Identification of the Major Metabolites of Prochloraz in Rainbow Trout by Liquid Chromatography and Tandem Mass Spectrometry

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The metabolic pattern of the imidazole fungicide prochloraz [*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)-ethyl]imidazole-1-carboximide] was investigated in rainbow trout (*Oncorhynchus mykiss*). Following a single oral administration of [^{14}C]prochloraz, levels 4.3 ± 4.1 and 3.9 ± 1.8% of the dose were excreted in the bile after 48 h in male and female animals, respectively. Urinary radioactivity accounted for 1.3 ± 0.4 and 2.4 ± 1.1% of the dose over the same period in males and females. Metabolites from both matrices were separated by reversed-phase HPLC with radioactive detection and analyzed by positive and/or negative electrospray ionization mass spectrometry. No unchanged prochloraz was detected in the analyzed excreta. The major biotransformation products in bile were the aldehyde corresponding to the cleavage of the imidazole ring, N-2-(2,4,6-trichlorophenoxy)-ethylurea, and the glucuronide conjugate of 2,4,6-trichlorophenoxyethanol. In urine, the major metabolite was 2,4,6-trichlorophenoxyacetic acid. On the basis of enzymatic hydrolysis by β-glucuronidase and LC-MS analyses, this study demonstrates that rainbow trout are able to biotransform prochloraz, mainly as glucuronide conjugates.

Keywords: *Metabolism; prochloraz; trout; ESI-MS/MS*

INTRODUCTION

Prochloraz [*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)-ethyl]imidazole-1-carboximide] is a broad-spectrum protectant and eradicant fungicide belonging to the class of imidazoles widely used in agiculture. It is especially active against eyespot, leaf spot, powdery mildew, *Rhynchosporium*, and *Alternaria* and is employed in the treatment of cereal crops, oilseed rape, citrus, beets, and mushrooms. The fungicide properties of prochloraz are due to the binding of the imidazole moiety of the molecule to the iron atom of the cytochrome P450 responsive for lanosterol 14α-demethylation, causing the inhibition of the biosynthesis of ergosterol, an essential component of fungal cell membrane (*1*). However, the interactions of prochloraz with cytochrome P450 and related monooxygenases are not limited to plant pathogens, and several studies have shown that this compound may induce or inhibit various cytochrome P450-dependent enzyme activities in several vertebrate species, including fish (*2*−*9*).

Prochloraz undergoes different transformations. In plants, the primary metabolic step is a breaking of the imidazole ring with the formation of *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]-*N'*-formylurea and *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]urea, which are then degraded to 2,4,6-trichlorophenol, present as free and conjugated metabolites, together with traces of 2,4,6-trichlorophenoxyacetic acid (*10*). In rat, the major metabolites result from the opening of the imidazole ring to form *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)-ethyl]urea, which is then further metabolized to 2,4,6-trichlorophenoxyethanol and the corresponding acid (*11*, *12*). However, the biotransformation pathways of prochloraz in fish are still unknown. The aim of this work was to investigate the metabolic pathways of [^{14}C]prochloraz administered orally to rainbow trout (*Oncorhynchus mykiss*). A radio-HPLC method was developed for the analysis of metabolites present in urine and bile, which were further identified using ESI-MS^n experiments.

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MATERIALS AND METHODS

Chemicals. Prochloraz was a gift from Agrevo (Cambridge, U.K.) and was 99% pure. [*benzene*-^{14}C]Prochloraz was purchased from Isotopchim (Ganagobie, France). This radiochemical had a specific activity of 2886 MBq/mmol, and the purity was at least 98% as indicated by radio-TLC and radio-HPLC analyses. Unless otherwise indicated, all other chemicals and reagents were from Sigma Chimie (Saint Quentin Fallavier, France). Solvents used for extraction and HPLC analyses were of the highest commercial grade available from Prolabo (Fontenay sous Bois, France), Scharlau Chemie S.A. (Barcelona, Spain), or Merck (Nogent-sur-Marne, France). Ultrapure water from a Milli-Q system (Millipore, Saint Quentin en Yvelines, France) was used for HPLC eluent preparation.

