Metabolites of 4-*n*-Nonylphenol in Wheat Cell Suspension Cultures

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4-Nonylphenol, a metabolite of nonionic surfactants, was examined for its metabolism in cell cultures of wheat according to a standardized method. Four major radioactively labeled fractions were detected and isolated by chromatography on Sephadex LH-20 and semipreparative HPLC. Hydrolysis of the peaks resulted in two compounds which were purified by semipreparative HPLC. Using enzymatic hydrolysis, HPLC, GC−MS, and ESI-MS/MS, the chemical structures were elucidated. They were shown to be 4-(hydroxy)- and 4-(dihydroxy)nonylphenols which were glucosylated at the phenolic OH-group and further glucosylated, glucuronidated, and acylated with acetic acid or malonic acid. Except for C-1 and C-9, the hydroxylations occurred at all C atoms of the alkyl side chain.

Keywords: *Alkylphenol; nonionic surfactant; plant metabolism; Triticum aestivum; electrospray mass spectrometry; xenobiotics*

INTRODUCTION

4-Nonylphenol (4-NP), a metabolite of nonionic surfactants (alkylphenol polyethoxylates), is a compound of environmental concern, especially since estrogenic effects of alkylphenolic compounds, including 4-NP, recently have been reported in fish, birds, and mammals (Soto et al., 1991; White et al., 1994; Purdom et al., 1994). During the past two decades, 4-nonylphenol has been shown to accumulate in high amounts in anaerobically degraded sewage sludges and effluents from sludge treatment (Giger et al., 1981, 1984; BUA, 1988; Jobst, 1995). The application of sewage sludges to agricultural lands and, in addition, the use of nonylphenols in the formulation of pesticides (BUA, 1988; Holloway and Stock, 1990) raise the question of uptake and metabolism in terrestrial plants, particularly food crop plants.

The metabolic fate of ethoxylated *p-tert*-octylphenols, which are structurally related to the parent nonylphenol polyethoxylates, has been studied in barley plants (Stolzenberg et al., 1982). However, little is known about the metabolic behavior of 4-NP in plant systems. An uptake into pine needles has been reported only 1 h after spray application of 4-NP. After 62 days, the compound could not be detected in the needles (Sundaram et al., 1980).

As the quality of crop (food) plants is of concern, it is important to know the physiological processes to which an apparent disappearance can be attributed. Among others, one reason for nondetection may be due to the formation of metabolites (e.g. conjugates) or nonextractable residues, which in certain conditions may release the parent compound. Here we report on the identification of the soluble metabolites following application of 4-*n*-nonylphenol (4-*n*-NP) to cell suspension cultures of wheat. Technical 4-NP and 4-NP in environmental matrices comprise isomeric compounds differing in the structure of the alkyl side chain (BUA, 1988; Stephanou and Giger, 1982). However, the pattern of metabolites that result from a mixture of isomers applied to cell cultures is so complex that unambiguous results are extremely difficult to obtain. 4-n-NP was the only isomer available in ^{14}C-labeled form, so for our investigation this isomer was chosen.

MATERIALS AND METHODS

Material. Cell cultures of wheat (*Triticum aestivum* L. cv. Heines Koga II) were used and maintained as described previously (Langebartels and Harms, 1986). 4-*n*-Nonyl[^{14}C-UL]phenol was synthesized by Amersham-Buchler (Braunschweig, Germany) with a specific activity of 1.295 MBq/mg. Nonlabeled 4-*n*-NP was purchased from Ehrensdorfer (Augsburg, Germany). α-Glucosidase from yeast was obtained from Boehringer (Mannheim, Germany). β-Glucosidase from sweet almonds and β-glucuronidase from *Helix pomatia* were purchased from Sigma (Deisenhofen, Germany).

