Metabolism of 3,4-Dichloroaniline by the Basidiomycete *Filoboletus* Species TA9054

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The basidiomycete *Filoboletus* sp. TA9054 metabolized 3,4-dichloroaniline and formed several condensation products on solid media. In liquid cultures no oligomers were produced. Six metabolites were isolated by extraction, chromatography on silica gel, and preparative HPLC on DIOL or CN phase. They were identified by UV, mass spectroscopy, and ^{1}H nuclear magnetic resonance analysis as 3,3′,4,4′-tetrachloroazobenzene (compound **I**), 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene (**II**), 4-[(3,4-dichloroanilino)-4-(3,4-dichloroanilino)]-3,3′,4′-trichloroazobenzene (**III**), 2-(3,4-dichloroanilino)-*N*-(3,4-dichlorophenyl)-4-(chlorophenylene) (**IV**), 6-(3,4-dichloroaniline)-*N*-(3,4-dichlorophenyl)-4-(chlorophenylene) (**V**), and 3,5-bis(3,4-dichloroanlino)-1,4-chlorobenzoquinone (**VI**).

Keywords: *Basidiomycetes; Filoboletus species; 3,4-dichloroaniline; metabolism*

INTRODUCTION

Halogenated anilines are formed by microbial degradation of widely used acylanilide, phenylurea, and phenylcarbamate herbicides in agricultural soils as well as under laboratory conditions (Bartha, 1968; Bordeleau *et al.*, 1972; Belasco and Pease, 1969; Hughes and Cork, 1974; Chiska and Kearney, 1970). These liberated chloroanilines can be incorporated into biopolymers such as lignin and humic substances [Laurent, 1994; for review see Marco and Novak (1991)]. Several di- and oligomeric condensation products of 3,4-dichloroaniline (3,4-DCA) have been isolated from herbicide-treated soils and microbial cultures containing chlorinated anilines. It has been shown that the dimers 3,3′,4,4′-tetrachloroazobenzene (TCAB) (Bartha and Pramer, 1967; Belasco and Pease, 1969; Kearney and Plimmer, 1972; Sprott and Corke, 1971) and 1,3-bis(3,4-dichlorophenyl)triazene (Plimmer *et al.*, 1970) as well as the trimer 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene (Linke, 1970) are formed in soils.

The white rot fungus *Phanerochaete chrysosporium* was found to mineralize 3,4-DCA as well as lignin-bound residues under nitrogen-limited conditions (Arjmand and Sanderman, 1985, 1986) and to form conjugates with endogenous metabolites (Arjmand and Sanderman, 1987; Hallinger *et al.*, 1988). Lignin peroxidase, isolated from submerged cultures of *P. chrysosporium*, was shown to dimerize 3,4-DCA to form TCAB and *N*-(3′,4′-dichlorophenyl)-*p*-2-chlorobenzoquinone imine (Pieper *et al.*, 1992). Peroxidase L-3 and an aniline oxidase from the soil fungus *Geotrichum candidum* dimerize several halogen and alkylanilines to azo compounds (Bordeleau and Bartha, 1972). 3,3′,4,4′-Tetrachloroazoxybenzene is produced in cultures of *Fusarium oxysporum* (Kaufman *et al.*, 1972).

Since there are only a few investigations on the metabolism of chlorinated anilines by basidiomycetes except for *P. chrysosporium*, we screened wood- and plant-inhabiting basidiomycetes for degradation of 3,4-DCA. *Filoboletus* sp. TA9054 was found to form several yellow and red pigments on solid medium in the presence of 3,4-DCA. *Filoboletus* species are widespread in eastern Asia and Australia, growing as small clusters on wood. Because of the poroid hymenophor, the genus has been placed in the tribe Myceneae family Tricholomaceae, Agaricales, Basidiomycetes. Strain TA9054 produces the antiviral compound filoboletic acid (Simon *et al.*, 1994). This study presents the isolation and identification of six oligomeric metabolites formed by the fungus. Four of the compounds have not been obtained from microbial cultures before.

