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A Novel Metabolic Pathway for Degradation of 4-Nonylphenol Environmental Contaminants by *Sphingomonas xenophaga* Bayram

ipso-HYDROXYLATION AND INTRAMOLECULAR REARRANGEMENT*

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Several nonylphenol isomers with α-quaternary carbon atoms serve as growth substrates for *Sphingomonas xenophaga* Bayram, whereas isomers containing hydrogen atoms at the α-carbon do not (Gabriel, F. L. P., Giger, W., Guenther, K., and Kohler, H.-P. E. (2005) *Appl. Environ. Microbiol.* 71, 1123–1129). Three metabolites of 4-(1-methyl-octyl)-phenol were isolated in mg quantities from cultures of strain Bayram supplemented with the growth substrate isomer 4-(1-ethyl-1,4-dimethyl-pentyl)-phenol. They were unequivocally identified as 4-hydroxy-4-(1-methyl-octyl)-cyclohexa-2,5-dienone, 4-hydroxy-4-(1-methyl-octyl)-cyclohex-2-enone, and 2-(1-methyl-octyl)-benzene-1,4-diol by high pressure liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. Furthermore, two metabolites originating from 4-*n*-nonylphenol were identified as 4-hydroxy-4-nonyl-cyclohexa-2,5-dienone and 4-hydroxy-4-nonyl-cyclohex-2-enone by high pressure liquid chromatography-mass spectrometry. We conclude that nonylphenols were initially hydroxylated at the *ipso*-position forming 4-alkyl-4-hydroxy-cyclohexa-2,5-dienones. Dienones originating from growth substrate nonylphenol isomers underwent a rearrangement that involved a 1,2-C,O shift of the alkyl moiety as a cation to the oxygen atom of the geminal hydroxy group yielding 4-alkoxyphenols, from which the alkyl moieties can be easily detached as alcohols by known mechanisms. Dienones originating from nongrowth substrates did not undergo such a rearrangement because the missing alkyl substituents at the α-carbon atom prevented stabilization of the putative α-carbocation. Instead they accumulated and subsequently underwent side reactions, such as 1,2-C,C shifts and dihydrogenations. The *ipso*-hydroxylation and the proposed 1,2-C,O shift constitute key steps in a novel pathway that enables bacteria to detach α-branched alkyl moieties of alkylphenols for utilization of the aromatic part as a carbon and energy source.

Technical nonylphenol is mainly used for the production of nonylphenol polyethoxylates, which are important nonionic surfactants for industry and agriculture with an annual production volume of approximately 650,000 tons (1). The complex technical mixture consists of at least twenty-two 4-nonylphenol isomers (2–4), which differ in the structure of the nonyl substituent. Most of the 4-nonylphenol found in the environment originates from microbial degradation of nonylphenol polyethoxylate surfactants, and little is known about its further degradation (5). 4-Nonylphenol is toxic for aquatic organisms (6, 7) and acts as an endocrine disruptor in mammals, fish, and other species (8, 9).

The availability of individual pure nonylphenol isomers has triggered several studies on the bacterial metabolism of 4-nonylphenols (10–12). *Sphingomonas xenophaga* Bayram (10), *Sphingomonas* TTNP3 (13), and *Sphingomonas cloacae* (14) were isolated for their ability to grow with 4-nonylphenols as sole carbon and energy source. Sphingomonads, a group of Gram-negative bacteria recognized as a discrete genus for approximately 15 years, are well known for their extraordinary metabolic capabilities to degrade many otherwise recalcitrant chemicals. They are widely distributed in nature and found in various soils and aqueous environments, and representatives have been isolated from deep subsurface sediments (15).

The three nonylphenol metabolizing strains release the nonyl side chains of the growth substrate isomers as C_9 alcohols with unchanged carbon connectivity into the culture fluids (Fig. 1*A*) and most likely do not further metabolize these alcohols (10, 11, 16). However, these bacteria seem to be able to completely mineralize the aromatic moiety of those nonylphenol isomers that serve as growth substrates (10, 11).

Growth experiments with strain Bayram showed that nonylphenol isomers with a quaternary carbon atom served as growth substrates, whereas the isomers containing one or two hydrogen atoms in benzylic position did not (10). However, the latter isomers were cometabolically transformed in experiments in which growth isomers were present. Furthermore, differential degradation was clearly evident because isomers with a more highly branched alkyl side chain were degraded much faster than those with less complex branching (10).

Several bacteria (17, 18) and fungi (19) convert α-secondary 4-alkylphenols to the corresponding α-keto derivatives and then by a Baeyer-Villiger mechanism introduce an oxygen atom between the carbon ring and the acyl moiety. The resulting esters are easily cleaved by a hydrolytic mechanism, whereby the original alkyl moiety leaves as an alkanoic acid (Fig. 1*B*). Alkylphenols are also degraded through *meta*-fission pathways in which the aromatic ring is opened by

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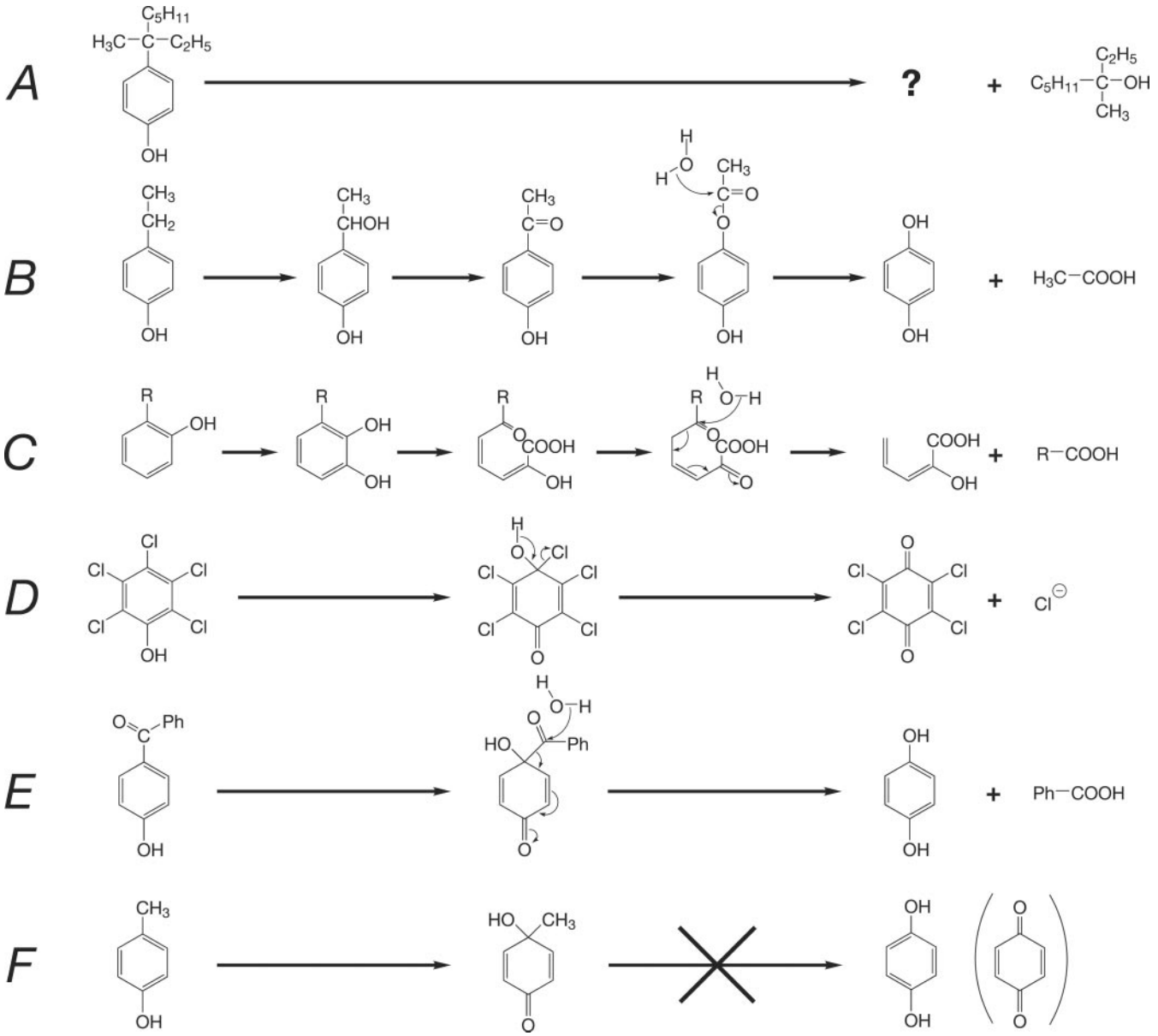

