XENOBIOTICA , 1998, VOL. 28, NO. 8, 745–757

Characterization of biliary metabolites of 4-*n*-nonylphenol in rainbow trout (*Oncorhynchus mykiss*)

R. THIBAUT, L. DEBRAUWER, D. RAO and J.-P. CRAVEDI*
Laboratoire des xénobiotiques, INRA, BP3, 31931 Toulouse Cedex 9, France

Received 13 January 1998

1. [R-2,6-^{3}H]-4-*n*-nonylphenol was synthesized and a single dose (5 mg, 1850 KBq) orally administered to rainbow trout. After 48 h, the radioactivity present in the bile amounted 5.5%. More than ten biliary metabolites were separated by hplc and collected for subsequent mass spectrometry analysis. The metabolic profile was totally modified by β-glucuronidase hydrolysis, showing that most of the metabolites were glucuronic acid conjugates.

2. Conjugated metabolites were identified by lc-ms analysis and their aglycones were analysed by gc-ms analysis as TMS and acetyl derivatives.

3. The major metabolite accounted for 52± 11% of the biliary radioactivity and was identified as nonylphenol-glucuronide.

4. Nonylphenol was hydroxylated at both ω and ω-1 positions of the alkyl chain, giving 9-hydroxynonylphenol and 8-hydroxynonylphenol.

5. 9-Hydroxynonylphenol was oxidized to the corresponding acid, and subsequently β-oxidized, yielding 7-(4-hydroxyphenyl)heptanoic acid, 5-(4-hydroxyphenyl)pentanoic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(4-hydroxyphenyl)-2-propenoic acid.

Introduction

Alkylphenols are compounds widely used as plastic additives and for manufacture of surfactants. Significant amounts of these chemicals have been found in the aquatic environment especially in sediments in which concentrations as high as 3 ppm of nonylphenol (NP) have been reported (Naylor *et al*. 1992). NP is the predominant component of alkylphenolic chemicals in the environment, and Giger *et al*. (1984) reported that concentrations of 4-NP in digested sewage sludge may be $>$ 2500 ppm whereas the level of octylphenol and decylphenols were substantially lower. Although some of the NP present in the environment is known to be released from plastic material, the main origin for this contaminant is from the microbial breakdown of nonylphenol polyethoxylates, a large group of nonionic surfactants (Ahel *et al*. 1994).

Because of its lipophilic characteristic, NP shows a considerable potential to bioaccumulate in freshwater organisms. Its estimated bioconcentration factor in the macrophyte algae *Cladophora glomerata* reached up to 10000 (Ahel *et al*. 1993) whereas the bioconcentration factor in fish ranged from 13 to 1300 (McLeese *et al*. 1981, Ekelund *et al*. 1990, Ahel *et al*. 1993). These high values could be due to the fact that some aquatic species may have some difficulties in metabolizing these compounds.

The oestrogenic properties of NP were demonstrated by Soto *et al*. (1991) on the basis of *in vitro* experiments with MCF_7 breast tumour cells and *in vivo* studies in the

* Author for correspondence.

0049–8254/98 $12.00

ovariectomized rat. NP induced both cell proliferation and the progesterone receptor in human MCF_7 cells and also triggered mitotic activity in rat endometrium, and the uterotrophic effect was confirmed by Lee and Lee (1996) in the immature female rat. In fish, induction of vitellogenin synthesis by NP was reported both *in vitro* and *in vivo* (Jobling and Sumpter 1993, Flouriot *et al.* 1995, Lech *et al.* 1996). Recently Jobling *et al.* (1996) reported that exposure of the male rainbow trout to NP caused inhibition of testicular growth. To understand the effects of NP on fish and the possible consequences for human consumers, it is essential to elucidate fully the metabolic fate of this compound. Only few data concerning the metabolism and disposition of NP in fish are available. Lewis and Lech (1996) studied the uptake and disposition of NP in trout in waterborne exposure experiments. They found in trout exposed for 14 h to 36 ppb ^{14}C-NP that radioactivity was mainly concentrated in the bile, liver and kidney, whereas a lower level was observed in muscle. Meldalh *et al.* (1996) demonstrated with a mixture of three isomers of NP, namely 2-, 3- and 4-(4-hydroxyphenyl)-nonane that NP was biotransformed in trout via ω-1 hydroxylation and conjugation with glucuronic acid.

We have previously studied the disposition and excretion route of [^{3}H]-4-*n*-NP in rainbow trout (Thibaut *et al.* in press). Forty-eight hours after a single *per os* administration of this labelled chemical, the highest radioactivity concentration was found in bile; however, the metabolites were not precisely identified. In the present study we investigated the *in vivo* biotransformation pathways of 4-*n*-NP in rainbow trout by determining the chemical structure of metabolites excreted in bile.

Materials and methods

Chemicals and biochemicals

3-(4-Hydroxyphenyl)-propionic acid was purchased from Fluka (Buchs, Switzerland), β-glucuronidase from bovine liver (sulphatase-free, type B-1) and MS 222 (tricaine methanesulphonate) were from Sigma (Saint Quentin Fallavier, France).

*Chemical synthesis of 4-*n*-NP and [R-2,6-^{3}H]-4-*n*-NP*

Technical NP and NP residues from the environment are isomeric compounds differing in the position and the structure of the alkyl side chain. The metabolic pattern from such a mixture is generally complex, resulting in complex data. To simplify the analysis of metabolic products, tritiated 4-*n*-NP was synthesized and used in the current study to investigate the metabolism of NP by rainbow trout.

4-*n*-NP was synthesized according to the procedure described by Corti *et al.* (1995) and characterized by gc-ms analysis. Gc showed only one peak and stated the chemical purity of 4-*n*-NP. 2,6-Dibromononylphenol was synthesized as follows: a mixture of 1 g 4-*n*-NP and 400 μl Br_2 in 7 ml acetic acid was stirred at room temperature for 24 h and the reaction mixture evaporated to dryness. The residue was chromatographed on tlc using a solvent consisting of a mixture of toluene/ethyl acetate (95:5 v/v). One spot was observed at $R_f = 0.87$ corresponding to a product less polar than NP. This product was analysed by gc-ms. The EI-mass spectrum gave a molecular ion at m/z 376 exhibiting an isotopic pattern characteristic of a dibrominated compound. Diagnostic fragment ions were observed at m/z 263 $[M\text{-}C_8H_{17}]^+$ and m/z 185 $[M\text{-}C_8H_{16}Br]^+$. Based on these data the product was identified as 2,6-dibromononylphenol; 1.2 g 2,6-dibromononylphenol was obtained under these conditions.

[R-2,6-^{3}H]-4-*n*-NP was synthesized by Isotopchim (Ganagobie-Peyruis, France) by debromination of 2,6-dibromononylphenol with tritium gas catalysed by Palladium on activated carbon (Pd 10%, Aldrich, St Quentin Fallavier, France). The labelled compound showed the same chromatographic (tlc, hplc) behaviour as standard 4-*n*-NP (data not shown). Its radiochemical purity, as determined by hplc, was > 98% and its specific activity was 1720 GBq/mmol.

