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Isomer-specific Degradation and Endocrine Disrupting Activity of Nonylphenols

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Abstract

Degradation of technical nonylphenol by *Sphingobium xenophagum* Bayram led to a significant shift in the isomers composition of the mixture. By means of gas chromatography–mass spectrometry, we could observe a strong correlation between transformation of individual isomers and their α-substitution pattern, as expressed by their assignment to one of six mass spectrometric groups. As a rule, isomers with less bulkiness at the α-carbon and those with an optimally sized main alkyl chain (4 to 6 carbon atoms) were degraded more efficiently. By mass spectrometric analysis, we identified the two most recalcitrant main isomers of the technical mixture (Group 4) as 4-(1,2-dimethyl-1-propylbutyl)phenols (NP_{193a} and NP_{193b}), which are diastereomers with a bulky α-CH_3, α-$CH(CH_3)C_2H_5$ substitution. Our experiments with strain Bayram show that the selective enrichment of isomers with bulky α-substitutions observed in NP fingerprints of natural systems can be caused by microbial *ipso*-hydroxylation. Based on the yeast estrogen assay (YES), we established an estrogenicity ranking with a variety of single isomers and compared it to rankings obtained with different reporter cell systems. Structure–activity relationships derived from these data suggest that Group 4 isomers have a high estrogenic potency. This indicates a substantial risk that enrichment of highly estrogenic isomers during microbial degradation by *ipso*-substitution will increase the specific estrogenicity of aging material.

Introduction

Nonylphenols (NPs) are well known toxic and endocrine disrupting environmental contaminants formed during microbial degradation of nonylphenol polyethoxylates, an important class of non-ionic surfactants (*1, 2*). Because nonylphenol polyethoxylates are originally synthetized from technical nonylphenol (tNP), the nonylphenol (NP) released during the microbial metabolism of the surfactant is the original technical material. tNP is a complex mixture of more than 100 isomers that differ in the structure and the position of the alkyl moiety attached to the phenol ring (*3*). More than 90% of the mixture consists of *para*-substituted NPs (4-NPs) (*4, 5*).

Given the amphiphilic nature and the branched alkyl side chain of NP, it was initially assumed that only bacterial consortia were able to use those compounds for growth (*6*). Yet in the last few years, several bacterial strains able to grow with tNP as the sole source of carbon and energy and belonging to the Sphingomonad group were isolated from activated sludge of sewage treatment plants (*6-9*). Surprisingly, these isolates do not metabolize NP by oxidative attack on the alkyl side chain, but rather release the alkyl moiety as a volatile alcohol derivative with unchanged carbon connectivity and most likely utilize only the aromatic ring as a carbon and energy source (*8, 10-12*) (Figure 1, A). Growth experiments with strain Bayram showed that α-quaternary NP isomers serve as growth substrates, whereas NPs containing α-hydrogens do not. Nevertheless, such isomers are cometabolically transformed to *para*-hydroxylated metabolites with retained alkyl moieties (Figure 1 B) (*8, 13*). These findings, together with elaborate ^{18}O labeling experiments led to the elucidation of a degradation pathway. NPs are hydroxylated at the *ipso*-position to produce 4-alkyl-4-hydroxy-cyclohexa-2,5-dien-1-ones (quinols), from which α-quaternary alkyl moieties are able to detach as transient alkyl carbocations (*13-15*). The electrophilic reaction of

the carbocation with a water molecule then yields the corresponding nonanol (Figure 1A). As the carbocation only forms if sufficiently stabilized by α-alkyl branching, side chains containing α-hydrogens are not released (Figure 1B). This partially explains the divergent biodegradative behavior of NP isomers with different α-substitutions.

As early as 1938, it was realized that the phenanthrene ring system in estradiol is not a prerequesite for estrogenic activity. It could be shown that several *para*-substituted monophenols (e.g. 4-propenylphenol, 4-*tert*.-amylphenol, 4-cyclohexylphenol) and substances containing two phenol groups joined by a carbon chain (e.g. 4,4'-dihydroxystilbene) mimic the activity of estradiol, causing vaginal cornification in ovariectomized rats (*16*). But it took almost half a century before it was realized that NP is an estrogenic disruptor (*17, 18*). Recently, estrogenic activities of different NP isomers were shown to vary depending on the structure of the alkyl side chain (*19-22*). This implies that changes in the isomer composition of NP mixtures will have an effect on the specific estrogenic potency of the material.