FIG. 1. **Biochemical strategies for detaching substituents in substituted phenols.** *A*, degradation of α-quaternary nonylphenols by *Sphingomonas* strains (10, 11, 16). *B*, transformation of the α-methylen group to a keto group, Baeyer-Villiger reaction, and hydrolysis (17, 19). *C*, *meta*-cleavage pathway for 2-alkylphenols (*R* = propyl, isopropyl, 2-*sec*-butyl) (20–22). In principle, dihydroxylation of 4-alkylphenols to 4-alkylpyrogallols would channel the substrate toward the same pathway. *D*, oxidative dehalogenation by *ipso*-substitution (27). *E*, proposed mechanism for *ipso*-substitution of *p*-benzoylphenol by rat liver microsomes (23). *F*, *ipso*-attack of *p*-cresol by rat liver microsomes (23).

dioxygenases before the *meta*-cleavage product is hydrolyzed to 2-hydroxypent-2,4-dienoic acid and an alkanoic acid derived from the original alkyl side chain and the ring anchor carbon (20–22) (Fig. 1*C*). However, a pathway for the metabolism of alkylphenols through detachment of the alkyl substituent as an alcohol (Fig. 1*A*) has not yet been reported.

Although never shown for the detachment of complex alkyl groups, *ipso*-substitution represents a general method by which bacteria and rat liver microsomes oxidatively detach substituents, such as halogen and nitro groups in *ortho*- or *para*-substituted phenols (23–27). *ipso*-Substitution starts with the hydroxylation of the ring anchor carbon, to which the substituent is attached (*ipso*-attack) (Fig. 1, *D* and *E*). The substituent is then eliminated either as an anion or as a cation; depending on whether the ring-substituent bond electrons in the cyclohexadienone derivative remain with the substituent or with the carbon ring, *p*-benzoquinone or hydroquinone is formed (23) (Fig. 1, *D* and *E*, respectively). This article describes the bacterial metabolism of several 4-nonylphenol isomers. Intermediates identified by NMR spectroscopy and mass spectrometry point to a novel pathway based on *ipso*-hydroxylation.

EXPERIMENTAL PROCEDURES

4-Nonylphenol Isomers—The acronyms for the various 4-nonylphenol isomers (Fig. 2) used in this study are based on the systematic numbering system proposed by Guenther and co-workers.[1] The source and the purity of the nonylphenol isomers utilized in this study has been described elsewhere (10).

Media and Growth Conditions—The preparation of LB and minimal media and the set up of the degradation experiments with strain Bayram have been described in detail elsewhere (10). Because nonylphenols are poorly soluble in aqueous medium, it was necessary to work up a whole culture vial for each time point measured. To study cometabolic degradation, several culture vials containing each 3 ml of minimal

[1] K. Guenther, E. Kleist, and B. Thiele, manuscript in preparation.