Analytical High-Performance Liquid Chromatography (HPLC). Analytical separation was obtained on a Merck HPLC system (655 A-12 LC and L 5000 LC-Controller, Merck, Darmstadt, Germany) equipped with a Merck UV spectrophotometer (L 4000) using a 4 μM Lichrospher C_{18} column (250 mm × 4 mm i.d., Manucart, Merck), with 5% methanol and 2% formic acid in water (v/v/v) as solvent A and 100% methanol as solvent B at a flow rate of 1 mL/min. The following elution program was used: Starting with 35% solvent B in A for 5 min, a linear gradient to 50% solvent B in A in 10 min was applied followed by a linear gradient to 90% solvent B in A within 15 min. These conditions were held for 5 min. Then 100% solvent B was reached after 2 min and kept for another 7 min. Elution was monitored at UV 280 nm and, simultaneously, by a radioactivity monitor (LB 506 C-1) equipped with a Z-2000 cell (Berthold, Wildbad, Germany) using 4 mL of Hydroluma (Baker, Deventer, The Netherlands)/mL effluent as scintillation cocktail.

Preparative Isolation of Metabolites. Fifty Erlenmeyer flasks with wheat cell cultures were incubated with 100 μM 4-*n*-NP for 2 days at the end of the linear growth phase. One control culture was incubated with the same amount of 4-*n*-NP including 10×10^6 dpm of radioactivity. Cells treated with labeled and nonlableled 4-*n*-NP were extracted separately according to the same method. They were harvested by filtering, and their fresh weights were determined. Calculated on the basis of a cell water content of 90% (w), dichloromethane and methanol were added to give a ratio of dichloromethane/methanol/water of 2:1:0.8 (v/v/v). The cells were homogenized using an Omni Mix (Omni Int., Waterbury, CT) for 5 min. The homogenate was stirred for 1 h at room temperature and

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filtered, and the residue was re-extracted twice with dichloromethane/methanol/water (2:1:0.8 v/v/v). The combined extracts were separated into two phases by adding 265 μL of water and 265 μL of dichloromethane per milliliter of cell extract. The upper methanol−water phase and the lower dichloromethane phase were separated using a separation funnel.

The methanol−water phases of the treatments with labeled and nonlabeled 4-*n*-NP were combined and evaporated to a few milliliters. This extract was subjected to chromatography on Sephadex LH-20 (glass column; 90 × 2 cm i.d.; Pharmacia, Freiburg, Germany) using methanol as solvent. Fractions of 10 mL were collected, and aliquots of every second one were analyzed for radioactivity by liquid scintillation counting. The radioactivity-containing samples were combined in three major fractions, which were subjected to semipreparative HPLC on a 5 μm C_{18} RP column (Nucleosil, 250 mm × 8 mm i.d.; Techlab, Erkerode, Germany) using 2% formic acid/5% methanol in water as solvent A and 100% methanol as solvent B. Starting conditions for chromatography were 15% solvent B in A, which was applied isocratically for 5 min, followed by a linear gradient to 40% B in A within 25 min and then within 50 min to 100% solvent B in A using a flow rate of 3 mL/min. Elution was monitored at UV 280 nm and, simultanously, by a radioactivity monitor (equipped with a YG-400 cell, Berthold, Germany). The radioactively labeled substances were collected and concentrated under reduced pressure.

Hydrolyses. For analytical enzymatic hydrolyses, aliquots of methanol−water phases or purified metabolites were evaporated to dryness and redissolved in 1.5 mL of sodium acetate buffer (100 mM; pH 5.0). β- or α-glucosidase or β-glucuronidase, one unit (U; unit of enzyme activity = micromoles per minute) each, was added in 50 μL, and the assays were incubated for 24 h at 30 °C. The reaction was stopped by adding 500 μL of methanol. Aliquots of 1 mL of each of the reaction mixtures were partitioned against 500 μL of dichloromethane and the dichloromethane phases used for TLC or HPLC analyses.

For analytical acid hydrolysis, a methanol−water phase of a cell extract or purified metabolites were evaporated to dryness and incubated in 4 N HCl at 90 °C for 4 h. The reaction was stopped by deep-freezing and treated in the same way as enzymatic hydrolyses.

For preparative hydrolysis one-fourth of the preparative methanol−water extract was concentrated to approximately 3 mL and diluted with 7 mL of sodium acetate buffer (pH 5.0; 100 mM). Ten units of β-glucuronidase was added, and the mixture was incubated at 30 °C for 24 h. Ten milliliters of methanol was added, and the mixture was evaporated to dryness. To remove excess salt and protein, the residue was extracted three times with a few milliliters of 80% aqueous MeOH. The extraction was monitored by scintillation counting. The extracts were combined and concentrated under vacuum to 1 mL and used for semipreparative HPLC as described above.