In most environmental monitoring studies NP isomeric mixtures have been treated as single compounds. Yet more recently, several investigations showed that the isomeric composition of NP mixtures in natural systems may significantly differ from that of technical mixtures (*19, 23-25*). In certain matrices however, no marked differences were observed (*26-29*). The finding that strain Bayram differentially degrades nonylphenol isomers, indicates that selective microbial metabolism can lead to changes in the isomer composition of 4-nonylphenol mixtures in the environment (*8*). Furthermore, during composting of biosolids the transformation of α-methyl-α-propyl substituted NPs was significantly slower than that of isomers with less bulkiness at the α-carbon (*25*).

Here we show that metabolism of tNP by *Sphingobium xenophagum* Bayram indeed leads to prominent changes in the isomeric composition. As a rule, isomers with less bulkiness at the α-position were degraded faster. This structure–biodegradability relationship is able to explain the formation of the specific NP isomeric patterns observed in complex natural systems (*23, 25*). Furthermore, estrogenicity assays with a wide variety of single NP isomers indicate that differential *ipso*-hydroxylation of NP mixtures may markedly affect the specific estrogenicity (activity per amount of the mixture) of the aging material.

Experimental Section

Nonylphenols. The abbreviations used for the various 4-NP isomers are based on the systematic numbering system proposed by Guenther *et al.* (*30*). For the sake of simplicity, we did generally not mention the anchor position of the alkyl moiety (e.g. NP_{194} instead of 4-NP_{194}). The technical nonylphenol (tNP) mixtures used in the degradation experiment and the yeast estrogen assay were purchased from Fluka (Buchs, Switzerland; 85% pure as referred to the amount of *para*-isomers) and Schenectady International, Inc. (Schenectady, NY, USA; 95% purity), respectively. Beside of NP_{93}, NP_{194}, NP_{10}, and NP_{70}, NPs were > 99% pure (see Table 2 and Supporting Information).

Media and growth conditions. For degradation experiments with tNP we used 300 ml Erlenmeyer culture flasks containing each 50 ml of minimal medium and 1 mg/ml tNP, which was added as described previously (*8*), except that the solvent was acetone instead of *n*-hexane. We inoculated each flask with 1 ml of a preculture of *Sphingobium xenophagum* Bayram (OD_{546} of 0.38) that was grown for six days in minimal medium with 1 mg/ml tNP as sole carbon and energy source. Incubation took

place on a rotatory shaker (250 rpm) at 25°C. To monitor the composition of undegraded NP we set up a series of identical vessels, each being sacrificed at the appropriate day by shock freezing at –80°C (incubation for 9, 18, and 27 days, respectively; at day 9, two vessels were sacrificed instead of one; a control vessel was frozen immediately after starting the incubation). A sterile control was incubated until the end of the experiments. A degradation experiment with strain Bayram and a mixture of the *para*-isomers NP_{194}, NP_{112}, NP_{111}, NP_{152}, and NP_{65} was performed in screw cap cylindrical glass culture vials, each containing 3 ml of liquid minimal medium and 1 mg/ml per isomer as the sole source of carbon and energy (Table S1) (*8*).

Analytical procedures. The frozen cultures were thawed and we added 1.0 mg and 500 μg of 4-*tert.*-octylphenol (4-(1,1,3,3-tetramethylbutyl)phenol) dissolved in 2-propanol (25 μl) as internal standards to the 50-ml and 3-ml cultures, respectively. Nonylphenol was then extracted thrice (twice in the case of the 3-ml cultures) by vigourously stirring the cultures for 5 min with 20 ml (2 ml) of CH_2Cl_2 on a magnetic stirrer. After phase separation an aliquot was withdrawn for GC-MS analysis. Samples were diluted with CH_2Cl_2 to obtain appropriate internal standard concentrations.

We used a GC 8060 gas chromatograph (Fisons instruments, Milan, Italy) coupled to an MD 800 quadrupole mass spectrometer (Fisons instruments, Manchester, UK) for GC-MS analysis. The injector was operated in splitless mode at 270°C. Two microliters of the sample were injected by an A200S autosampler (CTC Analytics, Zwingen, Switzerland). Separations were achieved on a DB-17 MS capillary column (60 m, 0.25 mm internal diameter, 0.25-μm film thickness; J&W Scientific, Folsom, CA, USA) connected to deactivated pre- and transfer columns (ca. 2 m each). The following gradient program was applied: isothermal at 50°C for 1.5 min, 10°C/min to 165°C, and 0.6°C/min to 175°C, which was held for 30 min. The interface and source temperatures

