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Metabolism of a nonylphenol isomer by *Sphingomonas* sp. strain TTNP3

Abstract For elucidation of the metabolism of the endocrine disruptor nonylphenol by *Sphingomonas* sp. strain TTNP3, the degradation of an isomer of nonylphenol, 4(2′,6′-dimethyl-2′-heptyl)-phenol, has been studied. As in the case of 4(3′,5′-dimethyl-3′-heptyl)-phenol, the metabolism of this nonylphenol isomer leads to the formation of the NIH-shifted product 2(2′,6′-dimethyl-2′-heptyl)-1,4-benzenediol (NIH: National Institute of Health), but also to the alkoxy derivative 4(2′,6′-dimethylheptan-2′-yloxy)phenol as additional metabolite. To the best of our knowledge, this is the first report describing the formation of alkoxyphenol as a degradation product of nonylphenol. Additionally, these results provide for the first time evidence for slight differences in the biodegradation of the isomers of nonylphenol.

Keywords Nonylphenol · Single isomer · Metabolites · Alkoxyphenol · Degradation · *Sphingomonas* · Endocrine disruptor

Introduction

The endocrine disrupting chemical nonylphenol is rather persistent in the environment (Soto et al. 1991; Giger et

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al. 1987). The low biodegradability of this xenobiotic can be attributed to its strong lipophilicity leading to sorption to materials such as sediments and humic acids (Vinken et al. 2004; Ying et al. 2003). Furthermore, most isomers of the technical mixture of nonylphenol (t-nonylphenol) possess a recalcitrant structure, i.e., a quaternary alpha-carbon on their branched alkyl chain (Wheeler et al. 1997; Van Ginkel et al. 1996). Bacteria, which degrade nonylphenol, belong to the Sphingomonas genus or to closely related ones such as Pseudomonas and Sphingobium (Corvini et al. 2004c).
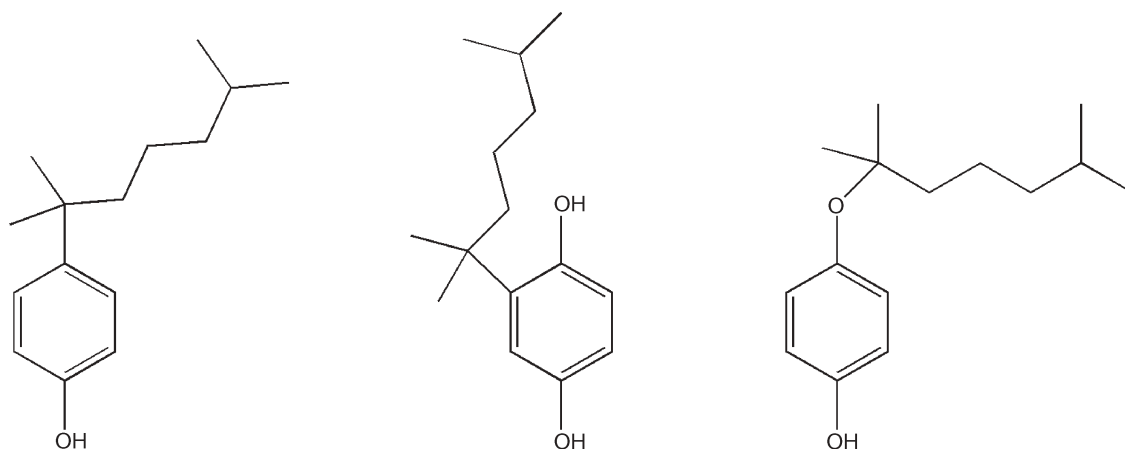
Until now, the metabolism of nonylphenol has only been investigated in *Sphingomonas* sp. strain TTNP3 (S. TTNP3), which uses it as growth substrate (Corvini et al. 2004a). This strain produces nonanols as end-metabolites from the alkyl chain moiety of nonylphenol, by replacing the aromatic ring with a hydroxyl group. The mechanisms that lead to the formation of these end-products are unclear because they would imply the hydroxylation of the quaternary α-carbon atom. We detected aromatic ring dihydroxylated metabolites of the diastereomeric mixture of 4(3′,5′-dimethyl-3′-heptyl)-phenol (p353-nonylphenol) in intracellular fractions of S. TTNP3 grown on that single isomer of nonylphenol (Corvini et al. 2004b). These metabolites were identified as diastereomers of 2(3′,5′-dimethyl-3′-heptyl)-1,4-benzenediol (o353NHQ). They result from a hydroxylation-induced migration of the alkyl chain also called NIH shift mechanism: firstly reported by scientists of the National Institute of Health. The hydroxylation of the aromatic ring at a quite unexpected position (C-4 atom) is followed by the migration of the alkyl chain to the adjacent carbon atom of the ring (Corvini et al. 2004c). However, intermediary steps that lead to the release of nonanol remain to be elucidated.