Mass Spectrometry. Compositional and Methylation Analyses of the 4-NP Derivatives by Gas Chromatography−Mass Spectrometry (GC−MS). Monosaccharides were analyzed by GLC on a Carlo Erba Mega Series instrument (Carlo Erba, Milan, Italy) after methanolysis, reacetylation, and trimethylsilylation as the corresponding methyl glycosides on a 30 m DB-1 capillary column according to the method of Chaplin (1982). Under these conditions the hydroxylated 4-NPs could also be observed as the corresponding pertrimethylsilylated derivatives. The glycoconjugates of 4-NP were permethylated, purified by chromatography on a Sephadex LH-20 column (210 mm × 4 mm i.d., solvent, ethyl acetate), hydrolyzed, reduced, and peracetylated as described by Nimtz et al. (1990).

All GC−MS analyses were performed on a double-focusing Kratos MS 50 fast scan mass spectrometer of EB geometry connected to a Carlo Erba gas chromatograph. GC−MS conditions: 1 μL on-column injections of the sample dissolved in cyclohexane on a 30 m × 0.25 mm i.d., 0.25 μm film thickness, DB-1 fused-silica capillary column with He as carrier gas. After injection, the oven temperature was held at 80 °C for 3 min and then linearly programmed to 300 °C at 10 °C min^{-1}. Effluents were directly transported to the mass spectrometer through heated capillary tubing. The mass spectrometer was operated in the EI mode at a resolution of 1500. The electron beam energy was 70 eV, the emission current 300 μA, the ion acceleration voltage 8 kV, and the ion source temperature 200 °C.

Electrospray MS (ESI-MS) of the Native 4-NP Metabolites. A Finnigan MAT TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan electrospray ion source (Finnigan MAT Corp., San Jose, CA) was used for electrospray mass spectrometry. The 4-*n*-NP derivatives were dissolved in methanol containing 0.1% ammonia and injected at a flow rate of 2 μL/min into the electrospray chamber. A voltage of −4.5 kV was applied to the electrospray needle. For collision-induced dissociation (CID) experiments, parent ions were selectively transmitted by the first mass analyzer and directed into the collision cell (argon was used as collision gas) with a kinetic energy set at −20 to −40 eV.

RESULTS AND DISCUSSION

Wheat cell suspension cultures were incubated with 1 ppm of 4-*n*-nonyl[^{14}C-UL]phenol according to a standardized method (Harms and Langebartels, 1986). The distribution of radioactivity in different fractions is documented in Table 1. After 2 days, more than 90% of the applied radioactivity can be extracted from the cells, the major proportion in the form of polar metabolites. Application of higher concentrations of 4-*n*-NP to the cells, as used in the preparative approach, resulted in a different distribution of radioactivity (Table 1). The HPLC separation of a methanol−water phase gives a complex pattern of compounds with three or four major fractions (Figure 1a). Hydrolyses of metabolites by HCl, β-glucuronidase, and β-glucosidase resulted in two major products (Figure 1b) which were less polar than the parent substance but more polar than 4-NP (retention time = 41.2 min). Treatment with α-glucosidase did not result in cleavage of the metabolites. These results indicate that the metabolites are conjugates, probably β-glycosides.

The metabolites and the products of the hydrolyses were isolated as described and submitted to GC−MS and ESI-MS.

Analysis of the Hydrolysis Products. The hydrolyzed metabolites of 4-*n*-NP were separated by RP-HPLC into two major peaks with only traces of unmodified 4-*n*-NP present (Figure 1b, peak III). On GC−MS analysis of a trimethylsilylated aliquot of peak II (Figure 1b) seven isomeric monohydroxylated derivatives of 4-*n*-NP could be separated, each peak yielding a molecular ion at *m*/*z* 380 (Figure 2a). Their individual structures could be elucidated by analyzing their fragmentation pattern (compare Figure 2b). Hydroxylation had taken place exclusively at the alkyl side chain of 4-*n*-NP (compare Table 2) resulting in the formation of asymmetric carbons. The enantioselectivity of this process, however, was not determined in the present investigation.