were set to 250 and 200°C, respectively. Ionization was performed by electron impact (70 eV). In the experiments with tNP and a mixture of selected nonylphenol isomers, data were acquired in the full scan mode (*m/z* 35 to 271) and by single ion monitoring (*m/z* 107, 121, 135, 149, 163, 177, 191, and 220), respectively. Peaks in Figure 2 and Table 1 were identified by comparing retention times, TIC peak intensities and fragmentation patterns to published (*5, 31*) and unpublished results (NP_{36}, NP_{37}, and NP_{119}; B. Thiele, V. Heinke, E. Kleist, and K. Guenther) and by cochromatography with available isomers (NP_{194}, NP_{36}, NP_{112}, NP_{111}, NP_{152}, NP_{65}, and NP_9). Our assignments of NP_{36}, NP_{37}, and NP_{119} are compatible with the results of Katase et al. (*32*). The same reference, together with mass spectrometric considerations (not shown), were used to assign peaks 9, 29, and 40 to NP_{38}, NP_{110a}, and NP_{110b}, respectively. We elucidated the structure of Group 4 isomers (peaks 27 and 30) by mass spectrometry (see Supporting Information). To calculate the amount of material remaining after incubation, relative peak areas (4-*tert*.-octylphenol as reference) at the end of the experiment were divided by those at the start. To quantify groups of isomers and individual isomers the TIC chromatogram and one or two characteristic *m/z* traces, respectively, were examined.

Yeast Estrogen Assay (YES). Details of the estrogen-inducible expression system in yeast and preparation of the medium components have been described previously (*33*). However, in the present study plates were shaken for 2 min before incubation. Assays with dilution series of the test samples were performed in duplicate and included an appropriate number of medium-only blanks. Because in our hands the sensitivity of the YES has increased over the last 12 years, direct comparisons of absolute potencies with those published elsewhere are not meaningful. Here, we compare the potency of individual isomers to that of the mixture (see below). As this is done in the same assay (and on more than one occasion), such data are robust and allow

to appraise if the order of relative potencies resulting from our experiments is consistent with rankings established by others.

Results and Discussion

Aging of technical nonylphenol. Strain Bayram significantly altered the isomer distribution profile of tNP when growing with this substrate as the sole carbon and energy source (Figure 2). It consumed 86% of the 4-NP isomers (retention time 32.7–40.5 min) and about 40% of the presumptive 2-NPs (retention time 25.5–32.6 min) within nine days of incubation. Thereafter degradation ceased, a phenomenon also described for strain TTNP3 (*12*) and possibly related to the accumulation of chemically reactive 2-nonyl-*p*-quinones derived from corresponding alkyl-hydroquinone metabolites (*4, 13*). We observed a remarkable correlation between the percentage metabolized of an individual isomer and its substitution pattern at the α-carbon, indicated by assignment of the isomer to one of six mass spectrometrical groups. Isomers are classified into those groups according to the set of fragment ions (*m/z* 107, 121, 135, 149, 163, 177, and 191) produced by homolytical cleavage of the α-substituents (*5, 31*) (Tables 1 and S1). For Group 4 isomers (peaks 27 and 30, Figure 2), we propose a structure (NP_{193a} and NP_{193b}, pair of diastereomers) that accounts for the characteristic mass spectrum, which displays intensive peaks at *m/z* 163, 121, and 107, and smaller peaks at *m/z* 177, 161, 147, 134, 133. Our structure assignments accord with those recently published by Katase et al., which are based on ^{1}H- and ^{13}C-NMR analysis (*32*) (see Supporting Information).

The percentage that was metabolized ranged from 31% in the case of the isomers 27 and 30 (Group 4) to about 100% for the α,α-dimethyl isomers (Group 1) (Table 1). Group 5 isomers (α-methyl, α-*n*-propyl) proved to be more recalcitrant than Group 2

isomers (α-methyl, α-ethyl). Results of a degradation experiment with a mixture of selected α-quaternary 4-NP isomers as substrates confirmed these findings. In this experiment the sequence of percentage metabolized ($NP_{112} > NP_{111a} > NP_{111b} > NP_{65} > NP_{194} \cong NP_{152}$, Table S1) matched that from the experiment with tNP (Table 1).

Differential degradation of NP isomers is most likely caused by *ipso*-hydroxylation rates that vary between the isomers, and not so much by the ability of the alkyl moiety to detach as a carbocation. Inability to detach would result in an accumulation of the cyclohexadienone intermediates with subsequent formation of dead end metabolites (Figure 1B) (*13*) but should not be the cause for differential degradation of NP isomers in a mixture. The degradation ranking established for differential metabolism of tNP by strain Bayram shows that degradation was more effective when the α-position was less bulky: α-$(CH_3)_2$ (Group 1) > α-CH_3, α-CH_2CH_3 (Group 2) > α-CH_3, α-CH_2CH_3, β-CH_3 (Group 3) > α-CH_3, α-$CH_2CH_2CH_3$ (Group 5) > α-CH_3, α-$CH(CH_3)_2$ (Group 6) > α-CH_3, α-$CH(CH_3)C_2H_5$ (Group 4) (Table 1) (for convenience, the α-$CH(CH_3)C_2H_5$ and the α-$CH_2CH_2CH_3$ branches of Group 4 isomers are viewed as α-substituent and part of the main chain, respectively).