Biochemical synthesis of standards

NP-glucuronide was biochemically synthesized as follows: 3 mg microsomal protein prepared from guinea pig liver (phenobarbital-induced) were incubated with 250 nmol [R-2,6-^{3}H]-4-*n*-NP (370 KBq), 2.5 μmol UDPGA, 200 μl Tris-maleate buffer (0.5 M, pH 7.4, $MgCl_2$ 10 mM), at 37 °C for 2 h. The

incubates were stored at − 25 °C. NP and NP-glucuronide were separated by hplc and the identity of NP-glucuronide was confirmed by ms analysis (ESI-ms).

Animals and treatment

Immature male rainbow trout (*Oncorhynchus mykiss*, 180–200 g) were obtained from the Institut National de la Recherche Agronomique pisciculture at Donzacq (Landes, France). Before the experiment fish were held in dechlorinated tap water (14± 1 °C) and the photoperiod was 12-h light (06:00–18:00 h) and 12-h dark. The fish were fed once daily *ad libitum* with a commercial trout feed (Trouvit, France). Rainbow trout were starved 48 h before experiment. Three trout were anaesthetized with 100 mg/l MS 222 (tricaine methanesulphonate), force-fed a gelatin capsule containing 1.5 g feed and ^{3}H-NP (1850 KBq, 5 mg). Each fish was placed in a 5-litre, well-aerated aquarium maintained at 10 °C. After 48 h, fish were killed with an overdose of anaesthetic (MS 222, 0.5 g/l). The gall bladders were then excised and the bile extracted with 1 ml methanol.

Bile treatment

The three bile samples were analysed on hplc system to quantify the metabolites. For each sample, an aliquot of 20 µl was evaporated under nitrogen stream to dryness, redissolved in 0.02 M ammonium acetate buffer (pH 5) and injected into the hplc system. The metabolites were quantified by ^{3}H radioactivity monitoring.

For metabolite isolation, the three bile samples were pooled and evaporated to dryness then redissolved in 0.01 M ammonium acetate buffer (pH 5) and purified on C18 cartridges (Supelco Park, Bellefonte, PA, USA) by methanol elution and the methanol was evaporated under a nitrogen stream. Half of the residue was redissolved in 0.02 M ammonium acetate buffer (pH 5) and injected into the hplc system. The rest of the residue was redissolved in distilled water and diluted with 0.2 M sodium acetate buffer (pH 5) for β-glucuronidase hydrolysis. The reaction was carried out at 37 °C for 12 h. Methanol was added to stop the reaction and the precipitated protein was removed by centrifugation. The supernatant was evaporated under nitrogen stream, redissolved in 0.02 M ammonium acetate buffer (pH 5) and injected into the hplc system. The biliary metabolites and the products of hydrolysis were isolated after hplc separation. The collected fractions were evaporated by vacuum centrifugation, the residue redissolved in distillated water and purified on C18 cartridge with methanol elution for ms analysis.

Instrumentation

Radio-hplc. Hplc was performed on a 1100 Hewlett Packard hplc system equipped with a 250× 4 mm Nucleosil C18 (5 µm) reversed-phase column (Bischoff Chromatography, Germany) protected by a guard column ODS Hypersil (5 µm). The same hplc gradient elution system was used for the separation of biliary metabolites before and after β-glucuronidase treatment. Separations were performed at 1 ml/min with a mobile phase composed of (A) ammonium acetate buffer (20 mM, pH 5) and (B) 90% acetonitrile and 10% ammonium acetate buffer (20 mM, pH 5). The run was set as follows: 0–40 min, linear gradient from 100% A to 35% B; 40–55 min, linear gradient from 35% B to 100% B; 55–60 min, 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radiomatic 500 Flo one apparatus (Packard, Instrument Co., Downers Grove, IL, USA).

For metabolite isolation, the hplc system was coupled with a model FC-204 collector (Gilson Medical Electronics, Middleton, WI, USA) to collect the metabolites and radioactivity profiles were obtained by counting an aliquot of the collected fractions in a liquid scintillation counter.

Liquid scintillation counting. The measurement of radioactivity was carried out in a Tricarb 2200 CA liquid scintillation counter using Ultima Gold (all Packard) as scintillation cocktail.

Derivatization. Silylation of hydrolysed metabolites was achieved using 50 µl of a mixture of bis-silyl trifluoroacetamide (BSTFA) and trimethyl chlorosilane (TMCS) (99:1), and allowed to stand for 1 h at 65 °C. Acetylation was achieved using 100 µl acetic anhydride and 50 µl pyridine and the mixture heated at 80 °C for 2 h. After the reaction, the samples were evaporated to dryness under a stream of nitrogen and redissolved in methanol before injection.

Mass spectrometry. Identification of the metabolites was made by lc-ms for conjugated metabolites, whereas hydrolysed metabolites were analysed by gc-ms as trimethylsilyl (TMS) and acetyl derivatives. ESI-ms analyses on intact glucuronides were achieved on a Finnigan LCQ ion trap mass spectrometer (Thermo Quest, Les Ulis, France). Solution samples (typically 10 ng/µl in methanol/water, 50:50 v/v) were infused into the electrospray ionization source at 3 µl/min. The instrument was used in the negative-ion mode and scanned over a mass range from m/z 50 to 600. Ms-ms were determined on the $[M\text{-}H]^-$ ion of each intact glucuronide to enhance structural information. For gc-ms analysis, mass spectra were obtained on a Nermag R-10–10-T single quadrupole mass spectrometer (Delsi-Nermag,

Argenteuil, France) and the instrument was coupled to a Delsi DI 200 gas chromatograph (Delsi-Nermag) fitted with a BPX5 (25 m × 0.22 mm i.d. × 0.25 μm) capillary column (SGE, Villeneuve St Georges, France). Helium was used as the gc carrier gas at 1 ml/min and injections made in the splitless mode. The oven temperature was programmed as follows: 50 °C for 50 s, then from 50 to 230 °C at 25 °C.min^{-1}, and from 230 to 280 °C at 5 °C.min^{-1}. The injector and interface temperatures were set at 270 °C. EI mass spectra were generated at 70 eV with a source temperature of 220 °C.

Results

At 48 h after *per os* administration of 25 mg/kg [^{3}H]-4-*n*-NP to rainbow trout, 5.5 ± 5.6% of the administered radioactivity was stored in bile (data not shown). An hplc method was developed for the separation of 4-*n*-NP and related metabolites and a baseline separation of all metabolites and products of hydrolysis was obtained. A typical profile of metabolites in trout bile is given in figure 1A, showing the presence of more than ten metabolites more polar than 4-*n*-NP, but no trace of the parent compound.

After enzymatic treatment of the bile with β-glucuronidase, the metabolic profile was dramatically modified, resulting in products less polar than the parent metabolites (figure 1B), suggesting the presence of glucuronide conjugates. The identification of six major products of hydrolysis as the TMS and acetylated derivatives by gc-ms analysis was investigated before the identification of their parent metabolites by lc-ms analysis. Three metabolites (R_t = 6.6, 18.0 and 28.0 min) remained unidentified because the low quantities collected.