In order to further investigate the metabolism of nonylphenol, another isomer, the 4(2′,6′-dimethyl-2′-heptyl)-phenol (p262-nonylphenol, Fig. 1a), has been used for the present work. Differently to the p353-nonylphenol, the carbon α of p262-nonylphenol is substituted with a hexyl and two methyl groups (Vinken et al. 2002).

Fig. 1 Chemical structure of 4(2′,6′-dimethyl-2′-heptyl)-phenol (p262-nonylphenol) (**a**), and of its metabolites of degradation by *Sphingomonas* sp. strain TTNP3 2(2′,6′-dimethyl-2′-heptyl)-1,4- benzenediol (o262NHQ) (**b**), and 4-(2,6-dimethylheptan-2-yloxy) phenol (p262NOP) (**c**)

Experimental

Cell extract preparation

S. TTNP3 precultures and cultures were prepared as described previously (Corvini et al. 2004a). For cultures with p262-nonylphenol (1 g/l), bacteria were incubated in a mineral medium. Crude cell extracts were obtained after washing and sonication of 1.4 l of a 6 days culture of *S.* TTNP3. After acidification to pH 2–3 and extraction with ethyl acetate, the extract was dried over Na_2SO_4 (Corvini et al. 2004b, c).

Syntheses

p262-nonylphenol was synthesized by a Friedel–Crafts alkylation of phenol with 2,6-dimethyl-2-heptanol (nonanol, 99%; Acros, USA) and BF_3 as a catalyst (Vinken et al. 2002).

For the syntheses of 2(2′,6′-dimethyl-2′-heptyl)-1,4-benzenediol (o262NHQ) and 4-(2,6-dimethylheptan-2-yloxy)phenol (p262NOP), hydroquinone (99%; Fluka, Switzerland) was used instead of phenol, leading to a mixture of the two products, which were purified by means of semi-preparative high performance liquid chromatography (HPLC). Experimentally, 1,280 mg of hydroquinone, 500 μl of 2,6-dimethyl-2-heptanol, 10.0 ml of diethyl ether (Acros), and 2.0 ml of BF_3–ether complex were mixed together. The reaction was allowed to run for 120 min at 60°C with stirring. The other steps of the synthesis was carried out as described previously (Vinken et al. 2002). The reaction products were dissolved in 680 μl methanol and the two products were purified by semi-preparative HPLC with online ultra-violet (UV) diode-array detection (DAD) (HP 1100, Agilent Technologies). Separation was achieved on a Eurospher-100 C18 column (8×250 mm, particle size 5 μm, Knauer, Germany) at 35°C using a gradient from 25:75 to 0:100% water–methanol v/v in 22 min and a flow rate of 3 ml/min. One hundred microliters of the syntheses products was injected repeatedly, and the peaks at retention times 9.5 and 17.5 min were collected separately. In the combined HPLC cuts of the peaks methanol was evaporated under a gentle N_2 stream. The remaining aqueous phase was acidified with HCl and extracted two times with two volumes of ethyl acetate. The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The yields of the two products determined by gas chromatography coupled to mass spectrometry (GC–MS) analyses were 50.7 mg of o262NHQ with a purity of 99% and 6.3 mg of p262NOP with a purity of 99%, respectively. GC–MS, [1]H-nuclear magnetic resonance (NMR) and ^{13}C-NMR were used for identification and characterization of the reaction products (Vinken et al. 2002).