The NP *ipso*-hydroxylase activity of strain Bayram has been grouped with the flavin monooxygenase enzyme family (*9*). Hence, we would expect an electrophilic oxygen species as hydroxylating agent. Bulky α-alkyl substituents enhance the electron density at the *ipso*-position and, therefore, should facilitate the electrophilic addition of that oxygen species. However, the opposite is observed. From these results we conclude that *ipso*-hydroxylation rates are most likely governed by steric rather than by electronic effects. On the one hand, bulky α-alkyl substituents sterically hinder hydroxylation at the neighboring *ipso*-position and on the other hand they electronically promote the cleavage of the alkyl moiety once the quinol intermediate has been formed (*8*). The

length of the main alkyl chain also seems to play an important steric role, because isomers with little bulkiness at the α-position but lengthy alkyl chains (NP_9, NP_2 and NP_1) are relatively slowly transformed (Table 1, Figure 1B) (*8*).

NP fingerprints. Differential microbial metabolism of tNP mixtures in the environment will ultimately lead to changes in the relative composition of aged material. Unraveling the principles that govern the reshaping of isomer distribution patterns will help us to understand how certain isomeric fingerprints observed in natural systems have formed. Indeed, careful analysis of isomer distribution patterns in river water samples at different locations around Tokyo Bay (*23*), leads to the hypothesis that the nonylphenol contaminants underwent microbial degradation by *ipso*-substitution (Figures S2, S3, S4). In this context, it is interesting to note that *Sphingomonas cloacae*, one of the first strains known to degrade NPs by detaching the alkyl side chain, was isolated at a sewage-treatment plant in Tokyo, i.e. in close proximity of the sampling sites (*7*). Furthermore, an *ipso*-substitution degradation mechanism may be called upon to explain the relative enrichment of NPs with a bulky α-substitution during composting of biosolids (*25*).

We believe that different environmental matrices are characterized by distinct isomeric fingerprints that depend on the grade of aging and on the dominating aging process (e.g. metabolism by specific NP degrading microbial communities). In certain matrices, when transformation processes with little discrimination between individual isomers prevail (*ortho*-hydroxylation may be an example), or when transformation processes are absent, NP fingerprints will be similar to those of technical mixtures. Diminished bioavailability by rapid adsorption to solids may explain the lack of isomeric shifts reported for some sediment (*27, 28*). Changes of isomeric fingerprints will be less obvious in systems such as aerobic basins of sewage treatment plants (*29*),

in which the rate of formation of NP from NP polyethoxylates is much higher than the rate of degradation of NP.

Estrogenicity of NP isomers. All NP samples were assayed over a concentration range of 0.13 mM down to 0.24 μM. Figure 3 shows the response of the yeast estrogen screen (YES) to various concentrations of each NP sample. The NP isomers generally produced full dose response curves over a range of two orders of magnitude, with the most potent isomer (NP_{93}) being only 1690 times less potent than 17β-estradiol, and nearly twice as potent as the technical mixture of isomers (Figure 3, Table 2).

A pioneering study of the structural properties of alkylphenols that are associated with estrogenic activity concluded that the branching on the α-carbon may be an important feature for estrogenic activity (*34*). Since all the isomers at our disposal were α-substituted, and because a difference in estrogenicity of two isomers inherently can be interpreted by positive and negative effects (Table 3), we could not derive a unequivocal correlation between α-substitution pattern and estrogenicity from our data. However, isomers that can be derived from a particular α-substituted isomer by shifting bulky groups that surround the α-position partly or entirely to the β- or γ-position always showed higher activities (compare NP_9 with NP_{10}, NP_{65} with NP_{70}, NP_{112} with NP_{93}/NP_{95}, NP_{152} with NP_{111}, Table 3 A–D, respectively). These data can be explained by a positive effect of β- and γ-substituents, by a negative effect of bulkiness at the α-position, or by a positive effect of distributing α-centered bulkiness along the proximal part of the main chain. The two most potent isomers, NP_{93} and NP_{70}, both have a β-alkyl branch and a main chain length of 5 and 6 carbon atoms, respectively. Isomers with a similar chain length (4 to 6 carbon atoms) but without β-substituents (NP_{65}, NP_{111}, NP_{112}, NP_{152}, NP_{170}, NP_{194}) and isomers with β-substituents but with a longer chain length (NP_{10}) only reached medium levels of estrogenicity (relative potency 0.80–0.25),

even though they have a quaternary α-carbon. This suggests that not α-substitution, but β-substitution together with an optimal length of the main chain is the key for high estrogenic activity. Lack of both of these structural characteristics resulted in very low activities (relative potencies < 0.04 for NP_9, NP_2, and NP_1).