MATERIALS AND METHODS

Microorganism and Culture Conditions. Fruiting bodies of *Filoboletus* sp. TA 9054 were collected in Queensland, Australia. The mycelial culture was obtained from spore prints. The strain was maintained on YMG agar containing 4 g of yeast extract, 10 g of malt extract, 4 g of glucose and 15 g of agar per liter. The pH was adjusted to 5.5 before sterilization. Subcultures were made every 12 months.

For the production of pigments the fungus was incubated on YMG or MM1 agar, containing 10 g of glucose, 1 g of $(NH_4)_2SO_4$, 0.1 g of $NaCl_2$, 0.1 g of $MgSO_4$, 0.125 g of K_2HPO_4, 0.875 g of KH_2PO_4, and 15 g of agar, for 10 and 15 days, respectively. Agar cylinders (9 mm diameter) of well-grown cultures were cut out, and five plugs were transferred to one Petri dish each, containing MM1 agar and 3,4-DCA (0.8 mM). The cultures were incubated at 25 °C for 14−16 days. Submerged cultures were performed in 500 mL Erlenmeyer flasks containing 100 mL of YMG or MM1 medium, respectively, at 25 °C as agitated cultures at 120 rpm or as static cultures.

Isolation of Metabolites. For the isolation of 3,4-DCA polymers the agar was cut into small pieces and repeatedly extracted with acetone until no coloring of the solvent occurred. The extracts were combined, and the solvent was removed under vacuum. This crude extract was dissolved in toluene and applied onto a silica gel column (300 × 50 mm) with toluene as mobile phase. Depending on the respective compound, final isolation was achieved by preparative HPLC. Compounds **I**, **II**, **IV**, and **VI** were separated by using a LiChrosorb Diol column (Merck, Darmstadt, Germany; column dimensions 250 × 25 mm, particle size 7 μm), while compounds

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Table 1. Characterization of Metabolites of 3,4-Dichloroaniline Identified from Cultures of *Filoboletus* Species TA9054[a]

compd	*m/z* (HRMS) calcd	*m/z* (HRMS) found	formula	color	UV_{max} (nm)	R_f (TLC)	Rt (min) (HPLC)
I	317.9285	317.9285	$^{12}C_{12}{}^{1}H_{6}{}^{14}N_{2}{}^{35}Cl_{4}$	yellow	239, 332	0.87	16.52
II	442.9317	442.9325	$^{12}C_{18}{}^{1}H_{10}{}^{14}N_{3}{}^{35}Cl_{5}$	yellow-orange	272, 302, 406	0.77	17.16
III	567.9350	567.9355	$^{12}C_{24}{}^{1}H_{14}{}^{14}N_{4}{}^{35}Cl_{6}$	orange	299, 418	0.81	17.91
IV	443.9157	443.9129	$^{12}C_{18}{}^{1}H_{9}{}^{16}O^{14}N_{2}{}^{35}Cl_{5}$	red	285, 503	0.51	14.52
V	443.9157	443.9157	$^{12}C_{18}{}^{1}H_{9}{}^{16}O^{14}N_{2}{}^{35}Cl_{5}$	red	275, 497	0.49	13.56
VI	459.9106	459.9116	$^{12}C_{18}{}^{1}H_{9}{}^{16}O_{2}{}^{14}N_{2}{}^{35}Cl_{5}$	yellow	286, 382	0.38	12.51

[a] The R_f value of 3,4-dichloroaniline was 0.25 and retention time on HPLC was 6.7 min.