Peak 1 (R_t = 48.4 min) accounted for 52.0 ± 11.4% of the biliary radioactivity and coeluted with authentic NP-glucuronide on hplc. After β-glucuronidase hydrolysis, the retention time of peak 1 shifted to 57.5 min in our hplc system. This value corresponds to the retention time of standard 4-*n*-NP, suggesting the presence of NP-glucuronide in bile. ESI-mass spectrum (negative ionization) of metabolite M1 yielded a quasi-molecular ion at m/z 395 $(M\text{-}H)^-$ (figure 2), confirming that M1 was the NP-glucuronide.

Peak 2 (R_t = 38.5 min) accounted for 2.0 ± 0.8% of the biliary radioactivity. After β-glucuronidase hydrolysis and subsequent gc-ms analysis as the TMS derivative, two isomeric monohydroxylated derivatives of 4-*n*-NP were separated, each peak yielding a molecular ion at m/z 380. The structures were determined by analysing the fragmentation pattern. For the first aglycone derivative (M2a) (gc R_t = 12.20 min), fragment ions at m/z 205 $[M\text{-}TMSOH\text{-}C_6H_{13}]^+$, 179 $[C_7H_6OTMS]^+$, 103 $[TMSOCH_2]^+$ and 73 $[(CH_3)_3Si]^+$ were obtained (figure 3a). For the second aglycone derivative (M2b) (gc R_t = 11.38 min), fragment ions at 290 $[M\text{-}TMSOH]^+$, 205 $[M\text{-}TMSOH\text{-}C_6H_{13}]^+$, 179 $[C_7H_6OTMS]^+$, 147 $[(CH_3)_2SiOSi(CH_3)_3]^+$, 117 $[TMSOCH_2CH_2]^+$ and 73 $[(CH_3)_3Si]^+$ were observed (figure 3b). The fragment ion at m/z 147 indicated the presence of two hydroxy groups and m/z 179 suggested that there was no hydroxylation on the phenol ring of these molecules. The fragment ion at m/z 103 showed the presence of a hydroxy group at ω position of the alkyl chain of M2a. A hydroxylation occurred at ω-1 position of the alkyl chain of M2b as demonstrated by the fragment ion at m/z 117. Based on these data the above two metabolites were identified as 9-hydroxynonylphenol-glucuronide (M2a) and as 8-hydroxynonylphenol-glucuronide (M2b).

Peak 3 (R_t = 32.2 min) accounted for 13.9 ± 2.3% of the biliary radioactivity. The corresponding aglycone eluted at 47.8 min on hplc and yielded, after trimethylsilylation and gc-ms analysis a molecular ion at m/z 352. The fragment ions at m/z 205 $[M\text{-}TMSOH\text{-}C_4H_9]^+$, 179 $[C_7H_6OTMS]^+$, 147 $[(CH_3)_2$

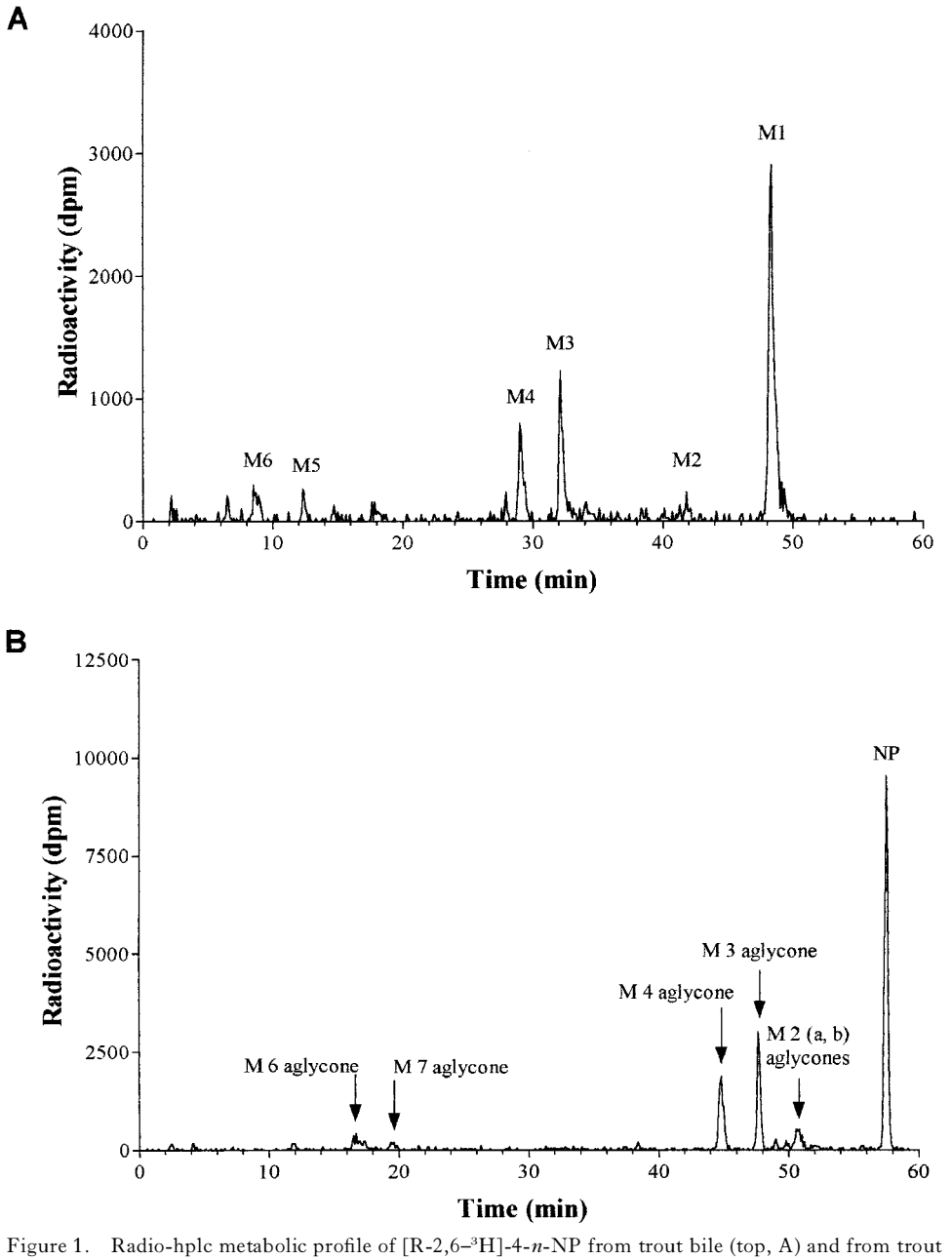

Figure 1. Radio-hplc metabolic profile of [R-2,6–^{3}H]-4-*n*-NP from trout bile (top, A) and from trout bile incubated with β-glucuronidase (bottom, B).