Despite the difference of reporter cell systems used, and except for NP_{152}, our estrogenicity ranking was identical to those established by Shioji et al. and Preuss et al. (Table 2) (*21, 22*). Kim et al. found a divergent ranking for tNP, NP_{152}, NP_{65}, and NP_9, although they used the same test system as we did (*19, 20*). However, they did not synthesize pure test isomers, but purified them from tNP by preparative HPLC, a procedure that probably leads to significant contamination with other NP isomers (*21*). Unfortunately, one of the samples that showed highest estrogenicity (NP_{70}) in our test system was contaminated with small amounts of eight other 4-NP isomers and several other, non-estrogenic compounds. However, since all of the NP contaminants contained a tertiary α-carbon atom, our conclusion that quaternary α-carbons are not essential for high estrogenicity remains unaffected (Supporting Information).

In agreement with Shioji et al., we favor the hypothesis that an optimal length of the main alkyl chain (4 to 6 carbon atoms) and bulkiness around the β- and γ-position is needed for NP isomers to exhibit high estrogenicity, whereas the presence of α-substituents is less important (17β-estradiol itself contains a tertiary α-carbon in *para*-position, not a quaternary one). To explain the positive effect of β-substituents, it was suggested that a β-anchored alkyl branch might interact with a cavity of the estrogen receptor (ER), located around the C ring of 17β-estradiol (E_2) in the ER-E_2 complex (*22*). Although the length and breadth of the E_2 skeleton are well matched by the receptor, there are indeed large unoccupied cavities adjacent to the 7α- and 11β-positions of E_2 (*35, 36*). These cavities allow appropriately positioned steric groups of

certain sizes to fit, and are of great importance for binding xenoestrogens, including DES-like chemicals, diphenylmethanes, and biphenyls (*36*).

Structure–property relationships. Growth experiments with strain Bayram and yeast estrogen assays (YES) showed that a lengthy main alkyl chain had a negative effect on degradability as well as estrogenic potency of a NP isomer. Because alkyl moieties with lengthy main chains intrinsically tend to have little branching, our former conclusion that the more highly branched the alkyl substituent the faster it degrades, still holds as a general principle (*8*). However, the rule does not hold for isomers that have bulky substituents at the α-position. Indeed, α-substitution appears to have a profound effect on *ipso*-hydroxylation rates, as isomers with increased bulkiness around the α-position tended to be more recalcitrant. This applies well to the newly identified NP isomers NP_{193a} and NP_{193b} (Group 4), which have bulky α-CH_3, α-$CH(CH_3)C_2H_5$ substitutions and among the main isomers in tNP are the most recalcitrant. Because NP_{119}, NP_{193a}, and NP_{193b} are characterized by a β-alkyl substituent and an optimal size of the main chain, we expect these isomers to have a high estrogenic potency. However, Kim et al. recently reported that in contrast to NP_{119}, NP_{193} did not show any estrogenic activity at all. It needs to be noted though, that the tested compounds were isolated from tNP by fractionation with HPLC and GC-PFC, which may affect their purity (*19*). If this somewhat surprising result is verified, one will have to conclude that in certain nonylphenol isomers, bulky α-substitutions have a negative effect on estrogenicity ("4-(di-*t*-butylmethyl)phenol" however, is highly estrogenic (*22*)).

Estrogenicity of tNP mixtures and the effect of aging. Among the 18 main NP isomers of tNP (Table 1), 11 isomers have been synthetized and tested for estrogenicity so far (this study, (*22*)). All these isomers were less estrogenic than the technical mixture. These findings imply that certain main isomers that have not been tested yet

for estrogenicity are at least moderately more estrogenic than the technical mixture or, alternatively, that certain minor isomers are highly estrogenic. For instance, it needs to be shown whether highly estrogenic isomers, such as 4-(1,2,2,3,3-pentamethylbutyl)phenol (NP_{167}), that appears to be 153 times more potent than tNP (*22*), are minor components of tNP. Obviously, it would not require much of such an isomer in a technical mixture to account for the latter's estrogenicity. Differential degradation of tNP mixtures certainly affects their specific estrogenicity (activity per amount of the mixture), although the degree and the quality of this effect cannot be fully estimated yet. But it is clear that if highly estrogenic isomers, such as NP_{167}, prove to be recalcitrant components of technical mixtures, then there is a substantial risk that microbial degradation by *ipso*-substitution will increase the specific estrogenicity of the material.