Animals. Six (three males and three females) rainbow trout (*O. mykiss*) weighing 300−350 g were held in the laboratory for at least 48 h before use and were not fed during the experiment. Fish were surgically prepared for urine collection as previously described (*13*). After recovery (24 h), animals were force-fed a gelatin capsule containing 1.5 g of commercial feed and 2 mg (0.35 MBq) of [^{14}C]prochloraz, and each fish was placed in a 5-L aquarium supplied with running dechlorinated and well-oxygenated fresh water at 15 ± 1 °C. Urine was collected during 48 h, and then fish were sacrificed and the gall bladder was excised. The total volumes of urine and bile were determined, and an aliquot was removed for radioactivity measurement. The remaining volumes were stored at −18 °C until analysis.

Sample Processing. Aliquots from each sample were evaporated to dryness with a Speed-Vac concentrator (Savant Instruments Inc., Farmingdale, NY) and then dissolved in acetonitrile/water (25:75, v/v) before HPLC analysis for metabolite profiling. Identification of metabolites was performed on pooled urine or bile samples.

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Table 1. Relative Amounts of [^{14}C]Prochloraz-Derived Material Excreted in the Bile of Rainbow Trout 48 h after a Single Oral Dose (2 mg)[a]

peak[b]	t_R (min)	males	females
B1	3.3	0.30 ± 0.12	0.17 ± 0.03
B2	7.6	0.74 ± 0.68	0.32 ± 0.02
B3	10.0	0.92 ± 0.42	0.87 ± 0.24
B4	16.0	0.93 ± 0.89	0.84 ± 0.28
B5	19.3	0.25 ± 0.24	0.40 ± 0.14
B6	26.3	0.92 ± 0.59	1.10 ± 0.23
total[c]		4.3 ± 4.1	3.9 ± 1.8

[a] Values are expressed as percent dose and are means ± SD from three animals. [b] Peaks are assigned as indicated in Figure 1a. [c] Corresponds to the total amount of radioactivity excreted in bile.

Radioactivity Counting. Aliquots of urine and bile were counted directly in a Packard Tricarb scintillation counter with Ultimagold (Packard, Downers Grove, IL) as the scintillation cocktail.

HPLC. HPLC analyses were carried out on a Hewlett-Packard HP1100 system (Hewlett-Packard, Les Ulis, France) connected to a Radiomatic Flo-one/β A500 radioactivity detector (Radiomatic, La-Queue-Les-Yvelines, France) using the Flow-Scint II scintillation cocktail (Packard Instruments Co., Downers Grove, IL) and a Gilson 202 fraction collector (Gilson, Villiers-Le-Bel, France) for the metabolite purification. The HPLC column used was an ODS2 Spherisorb column (250 × 4 mm, 5 μm) from Colochrom (Gagny, France), and the following gradient elution was used: from 92% A and 8% B to 70% B (30 min) and then 100% B (35 min), with A = acetonitrile/water/acetic acid (20:80:0.1, v/v/v) and B = acetonitrile/water/acetic acid (90:10:0.1, v/v/v). The flow rate was 1 mL/min. The abundance of metabolites in radio-HPLC chromatograms was determined as a percentage of the total of all peaks with an area >100 dpm.

Enzymatic Hydrolyses. To determine which metabolite peaks represented conjugates in HPLC chromatograms, samples were treated by β-glucuronidase from *Escherichia coli* (type VII, Sigma Chimie, St Quentin Fallavier, France) and sulfatase from *Aerobacter aerogenes* (type VI, Sigma Chimie). Hydrolysis conditions were as indicated by the supplier, except that 20 μL of 10% D-saccharic acid-1,4-lactone was added during incubations with sulfatase to inhibit possible β-glucuronidase activity.

Mass Spectrometry. Metabolite identification was carried out on a Finnigan LCQ ion trap mass spectrometer (Finnigan, Les Ulis, France) using electrospray as the ionization technique. Collected metabolites were dissolved in a methanol/water (50:50, v/v) mixture to obtain 5 ng/μL solutions. These solutions were infused at a flow rate of 3 μL/min into the ionization source. Both negative and positive ionization modes were employed depending on the metabolite to be analyzed. Typical ESI needle voltages used were ±5 kV depending on the ionization mode employed. The heated transfer capillary was set to 200 °C. All spectra were acquired in the normal scan mode (unit resolution) under automatic gain control conditions. MS^n analyses were performed using helium as collision gas with collision energies optimized for each compound to gain maximal structural information.