Metabolite purification

In order to further identify the metabolite M2, the organic extract was injected repeatedly into a Waters HPLC device (two Waters 510 pumps, a Waters WISP auto sampler 712 and a Waters UV detector 486) coupled to a Bruker Esquire-LC mass spectrometer. A reverse phase RP18e column (250×4 mm, 5 μm; Merck) was used for the chromatographic separation. The eluent consisted of acetonitrile (Baker, The Netherlands) (A) and water prepared by using a Milli-Q purification system from Millipore (Milford, CT) (B). The initial composition of 65% A was increased to 80% within 15 min and kept at this composition for 5 min. The percentage of A was then further increased within 5 min to 95% and kept at this composition for 10 min. The flow rate was 0.6 ml/min and the wavelength of the UV detector 270 nm. Twenty microliters of the extract was injected repeatedly (10 times), and the peak corresponding to M2 was collected, i.e., 480 μg.

NMR analyses

The combined HPLC cuts of each peak were evaporated to dryness under nitrogen, re-dissolved in 200 μl of CDCl$_3$ and transferred to a capillary NMR tube. M2 was analyzed by NMR and compared to the parent compound p262-nonylphenol. NMR measurements were carried out using a Bruker Avance DRX 600 (Bruker BioSpin, Germany) equipped with a 2.5 mm ^{1}H/^{13}C inverse-dual probe head with z-gradient. All types of spectra were acquired with standard Bruker pulse sequences. ^{1}H-NMR spectra: zg 30, sweep width 6313.131 Hz, 32 K data points, 2.4 s acquisition delay, 64 scans; 2D NOESY (Nuclear Overhauser Enhancement SpectroscopY) spectrum: noesytp, spectrum size 2 K× 256 data points, 16 scans; 2D (two-dimensional) COSY (COrrelation SpectroscopY) spectra: cosygs, spectrum size 2 K × 256 data points, 1 scan; 2D HMQC (Heteronuclear Multiple Quantum Coherence) spectrum: invieagssi, spectrum size 2 K× 400 data points, 8 scans; 2D HMBC (Heteronuclear Multiple Bond Correlation) spectrum: inv4gslplrnd, spectrum size 2 K × 512 data points, 16 scans. The average CH long-range coupling was set to 7.7 Hz. The 2D spectra were processed as 2 K× 1 K data matrices. Shifted squared sine window functions were applied in both dimensions.

Mass spectrometry analyses

Gas chromatography-mass spectrometry

Analyses of the purified M2 were performed as described previously (Vinken et al. 2002). Prior to GC–MS analyses, a derivatization step with N-methyl-N-trimethylsilyltrifluoroacetamide (Fluka, Germany) was carried out.

HPLC–DAD–MS

The extracts were analyzed by HPLC with UV DAD (HP 1100; Agilent Technologies) and electrospray ionization mass spectrometry detectors in series (ESI-MS, SSQ 7000; Finnigan MAT) (Corvini et al. 2004c).

HPLC-tandem mass spectrometry (LC–MS/MS)

Analyses of the purified M2 fraction were carried out with the Waters HPLC device coupled to the Bruker Esquire-LC mass spectrometer in the flow injection mode using an acetonitrile/water (50:50) mixture and a flow-rate of 400 μl/min. MS ionization parameters were: ESI source, negative-ion mode; skim voltage varied between 15.2 and 36.2 V; dry gas: 5 l/min nitrogen at 300°C; nebulizing gas: N$_2$ 15 psi. Scan range m/z=50–800; accumulation cut-off m/z=50; 20 averages per spectrum.