$SiOSi(CH_3)_3]^+$ and 103 $[TMSOCH_2]^+$ were observed. These fragment ions characterized a metabolite hydroxylated at the ω position of the alkyl chain. The molecular weight suggested a shorter alkyl chain than for M2a. Based on these data 7-hydroxyheptylphenol was proposed for the structure of the aglycone moiety. The structure of metabolite M3 aglycone was confirmed by gc-ms analysis of the acetylated derivative of the putative 7-hydroxyheptylphenol. The EI-mass spectrum exhibited a molecular ion at m/z 292 and showed fragment ions at m/z 250 [M-

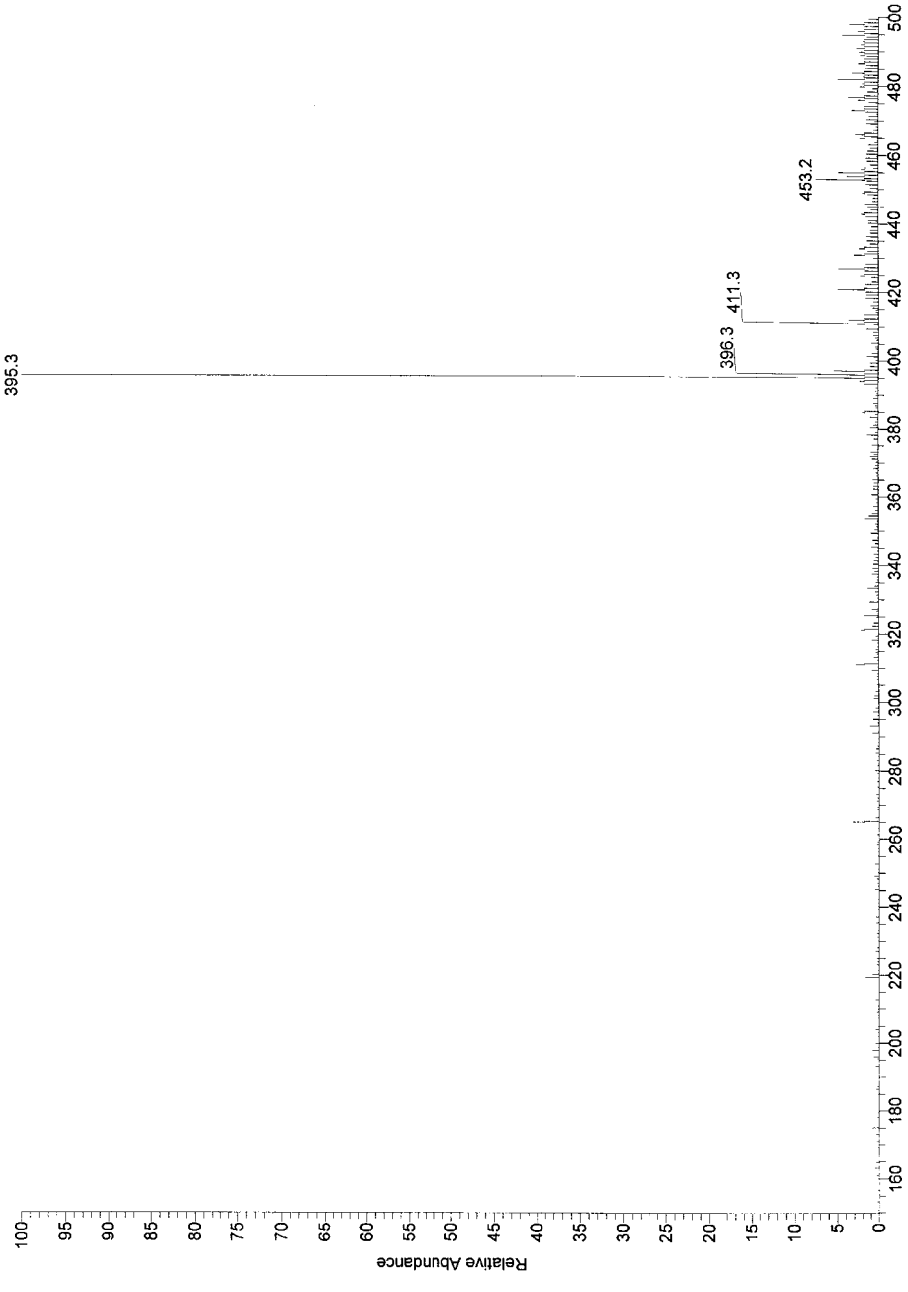

Figure 2. ESI-ms (negative ionization) of M1.

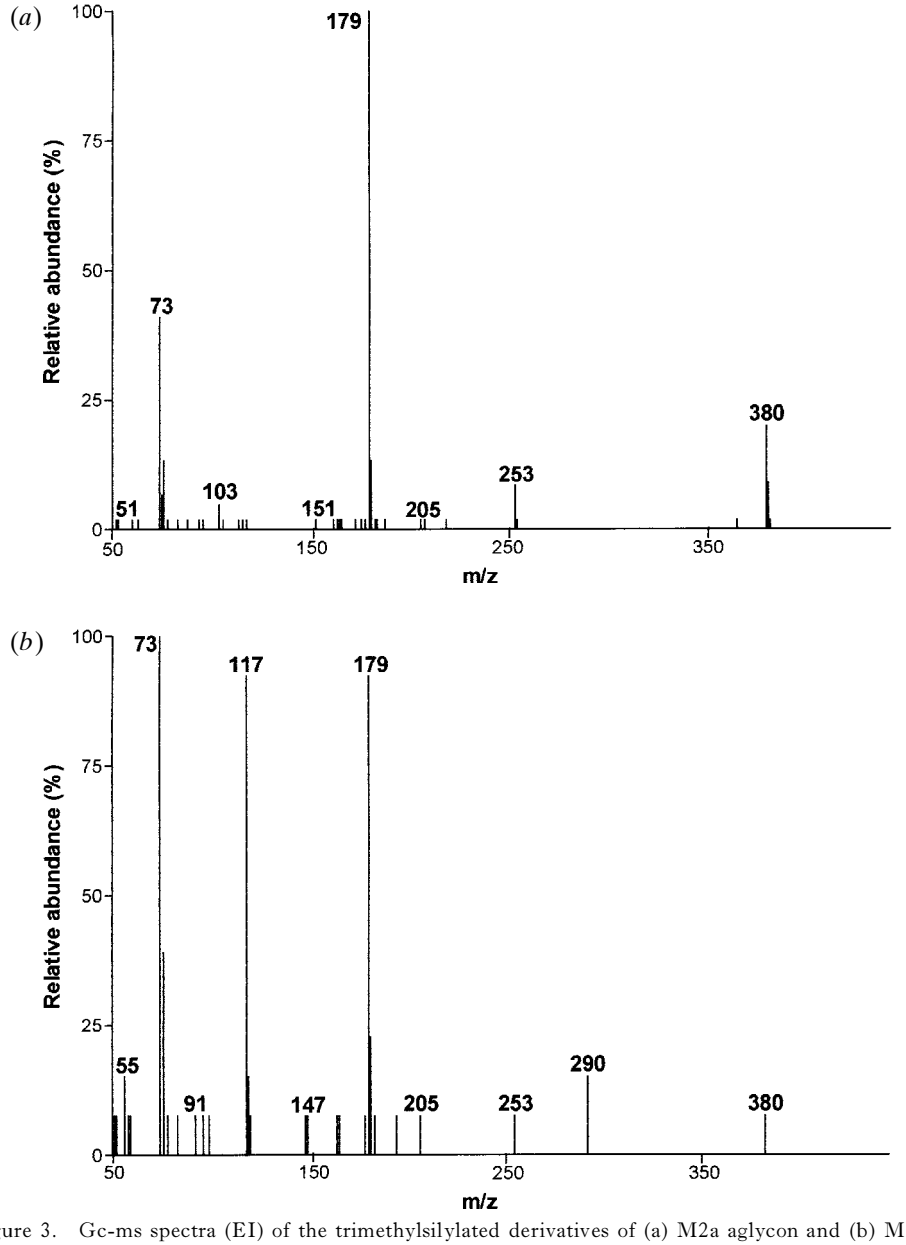

Figure 3. Gc-ms spectra (EI) of the trimethylsilylated derivatives of (a) M2a aglycon and (b) M2b aglycon.

