300\ \text{M}^{-1}\ \text{cm}^{-1}$) as

1 described by Tien and Kirk (1984). The assay was conducted at 30°C in 1 mL of a solution
2 containing 2 mM veratryl alcohol in 100 mM phosphate-citrate buffer pH 3. Manganese
3 peroxidase (MnP, EC 1.11.1.13) activity was also determined spectrophotometrically by the
4 methods of Paszczyński *et al.* (1986) with vanillylacetone ($\epsilon_{334} = 18\,300\text{ M}^{-1}\text{ cm}^{-1}$) as a
5 substrate, at 30°C. The reaction mixture (1 mL) contained 0.1 mM vanillylacetone and 0.1
6 mM MnSO₄. Both reactions were started by adding hydrogen peroxide. Laccase (Lac, EC
7 1.10.3.2) activity was measured as the oxidation of 2,2'-azinobis(3-ethylbenzthiazoline-6-
8 sulfonic acid) (ABTS, $\epsilon_{420} = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$) according to the method of Wolfenden and
9 Wilson (1982). The assay was conducted at 30°C in 1 mL of a solution containing 1 mM
10 ABTS in 100 mM phosphate-citrate buffer pH 3. Enzymatic activities were expressed in units
11 mL⁻¹ (U mL⁻¹).

12 DKN transformation by purified oxidases was assayed in 100 mM phosphate-citrate buffer
13 pH 3 or 5. The solution (1 mL final) contained 10 µM DKN (including 1.8 kBq labelled
14 herbicide) dissolved in 20 µL acetone. It was incubated at 30°C under stirring. Enzymatic
15 transformation of DKN is expressed in nmoles h⁻¹ U⁻¹. A solution of hydrogen peroxide (15
16 mM) was continuously added (20 µL h⁻¹) during incubations with peroxidases. Controls
17 (where at the least one component was omitted) were also performed.

18
19 **Analytical procedures for herbicide and transformation products.** Fractions of the filtered
20 aqueous media resulting from *in vivo* degradation studies were concentrated on a C₁₈ guard
21 column MCH-10 (3 cm x 4 mm ID; Varian, Les Ulis, France) at a flow rate of 1 mL min⁻¹
22 with an isocratic pump (Mougin *et al.*, 1994). The guard column, on-line interfaced with the
23 HPLC through an electric valve, was used to load the sample for analysis. Elution of DKN
24 and transformation products was then achieved from the guard column onto the analytical
25 column TSK ODS-80TM (25 cm x 4.6 mm ID; Varian) with a pump delivering a solvent

1 system composed of water/acetonitrile/H₃PO₄ (99:1:0.25 v/v/v) at 1 mL min⁻¹ for 3 min.
2 During the next 15 min, the solvent mixture was linearly brought to 0:100:0.25 (v/v/v). These
3 solvent conditions were then held for 12 min. Radioactivity (radiochemical detector) and A₂₅₄
4 (UV detector) of the column eluate were monitored. Radioactivity remaining in the fungal
5 biomass was assessed by digestion of filtered mycelia with 2 mL Optisolve solubilizer
6 (EG&G, Evry, France) for 5 h. Finally, mixtures were supplemented with 10 mL Optiphase 3
7 (EG&G) and liquid scintillation counting was achieved 24 h later. Labelled carbon dioxide
8 evolved from the cultures was determined by liquid scintillation counting of 500- μ L aliquots
9 of the sodium hydroxide solutions.

10 Degradation studies with purified enzymes were analyzed by injecting 100- μ L aliquots of the
11 incubation media through the HPLC with the chromatographic conditions described above.

12

13 **Experimental design.** Each experiment was done in triplicate. Results are expressed as
14 means \pm SD.

15

16 RESULTS

17

18 **Hypothetical pathway for DKN cleavage.** In a first time, we have searched a pathway for
19 chemical DKN transformation to the BZA metabolite. In the proposed pathway, the parent
20 compound **2** undergoes two successive reactions in the presence of the oxidant
21 dimethyldioxirane to release the acid (Figure 2). After 5 min, it gives a mixture of two
22 compounds: the epoxide **4** and the lactone **5**. Compound **4**, unstable, is rapidly converted into
23 **5**. After a night of contact, the lactone **5** is cleaved to BZA **3** and compound **6**, with a release
24 of cyanide. Both metabolites **3** and **6** were then derivatized into **7** and **8** for structural analysis.