Peak I could be separated into four clearly defined dihydroxylated derivatives after trimethylsilylation (compare Table 2). The exact structure of some minor not well resolved isomers could not be determined; hydroxylation at the aromatic ring, however, could be excluded in all cases.

Analysis of the Native Metabolites. The native metabolites were separated by RP-HPLC into four major fractions (Figure 1). Each fraction was subjected to compositional analysis (Table 2). Glucose (Glc) could be detected in all fractions, whereas glucuronic acid

Table 1. Distribution of Radioactivity (Percent) from 4-*n*-Nonyl(^{14}C-UL)phenol Applied to Wheat Cell Cultures (n = 5)

	100 μM[a]	4.5 μM[a] (=1 ppm)
medium	22.9 ± 6.1	9.3 ± 2.2
cell extract	61.4 ± 5.4	91 ± 5.3
(a) dichloromethane phase	(4.4) ± 1.4	(3.5) ± 2.0
(b) methanol−water phase	(57.0) ± 6.8	(87.5) ± 6.1
nonextractable residue	22.5 ± 3.8	10.3 ± 4.3
total recovery	106.8 ± 12.4	110.4 ± 11.7

[a] Applied concentration.

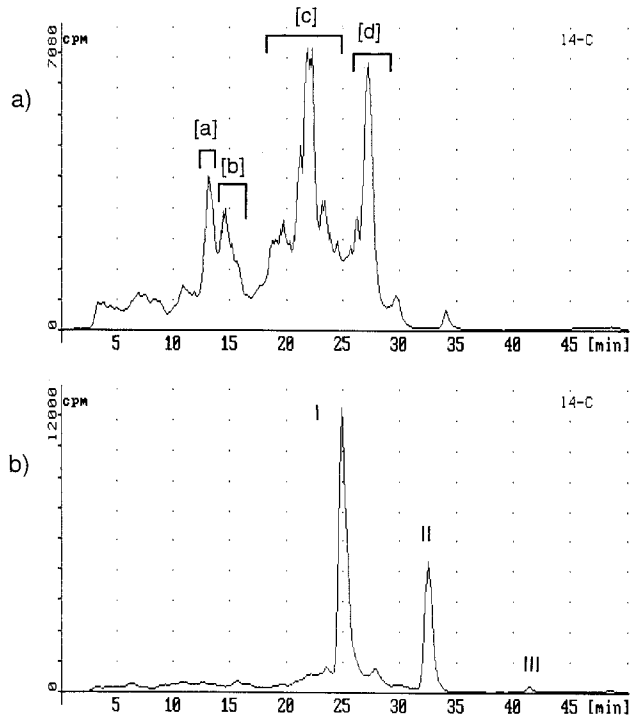

Figure 1. HPLC trace of a crude methanol−water extract of a wheat cell culture incubated with 4-*n*-nonyl(^{14}C-UL)phenol (a) and the chromatogram of the respective extract after hydrolysis with HCl (b).

Table 2. Compositional Analyses of the Metabolite Fractions of 4-*n*-NP

fraction[a]	Glc	GlcA	position of OH-groups on 4-NP
a	++[b]	+[c]	5, 7 (70%)[d]; 3, 7 (20%); 4, 7 (10%)
b	++	+	4, 8 (80%)
c	++	−[e]	7,-H_2O (5%);[f] 4, 7 (40%); 5, 7 (25%); 4, 8 (30%)
d	++	+	4 (50%); 5 (10%); 6 (15%); 7 (20%); 8 (3%)

[a] a−d, fractions according to Figure 1b. [b] Major constituent. [c] Minor constituent. [d] Relative amount of the compound in the respective fraction. [e] Not detectable. [f] Resulting in a double bond.

(GlcA) was observed in all but fraction c. The 4-NP derivatives detected in these individual HPLC fractions were either monohydroxylated or dihydroxylated derivatives (in fraction c small amounts of a monohydroxylated derivative with one additional double bond were detected) but were not homogeneous with respect to the position of the OH-group(s). Negative ion ESI-MS of these fractions was used to further characterize the metabolites.