$CH_2CO]^+$, 208 $[M\text{-}2(CH_2CO)]^+$ and 107 $[C_7H_6OH]^+$ (figure 4). The ESI-mass spectrum (negative ionization) of M3 yielded a quasi-molecular ion at m/z 383 $[M\text{-}H]^-$. Based on these data, 7-hydroxyheptylphenol-glucuronide was proposed as possible structure for M3.

Peak 4 (R_t = 29.1 min) accounted for 9.4± 4.9% of the biliary radioactivity. After enzymatic treatment with β-glucuronidase, peak 4 shifted to 44.8 min on our hplc system. This data suggested that M4 was a glucuronide conjugate as subsequently confirmed by the lc-ms analysis. The ESI-mass spectrum (negative-

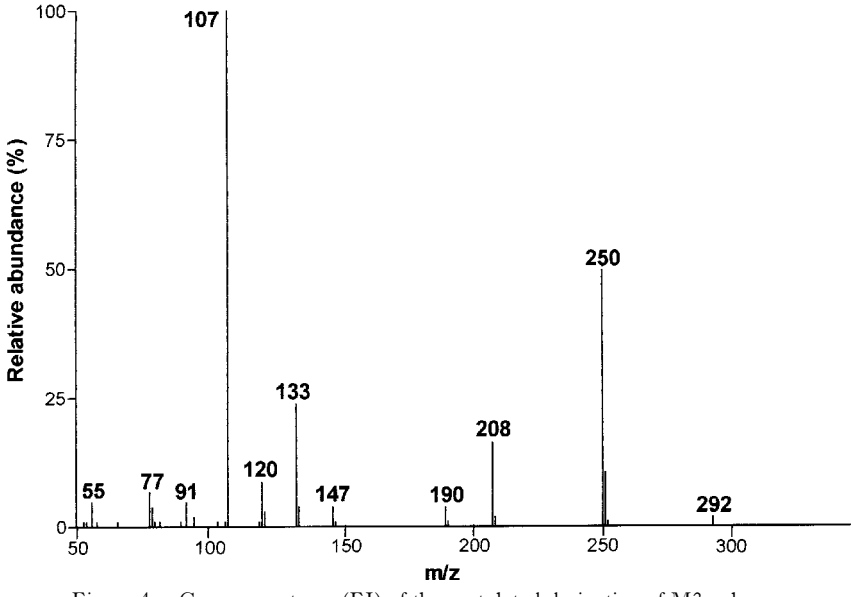

Figure 4. Gc-ms spectrum (EI) of the acetylated derivative of M3 aglycon.

ionization) was: m/z 397 $[M\text{-}H]^-$, m/z 419 $[M + Na\text{-}2H]^-$ and 221 $[M\text{-}H\text{-}176]^-$. The fragment ion at m/z 221 corresponded to the loss of the glucuronic acid and confirmed that M4 was a glucuronide conjugate. The EI-mass spectrum of the TMS derivative of the M4 aglycone exhibited a molecular ion at m/z 366 suggesting a shorter alkyl chain than M2. The same fragment ions at m/z 205, 179, 147 and 73 as those described for M2a and M2b aglycons EI-mass spectrum were observed. These spectra showed that no hydroxylation had occurred on the phenol ring and that the M4 aglycone was bis-trimethylsilylated. We suggest that one of the two TMS groups is linked to an acid function and 7-(4-hydroxyphenyl)heptanoic acid as a possible structure for the M4 aglycone. The gc-ms analysis of acetylated and trimethylsilylated derivatives of the M4 aglycone was then undertaken. The EI-mass spectrum exhibited a molecular ion at m/z 336, which was in agreement with the expected structure in which the phenolic function was acetylated and the carboxylic acid group was silylated. Main characteristic fragment ions were detected at m/z 294 $[M\text{-}CH_2CO]^+$, 204 $[M\text{-}CH_2CO\text{-}TMSOH]^+$, 107 $[C_7H_6OH]^+$ and 75 $[HOSi(CH_3)_2]^+$ (figure 5). These data corroborate the proposed structure for M4 as being 7-(4-hydroxyphenyl)heptanoic acid-glucuronide.

Peak 5 (R_t = 12,5 min) accounted for 2.0± 1.5% of the biliary radioactivity. Because of the coelution in our hplc system of peak 5 with an endogenous compound, no direct lc-ms analysis could be interpreted in this case. Gc-ms analysis was investigated after enzymatic hydrolysis of fraction 5 and the product of hydrolysis was trimethylsilylated. The EI-mass spectrum yielded a molecular ion at m/z 338 and showed fragment ions at m/z 179 $[C_7H_6OTMS]^+$, 147 $[(CH_3)_2 SiOSi(CH_3)_3]^+$ and 73 $[(CH_3)_3Si]^+$. The molecular mass and the fragmentation pattern were in agreement with the bis-trimethylsilyl derivative of 5-(4-hydroxyphenyl)pentanoic acid. Based on these data M5 was identified as 5-(4-hydroxyphenyl)pentanoic acid-glucuronide.

Peak 6 (R_t = 8.7 min) accounted for 4.5± 1.4% of the biliary radioactivity and coeluted, after enzymatic hydrolysis, with authentic 3-(4-hydroxyphenyl)propionic

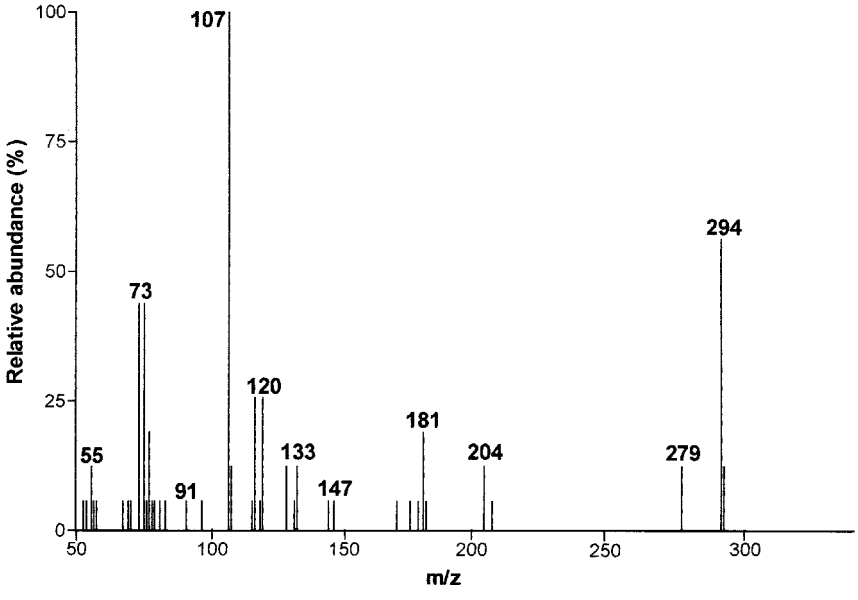

Figure 5. Gc-ms spectrum (EI) of the acetylated and trimethylsilylated derivative of M4 aglycon.