1 We have shown that an oxidative mechanism can chemically transform DKN to BZA. The
2 pathway is consistent with the possible involvement of fungal oxidases in herbicide cleavage.

3

4 ***In vivo* degradation of the DKN derivative by the two fungal strains.** Cultures of *P.*
5 *chrysosporium* and *T. versicolor* were incubated with [Ring-UL-¹⁴C]DKN for 15 days. After
6 that period, the amounts of radioactivity in the medium corresponded to 98.0% of initial
7 radioactivity in the uninoculated controls, to 92.8% with *P. chrysosporium*, and to 81.2% with
8 *T. versicolor*. No herbicide mineralization was detected with any of the fungal strains.
9 Determining the radioactivity trapped by the fungal biomass gave always recoveries
10 comprised between 92.4 and 97.2% of initial amounts. The amounts of DKN (RT = 16.5 min)
11 in the medium decreased slightly from 91.7 to 84.1% in the uninoculated control (Figure 3).
12 In the presence of *P. chrysosporium* and *T. versicolor*, they were only 65.6 and 59.6% after
13 15 days. With *P. chrysosporium*, the decrease occurred after a 3-day lag phase, whereas it was
14 already started at this time with *T. versicolor*. With both fungal strains, DKN decrease
15 corresponded to the formation of two polar metabolites. One of them, the most abundant (M₁,
16 RT = 15.5 min), amounted to 24.6% of initial radioactivity in the presence of *P.*
17 *chrysosporium* and to 15.1% with *T. versicolor*. M₁ was detected in low amounts (less than
18 1.6%) in the uninoculated controls. The second (M₂, RT = 14.0 min) represented always less
19 than 3.7% of initial radioactivity.

20 Profiles of extracellular oxidase production by *P. chrysosporium* and *T. versicolor* are shown
21 in Figure 4 for 15 days. Both LiPs and MnPs were already detected after 3 days of culture.
22 They exhibited typical production profiles obtained for *P. chrysosporium* under these
23 incubation conditions (Mougin *et al.*, 1996). The LiPs exhibited the highest activity, with
24 maximal values (approx. 0.84 U mL⁻¹) between 6 and 9 days of incubation. MnP production
25 was more reduced. At the opposite, Lac was not produced by this strain at day 3, and showed

1 an activity of 0.12-0.18 U mL⁻¹ between days 6 to 12. Only Lac production was measured in
2 *T. versicolor* cultures. Production began after 3 days to maintain its level until the end of the
3 experiment. Activity values were similar to these reported for *P. chrysosporium*. All three
4 oxidative enzymes show a similar time course to DKN degradation.

5 We tried to identify the metabolites formed from the DKN derivative by the fungi. For that
6 purpose, medium fractions were pooled and concentrated on the guard column, prior to HPLC
7 analysis. The peaks were then collected during elution. M₁ co-chromatographed with an
8 authentic standard of BZA analog. After solvent extraction and derivatization, GC-MS
9 analysis confirmed its identity. M₂ could not be extracted by organic solvents, and its
10 structure was not checked by GC-MS.

11

12 **Degradation of the DKN derivative by fungal purified enzymes.** The DKN derivative was
13 incubated with fungal extracellular oxidases, without or with their specific redox mediator.
14 Both LiPs and MnPs presenting high specific activities were unable to catalyze the enzymatic
15 transformation of DKN, with or without mediators, during 12-hour incubations (data not
16 shown). Only Lacs transformed the herbicide, when incubated with 1 mM ABTS. Three
17 labelled metabolites were formed. One of them co-chromatographed with an authentic
18 standard of BZA analog during HPLC analysis. After solvent extraction and derivatization,
19 GC-MS analysis confirmed its identity as BZA. Another compound presented the same
20 retention time than compound M₂ formed *in vivo*. The third was still a more polar compound.
21 The reaction was then studied under different incubation conditions with Lacs purified from
22 *T. versicolor* cultures. That strain was preferentially used for enzyme production because it
23 secreted mainly Lacs in the extracellular fluid in the presence of 2,5-xylydine. The DKN was
24 cleaved at its higher rate (approx. 0.3-0.4 nmol h⁻¹ U⁻¹) in the presence of ABTS at acidic pH
25 3 (Table 1). High incubation temperatures (30 to 50 °C) allowed the higher enzymatic

1 activities. The rates also depended on ABTS concentration, being maximal with values higher
2 than 1 mM mediator. Compounds such as exogenous (1-hydroxybenzotriazole, 1-HBT) or
3 physiological (3-hydroxyanthranilic acid, 3-HAA, Eggert *et al.*, 1996) mediators were not
4 able to support the reaction.