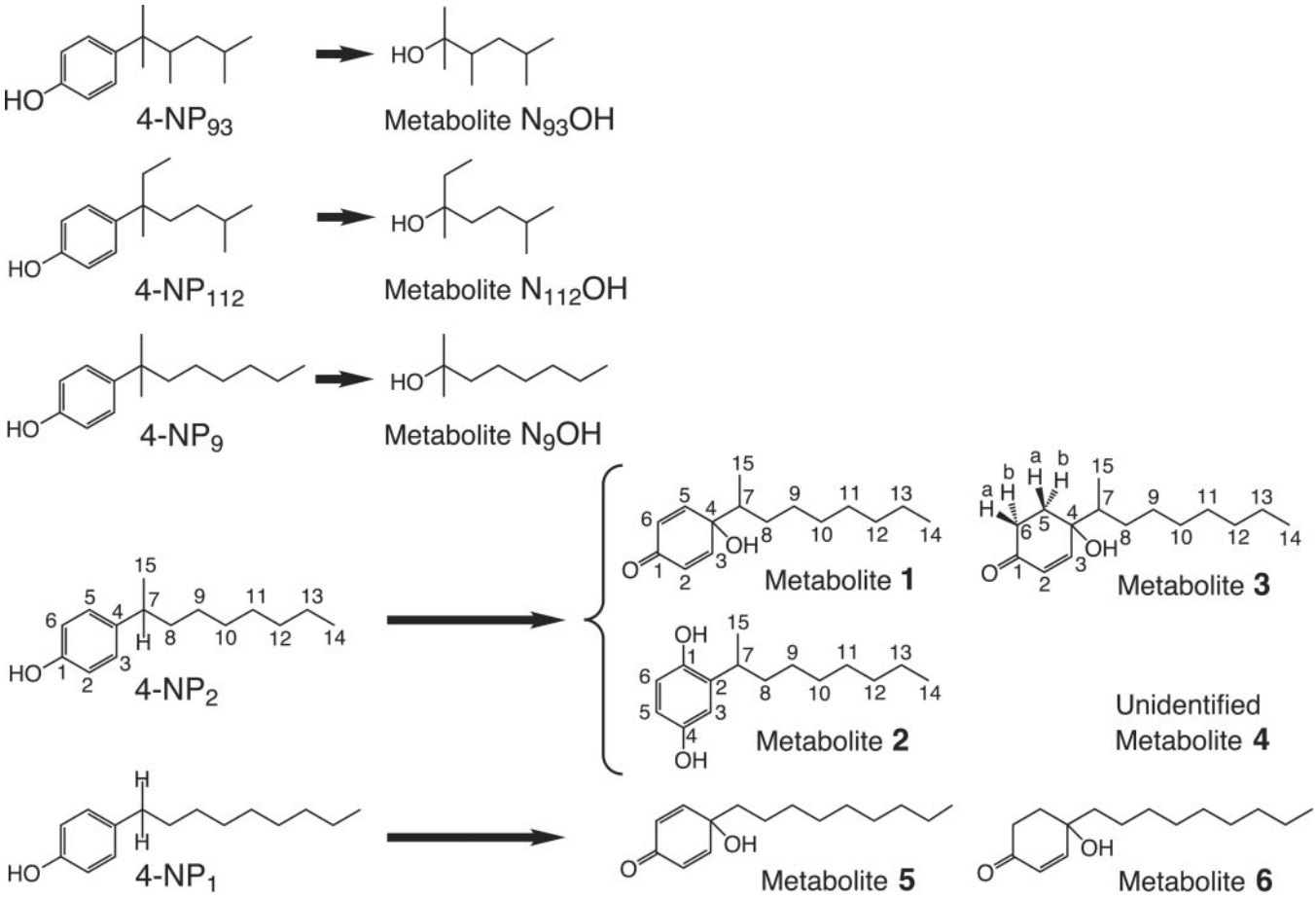

FIG. 2. **Metabolites formed during cometabolic degradation of 4-NP_2 and 4-NP_1 with growth substrate nonylphenols (the degradation of the isomers 4-NP_{93} and 4-NP_9 has been reported elsewhere (10)).** The atom numbering is according to that used for the assignments of chemical shifts. Both enantiomers of 4-NP_{112} are completely degraded, but questions with regard to enantioselectivity have not been addressed yet.

medium and a mixture of 4-NP_2 (or 4-NP_1)[2] and 4-NP_{112} (3 mg each) were inoculated and incubated for varying periods of time. Appropriate controls were included for all experiments. Inoculation was performed with 100 μl of a shaking culture of *S. xenophaga* Bayram, produced by maintaining the bacterium on minimal medium (3 ml) with 3 mg of technical nonylphenol or 4-NP_{112} as the sole carbon source.

Extraction of Nonylphenols and Metabolites from the Culture Vials—For work up, the frozen samples were thawed, and nonylphenols contained in the liquid cultures and sticking on the wall of the culture vials were extracted twice with 2 and 66 ml (preparative scale) , respectively, of CH_2Cl_2. Except for the experiment on the preparative scale, the cultures were acidified with 300 μl of 1 N HCl, prior to the extraction.

Analytical and Preparative HPLC—Dried culture extracts were analyzed as described elsewhere (detection wavelength of 277 nm) (10). To isolate metabolites, the extracts were separated by preparative reverse phase chromatography using a C18 column (VP 250/21 Nucleosil 7 C18, Macherey-Nagel) and isocratic elution conditions (CH_3CN/H_2O 7:3, 10 ml/min).

Mass Spectrometry—To detect C_9 alcohols derived from alkyl chains of nonylphenols, the CH_2Cl_2 extracts of the cultures were analyzed by gas chromatography-electron impact-MS, as described elsewhere (10). HPLC-TIS-MS and -MS/MS were used in the negative mode to get structural information about the UV active nonylphenol metabolites produced by the cometabolic transformation of 4-NP_2 and of 4-NP_1 (experiments with 3 mg of substrate). For injection, the samples were taken up in appropriate volumes of isopropanol. We used an HPLC system (Hewlett Packard 1100 series) that was connected to a triple quadrupole mass spectrometer through a Turbo Ion Spray interface (API 4000 liquid chromatography-MS/MS system; Applied Biosystems). The Analyst 1.3 software (Applied Biosystems, MDS Sciex) was utilized. The HPLC system was composed of an autosampler, a binary high pressure gradient pump, and a UV-visible detector and was equipped with a column oven and an on-line Vacuum degasser (DG4; Henggeler Analytic Instruments, Riehen, Switzerland). The eluents were prepared with HPLC grade CH_3OH (Multisolvent; Scharlau, Barcelona, Spain) and ultrapure H_2O (Milli-Q gradient A10; Switzerland Millipore AG, Volketswil). The HPLC conditions were identical to those for analytical HPLC-UV, except that the solvents were alkalinized with 1 μl of concentrated NH_3/100 ml to get higher signal intensities. The oven was set at 23 °C, and the eluate was monitored at 230 nm. For the Turbo Ion Spray ionization, nitrogen was used as curtain, nebulizer, and desolvation gas. The source temperature was set at 400 °C, and the ion spray voltage was set at −4500 V. Intensive signals were produced in MS spectra with a declustering potential of −30 V and an entrance potential of −12 V. Full scan spectra were acquired in the negative mode, scanning from 20 to 600 m/z. To obtain MS/MS spectra, a declustering potential of −30 to −20 V and an entrance potential of −12 to −10 V were used, depending on the time period window. Nitrogen was used as the collision gas, and a collision energy of −30 eV was selected. Full scan MS/MS spectra were acquired scanning from 20 to (i + 5) m/z, i being the m/z value of the mother ion. In the MS spectra, the $[M - H]^-$ peaks were identified and distinguished from cluster signals by referring to the unique $[(M)_2 - H]^-$ peaks.

Nuclear Magnetic Resonance—The ^1H and ^{13}C NMR spectra were recorded at 400.13 (100.61) MHz on a Bruker Avance-400 NMR spectrometer. The one-dimensional ^1H and ^{13}C NMR spectra and the $^1H,^1H$ and $^1H,^{13}C$ two-dimensional correlation experiments were performed using a 5-mm broadband inverse probe with z-gradient (100% gradient strength of 10 G cm^{-1}) and 90° pulse lengths of 6.5 μs (^1H) and 11.0 μs (^{13}C). The chemical shifts were referenced internally using the resonance signals of CHD_2CN at 1.93 (^1H) and of CD_3CN at 1.3 (^{13}C) ppm. The gradient-selected HSQC (HMBC) (28, 29) experiments were per-

[2] The abbreviations used are: NP, nonylphenol; HPLC, high pressure liquid chromatography; TIS, turbo ion spray; MS, mass spectrometry; $[M - H]^-$, quasimolecular ion; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; TOCSY, total correlation spectroscopy.

formed with selection of $^{1}H,^{13}C$ coupling constants of 145 (10) Hz, with gradient strengths of 80:20.1:11:−5 (50:30:40.1), applying a carbon decoupling field of 3.2 kHz for the HSQC experiments (globally optimized alternating phase rectangular pulses decoupling) (30). The HSQC-TOCSY (31) spectra were recorded applying mixing times of 100 or 20 ms, respectively. To record the NMR spectra, 42.0 mg of 4-NP_2, 9.1 mg of 2-(1-methyl-octyl)-benzene-1,4-diol (metabolite 2), and 22.0 mg of a mixture of 4-hydroxy-4-(1-methyl-octyl)-cylohexa-2,5-dienone (metabolite 1) and 4-hydroxy-4-(1-methyl-octyl)-cyclohex-2-enone (metabolite 3) were dissolved in CD_3CN (final volume, 600 μl), respectively.