acid on our hplc system (R_t = 16.8 min). These data suggested that the M6 was the 3-(4-hydroxyphenyl)propionic acid-glucuronide. After trimethylsilylation, authentic 3-(4-hydroxyphenyl)propionic acid and the M6 aglycone had a gc retention time of 8.4 min, both of them yielding identical EI-mass spectra with a molecular ion at m/z 310 and fragment ions at m/z 192 and 179. These data confirmed that M6 was the glucuronide conjugate of 3-(4-hydroxyphenyl)propionic acid.

Peak 7 eluted after enzymatic hydrolysis at 19.6 min on hplc (figure 1B). After trimethylsilylation, the M7 aglycone had a retention time of 8.5 min on gc and yielded on gc-ms a molecular ion at m/z 308. The EI-mass spectrum exhibited characteristic fragment ions at m/z 293 $[M\text{-}CH_3]^+$, 249 $[M\text{-}CH_3\text{-}CO_2]^+$, 219 $[M\text{-}TMSO]^+$ and 147 $[(CH_3)_2SiOSi(CH_3)_3]^+$. The fragment ion at m/z 147 confirmed the occurrence of two TMS groups on the molecule, whereas the m/z 249 fragment ion indicated the occurrence of a carboxylic acid function and m/z 219 ion was in agreement with a vinylic double bound. M7 was thus identified as 3-(4-hydroxyphenyl)-2-propenoic acid-glucuronide.

Discussion

In previous work, we demonstrated that 4-*n*-NP was extensively biotransformed in trout and that bile contained high levels of radioactivity (Thibaut *et al.*, in press). After β-glucuronidase treatment, most of the metabolites were hydrolysed, suggesting the presence of glucuronide compounds. The present study confirmed that all the biliary metabolic products of NP were glucuronic acid conjugates (figure 6). NP-glucuronide, the major metabolite, accounted for 52 ± 11% of the biliary radioactivity. This result was expected since Kobayashi *et al.* (1976) indicated that the biliary excretion of the glucuronide is one of the general detoxication mechanisms in fish for phenolic compounds. The major conjugate found in bile of goldfish exposed to phenol was identified as phenyl-glucuronide (Kobayashi *et al.* 1976, Layiwola 1983). However, Meldalh *et al.* (1996) did not find any nonylphenol-

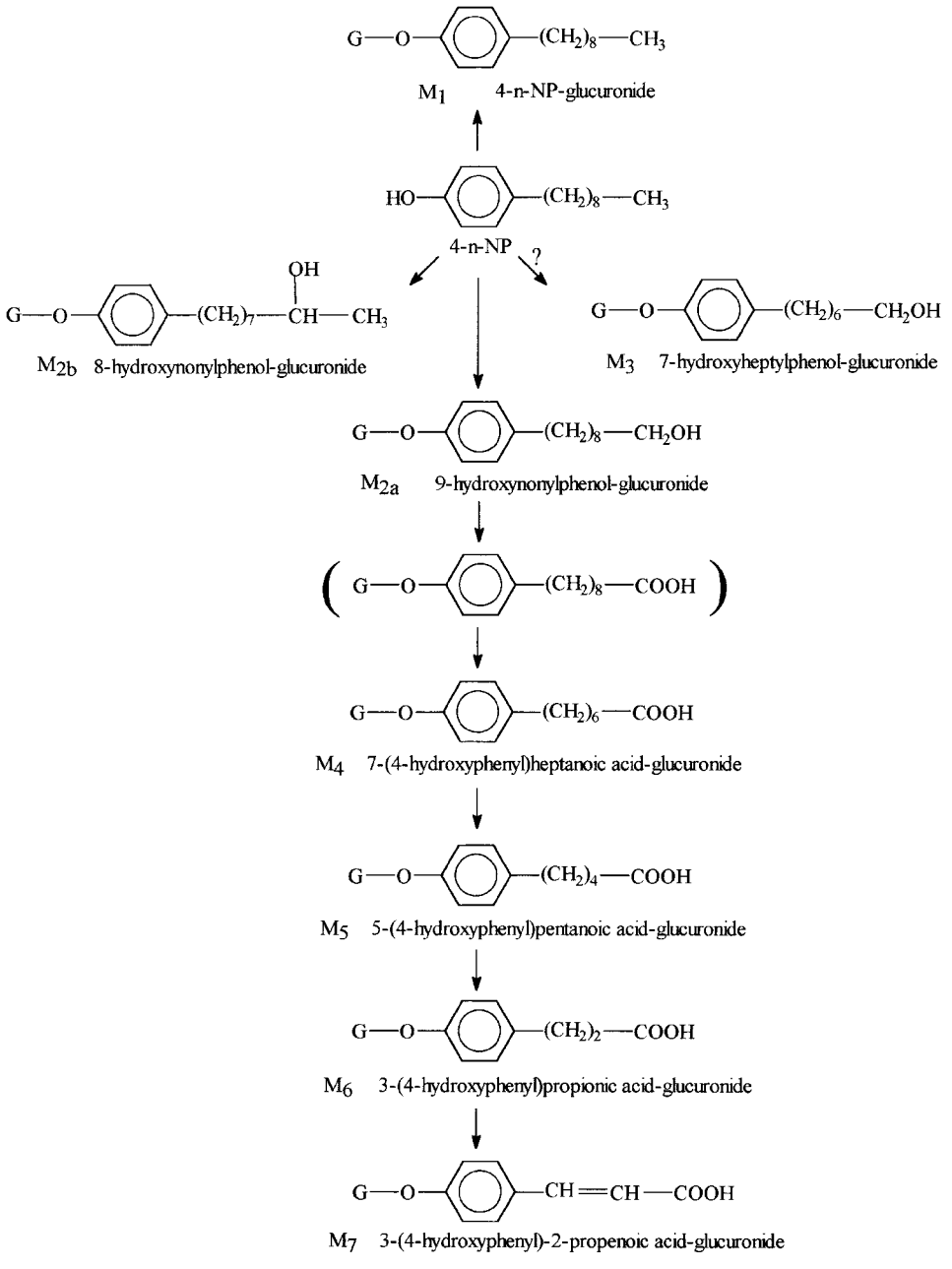

Figure 6 Proposed biotransformation pathways for the *in vivo* formation of biliary metabolites. Brackets indicate that the metabolite was not detected. G represents the glucuronide conjugate.

glucuronide in the bile of trout exposed to a mixture of three isomers of NP suggesting that the conjugation rates of NP may depend on the structure of the alkyl chain.