5

6 DISCUSSION

7

8 Fungal metabolism of xenobiotics by white-rot fungi has been studied for many years. In
9 most cases, they have been seen as efficient degraders (Barr and Aust, 1994). However,
10 among the bulk of existing chemical compounds metabolized, a few data are available
11 concerning the mechanisms involved in herbicide metabolism by these filamentous fungi. In
12 the present paper, we study the transformation of the active principle of the new herbicide
13 isoxaflutole, namely its ketonitrile (DKN) derivative, by two well-known white-rot fungi.

14 *P. chrysosporium* and *T. versicolor* were able to cleave shown to cleave the diketone in liquid
15 cultures, in producing the benzoic acid analog (BZA) also formed in tolerant plants (Viviani
16 *et al.*, 1998). *P. chrysosporium* was more efficient than *T. versicolor*. Nevertheless, herbicide
17 metabolism occurred mainly at the end of the fungal growing phase, and seemed correlated
18 with the production of extracellular oxidases, such as laccases, by the two strains. A similar
19 situation was recently reported for several PAHs metabolized by fungi producing oxidative
20 enzymes (Gramss *et al.*, 1999).

21 Enzymes involved in the diketone cleavage remain poorly characterized to date. Sakai *et al.*
22 (1986) reported a bacterial β -diketone hydrolase (EC 3.7.1.7) involved in the degradation
23 mechanism of poly(vinyl alcohol). Nevertheless, such an hydrolytic mechanism was not
24 relevant to the formation of BZA, because a methyl ketone should be formed instead of the

1 acid. Moreover, attempts to isolate a form of β -diketone hydrolase acting on DKN among
2 microorganisms were unsuccessful (J.-C. Thomas, Rhône-Poulenc, personal communication).
3 Because of the apparent correlation stated above and because of the high and non-specific
4 oxidizing potential of white-rot fungi, we looked for oxidative extracellular enzymes
5 produced by *P. chrysosporium* and *T. versicolor*. In dependence on their ionization potential
6 (IP), substrates can be directly oxidized by peroxidases or laccases, or by several
7 enzyme/mediator systems such as laccase/ABTS (Collins *et al.*, 1996). The initial one-
8 electron oxidation of xenobiotics is typical of wood-decay fungi such as white rot. Although
9 the reaction often leads to the formation of quinones, intermediates such as radical cations and
10 hydroxylated intermediates are possibly appearing. On the other hand, chemicals (mainly
11 methyl-, methoxy-, chloro- and bromophenols) can be submitted to oxidative coupling by
12 laccases, leading to polymeric products of increasing complexity (Gianfreda *et al.*, 1999).
13 Although the main role of fungal laccase in nature is to depolymerize lignin, examples of
14 bond cleavage among xenobiotics are not abundant. Chivukula *et al.* (1995) reported the
15 oxidation of phenolic azo dyes by laccase from *Pyricularia oryzae* to benzoquinone. The
16 reaction proceeded by the production of a carbonium ion in which the charge is localized on
17 the phenolic ring carbon bearing the azo linkage, and cleavage of the carbon- nitrogen bond.
18 In a similar way, we reported more recently the cleavage of the *N,N*-dimethyl-*N*-(4-
19 hydroxyphenyl)urea (a metabolite of phenylurea herbicide) to *p*-benzoquinone by laccases of
20 *T. versicolor* (Jolivalt *et al.*, 1999). In this paper, we describe a new laccase-mediated
21 pesticide cleavage. Nevertheless, results from our study with purified enzymes establish that a
22 direct attack of the laccase never occurred. Only ABTS acted as a potent redox mediator. As
23 the herbicide was also transformed *in vivo*, that implies the production and use by the fungus
24 of an unidentified redox mediator. Laccase production can be 6-fold increased *in vivo* by
25 treatment of *Trametes* cultures with 2,5-xylidine, without any significant diketonitrile

1 cleavage increase (data not shown). This result seems to indicate that the production of the
2 redox mediator is the limiting step in the reaction. On the other hand, we can not exclude the
3 involvement of other oxydases (*i.e.* intracellular oxygenases) in DKN breakdown, although
4 we failed to obtain any reaction with intact mycelia, or microsomal fractions (unpublished
5 results).