RESULTS

Cometabolic Transformation of 4-NP_2—A degradation experiment with the growth substrate 4-NP_{112} (70% transformation) showed that within 9 days of incubation, 39% of the nongrowth substrate 4-NP_2 was cometabolically transformed. Thereafter, between days 9 and 24 degradation slowed down to only 12 and 15% of the initial amounts of 4-NP_2 and 4-NP_{112}, respectively. Substantial amounts of 3,6-dimethyl-heptan-3-ol ($N_{112}OH$), the C_9 alcohol derived from the alkyl chain of 4-NP_{112}, were detected by gas chromatography-MS in the CH_2Cl_2 extracts of cultures that were incubated for 5 and 9 days, respectively. In contrast, no C_9 alcohol derived from 4-NP_2 was detectable. However, four metabolites, each with a characteristic UV spectrum, were found by HPLC-UV (Table I). The most polar compound, metabolite 2 (retention time, 11.8 min), had a UV absorption maximum at a higher wavelength (291 nm) than that of nonylphenol isomers (~277 nm; Table I). The metabolites 1 and 3, eluting at 15.0 and 15.5 min, were not completely separable and had absorption maxima at 232 and 221 nm, respectively. Metabolite 4 (retention time, 24.9 min), which has not yet been identified, eluted between 4-NP_{112} (retention time, 23.4 min) and 4-NP_2 (retention time, 27.0 min).

Isolation of Metabolites 1, 2, and 3—To isolate the UV active metabolites in sufficient amounts for structure determination, a degradation experiment with 100 mg of both 4-NP_2 and 4-NP_{112} in 100 ml of minimal medium was carried out. Preparative HPLC of the CH_2Cl_2 extract of a culture incubated for 9 days yielded 9.2 mg of metabolite 2 (8.4 mol-%), 22.3 mg of a mixture of metabolite 1 (9.1%) and metabolite 3 (11.2%) (metabolite 1 and metabolite 3 were collected in the same fraction; their relative amounts were determined by comparing relative signal intensities in the ^{1}H NMR spectra), and 2.2 mg of a fraction containing mainly metabolite 4.

TIS-MS/MS Spectra of Nonylphenol Isomers—For the correct interpretation of the TIS-MS/MS spectra of the four metabolites, it was necessary to carefully analyze the spectra of the 4-nonylphenol isomers that served as growth and nongrowth substrates. Under MS/MS conditions, the isomers 4-NP_{112}, 4-NP_2, and 4-NP_1 produced prominent signals at *m/z* 219 for the quasimolecular ions ($[M - H]^-$) with relative intensities of 100, 100, and 90%, respectively. The three nonylphenols showed characteristic spectra that were dominated by signals corresponding to the homolytic cleavage of the benzylic substituents and the subsequent loss of hydrogen atoms or alkyl groups in β-position, thereby yielding nonradical, α,β-unsaturated anions, for which usually more intensive signals were observed (Table I).

NMR Analysis of 4-NP_2—The ^{1}H and ^{13}C chemical shifts of the substrate 4-NP_2 and metabolites 1, 2, and 3 were assigned by one- and two-dimensional NMR experiments (Table II). The following analysis of the NMR data of 4-NP_2 was a prerequisite for the correct elucidation of the structures of the 4-NP_2-derived metabolites. The $^{1}H,^{13}C$-HMBC correlations used for the chemical shift assignments are shown in Table III, together with the resolved $^{1}H,^{1}H$ coupling constants. By analyzing the $^{1}H,^{13}C$-HSQC and -HMBC spectra (correlations over one and 2−3 H–C bonds, respectively), the framework comprising the carbons and protons at positions 1–8 and 15 could be easily reconstituted in all instances. On the other hand, the interpretation and adequate assignment were not always straightforward for the positions 9–14. Because of the overlapping of signals situated ~1.25 ppm (^{1}H) and 30 ppm (^{13}C), the expected correlation signals were sometimes hidden beyond dominant cross-peaks; also, the differences in chemical shifts of successive atoms in the chain were at times so small that not enough resolution was achieved for a confident assignment of all peaks. In the case of 4-NP_2, the assignment of the carbon atoms at positions 10–12 to the ^{13}C resonances at 30.0, 30.4, and 32.6 ppm remained unclear. We therefore performed HSQC-TOCSY experiments (31), using mixing times of 20 and 100 ms. Fig. 3 shows expanded regions with the correlation signals between the methyl protons H-14 of 4-NP_2 and the neighboring carbon atoms for both mixing times. With the long period, the magnetization was transferred to the whole carbon skeleton (C-(9–14); Fig. 3*b*). In contrast, the short mixing allowed a transfer only to the carbons at 14.4 (C-14), 23.4 (C-13), 32.6 ppm (C-12), and 30.0 ppm, the latter correlation being rather weak. The ^{13}C signal at 30.0 ppm could thus be assigned to C-11. The ^{13}C chemical shifts we determined for the carbon atoms of the alkyl chain corresponded well to those evaluated by a "chemical shift increment calculation" (32) (30.6, 30.0, 32.5, 23.1, and 14.0 ppm for C-10 through C-14).

Identification of Metabolite 2 as 2-(1-Methyl-octyl)-benzene-1,4-diol—The UV spectrum of metabolite 2 showed a characteristic absorption maximum at the same wavelength (291 nm) as that of 2-methyl-benzene-1,4-diol (33). This gave a first indication for a 2-alkyl-benzene-1,4-diol structure of metabolite 2. Analysis of the HPLC mass spectrometry data gave further evidence for the proposed structure because the $[(M)_2 - H]^-$ signal at *m/z* 471.9 (Table I) showed that metabolite 2 contained an additional oxygen atom when compared with nonylphenol. When the quasimolecular ion $[M - H]^-$ was submitted to MS/MS conditions (Table I), the fragments resulting from the cleavage of the benzylic bonds proved the presence of a 1-methyl-octyl substituent, suggesting that metabolite 2 was derived from 4-NP_2 and was identical with 2-(1-methyl-octyl)-benzene-1,4-diol (Fig. 2). Indeed, the MS/MS spectra of metabolite 2 and 4-NP_2 both showed intense signals corresponding to the cleavage of $^{\cdot}C_7H_{15}$ (*m/z* 136, 68% and *m/z* 120, 17%, respectively) and $^{\cdot}C_7H_{15}$ + $^{\cdot}H$ (*m/z* 135, 100% and *m/z* 119, 58%). Smaller signals corresponded to the abstraction of $^{\cdot}CH_3$ (*m/z* 220, 6% and *m/z* 204, 1%) and $^{\cdot}CH_3$ + $^{\cdot}H$ (*m/z* 219, 3% and *m/z* 203, 3%). The loss of $^{\cdot}C_9H_{19}$ was observed in the mass spectrum of metabolite 2 (*m/z* 108, 29%) but not in that of 4-NP_2; the presence of the additional oxygen atom in *ortho* position of metabolite 2 probably reduced the stability of the aryl-alkyl bond.