RESULTS

Biliary and Urinary Disposition. The biliary excretion of radioactivity amounted to 4.3 ± 4.1 and 3.9 ± 1.8% of the dose 48 h after [^{14}C]prochloraz administration to male and female rainbow trout, respectively (Table 1). The total cumulative urinary excretion of radiolabeled compounds after [^{14}C]prochloraz dosing accounted for 1.3 ± 0.4% of the dose in males, whereas the recovery in females was 2.4 ± 1.1% (Table 2).

Metabolic profiles obtained from the radio-HPLC analysis of bile and urine are displayed in panels a and b, respectively, of Figure 1 (metabolites are numbered in the order of their elution). Unchanged prochloraz is totally absent in bile as well as in urine (data not shown).

Table 2. Relative Amounts of [^{14}C]Prochloraz-Derived Material Excreted in the Urine of Rainbow Trout after a Single Oral Dose (2 mg)[a]

		males		females	
peak[b]	t_R (min)	0–24 h	24–48 h	0–24 h	24–48 h
U1	3.3	0.20 ± 0.18	0.11 ± 0.07	0.12 ± 0.08	0.03 ± 0.04
U2	5.0	0.06 ± 0.03	0.22 ± 0.01	0.14 ± 0.07	0.25 ± 0.12
U3	17.0	0.05 ± 0.02	0.38 ± 0.08	0.54 ± 0.41	1.14 ± 0.36
total[c]		1.3 ± 0.4		2.4 ± 1.1	

[a] Values are expressed as percent dose and are means ± SD from three animals. [b] Peaks are assigned as indicated in Figure 1b. [c] Corresponds to the total amount of radioactivity excreted in urine in 48h.

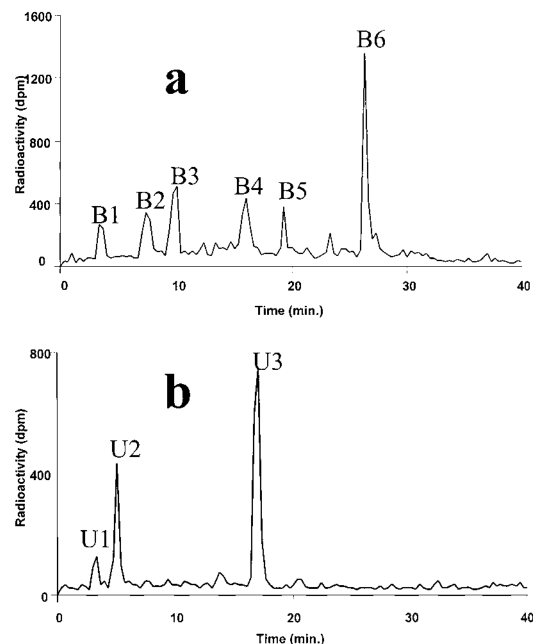

Figure 1. Representative radio-HPLC chromatograms obtained from (a) the bile and (b) the urine of [^{14}C]prochloraz-treated trout (0–40 min portion of the 60 min run).

Metabolite Identification. *Biliary Metabolites.* Metabolites B1 and B2 could not be successfully analyzed by mass spectrometry due to abundant contaminants (endogenous compounds, salts, etc.) coeluting with these very polar metabolites during the various purification steps, which very likely hindered the ionization process. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were assessed under both positive and negative ionization conditions, but no clear signal could be obtained whatever system was used. Due to the low amounts of biological material available, no further attempts could be undertaken for improving the sample preparation before the mass spectrometric analysis. To evidence a possible conjugation of these very polar metabolites, bile has been submitted to β-glucuronidase and sulfatase incubations and subsequent HPLC analyses. The biliary metabolic profile was not affected by the sulfatase treatment, indicating that no sulfate conjugate was present in the bile. On the other hand, on the HPLC profile obtained after β-glucuronidase treatment, metabolites B1, B2, and B3 completely disappeared, yielding several unresolved peaks eluted between 20 and 25 min (data not shown),