Table 2. Mass Spectral Data of the Isolated Compounds

metabolite	*m/z* (%)
I	318 (29) [M^+], 281 (3), 225 (4), 202 (5), 173 (40), 145 (100), 125 (9), 109 (17), 97 (16), 85 (18), 85 (6), 63 (5)
II	443 (48) [M^+], 298 (5), 270 (100), 235 (59), 200 (36), 164 (13), 145 (26)
III	570 (100) [$M^+ + 2$], 568 (53) [M+], 411 (7), 395 (41), 360 (8), 325 (8), 289 (10), 235 (15), 200 (12), 161 (6), 145 (18), 95 (7), 57 (8)
IV	446 (100) [$M^+ + 2$], 444 (52) [M+], 409 (19), 374 (44), 339 (14), 301 (8), 276 (7), 250 (10), 210 (8), 196 (17), 187 (15),161 (20), 145 (28), 109 (22), 69 (9), 58 (20), 43 (61)
V	446 (100) [$M^+ + 2$], 444 (52) [M+], 409 (29), 374 (44), 339 (24), 301 (11), 265 (10), 237 (12), 187 (17), 161 (10), 145 (30), 109 (24), 75 (19), 5 (27), 43 (71)
VI	460 (34) [M^+], 425 (31), 399 (6), 390 (6), 362 (5), 300 (12), 212 (100), 145 (35), 109 (25) 75 (19), 63 (10)

III and **V** were isolated by using a LiChrosorb CN column (Merck; column dimensions 250 × 25 mm, particle size 7 μm). The compounds were eluted with a linear gradient of cyclohexane−*tert*-butyl methyl ether (100:0 to 0:100, v/v, in 50 min) and a flow rate of 3 mL/min. The elution of the metabolites was followed at 210 nm.

Chemical Analysis. To analyze the transformation products, high-performance liquid chromatography (HPLC) was carried out on a Hewlett-Packard 1090 liquid chromatograph using a Merck RP18 LiChrosphere column (inner dimensions 125 × 4.6 mm). The HPLC elution profile consisted of a 20 min linear gradient of acetonitrile−water (30:70 to 100:0, v/v), followed by 10 min of 100% acetonitrile elution. The flow rate was 1 mL/min. UV detection operated with dual channel analysis at 210 and 280 nm. UV−visible absorption spectra were recorded with a Hewlett-Packard 1090 HPLC diode array detector.

Thin-layer chromatography (TLC) was performed on silica gel 60 $WF_{254}S$ (Merck), with toluene as mobile phase. Mass spectral analyses were performed with a JEOL SX102 spectrometer. The electron multiplier voltage was 1000 V, and the ionization chamber temperature was 250 °C. The spectra were recorded with electron impact ionization at 70 eV. The ^1H nuclear magnetic resonance (NMR) measurements were performed at 500 MHz in $CDCl_3$ (compounds **I**, **II**, **IV**, **V**, and **VI**) or acetone-d_6 (compound **III**) with a Bruker ARX500 apparatus.