6 The hypothetical pathway for DKN chemical oxidation to BZA implies the loss of the cyano
7 group of the molecule. In living organisms (bacteria, fungi and higher plants), the nitrile is
8 converted into the corresponding acid, with a possible release of ammonia as a by-product
9 (Harper, 1977; Stalker and McBride, 1987). Then, the acid can be submitted to oxidative
10 decarboxylation to give phenolic compounds (Buckland *et al.*, 1973).

11
12 In summary, this work shows that fungal laccases are able to cleave diketone compounds.
13 Nevertheless, the reaction is not due to a direct action of the enzyme on the chemical, but
14 requires the presence of a redox mediator acting as a diffusible electron carrier. *In vivo*,
15 identity of fungal endogenous mediators remains to be determined. Moreover, our results
16 should not prevent the involvement of other unknown enzymatic systems in the cleavage of
17 the diketone nitrile derivative of the herbicide isoxaflutole. For all these reasons, no attempts at
18 cloning the enzymes involved in the metabolic pathway of the herbicide will be started.

19
20 **Acknowledgements.** This study was funded by Rhône-Poulenc Agrochimie (Lyon, France).
21 We wish to thank Drs. A. Sailland and J.-C. Thomas for their helpful discussions.

22

1 LITERATURE CITED

2

3 Adam, W.; Bialas, J.; Hadjiarapoglou, L. A convenient preparation of acetone solutions of
4 dimethyldioxirane. *Chem. Ber.*, **1991**, *124*, 2377-2379.

5 Barr, D.P.; Aust, S.D. Mechanisms white rot fungi use to degrade pollutants. *Environ. Sci.*
6 *Technol.*, **1994**, *28*, 78A-87A.

7 Buckland, J.E.; Collins, R.F.; Pullin, E.M. Metabolism of bromoxynil octanoate in growing
8 wheat. *Pestic. Sci.*, **1973**, *4*, 149-162.

9 Chivukula, M.; Renganathan, V. Phenolic azo dye oxidation by laccase from *Pyricularia*
10 *oryzae*. *Appl. Environ. Microbiol.*, **1995**, *61*, 4374-4377.

11 Collins, P.J.; Kotterman, M.J.J.; Field, J.A.; Dobson, A.D.W. Oxidation of anthracene and
12 benzo(a)pyrene by laccases from *Trametes versicolor*. *Appl. Environ. Microbiol.*, **1996**,
13 *62*, 4563-4567.

14 Eggert, C.; Temp, U.; Dean, J.F.D.; Eriksson, K.-E.L. A fungal metabolite mediates
15 degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS*
16 *Letters*, **1996**, *391*, 144-148.

17 Gianfreda, L.; Xu, Feng.; Bollag, J.-M. Laccases: a useful group of oxidoreductive enzymes.
18 *Bioremed. J.*, **1999**, *3*, 1-25.

19 Gramss, G.; Kirsche, B.; Voigt, K.-D.; Gunther, Th.; Fristche, W. Conversion rates of five
20 polycyclic aromatic hydrocarbons in liquid cultures of fifty-eight fungi and the
21 concomitant production of oxidative enzymes. *Mycol. Res.*, **1999**, *103*, 1009-1018.

22 Harper, D.B. Enzymology of C-N cleavage by *Fusarium solani*. *Biochem. J.*, **1977**, *167*, 685-
23 692.

1 Jolivalt, C.; Raynal, A.; Caminade, E., Kokel, B.; Le Goffic, F.; Mougin, C. Transformation
2 of *N,N*-dimethyl-*N*-(hydroxyphenyl)ureas by laccase from the white rot fungus
3 *Trametes versicolor*. *Appl. Microbiol. Biotechnol.*, **1999**, *51*, 676-681.