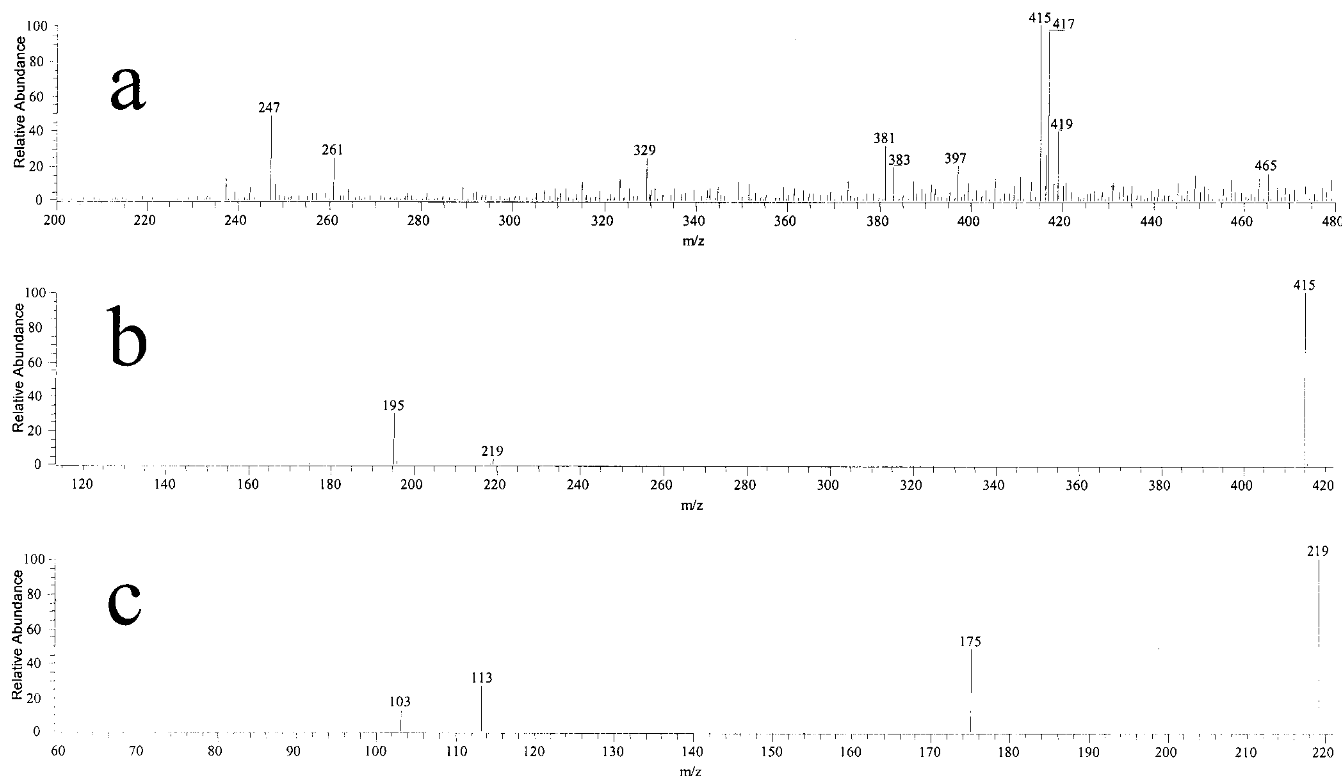

Figure 2. Mass spectrometric analysis of metabolite B3: (a) negative ESI-MS mass spectrum; (b) product ion MS/MS spectrum of ESI-produced [M − H]⁻ ion; (c) product ion MS/MS/MS spectrum obtained from the *m/z* 219 ion produced from the decomposition of the [M − H]⁻ ion.