Further research on the varying isomer composition and estrogenicity of NP mixtures during microbial degradation will be needed to show whether certain highly estrogenic isomers are recalcitrant and whether increasing specific estrogenicity is important with regard to risk assessment of aged tNP mixtures.

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Supporting Information Available

In the SI section, we present a detailed analysis of the GC-MS chromatograms of tNP and aged tNP, and of the NP isomer patterns observed by Horii et al. in contaminated river water around Tokyo Bay (*23*). We also show the results of a degradation experiment with strain Bayram and a defined mixture of NP isomers. Extraction efficiencies are mentioned. Furthermore, we present a detailed analysis of impurities in the NP_{10} and NP_{70} samples, and a structural proposal for Group 4 isomers. This information is available free of charge via the Internet at http://pubs.acs.org.

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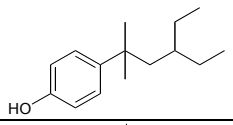
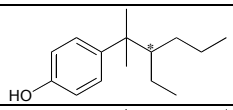
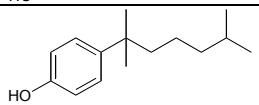
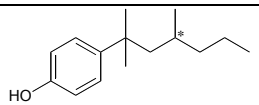
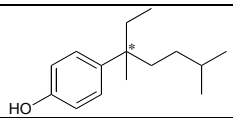
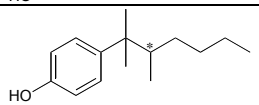
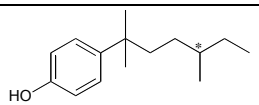
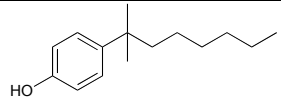
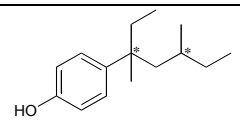
TABLE 1. Degradation of tNP by *S. xenophagum* Bayram in minimal medium. The degradation of individual isomers correlated well with their assignment to one of the six mass spectrometrical groups (Group 1 > Group 2 > Group 3 > Group 5 > Group 6 > Group 4). Isomers are listed according to the amount degraded [%].

Peak in Figure 2	NP isomer	Retention time [min] (Figures 2A and S1A)	Structure	Mass spectrometrical group (*5*, *31*)	Degradation after 9 days of incubation [%]	Corresponding peak in ref. (*32*)	Corresponding peak in refs. (*5*, *31*)
14	NP_{128}	34.8		1	99.7	3 (D)	5
26	NP_{119}	37.0		1	99.7	6 (I)	10
9	NP_{38}	34.1		1	99.3	3 (C')	3
7	NP_{36}	33.7		1	98.8	2 (B)	2
13	NP_{112}	34.5		2	98.2	3 (C)	4B
31	NP_{35}	38.7		1	97.6	10 (M)	14
16	NP_{37}	35.19		1	96.6	4 (F)	7
33	NP_{9}	38.91		1	96.1	11 (O)	17
17	NP_{111a}	35.25		2	94.0	4 (E)	6
19	NP_{111b}	35.7		2	90.4	5 (G)	8

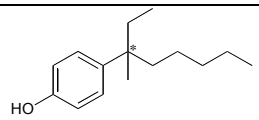
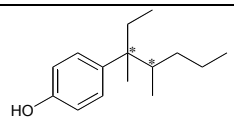
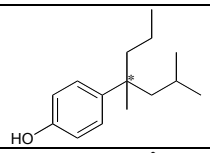
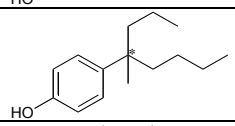
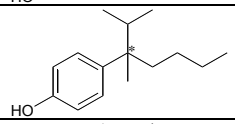
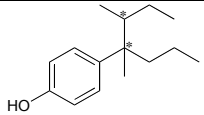
32	NP_{65}	38.84		2	81.0	11 (N)	16
29	NP_{110a}	38.1		3	77.5	8 (K)	12C
40	NP_{110b}	39.9		3	76.3	12 (P)	18
1	NP_{194}	32.9		5	61.8	1 (A)	1
24	NP_{152}	36.5		5	56.2	6 (H)	9
28	NP_{143}	37.8		6	52.2	–	12B
27	NP_{193a}	37.4		4	31.3	7 (J)	11
30	NP_{193b}	38.3		4	30.5	9 (L)	13