Fraction a yielded a dominant deprotonated molecular ion at m/z 589 corresponding to [4-*n*-NP + 2O + Glc + GlcA − $2H_2O$] (Figure 3a). After CID, an intense fragment at m/z 251 [4-*n*-NP + 2O] was observed, confirming the presence of a dihydroxylated 4-*n*-NP derviative as aglycon. A fragment at m/z 337 [Glc +

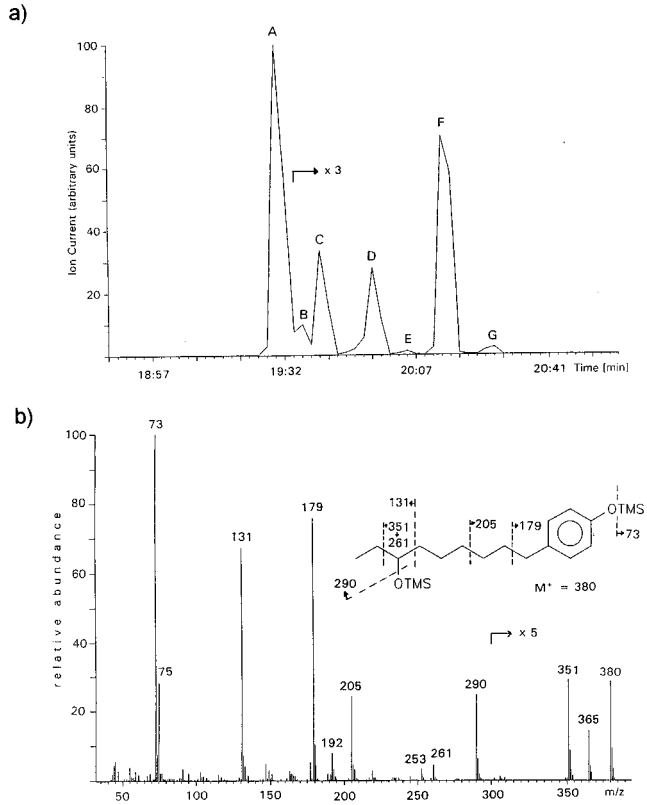

Figure 2. GC−MS analysis of trimethylsilylated peak II (a) and fragmentation pattern of peak F in the resulting chromatogram (b).

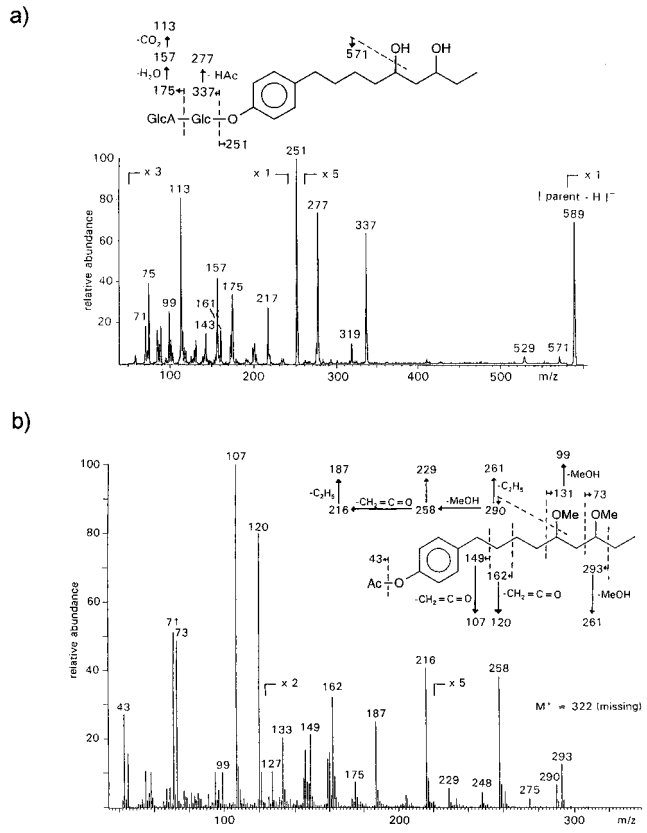

Figure 3. MS of fraction a after CID of the parent ion 589 (a) and after methylation analysis (b).