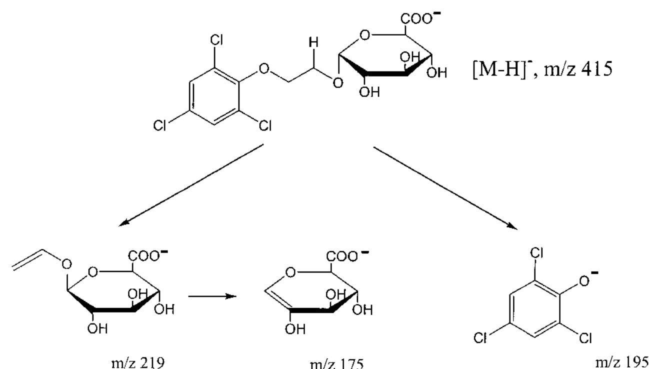

Figure 3. Proposed mechanism for the decomposition of the *m/z* 415 [M − H]⁻ ion of metabolite B3.

indicating that these polar peaks corresponded to mixtures of unresolved glucuronide conjugates eluted very early in the metabolic profile. However, due to the very limited amounts available, we failed in our attempts to identify the corresponding aglycons.

The acidic properties of metabolite B3 guided its MS analysis toward the use of negative ESI. The ESI-MS spectrum of metabolite B3 is presented in Figure 2a. The characteristic isotopic pattern of a triply chlorinated [M − H]⁻ species was observed at *m/z* 415/417/419 (Figure 2a). The MS/MS spectrum obtained from the isolation and collisional activation of the *m/z* 415 ion into the ion trap device is presented in Figure 2b. This spectrum exhibited the characteristic pair of complementary daughter ions at *m/z* 219 and 195 (Figure 3). The latter corresponded to the trichlorophenoxy anion, as it could be confirmed by the occurrence of an *m/z* 197 daughter ion generated by decomposition of the isotopic *m/z* 417 parent ion (indicating that it corresponded to the chlorinated moiety of the molecule), whereas the *m/z* 219 complementary daughter ion remained unchanged. The MS to the third experiment carried out from the *m/z* 219 daughter ion (Figure 2c) led to the decomposition of this ion into the *m/z* 175 (Figure 3) and *m/z* 113 fragment ions, which can be considered as characteristic of the presence of a glucuronic acid moiety in the negative ion tandem mass spectra of xenobiotic metabolites (*14*, *15*). From these results, metabolite B3 could be identified as the glucuronide conjugate of trichlorophenoxyethanol.

Metabolite B4 was analyzed by ESI in the positive mode. The MH^+ ion was located at *m/z* 283 and also exhibited the characteristic isotopic pattern corresponding to the presence of three chlorine atoms in the molecule. Submitted to collisional activation, the *m/z* 283 ion decomposed into fragment ions at *m/z* 265, 240, and 87. The observed decompositions could be considered as characteristic of *N*-2-(2,4,6-trichlorophenoxy)-ethylurea and could be interpreted as follows: (i) the initial protonation of the enolized urea carbonyl function followed by a hydrogen rearrangement from one urea amino function to the oxygen atom led to the $[MH - H_2O]^+$ ion at *m/z* 265; (ii) the scission of the amide bond between the carbonyl and the secondary amine function of the molecule, accompanied by a hydrogen transfer from the primary amine function to the secondary one, gave rise to the *m/z* 240 daughter ion; and (iii) the formation of the *m/z* 87 fragment ion corresponded to the loss of the trichlorophenol neutral moiety from the MH^+ ion. Accordingly, metabolite B4 was identified as *N*-2-(2,4,6-trichlorophenoxy)ethylurea.

Under negative ESI conditions, metabolite B5 gave a trichlorinated [M − H]⁻ ion at *m/z* 309. The MS/MS spectrum of this parent ion showed only one decomposition route, leading to the *m/z* 195 ion. The identity of the *m/z* 195 daughter ion was established as the