RESULTS AND DISCUSSION

During a screening of 250 basidiomycetes for the degradation and metabolization of 3,4-DCA, 21 strains (8.4 %) belonging the genera *Collybia* (1 strain), *Cyathus* (4), *Filoboletus* (1), *Gloeophyllum* (1), *Marasmius* (3), *Merulius* (1), *Phellinus* (1), *Schizophyllum* (1), *Sterum* (1), and *Stropharia* (1) and 6 unidentified strains were found to be able to metabolize the compound. *Filoboletus* sp. TA9054 produced several yellow and red pigments that were less polar than the original compound under the chosen conditions (Table 1). Growing on mineral salt agar (MM1) with glucose as carbon source, the strain metabolized 3,4-DCA up to a concentration of 1.2 mM. Concentrations higher than 0.2 mM were toxic to the fungus and inhibited growth, but not metabolism of the compound, and 1.3 mM 3,4-DCA in the medium was lethal to the fungus. The transformation of 3,4-DCA started at the fifth day of incubation, indicated by colored zones around the agar plugs. After 15−17 days, 1 mM of 3,4-DCA was completely removed from the agar as shown by HPLC analysis. *Filoboletus* sp. could not use the compound as sole carbon and energy source. On complex agar (YMG), as well as in agitated and static liquid cultures, no disappearance of 3,4-DCA and formation of oligomers occurred. The reasons for this are not known. However, this may be an important aspect of screening of basidiomycetes for biodegradation or metabolism of xenobiotic compounds, since only little is known about environmental factors that influence the metabolism of xenobiotic compounds by basidiomycetes. Similar results were obtained from cultures of the basidiomycetes *Schizophyllum commune*, *Schizophyllum fasciatum*, and *Auriculariopsis ampla* incubated in the presence of 3,4-DCA. Only on solid media is the compound able to induce the formation of indigo in these species (Schwarz *et al.*, 1992). Investigations with *P. chrysosporium* revealed that culture conditions such as shaking (Jäger *et al.*, 1985; Reid *et al.*, 1984), oxygen concentration, and pH (Kirk *et al.*, 1978) may affect lignin and pollutant degradation (Hammel *et al.*, 1992; Leisola and Fiechter, 1985). Furthermore, the formation of ligninolytic enzymes occurred in response to nitrogen (Keyser *et al.*, 1978), carbon, or sulfur deprivation (Jeffries *et al.*, 1981) as well as in the presence of low molecular inducers such as veratryl alcohol and veratric acid (Lundell *et al.*, 1990) or detergents in the medium (Jäger *et al.*, 1985).

In contrast to 3,4-DCA, monochloroanilines, 2,5-, 2,6-, and 3,5-dichloroaniline, 3,4-difluoroaniline, and the herbicides linuron and diuron were not metabolized by the fungus, although no growth inhibition up to 1 mM of the appropriate compound was observed. Furthermore, 2,3,4-, 2,4,5-, and 3,4,5-trichloroaniline were toxic to the fungus at concentrations of 0.1 mM and no metabolite formation was observed.

For the production of the pigments, the cultures were harvested after 15 days of incubation and the compounds were isolated as described under Materials and Methods. The structure determination of the isolated derivatives of 3,4-DCA has been carried out with a combination of high-resolution mass spectrometry (Tables 1 and 2) and ^1H NMR (Table 3) and UV (Table 1) spectroscopy. The chemical structures of the isolated compounds are shown in Figure 1. Because of the limited amounts of the derivatives available, it has not