GlcA − $2H_2O$] indicated a linear arrangement of the two carbohydrate residues. The exact type of linkages present in this 4-*n*-NP conjugate was elucidated by methylation analysis (Jansson et al., 1976). Detection

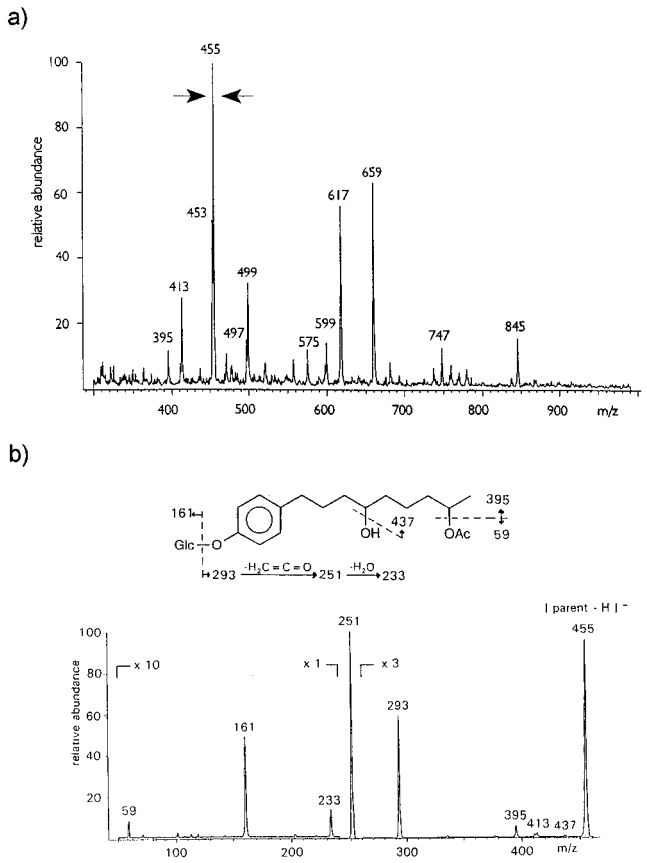

Figure 4. ESI mass spectrometry of fraction c (a) and fragmentation pattern of one peak (M 455) (b).

of only small amounts of 1,5-diacetyl-2,3,4,6-tetramethylglucitol corresponding to terminal Glc, but large amounts of 1,2,5-triacetyl-3,4,6-trimethylglucitol characteristic for 2-substituted Glc indicated the presence of a GlcA 1−2 Glc-R-motif. This partial structure is predominantly bound to the phenolic OH-group of the dihydroxy-4-*n*-NP moiety, since 4-(5,7-dimethoxynonyl)-acetylphenol was the dominant 4-*n*-NP derivative detected (Figure 3b). This agrees with the data from the compositional analysis (Table 2).

The compositional data for fraction b clearly show the presence of a differently substituted dihydroxy-4-*n*-NP derivative, whereas no differences were observed by ESI-MS, indicating the presence of an isomeric structure differing only in the position of the OH-groups on the side chain of 4-*n*-NP.