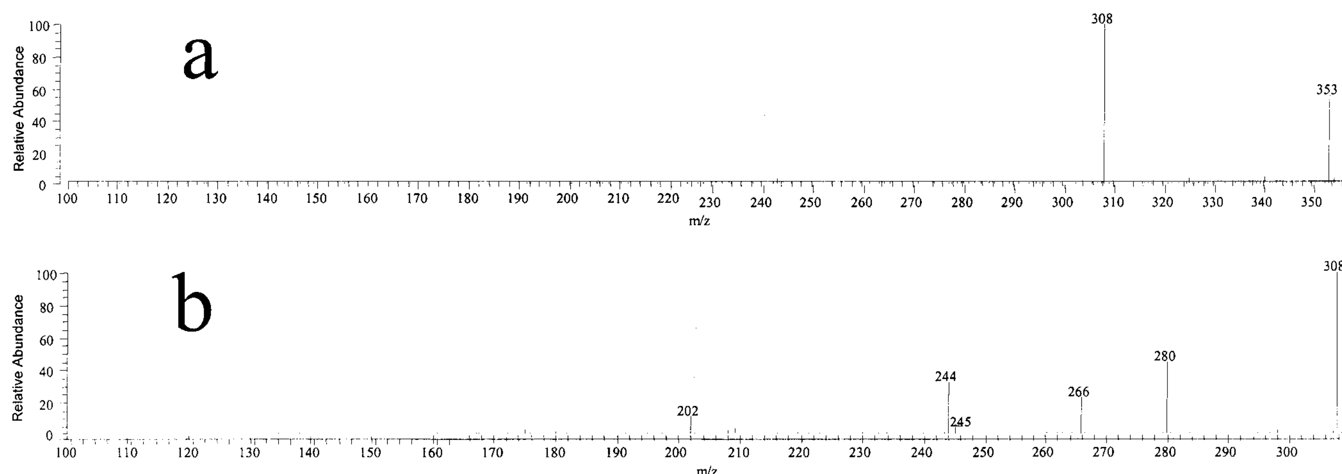

Figure 4. Tandem mass spectrometric analysis of metabolite B6: (a) product ion MS/MS spectrum of ESI-produced MH^+ ion; (b) product ion MS/MS/MS spectrum obtained from the *m*/*z* 308 ion produced from the decomposition of the MH^+ ion.

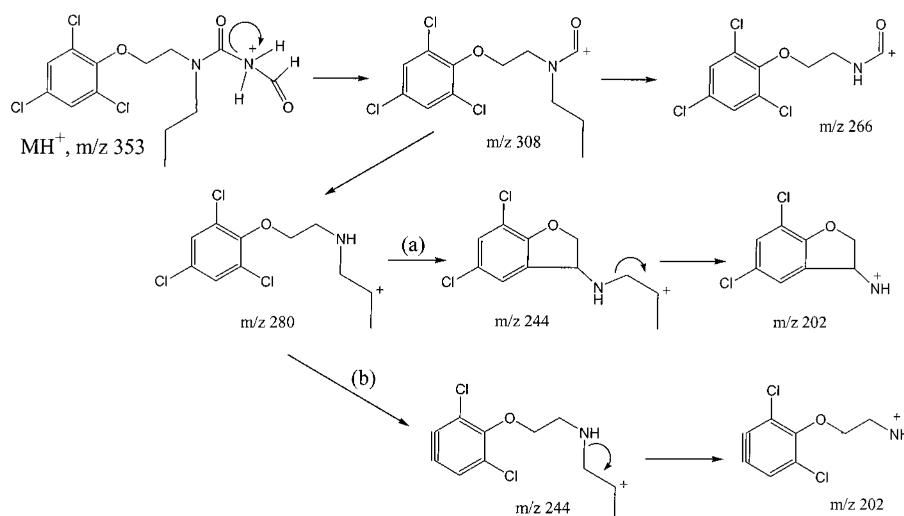

Figure 5. Proposed decomposition pathways of the *m*/*z* 353 MH^+ ion of metabolite B6 submitted to sequential MS^n experiments into the trap.

trichlorophenoxy anion, in the same manner as for metabolite B3. From this result, *N*-2-(2,4,6-trichlorophenoxy)ethyl-*N*′-formylurea could be proposed as the structure of metabolite B5.