Results and discussion

Detection of the metabolites

On the basis of previous studies with p353-nonylphenol (Corvini et al. 2004c), intracellular extracts of cells of $S.$ TTNP3 grown on p262-nonylphenol were screened for the presence of metabolites of nonylphenol, and more particularly for the metabolite equivalent to o353NHQ, i.e., 2(2′,6′-dimethyl-2′-heptyl)-1,4-benzenediol (o262NHQ, Fig. 1b). HPLC–DAD–MS analyses of the extract were carried out. Peaks containing substances giving ions at m/z 235 (additional oxygen atom) were screened in the negative mode. Two peaks showed this mass in the chromatogram, a small peak at 9.8 min corresponding to metabolite M1 (0.5 mg) and a larger peak at 15.5 min for

Table 1 ^{1}H-NMR data of p262-nonylphenol and ^{1}H-NMR and ^{13}C-NMR data of the metabolite M2
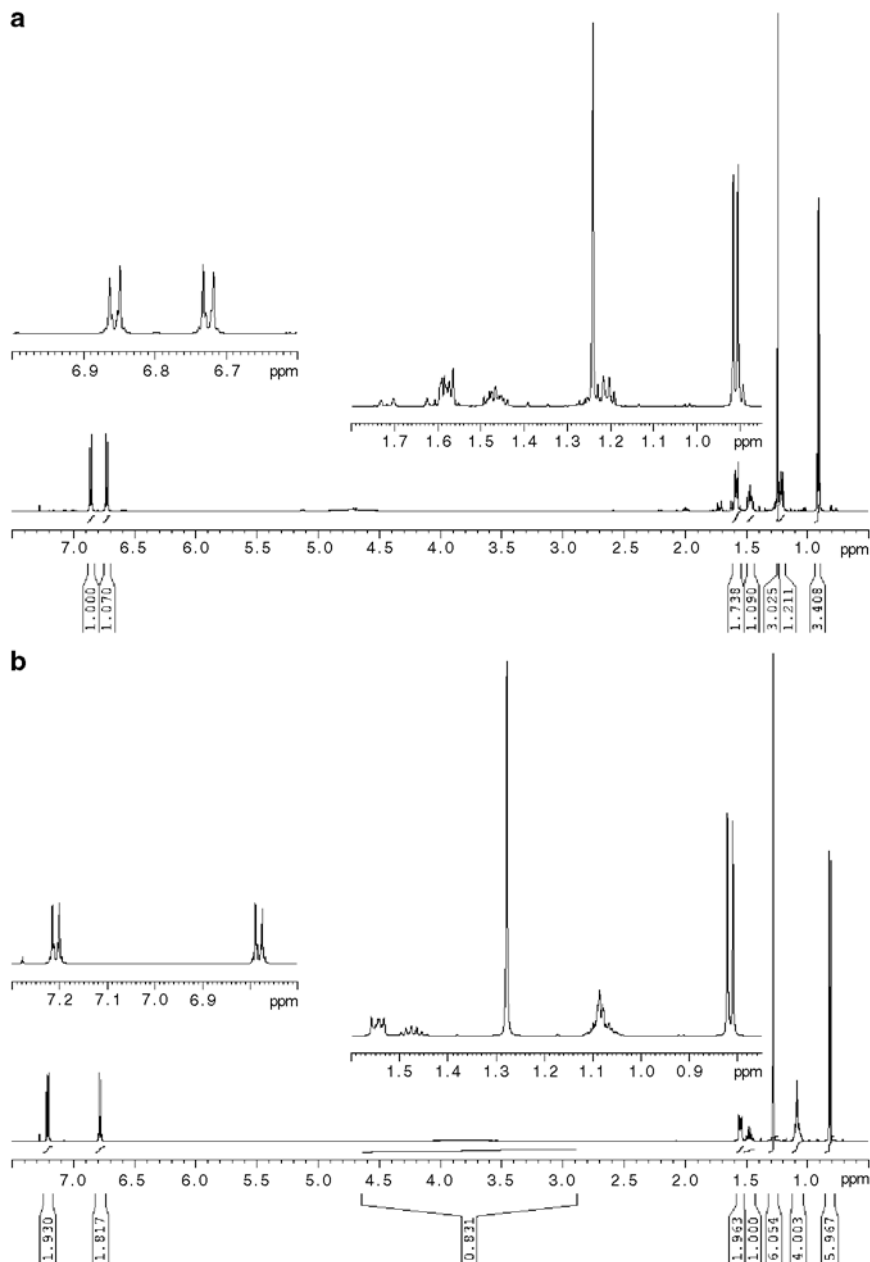
p262-nonylphenol		M2			
Proton	Chemical shifts δ (ppm) and coupling constants J (Hz)	Proton	Chemical shifts δ (ppm) and coupling constants J (Hz)	Carbon	Chemical shifts δ (ppm)^{bss}
H$_3$-1′	1.278, s	H$_3$-1′	1.241, s	C-1′	26.4
CH$_3$-2′	1.278, s	CH$_3$-2′	1.241, s	CH$_3$-2′	26.4
				C-2′	80.5
H$_2$-3′	1.52–1.57, m	H$_2$-3′	1.55 - 1.607, m	C-3′	42.2
H$_2$-4′	1.03–1.13, m	H$_2$-4′	1.466, m	C-4′	22.1
H$_2$-5′	1.03–1.13, m	H$_2$-5′	1.209, dt, 7.2	C-5′	39.5
H-6′	1.475, m	H-6′	1.55–1.607, m	C-6′	28.0
CH$_3$-6′	0.816, d, 6.6	CH$_3$-6′	0.911	CH$_3$-6′	22.7
H$_3$-7′	0.816, d, 6.6	H$_3$-7′	0.911	C-7′	22.7
				C-1	151.6
H-2	6.782, pd	H-2[a]	6.73, pd	C-2	115.4
H-3	7.208, pd	H-3	6.86, pd	C-3	125.4
				C-4	148.6
H-5	7.208, pd	H-5	6.86, pd	C-5	125.4
H-6	6.782, pd	H-6	6.73, pd	C-6	115.4