4 Lesage-Meesen, L.; Delattre, M.; Haon, M.; Thibault, J.F.; Colonna Ceccaldi, B.; Brunerie,
5 P.; Asther, M. A two-step bioconversion process for vanillin production from ferulic
6 acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. *J. Biotechnol.*, **1996**,
7 *50*, 107-113.

8 Luscombe, B.M.; Pallett, K.E.; Loubiere, P.; Millet, J.-C.; Melgarejo, J.; Vrabel, T.E. RPA
9 201772, a novel herbicide for broad leaf and grass weed control in maize and sugarcane.
10 *Proc. Brighton Crop Protect. Conf.-Weeds*, **1995**, *1*, 35-42.

11 Mougin, C.; Laugero, C.; Asther, M.; Dubroca, J.; Frasse, P.; Asther, M. Biotransformation of
12 the herbicide atrazine by the white rot fungus *Phanerochaete chrysosporium*. *Appl.*
13 *Environ. Microbiol.*, **1994**, *60*, 705-708.

14 Mougin, C.; Pericaud, C.; Malosse, M.; Laugero, C.; Asther, M. Biotransformation of the
15 insecticide lindane by the white rot basidiomycete *Phanerochaete chrysosporium*.
16 *Pestic. Sci.*, **1996**, *47*, 51-59.

17 Pallett, K.E.; Little, J.P.; Sheekey, M.; Veerasesaran, P. The mode of action of isoxaflutole. I.
18 Physiological effects, metabolism, and selectivity. *Pestic. Biochem. Physiol.*, **1998**, *62*,
19 113-124.

20 Paszczynski, A.; Huynh, V.B.; Crawford, R. Comparison of ligninase-1 and peroxidase M₂
21 from the white rot fungus *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.*,
22 **1986**, *244*, 705-765.

23 Sakai, K.; Hamada, N.; Watanabe, Y. Degradation mechanism of poly(vinyl alcohol) by
24 successive reactions of secondary alcohol oxidase and β -diketone hydrolase from
25 *Pseudomonas sp.* *Agric. Biol. Chem.*, **1986**, *50*, 989-996.

- 1 Stalker, D.M.; McBride, K.E. Cloning and expression in *Escherichia coli* of a *Klebsiella*
2 *ozaenae* plasmid-borne gene encoding a nitrilase specific for the herbicide bromoxynil.
3 *J. Bacteriol.*, **1987**, *169*, 955-960.
- 4 Tien, M.; Kirk, T.K. Lignin-degrading enzyme from *Phanerochaete chrysosporium*:
5 purification, characterization and catalytic properties of a unique H₂O₂-requiring
6 oxygenase. *Proc. Natl. Acad. Sci. USA*, **1984**, *81*, 2280-2284.
- 7 Viviani, F.; Little, J.P.; Pallett, K.E. The mode of action of isoxaflutole. II. Characterization
8 of the inhibition of carrot 4-hydroxyphenylpyruvate dioxygenase by the diketone nitrile
9 derivative of isoxaflutole. *Pestic. Biochem. Physiol.*, **1998**, *62*, 125-134.
- 10 Wolfenden, B.S.; Wilson, R.L. Radical cations as a reference chromogens in kinetics studies
11 of one electron transfer reactions. *J. Chem. Soc. Perkin Trans. II*, **1982**, 805-812.

12

13

1 **Table 1.** Laccase Activity towards DKN Derivative versus various
 2 Incubation Conditions

incubation conditions		enzymatic activity (nmol h ⁻¹ U ⁻¹)
controls	without Lac	0.05
	without ABTS	0.02
pH	3	0.36
	5	0.08
	7	0.06
temperature (°C)	20	0.16
	25	0.28
	30	0.43
	40	0.45
	50	0.44
	60	0.29
ABTS concentration (mM)	0	0.02
	0.1	0.08
	0.2	0.13
	0.5	0.23
	1.0	0.36
	2.0	0.46
mediator (1.0 mM)	ABTS	0.28
	3-HAA	0.03
	1-HBT	0.04

3 ABTS, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); 3-HAA,
 4 3-hydroxyanthranilic acid; 1-HBT, 1-hydroxybenzotriazole.

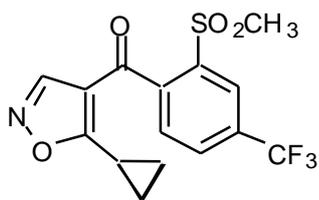
5 Results are expressed as means of triplicates. The standard deviation is less
 6 than 10% of the mean in all cases.