Metabolite B6 was investigated using positive ESI and exhibited an MH^+ ion at *m*/*z* 353. Here again, the isotopic pattern of the MH^+ ion showed the presence of three chlorine atoms in the molecule. This *m*/*z* ratio was consistent with a structure arising from the opening of the imidazole ring followed by an oxidation step leading to the formation of an aldehyde function. The collisionally activated dissociation (CAD) spectrum acquired from the isolated *m*/*z* 353 parent species displayed a daughter ion at *m*/*z* 308 (Figure 4a). The formation of this fragment ion corresponded to the scission of the amide bond resulting in the loss of the NH_2COH neutral species as proposed in Figure 5. More structural information could be obtained by isolation and subsequent resonant excitation of the *m*/*z* 308 fragment ion into the ion trap. The resulting MS^3 product ion spectrum is reported in Figure 4b, and a decomposition pathway of this ion is proposed in Figure 5. As a matter of fact, from the selected *m*/*z* 308 daughter ion, two competitive decomposition processes could occur, that is, the loss of neutral CO or propene species, which led to the formation of the *m*/*z* 280 or 266 fragment ions, respectively. The *m*/*z* 266 fragment ion did not undergo further decomposition at this MS stage, whereas some consecutive fragmentation processes were observed from the *m*/*z* 280 ion. A loss of HCl from the *m*/*z* 280 ion explained the occurrence of the *m*/*z* 244 ion in the MS^3 CAD mass spectrum. This elimination can be interpreted by considering two different mechanisms as indicated in Figure 5. The first process may concern the elimination of HCl involving the ortho chlorine atom and a hydrogen atom from the ethyl moiety of the ion, accompanied by a ring closure (route a, Figure 5), whereas a second route can be considered by direct elimination of HCl on the aromatic ring (route b, Figure 5), as previously described from various chlorinated aromatic compounds (*16*–*19*). Finally, a consecutive loss of propene can lead to the *m*/*z* 202 ion. On the basis of these data, metabolite B6 could be identified as *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]-*N*′-formylurea.

Urinary Metabolites. As in the case of the biliary metabolites, U1 and U2 (Figure 1b) could not be analyzed by mass spectrometry owing to their highly hydrophilic properties, which prevented an efficient purification. Trials have been carried out using the same approach as for biliary polar metabolites, but no chlorinated species could be detected for those metabolites. Thus, only the major urinary metabolite U3 could be

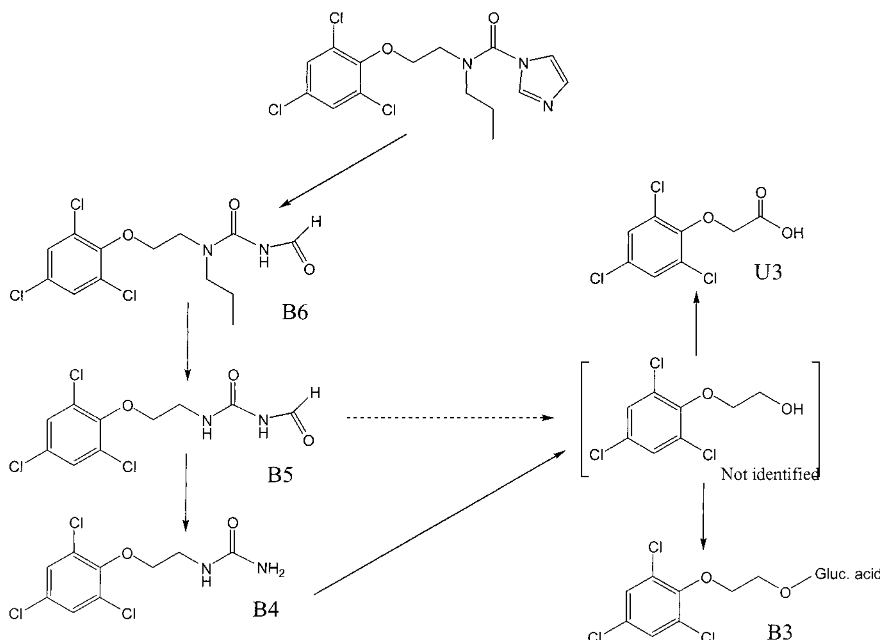

Figure 6. Proposed metabolic pathways of prochloraz in trout.

investigated using ESI-MS. The negative ion mass spectrum of this metabolite exhibited the $[M - H]^-$ ion at *m/z* 253. Submitted to resonant excitation processes, this ion decomposed into the characteristic trichlorophenoxy *m/z* 195 ion, indicating that U3 should correspond to the 2,4,6-trichlorophenoxyacetic acid. This was confirmed by the analysis of a reference standard compound, which presented the same decomposition mass spectrum as well as the same chromatographic retention time as metabolite U3.