s singlet; pd pseudo-doublet; dt double triplet; m m multiplet. For atom positions see Fig. 1a, c
[a] Assignments of the aromatic protons were confirmed by the 2D NOESY spectrum (data not shown)
[b] ^{13}C chemical shifts were determined from the 2D HMQC and 2D HMBC spectra (data not shown)

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Fig. 2 a ^{1}H NMR spectrum of the metabolite M2 of *p*262-nonylphenol recorded on a Bruker Avance DRX 600 spectrometer, indicating a para disubstituted aromatic compounds. For details concerning chemical shifts and coupling constants, see Table 1. **b** ^{1}H NMR spectrum of *p*262-nonylphenol (parent compound). Comparison of this spectrum with the ^{1}H NMR spectrum of the metabolite M2 (**a**) showed a shift of the aromatic protons of M2 to higher fields and alkyl protons to lower fields. For details, see Table 1

metabolite M2 (7.2 mg). M1 was identified as *o*262NHQ by comparison with the authentic compound (data not shown). *o*262NHQ results from a NIH shift, as previously reported for the biodegradation of *p*353-nonylphenol (Corvini et al. 2004c).

NMR analysis of metabolite M2

In order to further identify M2, the organic fraction of the cell extract was purified by HPLC and prepared for NMR analyses. The ^{1}H NMR data of the parent compound *p*262-nonylphenol and the ^{1}H and ^{13}C NMR chemical shifts and coupling constants of the metabolite M2 are listed in Table 1.

The ^{1}H NMR spectrum of M2 (Fig. 2a) is typical for a *para*-disubstituted aromatic compound in the chemical shift range of aromatic protons, proving that no additional substituent was introduced to the aromatic ring system.

A comparison with the proton spectrum of *p*262-nonylphenol (Fig. 2b) shows high-field shifts of 0.35 and 0.05 ppm, respectively, for the aromatic protons, and, as a rule, a low-field shift can be recognized for the protons of the alkyl group, which is particularly large for H$_2$-4′.

The only exception is the singlet of the two equivalent methyl groups at C-2′, which is shifted by 0.04 ppm to the higher field. Since no additional substituent can be recognized at the alkyl group, a rearrangement reaction can be assumed, that would include the introduction of a heteroatom (oxygen) between the alkyl group and the aromatic ring.