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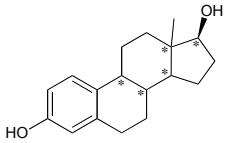
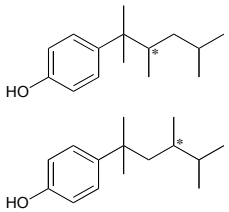
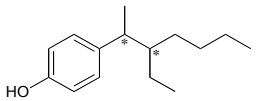
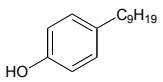
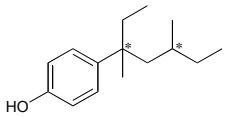
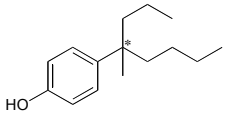
TABLE 2. Structures and estrogenicities of 17β-estradiol and NP isomers assayed in this as well as other studies (*20-22*). Potencies relative to that of the technical mixture are shown. In the cases where the test samples represented mixtures of two isomers, we also pointed out the minor component and the relative abundances. The test assay and the tNP mixture used in the different studies, and the relevant concentrations to determine relative potencies are indicated.

Test compound	Main chain length	Structure	Kim et al. 2004 (*20*)	Shioji et al. 2006 (*22*)	Preuss et al. 2006 (*21*)	This study
Estrogenicity assay			YES	Yeast Two-Hybrid Assay	MVLN	YES
Commercial source of tNP			Tokyo Kasei Koygo Co.	Kishida	Fluka	Schenectady International, Inc.
Concentrations used to calculate relative potencies			Minimal effective concentrations	concentrations producing same effect as EC_{10} of E_2	EC_{20} of test compounds	concentrations producing same effect as EC_{50} of E_2
			Relative potencies			
17β-Estradiol			54825	538	84615	3168
NP_{93} (81%) NP_{95} (17%)	5 5		–	–	–	1.87
NP_{70a} (43.1%) NP_{70b} (40.5%)	6		–	–	–	1.68
techn. NP			1.00	1.00	1.00	1.00
NP_{111a} (44.9%) NP_{111b} (55.1%)	5		–	–	0.81	0.78
NP_{152}	5		1.09	0.080	–	0.70

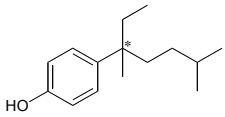
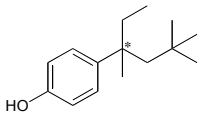
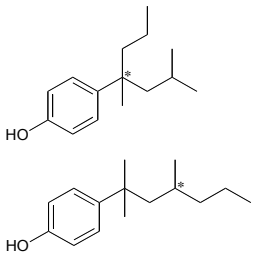
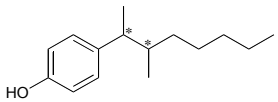
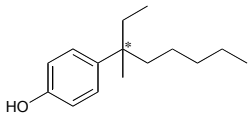
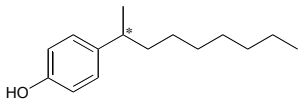
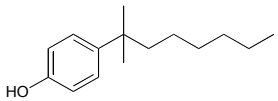
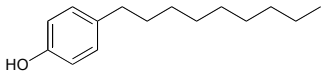
NP_{112}	5		–	–	0.59	0.60
NP_{170}	4		–	0.76	–	0.56
NP_{194} (86%) NP_{36} (14%)	4 6		0.91 (HPLC-Fraction with NP_{194})	–	–	0.49 (NP_{194} + NP_{36})
NP_{10a} (32.4%) NP_{10b} (60.3%)	7		–	–	–	0.45
NP_{65}	6		2.0	0.11	0.52	0.25
NP_2	8		–	0.083	–	0.038
NP_9	7		3.2	0.070	–	0.023
NP_1	9		0.61	–	–	0.022

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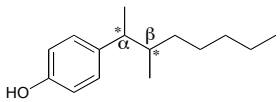
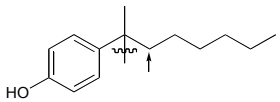
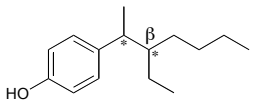
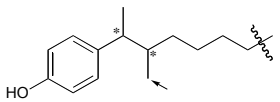
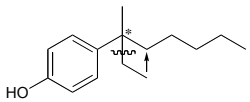
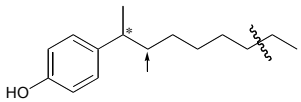
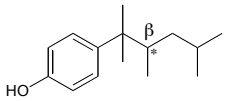
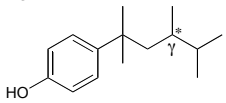
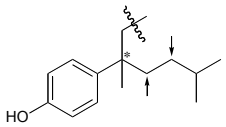
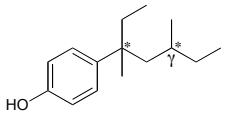
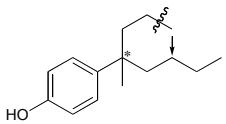
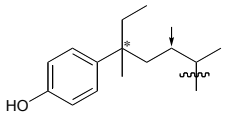
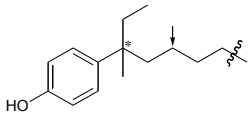
TABLE 3. Effects of β- and γ-substituents on estrogenicity: Comparison of β- and γ-substituted NPs serving as references (acronyms in bold and italic), with structurally related isomers (A–D). Reference isomers can be derived from related isomers by shifting of a group to the β- and γ-position (abstraction and relocation of the migrating group are indicated by wavelines and arrows, respectively). Besides positive effects of β- and γ-substituents, the increase in estrogenicity associated with the transposition might also be explained by negative effects (column at the far right). The clear potency difference between NP_{65} and NP_{112} seems to indicate that also δ-substituents have a positive effect on estrogenicity (D).