The proposed structure of metabolite 2 (Fig. 2) was confirmed by NMR analysis. The interpretation of the NMR data was done in analogy to that of the data for 4-NP_2 (Tables II and III). The correlations of H-3 to C-(1,5,7), which were observed in the HMBC spectrum, were used to prove that the alkyl residue was connected to the aromatic carbon atom at 136.0 ppm (C-2).

Identification of Metabolites 1 and 3 as 4-Hydroxy-4-(1-methyl-octyl)-cyclohexa-2,5-dienone and 4-Hydroxy-4-(1-methyl-octyl)-cyclohex-2-enone, Respectively—Because metabolites 1 and 3 could not be completely base line-separated, the HPLC-TIS-MS spectra were recorded at the left-hand and the right-hand slopes of the two chromatographic peaks, respectively; these spectra did not interfere with each other. The signals at *m/z* of 471.8 (2.4%) and 235.3 (100%) in the TIS-MS spectrum of metabolite 1 (Table I) were strong indicators for the presence of

TABLE I

HPLC-UV, -TIS-MS, and -TIS-MS/MS analysis of nonylphenols and UV active metabolites

The UV active metabolites have been listed according to their retention time. The unidentified metabolite 4 is not represented.

Compound (acronym)	Compound	Retention time	UV absorption maxima	TIS-MS analysis (*m/z* of $[(M)_2 - H]^-$ and $[M - H]^-$)[a]	TIS-MS/MS analysis[b]	
					m/z of daughter ions of $[(M)_2 - H]^-$	*m/z* of daughter ions of $[M - H]^-$
		min	*nm*			
4-NP_{112}	4-(1-ethyl-1,4-dimethyl-pentyl)-phenol	23.4	277, 224, 203	439 (3.6); 219 (100)	219 (100, $[M - H]^-$).	219 (100, $[M - H]^-$); 190 (3.7, $[M - H]^- - \cdot C_2H_5$); 189 (11, $[M - H]^- - \cdot C_2H_5 - \cdot H$); 148 (12, $[M - H]^- - \cdot C_5H_{11}$); 147 (19, $[M - H]^- - \cdot C_5H_{11} - \cdot H$); 133 (93, $[M - H]^- - \cdot C_5H_{11} - \cdot CH_3$).
4-NP_2	4-(1-methyl-octyl)-phenol	27.0	278, 224, 204	439 (4.0); 219 (100)	219 (100, $[M - H]^-$).	219 (100, $[M - H]^-$); 204 (0.9, $[M - H]^- - \cdot CH_3$); 203 (2.7, $[M - H]^- - \cdot CH_3 - \cdot H$); 121 (1.0); 120 (17, $[M - H]^- - \cdot C_7H_{15}$); 119 (58, $[M - H]^- - \cdot C_7H_{15} - \cdot H$).
4-NP_1	4-*n*-nonylphenol	28.5	279, 225, 206 *(sh)*[c]	439 (1.7); 219 (100)		219 (90, $[M - H]^-$); 107 (2.7); 106 (100, $[M - H]^- - \cdot C_8H_{17}$).
Metabolite 2	2-(1-methyl-octyl)-benzene-1,4-diol	11.8	292, 216 (*sh*), 203	471 (53); 235 (22)	236 (74); 235 (100, $[M - H]^-$).	235 (99, $[M - H]^-$); 234 (54); 233 (1.1); 220 (6.4, $[M - H]^- - \cdot CH_3$); 219 (3.2, $[M - H]^- - \cdot CH_3 - \cdot H$); 150 (1.9); 137 (1.6); 136 (68, $[M - H]^- - \cdot C_7H_{15}$); 135 (100, $[M - H]^- - \cdot C_7H_{15} - \cdot H$); 134 (9.4); 122 (1.2); 121 (2.6); 108 (29, $[M - H]^- - \cdot C_9H_{19}$); 107 (6.0).
Metabolite 1	4-(1-methyl-octyl)-4-hydroxy-cyclohexa-2,5-dienone	15.0	232	471 (2.4); 235 (100)		235 (0.33, $[M - H]^-$); 234 (0.10); 220 (0.18, $[M - H]^- - \cdot CH_3$); 136 (1.3, $[M - H]^- - \cdot C_7H_{15}$); 135 (1.0, $[M - H]^- - \cdot C_7H_{15} - \cdot H$); 134 (0.12); 109 (0.11); 108 (100, $[M - H]^- - \cdot C_9H_{19}$); 81 (0.1).
Metabolite 3	4-(1-methyl-octyl)-4-hydroxy-cyclohex-2-enone	15.5	221	475 (4.9); 237 (100)	238 (34); 237 (19, $[M - H]^-$); 111 (6.7); 110 (100, $[M - H]^- - \cdot C_9H_{19}$).	237 (1.2, $[M - H]^-$); 219 (0.9, $[M - H]^- - H_2O$); 209 (1.5, $[M - H]^- - CO$); 207 (4.5, $[M - H]^- - CO - 2 \cdot H$); 205 (1.2); 111 (3.6); 110 (100, $[M - H]^- - \cdot C_9H_{19}$); 109 (1.8); 108 (12, $[M - H]^- - \cdot C_9H_{19} - 2 \cdot H$); 83 (2.0); 81 (2.1); 80 (1.9).
Metabolite 5	4-nonyl-4-hydroxy-cyclohexa-2,5-dienone	16.2	230	471 (3.5); 235 (100)	236 (5.9); 235 (7.9, $[M - H]^-$); 109 (15); 108 (100, $[M - H]^- - \cdot C_9H_{19}$).	235 (1.2, $[M - H]^-$); 234 (1.0); 122 (2.3, $[M - H]^- - \cdot C_8H_{17}$); 121 (1.8); 109 (1.7); 108 (100, $[M - H]^- - \cdot C_9H_{19}$).
Metabolite 6	4-nonyl-4-hydroxy-cyclohex-2-enone	16.7	221	475 (1.3); 237 (60)	238 (65); 237 (93, $[M - H]^-$); 235 (1.4); 111 (4.9); 110 (100, $[M - H]^- - \cdot C_9H_{19}$); 109 (3.0).	237 (1.1, $[M - H]^-$); 219 (4.4, $[M - H]^- - H_2O$); 209 (1.3, $[M - H]^- - CO$); 207 (6.6, $[M - H]^- - CO - 2 \cdot H$); 205 (1.0); 181 (1.8); 111 (5.1); 110 (100, $[M - H]^- - \cdot C_9H_{19}$); 109 (3.4); 108 (15, $[M - H]^- - \cdot C_9H_{19} - 2 \cdot H$); 84 (1.4); 83 (1.5); 81 (4.0); 80 (1.6).

[a] The two TIS-MS signals that are relevant for the determination of the molecular weight of the analytes are presented (for clarity, TIS-MS signals of clusters are not shown). Relative abundances as a percentage and interpretations are given in parentheses.

[b] The most prominent TIS-MS/MS signals are presented. Relative abundances as a percentage and interpretations are given in parentheses.