1 **Figure 1.** Structure of isoxaflutole **1**, diketetonitrile (DKN) **2** and benzoic acid (BZA) **3**.

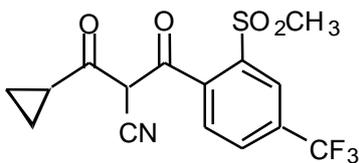
2

3

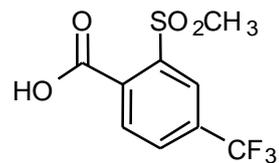
4



1



2



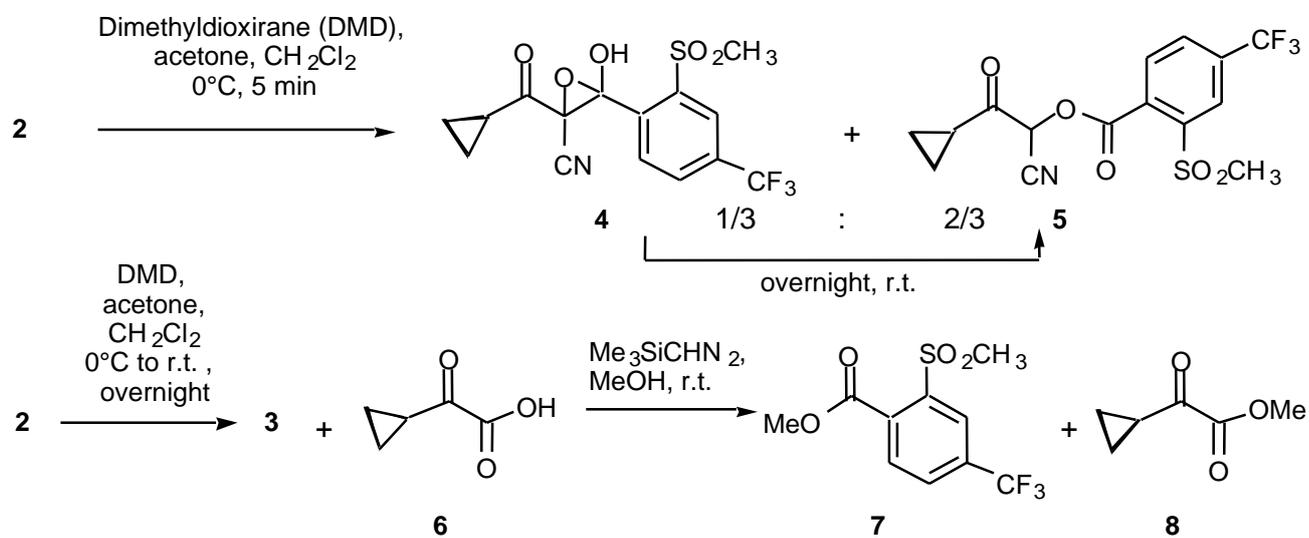
3

5

6

1 **Figure 2.** Hypothetical pathway for DKN chemical cleavage.