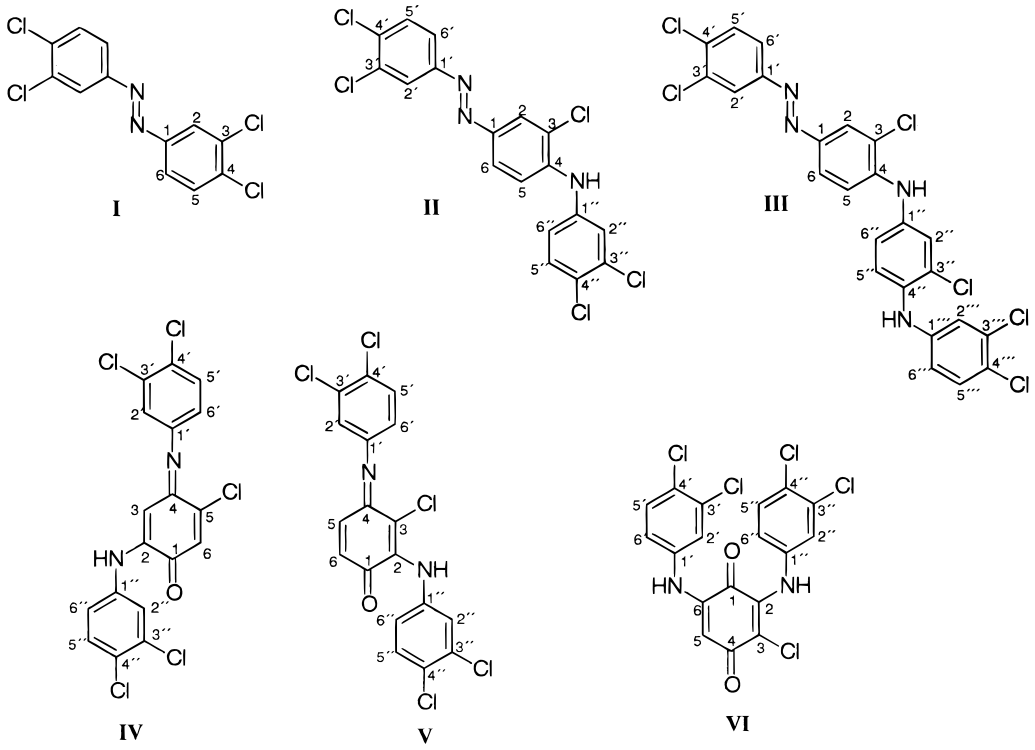

Figure 1. Proposed structures of oligomeric metabolites of 3,4-dichloroaniline, isolated from agar cultures of *Filoboletus* sp. TA9054: **I**, 3,3′,4,4′-tetrachloroazobenzene (TCAB); **II**, 4-(3,4-dichloroanilino)-3,3′,4′-trichloroazobenzene; **III**, 4-[(3,4-dichloroanilino)-4-(3,4-dichloroanilino)]-3,3′,4′-trichloroazobenzene; **IV**, 2-(3,4-dichloroanilino)-*N*-(3,4-dichlorophenyl)-4-(chlorophenylene); **V**, 6-(3,4-dichloroanilino)-*N*-(3,4-dichlorophenyl)-4-(chlorophenylene); **VI**, 3,5-bis(3,4-dichloroanlino)chlorobenzoquinone.

Table 3. ^{1}H (500 MHz) NMR Data (δ; Multiplicity; *J*) for Compounds I−VI, Compounds I, II, IV, V, and VI in $CDCl_3$, and Compound III in CD_3COCD_3 [The Solvent Signals (7.26 and 2.05 ppm) Were Used as Reference, and the Coupling Constants *J* Are Given in Hertz]

	metabolite					
H	**I**	**II**	**III**	**IV**	**V**	**VI**
2	8.02; d; 2.2	8.02;[a] d; 2.1	8.02; d; 2.1			
3				6.27; s		
5	7.62; d; 8.5	7.28; d; 8.8	7.42; d; 8.6		6.62; d; 10.1	6.02; s
6	7.79; dd; 2.2, 8.5	7.81; dd; 2.1, 8.8	7.87; dd; 2.1, 8.6	7.02; s	7.07; d; 10.1	
2′		7.98;[a] d; 2.1	8.02; d; 2.1	7.18;[a] d; 2.5	7.09;[a] d; 2.5	7.38;[a] d; 2.2
5′		7.58; d; 8.5	7.78; d; 8.6	7.38;[b] d; 8.7	7.47;[b] d; 8.5	7.50;[b] d; 8.5
6′		7.74; dd; 2.1, 8.5	7.87; dd; 2.1, 8.6	6.89;[c] dd; 2.5, 8.7	6.73;[c] dd; 2.5, 8.5	7.12;[c] dd; 2.2, 8.5
2″		7.37; d; 2.5	7.51;[a] d; 2.2	7.05;[a] d, 2.3	7.01;[a] d; 2.5	7.21;[a] d; 2.2
5″		7.43; d, 8.6	7.35;[b] dd; 2.2, 8.6	7.48;[b] d; 8.4	7.38;[b] d; 8.7	7.42;[b] d; 8.5
6″		7.09; dd; 2.5, 8.6	7.47;[c] d; 8.6	6.77;[c] dd; 2.3, 8.4	6.84;[c] dd; 2.5, 8.7	6.97;[c] dd; 2.2, 8.5
2‴			7.22;[a] d; 2.2			
5‴			7.05;[b] dd; 2.1, 8.6			
6‴			7.37;[c] d; 8.6			
N′H						8.05;[d] br s
N″H		6.44; br s	6.41;[d,e] br s	7.08; brs	7.00; br s	7.95;[d] br s
N‴H			5.99;[d,e] br s			

[a−d] Interchangeable. [e] Chemical shift in $CDCl_3$.

been possible to record ^{13}C NMR spectra or ^{1}H−^{13}C correlation spectra.