[c] Shoulder.

an additional oxygen atom when compared with the parent substrate 4-NP_2, suggesting that this metabolite was a monooxygenation product of 4-NP_2. The UV spectrum of metabolite 1 with its single absorption maximum at λ_{max} of 232 nm indicated that the compound was not aromatic. In the fragmentation spectrum of $[M - H]^-$, the base peak at *m/z* 108 was the only signal above 1.3% intensity. The base peak corresponded to an ion generated by homolytic cleavage of the entire alkyl group ($[M - H]^- - \cdot C_9H_{19}$), implying that the introduced oxygen atom profoundly effected the stability of the alkyl-carbon ring bond. The daughter ion, a carbon ring radical, was stable and did not yield fragment ions under MS/MS conditions. Furthermore, the set of signals with relatively small intensities at *m/z* 220, 136, and 135 (Table I) revealed that metabolite 1 contained a 1-methyl-1-octyl moiety. On the basis of these data, we propose metabolite 1 to be identical with 4-hydroxy-4-(1-methyl-octyl)-cyclohexa-2,5-dienone.

The quasimolecular ion of metabolite 3 at *m/z* 237 (Table I) indicated the presence of two additional hydrogen atoms when compared with that of metabolite 1, at *m/z* 235. Metabolites 3 and 1 showed a similar fragmentation behavior; base peaks at *m/z* 110 and 108 dominated their TIS-MS/MS spectra (Table I). Because the ion at *m/z* 108 corresponded to the carbon ring moiety of metabolite 1, we concluded by analogy that the two supplementary hydrogen atoms in metabolite 3 should be located on the carbon ring. This conclusion was supported by the UV spectrum of metabolite 3, which in comparison with the spectrum of metabolite 1 showed a shift of the absorption maximum to a lower wavelength (221 nm), indicating the absence of the cross-conjugated double-bond system of metabolite 1. We hence proposed that metabolite 3 was identical to 4-hydroxy-4-(1-methyl-octyl)-cyclohex-2-enone (Fig. 2).

Because the metabolites 1 and 3 could not be completely separated by preparative HPLC, a fraction containing both

TABLE II
^1H and ^{13}C chemical shifts of 4-NP_2, 1, 2, and 3

Position[a]	δ							
	^1H				^{13}C[b]			
	4-NP_2	Metabolite 1	Metabolite 2	Metabolite 3	4-NP_2	Metabolite 1	Metabolite 2	Metabolite 3
	ppm							
1					155.8	186.8	148.2	200.2
2	6.71	6.11		5.82	115.9	129.2	136.0	129.0
3	7.01	6.78	6.56	6.75	128.8	152.1	114.6	155.2
4					140.1	72.9	151.2	72.4
5	7.01	6.80	6.43	1.83/2.10	128.8	152.4	113.6	31.1
6	6.71	6.09	6.58	2.28/2.57	115.9	128.8	116.7	34.5
7	2.59	1.69	3.01	1.62	39.8	42.6	32.8	43.0
8	1.50	0.89/1.65	1.4–1.6	1.05/1.65	39.3	31.5	37.7	31.0
9	1.11/1.22	1.19/1.41	1.14/1.24	1.19/1.43	28.5	28.7	28.4	28.8
10	1.23	1.27	1.23	1.28	30.4	30.4	30.4	30.6
11	1.22	1.26	1.23	1.28	30.0	29.96	30.0	30.03
12	1.22	1.26	1.23	1.26	32.6	32.58	32.6	32.64
13	1.25	1.28	1.25	1.28	23.4	23.38	23.4	23.39
14	0.86	0.88	0.86	0.88	14.4	14.4	14.4	14.4
15	1.15	0.86	1.11	0.88	23.0	14.1	21.4	14.7

[a] For atom numbers, see Fig. 2.
[b] The compounds 1 and 3 were analyzed as a mixture. Therefore, some of the ^{13}C chemical shifts are given with two decimals.

TABLE III
$^1H,^{13}C$ HMBC correlations used for NMR shift assignments in Table II and resolved $^1H,^1H$ coupling constants

Compound	HMBC correlations (w = weak)	$^1J(H,H)$
		Hz
4-NP_2	OH → C-(1, 2, 6); H-(2,6) → C-(1, 2, 4, 6); H-(3, 5) → C-(1, 2, 3, 5, 6, 7); H-(7) → C-(3, 4, 5, 8, 9, 15); H-(8) → C-(4, 9, 10, 15); H-(9) → C-(10); H-(13) → C-(12, 14); H-(14) → C-(12, 13); H-(15) → C-(4, 7, 8)	J(2,3) = 8.6; J(2,5) = 0.6; J(2,6) = 2.5; J(7,15) = 7.0; J(13,14) = 7.0
Metabolite 1	H-(2) → C-(1 (w), 4, 6); H-(3) → C-(1, 5, 6 (w), 7); H-(5) → C-(1, 2 (w), 3, 4 (w), 7); H-(6) → C-(1 (w), 2, 4); H-(7) → C-(3, 4, 5, 15); H-(8) → C-(9); H-(15) → C-(4, 7, 8)	J(7,15) = 6.6
Metabolite 2	H-(3) → C-(1, 4 (w), 5, 7); H-(5) → C-(1, 3, 4 (w)); H-(6) → C-(1, 2, 4); H-(7) → C-(1, 2, 3, 8, 9 (w), 15); H-(8) → C-(2, 9, 10, 15); H-(14) → C-(12, 13); H-(15) → C-(2, 7, 8)	J(3,5) = 3.0; J(5,6) = 8.5; J(7,15) = 7.0; J(13,14) = 7.0
Metabolite 3	H-(2) → C-(1 (w), 3 (w), 4, 6); H-(3) → C-(1, 4 (w), 5, 7); H-(5) → C-(1, 3, 4, 7, 6); H-(6) →C-(1, 2, 4, 5); H-(7) → C-(3, 4, 15); H-(8) → C-(7, 9); H-(15) → C-(4, 7, 8)	J(2,3) = 10.2; J(2,6b) = 0.6; J(3,5a) = 1.4; J(5a,5b) = 13.7; J(5a,6a) = 5.4; J(5a,6b) = 6.2; J(5b,6a) = 10.6; J(5b,6b) = 4.7; J(6a,6b) = 17.0; J(7,15) = 7.2

compounds with relative amounts of 45 and 55%, respectively, was used to perform the one- and two-dimensional NMR measurements. The chemical shift assignments for the framework comprising the ring moiety and the neighboring carbons at positions 7, 8, and 15 were established by means of HSQC and HMBC correlation experiments (Tables II and III). For metabolite 3, the $^1H,^1H$ coupling patterns of the protons at positions 5a, 5b, 6a, and 6b could be completely resolved (Table III; stereochemistry of metabolite 3 as shown in Fig. 2), whereas the relative configuration at C-4 remained unclear. Because two sets of ^1H and ^{13}C NMR signals were superimposed in the aliphatic regions (metabolites 1 and 3 formed nearly a one to one mixture), the chemical shifts of the alkyl moieties could not be assigned straightforwardly. In the HSQC-TOCSY experiment, the resonances at 1.05 ppm from one of the protons at position 8 of metabolite 3 did not overlap with other signals. The observed correlations to the carbons at 14.7, 28.8, 30.03, 30.6, 31.0, and 32.64 ppm were assigned to C-(15,9,11,10,8,12) by chemical shift arguments. Likewise, the proton pair attached to C-9 of metabolite 1 revealed correlations to the carbons at positions 8, 9, 10, 12, and 15 (assignments similar to above). Obviously, the ^1H and ^{13}C NMR chemical shifts of side chain protons and carbons in positions far enough from the six-membered ring did not vary considerably between different compounds (compare positions 9–14 of 4-NP_2, metabolites 1, 2, and 3; Table II).