Phase I metabolic pathways were also involved in the metabolism of NP as demonstrated by the oxidations on the alkyl side chain (figure 6). We found in bile treated with β-glucuronidase NP hydroxylated at both ω and ω-1 positions, namely

9-hydroxynonylphenol and 8-hydroxynonylphenol respectively (figure 6). This result was in agreement with those of Meldalh *et al.* (1996) who found with a mixture of three isomers of NP, that hydroxylation occurred on the C-8 position of the alkyl chain. Terminal hydroxylation of alkanes have been already described in fish and other species (Perdu-Durand and Tulliez 1985). Cravedi and Tulliez (1986a, b) showed (*in vivo*) that trout can hydroxylate the terminal carbon of heptadecane and the penultimate carbon of the alkyl chain of the naphthenic hydrocarbon dodecylcyclohexane leading to cyclohexyldodecane-2-ol. In addition, lauric acid was shown to be hydroxylated at both ω and ω-1 positions by liver microsomes from rat (Okita and Masters 1980, Orton and Parker 1981, Buhler *et al.* 1997) and from trout (Miranda *et al.* 1990, Buhler *et al.* 1997).

In our study, bile also contained carboxylic acid metabolites which are likely produced by the subsequent oxidation of ω-hydroxylated compounds as reported by Kusunose *et al.* (1969) and Perdu-Durand and Tulliez (1985) for alkanes. Similarly Cravedi *et al.* (1985) and Cravedi and Tulliez (1986b) reported that trout readily converted by ω-oxidation, dodecylcyclohexane and pristane into cyclohexyldodecanoic acid and pristanic acid respectively. In our case the presence of 9-(4-hydroxyphenyl)nonanoic acid was therefore expected but our attempts in detecting this acid were unsuccessful, probably because of the subsequent β-oxidation of this carboxylic acid.

As cyclohexyldodecanoic acid was reported to be converted to cyclohexylacetic acid in trout (Cravedi and Tulliez 1987), the β-oxidation of 9-(4-hydroxyphenyl)nonanoic acid may lead to *para*-hydroxybenzoic acid. This acid was not detected in our bile samples but some intermediary metabolites of β-oxidation, namely 7-(4-hydroxyphenyl)heptanoic acid, 5-(4-hydroxyphenyl)pentanoic acid, 3-(4-hydroxyphenyl)propionic acid and 3-(4-hydroxyphenyl)-2-propenoic acid, were present (figure 6). The presence of these intermediary metabolites suggests that in our case β-oxidation could be peroxisomal. It is well known that mitochondrial β-oxidation proceeds to completion, whereas peroxisomes may cycle a fatty acid through only a few turns of the β-oxidation spiral and thus generate not only acetyl-CoA units and NADH but also chain-shortened fatty acids (Lazarow 1978). Moreover, peroxisomal β-oxidation in contrast with mitochondrial β-oxidation is characterized by a very broad substrate specificity and can be regarded as an efficient pathway for detoxification of xenobiotics possessing hydrophobic aliphatic substituents. Xenobiotic compounds like ω-phenyl-fatty acids, possessing a bulky substituent at the ω-end appear unable to enter the mitochondrial matrix but may be chain-shortened by peroxisomes (Osmundsen *et al.* 1991). The main function of rat liver peroxisomes seems to be partial β-oxidation of very long chain fatty acids and xenobiotic compounds like phenyl fatty acids (Schulz 1991). Yamada *et al.* (1986) reported chain-shortened metabolites of *N*-(α-methylbenzyl)azelaamic acid (C_9) with odd numbers of carbons atoms (C_7 and C_5) in rat urine. Recently, Crockett and Sidell (1993) demonstrated that peroxisomal β-oxidation was a significant pathway for the catabolism of fatty acids in teleost fish.

An unexpected NP metabolite was isolated and identified unambiguously as 7-hydroxyheptylphenol. The presence of this metabolite could be related to an initial ω-2 hydroxylation (i.e. C-7 position) on the alkyl chain. This ω-2 hydroxylation was recently demonstrated in trout with lauric acid as substrate (Buhler *et al.* 1997). In our case, however, the pathway from the putative 7-hydroxynonylphenol to 7-hydroxyheptylphenol remains to be elucidated.

Meldalh *et al.* (1996) reported that the major metabolites from a mixture of three isomers of NP were hydroxylated in only one position of the alkyl chain (C-8) and consequently that only one cytochrome P450 was responsible for this metabolism. They showed that trout CYP1A1 may not be responsible for ω-1 hydroxylation and proposed as a candidate a non-specific cytochrome P450 fatty acid hydroxylase. Lee *et al.* (1996a, b) demonstrated that NP down-regulates and inhibits the catalytic activity of CYP1A and up-regulates and increases the catalytic activity of CYP3A in rat liver suggesting a possible role for these isozymes in the hepatic metabolism of NP. However, the modulation of fish cytochrome P450 by NP remains to be investigated. In trout, ω and ω-1 hydroxylation of lauric acid are carried out separately by distinct isozymes of cytochrome P450 designated LMC1 (CYP2M1) and LMC2 (CYP2K1) respectively (Miranda *et al.* 1990, Buhler *et al.* 1997). These cytochrome P450 forms are implicated in the metabolism of steroids and metabolize foreign compounds like benzphetamine and aflatoxin B1. They are other possible candidates for the ω and ω-1 oxidation of NP but additional studies will be necessary to confirm this further.

In summary, the present study indicates that 4-*n*-NP is biotransformed by trout and that phase I and II metabolic pathways are involved in its biotransformation. However, our study was focused on the normal isomer of nonylphenol congeners, and the metabolic fate of highly branched isomers of NP that could be encountered in the environment remains to be elucidated. For such isomers, β-oxidation is impaired making NP elimination more difficult, and we do not know if glucuronidation of the phenolic group is a sufficiently efficient metabolic pathway to eliminate NP. Moreover, the possible oestrogenic effect of the formed metabolites requires to be investigated in further studies.

Acknowledgement

Elisabeth Perdu is thanked for preparation of 4-*n*-nonylphenol glucuronide standard. This work was supported in part by the EC programme Environment and Climate (contract ENV4-CT96–0223)

References

Ahel, M., Giger, W. and Koch, M., 1994, Behaviour of alkylphenol polyethoxylate surfactants in aquatic environment—I. Occurrence and transformation in sewage treatment. *Water Research*, **28**, 1131–1142.

Ahel, M., McEvoy, J. and Giger, W., 1993, Bioaccumulation of lipophilic metabolites of nonionic surfactants in freshwater organisms. *Environmental. Pollution*, **79**, 243–248.

Buhler, D. R., Miranda, C. L., Deinzer, M. L., Griffin, D. A. and Henderson, M. C., 1997, The regiospecific hydroxylation of lauric acid by rainbow trout (*Oncorhynchus mykiss*) cytochrome P450. *Drug Metabolism and Disposition*, **25**, 1176–1183.