Compound **I** was the major product formed by the fungus and identified as TCAB. The elemental composition is $C_{12}H_6N_2Cl_4$, and since only three signals are visible in the ^{1}H NMR spectrum recorded in $CDCl_3$, the structure must be symmetrical. The two most important fragments in the mass spectrum of TCAB, each containing two chlorines, are *m*/*z* 145 (base peak) and 173. This is in agreement with the proposed structure of *trans*-3,3′,4,4′-tetrachloroazobenzene, which has been reported previously (Linke and Pramer, 1969; Pieper *et al.*, 1992). This compound is a common metabolite of 3,4-DCA, formed in soils as well as under laboratory conditions (Belasco and Pease, 1969; Sprott and Corke, 1970; Chiska and Kearney, 1970; Hughes and Cork, 1974). TCAB can be formed by bacteria containing nitrate reductase (Corke *et al.*, 1979). It is also formed by lignin peroxidase preparations from culture fluids of *P. chrysosporium* (Pieper *et al.*, 1992) and by a peroxidase and an aniline oxidase of *Geotrichum candidum* (Bordeleau and Bartha, 1972).

Compounds **II** and **III** are conjugates of TCAB. Compound **II** is a trimer, with the elemental composition $C_{18}H_{10}N_3Cl_5$. The presence of an azo group is suggested by both the MS and the NMR data, and the MS fragments with *m*/*z* 298 (containing three chlorines), 270 (three chlorines), and 145 (two chlorines) all originate from cleavage on both sides of the azo function. In the ^{1}H NMR spectrum the signals for three 1,3,4-trisubstituted aromatic systems are observed, of which two resemble those found in compound **I** and the third

resembles 3,4-DCA itself. The connection between the N-substituted 3,4-DCA and C-4, and not C-3, is indicated by the influence of the nitrogen on 5-H, which is upshifted compared to 5′-H.

The tetramer **III** has the elemental composition $C_{24}H_{14}N_4Cl_6$. The major MS fragment is obtained after the loss of $C_6H_3N_2Cl_2$ as with compounds **I** and **II**, suggesting the same azo group as in compound **II**. This is supported by the ^{1}H NMR data. In addition, two 1,3,4-trisubstituted aromatic systems and two amino protons can be observed. Due to overlapping with the solvent signal, the ^{1}H NMR spectrum of compound **III** was measured in acetone-d_6, which makes the comparison with the data for the other compounds obtained in $CDCl_3$ somewhat uncertain. However, 5-H is upshifted compared to 5′-H, indicating that N″ is attached to C-4 and not to C-3, and it is reasonable to assume that the aromatic substitution by N‴ has taken place on C″-4 and not on C″-3.

Formation of tri-, tetra-, and pentameric products of 4-chloroaniline was found during incubation with a *Trametes versicolor* laccase (Hoff *et al.*, 1985). At least the trimeric product was coupled by a nonenzymatic reaction of the dimer and 4-chloroaniline. To investigate whether coupling of TCAB with further 3,4-DCA molecules could happen abiotically, TCAB (10 mg/L) was incubated with 0.8 mM 3,4-DCA under the same conditions as described above. After 15 days, no disappearance of TCAB and 3,4-DCA was observed, indicating that both compounds were stable during abiotic incubation and the formation of the tri- and tetrameric compounds **II** and **III**, respectively, must be catalyzed by a fungal enzyme.