2



3

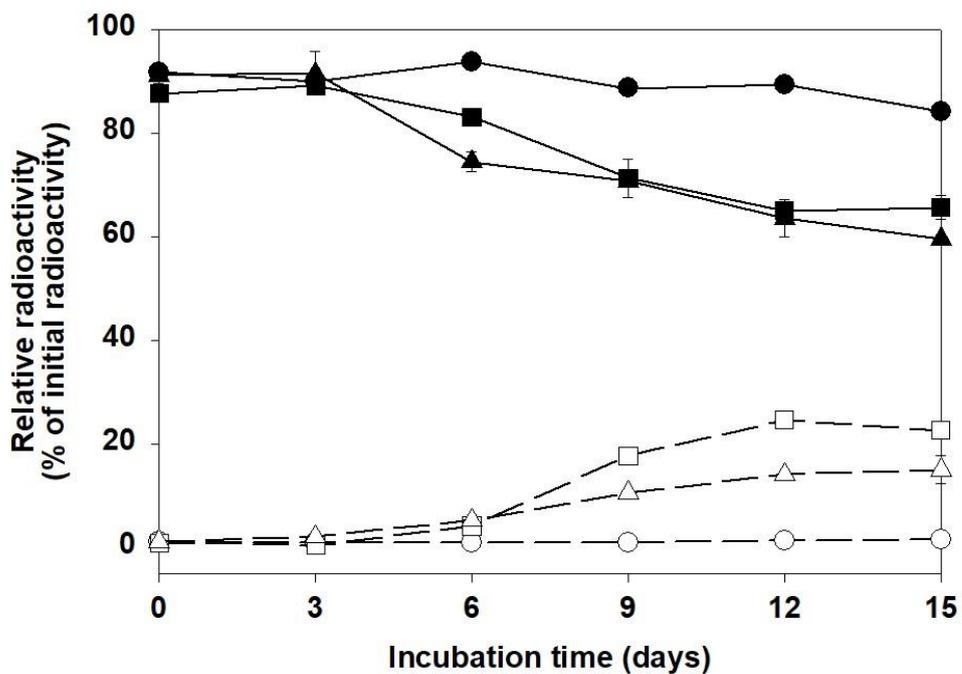
4

5

6

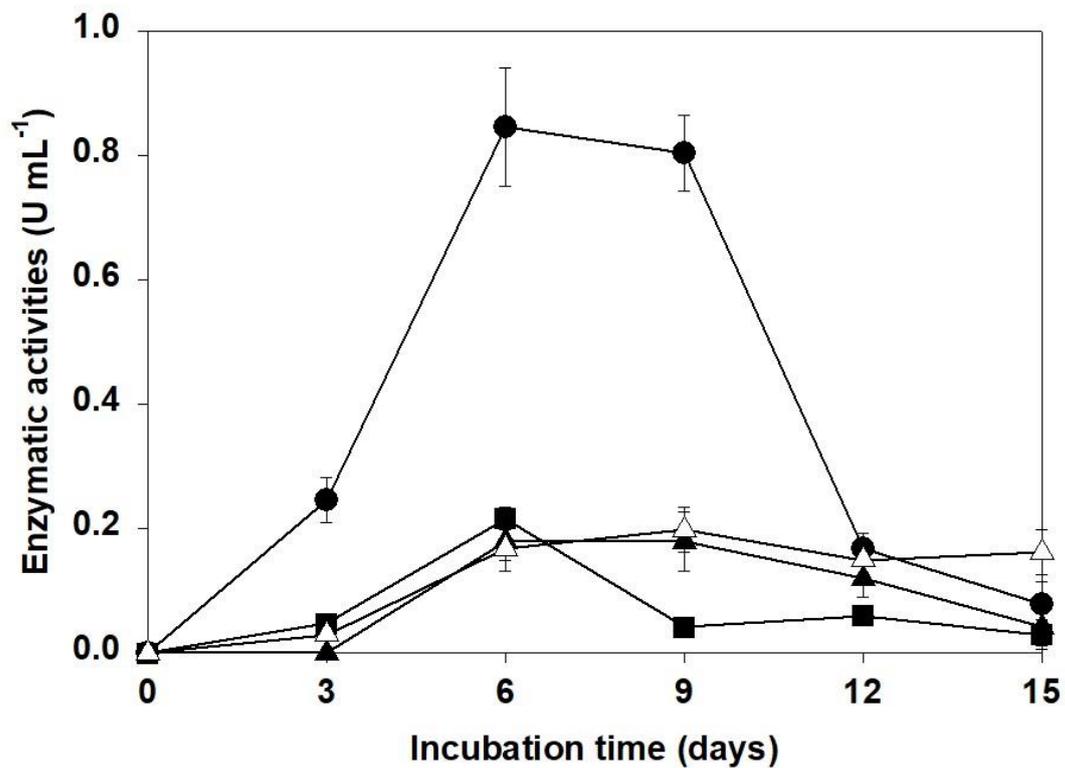
7

1 **Figure 3.** DKN depletion (black symbols) and BZA formation (white symbols) in
2 uninoculated controls (●) and in cultures inoculated with *P. chrysosporium* (■) and *T.*
3 *versicolor* (σ).



4
5
6
7
8

- 1 **Figure 4.** Extracellular enzyme production in cultures of *P. chrysosporium* (●, LiPs; ■, MnPs; σ, Lacs) and *T. versicolor* (Δ, Lacs).
- 2



- 3
- 4
- 5
- 6