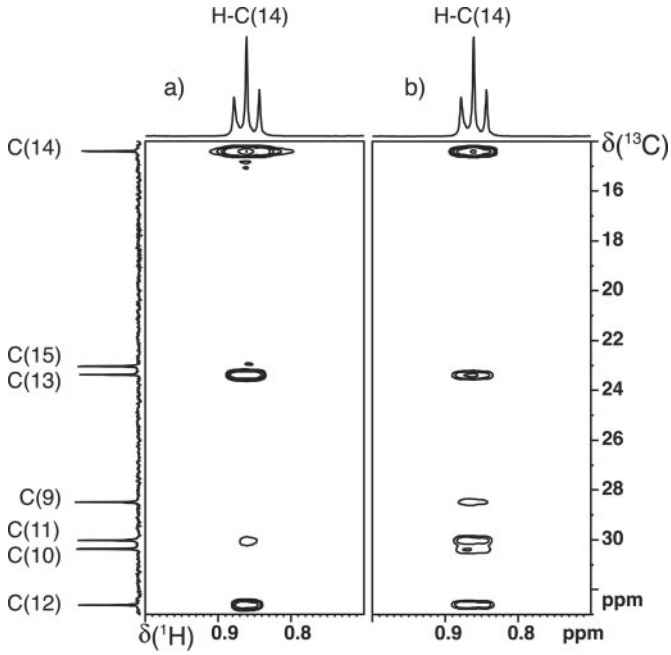

FIG. 3. **Two-dimensional HSQC-TOCSY spectra of 4-NP_2 with mixing times of 20 (*a*) and 100 ms (*b*).** The corresponding one-dimensional ^1H and ^{13}C NMR spectra are shown as projections.

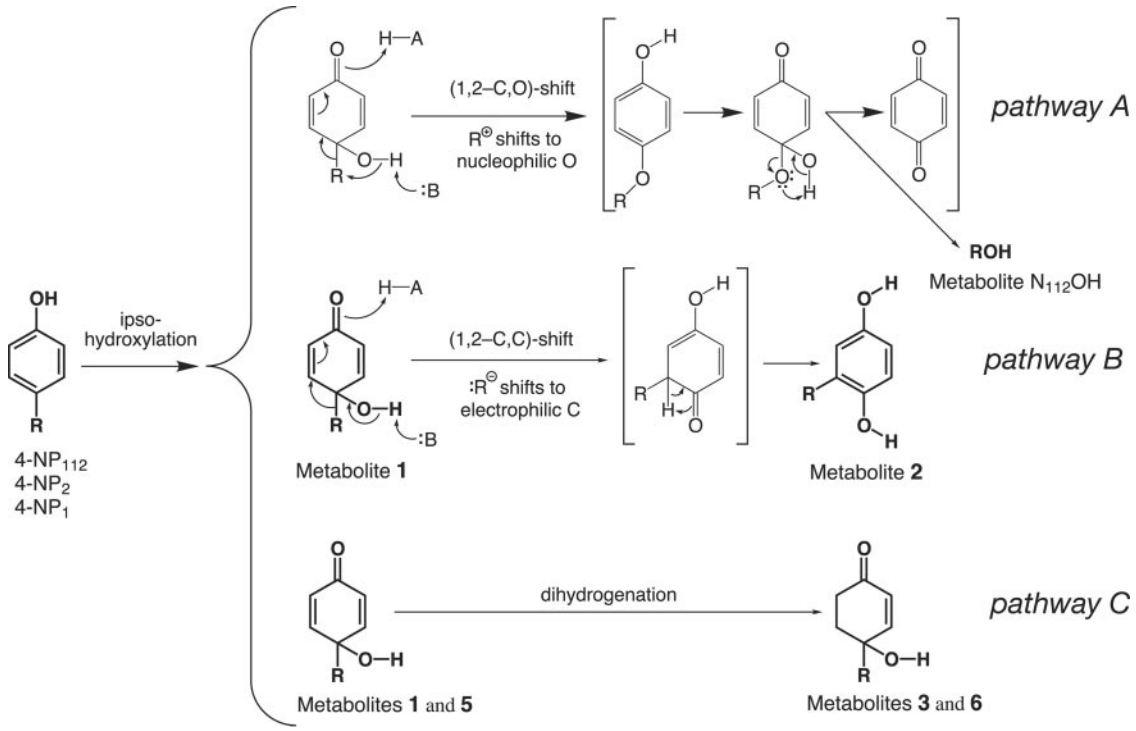

FIG. 4. **Proposed scheme for the degradation of nonylphenol isomers by strain Bayram.** *R* = alkyl moiety; the exact structures of the substrates and the identified metabolites (*bold type*) are depicted in Fig. 2. *H-A* and *:B* symbolize proton donors and acceptors, respectively. All of the isomers are initially *ipso*-hydroxylated to 4-alkyl-4-hydroxy-cyclohexa-2,5-dienones. The dienone derived from the growth substrate isomer 4-NP_{112} undergoes a rearrangement, in which the alkyl moiety shifts as a cation to the oxygen atom of the geminal hydroxy group (*pathway A*), forming a 4-alkoxyphenol as putative intermediate, from which the alkyl moiety can be easily detached as an alcohol by known mechanisms (36) (the dienones derived from the growth substrate isomers 4-NP_{93} and 4-NP_9 are expected to proceed accordingly). In the case of the isomers with benzylic hydrogens (4-NP_2 and 4-NP_1), the α-carbocation is unsufficiently stabilized by α-alkyl branching and the 1,2-C,O shift does not occur; the corresponding cyclohexadienones accumulate and undergo side reactions, a 1,2-C,C shift (NIH shift) and the dihydrogenation of a ring double bond (4-NP_2 follows *pathways B* and *C*, whereas 4-NP_1 follows *pathway C*).