In compound **IV** oxygen has been introduced, according to the elemental composition $C_{18}H_9ON_2Cl_5$ suggested by HRMS measurements. In the ^{1}H NMR spectrum, the signals for two 1,3,4-trisubstituted aromatic systems with chemical shifts corresponding to N-substituted 3,4-DCA are observed. In addition, two sharp singlets and a broad singlet (corresponding to a NH proton) are present. The absence of a second NH proton suggests that this nitrogen is part of a benzoquinone imine, which is reasonable in view of the UV absorption of compound **IV**. The lack of a ^{1}H−^{1}H long-range coupling between 3-H and 6-H, as well as their chemical shifts, supports the suggested substitution pattern. The *N*-(3,4-dichlorophenyl)-3-chloro-*p*-benzoquinone imine is the major product of a lignin peroxidase isolated from cultures of *P. chrysosporium* (Pieper *et al.*, 1992). The radicals formed by this peroxidase react to *N*-(3′,4′-dichlorophenyl)-*p*-2-chlorobenzoquinone diimine, followed by spontaneous hydrolysis, releasing the quinone imine.

The spectroscopic data of compounds **IV** and **V** are very similar, suggesting that compound **V** is a benzoquinone imine. The major difference is that the two singlets in the ^{1}H NMR spectrum of **IV** corresponding to 3-H and 6-H in the spectrum of **V** have been replaced by two doublets with a coupling constant of 10.1 Hz. This is in agreement with attachment of 3,4-DCA to the carbon between the carbonyl group and the chlorinated carbon, i.e. C-2 in structure **V** (Figure 1).

HRMS measurements of compound **VI** suggest that its elemental composition is $C_{18}H_9O_2N_2Cl_5$ with 13 unsaturated carbon atoms. The presence of two N-substituted 3,4-dichloroanilines in compound **VI** is suggested by the ^{1}H NMR data, with two 1,3,4-trisubstituted aromatic systems and two amino hydrogens (broad singlets at ∼8 ppm). This leaves C_6HO_2Cl and five unsaturated carbon atoms, which is consistent with a trisubstituted 1,4-benzoquinone, and the substitution pattern is supported by the chemical shift for the ninth proton, a singlet at 6.02 ppm.

In control experiments in the absence of *Filoboletus* sp., 3,4-DCA and 2-chloro-*p*-benzoquinone formed an unknown red compound in medium MM1, after 2 days of incubation, but compound **VI** was not formed under these conditions. Compound **VI** can be formed via two different pathways. *N*-(3,4-Dichlorophenyl)-3-chloro-*p*-benzoquinone imine may be cleaved abiotically to form 2-chloro-1,4-benzoquinone and 3,4-DCA, due to the fact that the benzoquinone imine is relatively unstable as shown by Pieper *et al.* (1992). Condensation with two 3,4-DCA in positions 2 and 6 would result in compound **VI**. The second possibility is a hydrolytic cleavage of compound **V** to form 3,4-DCA and 2-(3,4-dichloroanilino)-3-chlorobenzoquinone and subsequent coupling with 3,4-DCA.

Oligomerization of 3,4-dichloroaniline results in a net dechlorination of the aromatic rings in five of the six identified oligomers. The enzymes responsible for the oligomerization and dechlorination of 3,4-DCA are not known yet, but the diversity of the oligomeric metabolites formed as well as decoloring of the polymeric dye PolyR 478 on agar cultures (data not shown) indicated that it could be a peroxidase or laccase (Gold *et al.*, 1988; Hoff *et al.*, 1985). Further investigations on the nature of the enzyme are in progress.

The structure of *trans*-3,3′,4,4′-tetrachloroazobenzene is similar to that of 2,3,7,8-tetrachlorodibenzo[*p*]dioxin, and it might be expected to have similar toxicological properties (Poland and Knutson, 1982; Pothuluri *et al.*, 1991). The environmental significance of the new compounds presented in this work is still unknown, but the results demonstrate the diversity of oligomeric compounds that can be formed through microbial activities.

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