Corti, A., Frassinetti, S., Vallini, G., D'Antone, S., Fichi, C. and Solaro, R., 1995, Biodegradation of nonionic surfactants. I. Biotransformation of 4-(1-nonyl)phenol by a *Candida mal.tosa* isolate. *Environmental. Pollution*, **90**, 83–87.

Cravedi, J.-P., Le Bon, A.-M. and Tulliez, J., 1985, Isolation and characterisation of pristane metabolites formed *in vivo* by rainbow trout. *Marine Environmental. Research*, **17**, 125–129.

Cravedi, J.-P. and Tulliez, J., 1986a, Metabolism of *n*-alkanes and their incorporation into lipids in the rainbow trout. *Environmental. Research*, **39**, 180–187.

Cravedi, J.-P. and Tulliez, J., 1986b, Metabolites of the naphthenic hydrocarbon dodecylcyclohexane in rainbow trout liver and their incorporation into lipids. *Archives of Environmental. Contamination and Toxicology*, **15**, 207–213.

Cravedi, J.-P. and Tulliez, J., 1987, Urinary metabolites of dodecylcyclohexane in *Salmo gairdneri*: evidence of aromatization and taurine conjugation in trout. *Xenobiotica*, **17**, 1103–1111.

Crockett, E. L. and Sidell, B. D., 1993, Peroxisomal β-oxidation is a significant pathway for catabolism of fatty acids in a marine teleost. *American Journal. of Physiology*, **264**, 1004–1009.

Ekelund, R., Bergman, Å., Granmo, Å. and Berggren, M., 1990, Bioaccumulation of 4-nonylphenol in marine animals—a re-evaluation. *Environmental. pollution*, **64**, 107–120.

Flouriot, G., Pakdel, F., Ducouret, B. and Valotaire, Y., 1995, Influence of xenobiotics on rainbow trout estrogen receptor and vitellogin expression. *Journal. of Molecular Endocrinology*, **15**, 143–151.

Giger, W., Brunner, P.H. and Schaffner, C., 1984, 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. *Science*, **225**, 623–625.

Jobling, S. and Sumpter, J. P., 1993, Detergent components in sewage effluent are weakly estrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic toxicology*, **27**, 361–372.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P. and Sumpter, J. P., 1996, Inhibition of testiculary growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environmental. Toxicology and Chemistry*, **15**, 194–202.

Kobayashi, K., Kimura, S. and Shimizu, E., 1976, Studies on the metabolism of chlorophenols in fish—VIII. Isolation and identification of phenyl-β-glucuronide accumulated in bile of goldfish. *Japanese Society of Scientific Fisheries*, **42**, 1365–1372.

Kusunose, M., Ichihara, K. and Kusunose, E., 1969, Oxidation of *n*-hexadecane by mouse liver microsomal fraction. *Biochimica et Biophysica Acta*, **176**, 679–681.

Layiwola, P. J., Linnecar, D. F. C. and Knights, B., 1983, Hydrolysis of the biliary glucuronic acid conjugate of phenol by the intestinal mucus/flora of goldfish (*Carassius auratus*). *Xenobiotica*, **13**, 27–29.

Lazarow, P. B., 1978, Rat liver peroxisomes catalyze the β-oxidation of fatty acids. *Journal. of Biological. Chemistry*, **253**, 1522–1528.

Lech, J. J., Lewis, S. K. and Ren, L., 1996, *In vivo* estrogenic activity of nonylphenol in rainbow trout. *Fundamental. and Applied Toxicology*, **30**, 229–232.

Lee, P. C., Chakraborty Patra, S., Stelloh, C. T., Lee, W. and Struve, M., 1996a, Interaction of nonylphenol and hepatic CYP1A in rats. *Biochemical. Pharmacology*, **52**, 885–889.

Lee, P. C., Chakraborty Patra, S. and Struve, M., 1996b, Modulation of rat hepatic CYP3A by nonylphenol. *Xenobiotica*, **26**, 831–838.

Lee, P.C. and Lee, W., 1996, *In vivo* estrogenic action of nonylphenol in immature female rats. *Bulletin of Environmental. Contamination and Toxicology*, **57**, 341–348.

Lewis, S. K. and Lech, J. J., 1996, Uptake, disposition and persistence of nonylphenol from water in rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica*, **26**, 813–819.

McLeese, D. W., Zitko, V., Sergeant, D. B., Burridge, L. and Metcalfe, C. D., 1981, Lethality and accumulation of alkylphenols in aquatic fauna. *Chemosphere*, **10**, 723–730.

Meldalh, A. C., Nithipatikom, K. and Lech, J. J., 1996, Metabolism of several ^{14}C-nonylphenol isomers by rainbow trout (*Oncorhynchus mykiss*): *in vivo* and *in vitro* microsomal metabolites. *Xenobiotica*, 26, 1167–1180.

Miranda, C. L., Wang, J. L., Henderson, M. C., Williams, D. E. and Bulher, D. R., 1990, Regiospecificity in the hydroxylation of lauric acid by rainbow trout hepatic cytochrome P450 isozymes. *Biochemical. and Biophysical. Research Communications*, **171**, 537–542.

Naylor, C. G., Mieure, J. P., Adams, W. J., Weeks, J. A., Castaldi, F. J., Ogle, L. D. and Romano, R. R., 1992, Alkylphenol ethoxylates in the environment. *Journal. of the American Oil Chemist's Society*, **69**, 695–703.

Okita, R. T. and Masters, B. S. S., 1980, Effect of phenobarbital treatment and cytochrome P-450 inhibitors on the laurate ω- and (ω-1)-hydroxylase activities of rat liver microsomes. *Drug Metabolism and Disposition*, **8**, 147–151.

Orton, T. C. and Parker, G. L., 1982, The effect of hypolipidemic agents on the hepatic microsomal drug-metabolizing enzyme system of the rat. Induction of cytochrome(s) P-450 with specificity toward terminal hydroxylation of lauric acid. *Drug Metabolism and Disposition*, **10**, 110–115.

Osmundsen, H., Bremer, J. and Pedersen, J. I., 1991, Metabolic aspects of peroxisomal β-oxidation. *Biochimica et Biophysica Acta*, **1085**, 141–158.

Perdu-Durand, E.F. and Tulliez, J. E., 1985, Hydrocarbon hydroxylation system in liver microsomes from four animal species. *Food and Chemical. Toxicology*, **23**, 363–366.

Schulz, H., 1991, Beta oxidation of fatty acids. *Biochimica et Biophysica Acta*, **1081**, 109–120.

Soto, A. M., Justicia, H., Wray, J. W. and Sonnenschein, C., 1991, *p*-Nonylphenol: an estrogenic xenobiotic released from 'modified' polystyrene. *Environmental. Health Perspectives*, **92**, 167–173.

Thibaut, R., Debrauwer, L., Rao, D. and Cravedi, J.-P., 1998, Disposition and metabolism of [^{3}H]-4-*n*-nonylphenol in rainbow trout. *Marine Environmental. Research* (in press).

Yamada, J., Itoh, S., Horie, S., Watanabe, T. and Suga, T., 1986, Chain-shortening of a xenobiotic acyl compound by the peroxisomal β-oxidation system in rat liver. *Biochemical. Pharmacology*, **35**, 4363–4368.