Cometabolic Transformation of 4-NP_1 to Metabolite 5 (4-Hydroxy-4-nonyl-cyclohexa-2,5-dienone) and Metabolite 6 (4-Hydroxy-4-nonyl-cyclohex-2-enone)—In a further degradation experiment with strain Bayram, the linear nonylphenol isomer 4-NP_1 was cometabolically transformed when 4-NP_{112} served as the growth substrate. In this experiment 53% of the growth substrate was consumed within 9 days, whereas 20% of 4-NP_1 transformed within the same period of time. Gas chromatography-MS analysis of the culture extract revealed the presence of 3,6-dimethyl-heptan-3-ol, the C_9 alcohol derived from the growth substrate, whereas no traces of nonan-1-ol, the potential C_9 alcohol product of the cosubstrate, could be detected. This was a strong indication for the accumulation of intermediary metabolites during the experiment. Indeed, HPLC-UV analysis revealed the presence of two metabolites, metabolites 5 and 6, with properties similar to those of metabolites 1 and 3, respectively. Metabolite 5 derived from the cosubstrate 4-NP_1, and metabolite 1 derived from the incubation with the cosubstrate 4-NP_2 had identical UV spectra (λ_{max} = 232 nm) and the same molecular weight (quasimolecular ions at *m/z* 235; Table I); the same was true for metabolites 6 and 3 (λ_{max} = 221 nm, quasimolecular ions at *m/z* 237). Overall, the identity of the UV spectra and a detailed analysis of the MS data (Table I) lead us to the conclusion that metabolites 5 and 6 were 4-nonyl analogs of metabolites 1 and 3 and were identical to 4-hydroxy-4-nonyl-cyclohexa-2,5-dienone and 4-hydroxy-4-nonyl-cyclohex-2-enone, respectively.

DISCUSSION

Recent investigations of bacterial metabolism of 4-nonylphenol and 4-octylphenol (10–12, 34) showed that the alkyl moieties of the alkylphenol growth substrates were transformed to alkyl alcohols with retention of the structures of the original alkyl side chains. As outlined in the Introduction, conventional pathways of alkylphenol metabolism cannot explain the formation of such metabolites. Because we had available several pure 4-nonylphenol isomers, we planned to identify intermediates of the degradation pathway when strain Bayram grew on the 4-nonylphenol isomers 4-NP_{93}, 4-NP_{112}, and 4-NP_9 (Fig. 2) as the sole carbon and energy sources. Unfortunately, we could not detect any metabolites in such growth experiments except the C_9 alcohols derived from the nonyl side chains. Therefore, we designed experiments in which the nongrowth isomers 4-NP_2 and 4-NP_1 were cometabolically transformed in the presence of the growth substrate 4-NP_{112}. In such experiments, 4-NP_2 and 4-NP_1 were transformed to various metabolites that accumulated in the culture medium. The metabolites derived from 4-NP_2 were isolated in mg quantities, and their chemical structures were elucidated by NMR and MS analyses, whereas the metabolites derived from 4-NP_1 were identified solely by mass spectrometry. Fig. 2 shows the structures of the metabolites, and in Fig. 4 we propose a nonylphenol degradation scheme that is based on our experimental data. The structures of metabolites 1 and 5, both with an additional hydroxy substituent at the 4-position of the hexadienone ring, are strong indicators that the initial reaction was a hydroxylation at the anchor carbon atom of the alkyl substituent, an *ipso*-attack, forming 4-alkyl-4-hydroxy-cyclohexadienones. By analogy, we conclude that the growth substrate isomers 4-NP_{93}, 4-NP_{112}, and 4-NP_9 underwent the same initial *ipso*-hydroxylation. However, the intermediates derived from the growth substrate isomers did not accumulate, because they were immediately metabolized. At this stage of understanding, the most reason-

able suggestion for the next reaction step ultimately leading to cleavage of the nonyl moiety as C_9 alcohol is a rearrangement involving a 1,2-C,O shift of the alkyl moiety (Fig. 4, *pathway A*). We propose that the alkyl moiety formally moved as an alkyl cation to the nucleophilic oxygen atom of the geminal hydroxy substituent yielding a 4-alkoxyphenol. We can only speculate about the exact mechanism of the proposed reaction, but it seems reasonable that the rearrangement is facilitated by protonation of the keto group and deprotonation of the hydroxy group leading to separation of electric charges and to destabilization of the molecule. The proposed alkyl shift leads to charge neutralization and rearomatization. We believe that metabolites 1 and 5 did not undergo the 1,2-C,O shift of the alkyl moiety because the corresponding α-carbocations were not sufficiently stabilized by alkyl substituents in the α-position. However, they served as substrates for side reactions; metabolite 1 was converted to metabolites 2 and 3, whereas metabolite 5 was transformed to metabolite 6 (Figs. 2 and 4, *pathways B* and *C*).

The conversion of metabolite 1 into metabolite 2, a 2-alkylhydroquinone, involved a 1,2-C,C shift of the alkyl substituent, a reaction well known as NIH shift. Small amounts of an NIH shift product have recently been detected in the cell extracts of *Sphingomonas* strain TTNP3 grown on 4-(1-ethyl-1,3-dimethyl-pentyl)-phenol (4-NP_{111}) (35). However, the reported acidification of the lysate prior to extraction does not facilitate the interpretation of these results, because NIH shifts also occur nonenzymatically under acidic conditions (dienone-phenol rearrangement). To prevent nonenzymatic reactions, we carefully avoided acidification of the cultures prior to extraction of the metabolites in the experiment on preparative scale.

In principle, both the proposed 1,2-C,O shift and the alternative 1,2-C,C shift (NIH shift) would be effective in restabilizing the destabilized cyclohexadienone intermediate, because both lead to a charge neutralization and a rearomatization. The 1,2-C,O shift introducing an oxygen atom between the alkyl moiety and the carbon ring will lead to an intermediate (a 4-alkoxyphenol) that is very amenable to cleavage of the side chain as a C_9 alcohol with retention of the structure. In contrast, the 1,2-C,C shift is disadvantageous in terms of efficiency to break apart the molecule, because it leads to a product, a 2-alkylhydroquinone, in which the alkyl moiety remains connected to the carbon ring by a C–C bond (Fig. 4, *pathway B*). We propose that the NIH shift only becomes relevant when the C,O shift is unfavorable, as is the case for 4-nonylphenol isomers with a hydrogen atom at the benzylic α-carbon.

The 4-alkyl-4-hydroxy-cyclohexenones 3 and 6 were formed from the metabolites 1 and 5, respectively, by dihydrogenation of one of the two carbon-carbon double bonds of the ring. When Bayram degraded 4-NP_2 in LB medium, which is rich in reduced carbon compounds, the reduction of metabolite 1 was particularly efficient because large amounts of metabolite 3 accumulated, and no metabolite 1 could be detected (data not shown).

Here, we show that the initial reaction of the degradation pathway of nonylphenols was an *ipso*-hydroxylation producing 4-alkyl-4-hydroxy-cyclohexadienones. Microorganisms and rat liver microsomes use the same type of mechanism to detach different substituents in *ortho*- and *para*-substituted phenols. However, to our knowledge, *ipso*-substitution has never been described for the disconnection of an alkyl moiety as such. When rat liver P450 enzymes attack *p*-cresol at the *ipso*-position, 4-hydroxy-4-methyl-cyclohexa-2,5-dienone accumulates, because the methyl group is unable to leave either as an anion or as a cation (23) (Fig. 1*F*). *ipso*-Hydroxylation and the proposed 1,2-C,O shift constitute key steps in a novel pathway that enables bacteria to detach the alkyl substituent of an α-quaternary nonylphenol isomer as a C_9 alcohol and to utilize the ring as a source of carbon and energy.

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