Subjection of fraction c to ESI mass spectrometry afforded a very heterogeneous population of 4-*n*-NP conjugates (Figure 4a). Deprotonated molecular ions were observed at *m/z* 413 [4-*n*-NP + 2O + Glc − H_2O], 453 [4-*n*-NP + 3O + Glc + HAc − $3H_2O$], 455 [4-*n*-NP + 2O + Glc + HAc − $2H_2O$], 499 [4-*n*-NP + 2O + Glc + HMa − $2H_2O$], 575 [4-*n*-NP + 2O + 2Glc − $2H_2O$], 599 [4-NP + 2O + 2Glc + HAc − $4H_2O$], 617 [4-*n*-NP + 2O + 2Glc + HAc − $3H_2O$], 659 [4-*n*-NP + 2O + 2Glc + 2HAc − $4H_2O$], 747 [4-*n*-NP + 2O + 2Glc + 2HMa − $4H_2O$] and 845 [unknown composition]. MS/MS experiments were performed on the most prominent ions (compare Figure 4b). For the metabolites of lower molecular weight a relatively intense fragment corresponding to the free aglycon was observed, while the respective dehydrated fragment had a much lower intensity, suggesting a pre-eminent conjugation of the phenolic OH-group, since only at this position the elimination of the OH-group is unlikely. For metabolites of higher molecular weight, fragments generated by the sequential loss of Glc, HAc, or HMa, respectively, were found. However, no further evidence for an ordered mechanism of the conjugation process was observed; i.e., after the saturation of the phenolic OH-group, all further conjugating residues bind to OH-groups in a random manner. Since their number exceeds the number of OH-groups present on the 4-*n*-NP derivative for the larger molecules, OH-groups of Glc must also be occupied by another Glc residue, acetic or malonic acid, enhancing the number of possible isomers significantly.

Fraction d yielded a dominant molecular ion at *m/z* 573 corresponding to [4-*n*-NP + O + Glc + GlcA − $2H_2O$]. MS/MS experiments confirmed the presence of structures analogous to fraction a, displaying a distinct heterogeneity with respect to the position of the single OH-group on the alkyl side chain of 4-*n*-NP (compare Table 2).

These results confirm and extend the findings of Stolzenberg et al. (1982), who described monohydroxylated and glycosylated metabolites of a hexaethoxylated *p-tert*-octylphenol in barley plants. Generally the results are in accordance with the plant metabolism of other xenobiotics (Cole, 1983; Sandermann, 1994), during which hydroxylations first occur and subsequently conjugations. However, the sequence of hydroxylation and glycosylation in the metabolism of 4-*n*-NP cannot be elucidated from the data presented. The occurrence of free 4-(hydroxy)nonylphenol or a free 4-nonylphenol glucoside cannot be identified from the HPLC analyses. These metabolites would indicate whether the hydroxylation or the glucosylation on the phenolic OH is the first reaction occuring in wheat cell cultures. However, smaller peaks deriving from 4-*n*-nonyl[$U^{14}C$]phenol in the crude extract, possibly due to the presence of one or both of these compounds, are unidentified.

Hydroxylations of aliphatic side chains have been described previously, e.g. in the metabolism of flurenol *n*-butyl ester where the 2′- and the 3′-hydroxybutyl ester were formed (Wotschokowsky, 1972). Glycosylations and malonylations are among the most common conjugation reactions in plant metabolism of xenobiotics (Sandermann, 1994; Schmidt et al., 1994). It cannot be excluded that in the present investigation the occurrence of conjugates with HAc might indicate a loss of CO_2 from a HMa moiety. In contrast to animals, the occurrence of glucuronides in the metabolism of xenobiotics in plants so far has not been described and is reported here for the first time. However, it has been shown that enzymes involved in the metabolism of xenobiotics very often are closely related to enzymes of plant secondary metabolism (Sandermann, 1994). Glucuronides and glucuronosyltransferases in plant phenolic metabolism have been reported, e.g. glucuronides of hydroxycinnammoyl conjugates in cell cultures of Chenopodiaceae and the respective transferase (Bokern et al., 1987, 1991a,b), or flavonoid glucuronides, e.g. of quercetin and luteolin, and the respective enzymes (Möhle et al., 1985; Schulz and Weissenböck, 1988). Thus, the occurrence of glucuronides in plant metabolism of xenobiotics is not surprising. The role of conjuations with malonic acid or glucuronic acid in the metabolism of xenobiotics has been extensively discussed by Sandermann (1994).

ABBREVIATIONS USED

CID, collision-induced dissociation; ESI-MS, electrospray ionization mass spectrometry; GC−MS, gas chro-

matography−mass spectrometry; Glc, glucose; GlcA, glucuronic acid; HAc, acetic acid; HMa, malonic acid; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; 4-*n*-NP, 4-*n*-nonylphenol; 4-NP, 4-nonylphenol; RP-HPLC, reversed phase high-performance liquid chromatography; TLC, thin layer chromatography; U, μmol/min (enzyme activity).

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