The ^{13}C chemical shifts determined from the HMQC and HMBC spectra of the metabolite (data not shown) clearly confirm this assumption. The signal of C-2′, which could be identified in the HMBC spectrum by cross peaks with H_2-3′, H_2-4′ and the methyl groups at C-2′, showed a chemical shift of δ=80.5 ppm lying within the expected chemical shift range for quaternary carbons substituted by oxygen. Thus, the metabolite M2 was assumed to be 4-(2,6-dimethylheptan-2-yloxy)phenol (p262NOP) (Fig. 1c). Further comparisons of M2 NMR data with the ^1H and ^{13}C NMR spectra of the authentic compound p262NOP confirmed this identification.

Mass spectrometry analyses

The structure of the metabolite M2 has been verified by LC–MS/MS. The MS/MS spectrum showed a quasi-molecular ion peak [M−H]$^-$ at m/z=235 as expected, and an intensive fragment peak [M−H−C_9H_{19}]$^-$ at m/z=108 corresponding to an anion radical, which can be explained by the elimination of the alkyl residue (data not shown).

A derivatization reaction helped elucidate whether M2 was an alkoxylated form of p262-nonylphenol. If so, only the oxygen atom of the phenol hydroxy group should be trimethysilylated giving M$^+$ ion at m/z=308 (molecular weight of p262NOP = 236, once trimethysilylated). Derivatized M2 had a retention time of 21.9 min and a molecular ion at m/z=308. Characteristic MS fragments of M2 and of reference compound p262NOP were at m/z (% abundance) 308 (2), 293 (2), 223 (4), 182 (100), 167 (40) and 73 (14). The base peak was found at m/z=182 and this can be explained by a fragmentation between the oxygen atom and C2′ of the alkyl chain. The alkyl chain can be eliminated as an energetically favorable neutral fragment (C_9H_{18}) leading to the radical cation $C_9H_{14}O_2Si^{+\cdot}$. Likewise, the intensive fragment ion m/z=167 can be explained by the primary loss of a methyl radical from the trimethylsilyl moiety (m/z=293) followed by the loss of the neutral C_9H_{18}. The ion at m/z=223 could result from the cleavage between C-2′ (quaternary C atom) and C-3′ giving a $C_{12}H_{19}O_2Si^+$ ion. The ion at m/z=73 corresponds to the trimethylsilyl fragment.

Conclusion

p262-nonylphenol metabolism by S. TTNP3 leads to the production of a NIH-shift product o262NHQ and to a further metabolite, which has been identified as p262NOP. This confirms that p262-nonylphenol metabolism occurs as for p353-nonylphenol via an attack at an unexpected position of the aromatic ring, i.e., at C-4. However, no equivalent alkoxyphenol was detected in the case of p353-nonylphenol (data not shown). The main difference between these two isomers is the substitution of the carbon α of the alkyl chain. If the formation of nonanol, o262NHQ and p262NOP involves a carbocation intermediate at the position α, a different reactivity of the corresponding car-

bocation can be assumed. For the syntheses of these nonylphenol isomers, the same carbocations are formed from nonanols for the alkylation of the phenol ring. It was observed that the reactivity of the carbocation leading to p262-nonylphenol was much higher than that of the cation leading to p353-nonylphenol (Vinken et al. 2002). Furthermore, the synthesis of o353NHQ does not lead to the formation of alkoxyphenol as it is the case for the simultaneous preparation of o262NHQ and p262NOP.

Besides, many alkoxyphenol homologues to the p262NOP could be detected in the case of t-nonylphenol (data not shown). Despite the difficult gas chromatographic separation of all isomers of the t-nonylphenol (Corvini et al. 2004a), the number of peaks which were assumed to be alkoxyphenols (9 peaks) was not as high as the number of peaks to be assigned to nonylphenol isomers (15 peaks). Although previous work reported the absence of significant consumption differences by bacteria between the different isomers of nonylphenol (Corvini et al. 2004a), this study provides for the first time evidence for slight differences in the biodegradation of the isomers of nonylphenol.

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