Similarly to the bile, U1 and U2 were hydrolyzed by β-glucuronidase treatment of the urine, whereas metabolite U3 remained unchanged. However, the hydrolysis products remained unidentified due to the low quantities collected (data not shown). As in the case of bile, sulfatase treatment of urine did not affect the urinary metabolic profile.

DISCUSSION

Previous studies have shown that prochloraz was extensively metabolized by vertebrates. In rats exposed to labeled prochloraz, 41−68% of the dose was eliminated in urine after 96 h (*11*, *12*). The minor importance of urinary excretion in trout may be related to a low digestive absorption of this fungicide in fish and/or to the preferential elimination of metabolites across the gill. This excretion pathway was demonstrated to be efficient in fish for lipophilic compounds (*20*, *21*). The identification of prochloraz metabolites was first investigated in rats by Needham and Challis (*11*), but by indirect methods mainly based on β-glucuronidase/sulfatase treatment and subsequent GC-MS analysis of methyl ester or TMS derivatives (*11*, *12*). The parent compound was found to be extensively biotransformed initially by opening of the imidazole ring, yielding the aldehyde product followed by hydrolysis to the corresponding urea, the latter being hydrolyzed to an acid (2,4,6-trichlorophenoxyacetic acid), an alcohol (2-[2,4,6-trichlorophenoxy]ethanol), or a phenol (2,4,6-trichlorophenol). The alcohol and the phenol metabolites were present almost exclusively in the form of conjugates.

In this study we report for the first time the metabolism of an imidazole-based fungicide in fish. To this end, we have investigated the metabolites excreted in bile and urine of trout exposed to prochloraz. The low amount of radioactivity excreted by these routes during 48 h may be explained by the high residue levels recently observed in carcasses and viscera (*22*). The use of ESI associated with sequential ion trap MS^n experiments allowed the identification of most of the prochloraz metabolites (Figure 6). The screening and structural identification of the various metabolites studied in this work using automated processes such as data-dependent scanning as described for several other molecules (*23*−*26*) were rather difficult in this case. As a matter of fact, the properties of the metabolites were very different and forced us to use mass spectrometric conditions very different from those used for previously purified compounds. For example, metabolites B3 and B5 could be identified using negative ionization conditions, leading in particlar to the characteristic trichlorophenoxy daughter ion (*m/z* 195) when submitted to collisional activation. This fragment ion could be considered as diagnostic for the screening of prochloraz metabolites using LC coupled to negative ESI. Unfortunately, metabolites B4 and B6 gave no response under negative ion conditions, and only the characteristic isotopic pattern of the trichlorinated prochloraz allowed us to securely locate the metabolites as prochloraz-related species by scrutinizing the positive and/or negative ESI-MS spectra.

As reported for rat, the initial stage in the metabolism of prochloraz in rainbow trout was the cleavage of the imidazole ring, yielding the related aldehyde product followed by hydrolysis to the corresponding urea. The major metabolite identified in the urine was 2,4,6-trichlorophenoxyacetic acid. Nevertheless, several end products present in rat such as trichlorophenol and corresponding conjugates (*11*), or ring-hydroxylated metabolites (*11*, *12*), were not found in trout, suggesting that prochloraz metabolic pathways in fish could be limited in comparison with those in mammals. However, due to contamination problems, the most polar metabo-

lites could not be identified either in the bile or in the urine of the [^{14}C]prochloraz-treated trout, despite several attempts for further purification. Bile is known to constitute a very difficult matrix for mass spectrometric analyses, and several ion suppression phenomena have already been reported in similar situations (*23*). Nevertheless, the fact that these metabolites were hydrolyzed by β-glucuronidase but not by sulfatase suggests that B1, B2, U1, and U2 could correspond to glucuronides.

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