NP isomer		main chain length	α	β	γ	δ	Relative Potency	Possible negative effect
(A) Effect of β-substituents								
***NP*$_{10}$**		7	Me	Me			0.45	
NP_9		7	di-Me				0.023	Bulkiness adjacent to the α-position
(B) Effect of bulky β-substituents								
***NP*$_{70}$**		6	Me	Et			1.68	
NP_{10}		7	Me	Me			0.45	long main chain
NP_{65}		6	Me, Et				0.25	bulky α-substituent
NP_2		8	Me				0.038	long main chain

(C) Effect of β- or γ-substituents								
***NP*$_{93}$** (81%)		5	di-Me	Me		Me	1.87	
***NP*$_{95}$** (17%)		5	di-Me		Me	Me		
NP_{112}		5	Me, Et			Me	0.60	bulky α-substi-tuent
(D) Effect of γ-substituents								
***NP*$_{111}$**		5	Me, Et		Me		0.78	
NP_{152}		5	Me, Pr				0.70	bulky α-substi-tuent
NP_{112}		5	Me, Et			Me	0.60	
NP_{65}		6	Me, Et				0.25	

1

2

FIGURE 1. Diverging pathways in the metabolism of an α-quaternary (NP_{112}) (A) and an α-tertiary NP isomer (NP_2) (B) by strain Bayram (*8, 13, 15*). In both cases, the initial reaction is an *ipso*-hydroxylation, which yields 4-alkyl-4-hydroxycyclohexa-2,5-dien-1-one intermediates (quinols). Because of unsufficient stabilization by α-substitution, the alkyl moiety of α-tertiary alkyl-cyclohexadienones is not released as a cation. Hence, α-tertiary quinol intermediates accumulate and undergo side-reactions, e.g. a dienone–phenol rearrangement (NIH-shift)(*13*)(B). Bold, thin, and dotted reaction arrows symbolize putatively high, medium sized, and low reaction rates, respectively.

FIGURE 2. "Aging" of tNP as a result of degradation by *Sphingobium xenophagum* Bayram in minimal medium. GC-MS chromatograms (TIC) of the culture extracts correspond to the start of the experiment (A) and to day 9 of the incubation (B). Please note that differential degradation leads to a significant change in the isomers distribution of the NP mixture. Chromatograms of extracts from cultures sacrificed at 9 (duplicate), 18, and 27 days were nearly identical (B), indicating that no further degradation occurred after 9 days. In contrast, the isomer pattern in the noninoculated control was identical to that of nondegraded technical NP (A). Peaks of recalcitrant isomers (Groups 4, 6, and 5) are highlighted (encircled numbers). The great majority of peaks eluting before peak 1 most likely correspond to *ortho*-isomers (*5, 31*). Signals were numbered according to their retention time (Figures S1A and B). The vertical position of a mass spectrometrical group indicates the recalcitrance of its members to biodegradation by strain Bayram (Group numbers — encircled numbers at the far right — are listed according to increased recalcitrance).

FIGURE 3. Response of the yeast estrogen screen (YES) to a range of NP isomers. The graph depicts the logarithmic concentration of 17β-estradiol (E_2) serially diluted from 2.5 nM to 4.9 pM and of NP isomers diluted from 0.13 mM to 0.24 μM, plotted against the absorbance of the medium after a 3-day incubation at 32 ˚C. The blank (BL) shows the response of the assay in the absence of test compound (solvent alone; blanks run in parallel to E_2-assays are shown). Values represent mean ± standard deviation from duplicate wells. Structures of selected test compounds are shown. The vertical lines depict the concentrations used to determine the potencies of NP_{93}, NP_1 (the most and least potent isomers of our test series, respectively; Table 2), and 17β-estradiol, relative to that of tNP. The relative potency of a test NP isomer was assessed by dividing the concentration of tNP producing an OD of 1.737 (EC_{50} of 17β-estradiol) by that of the test isomer effecting the same response.

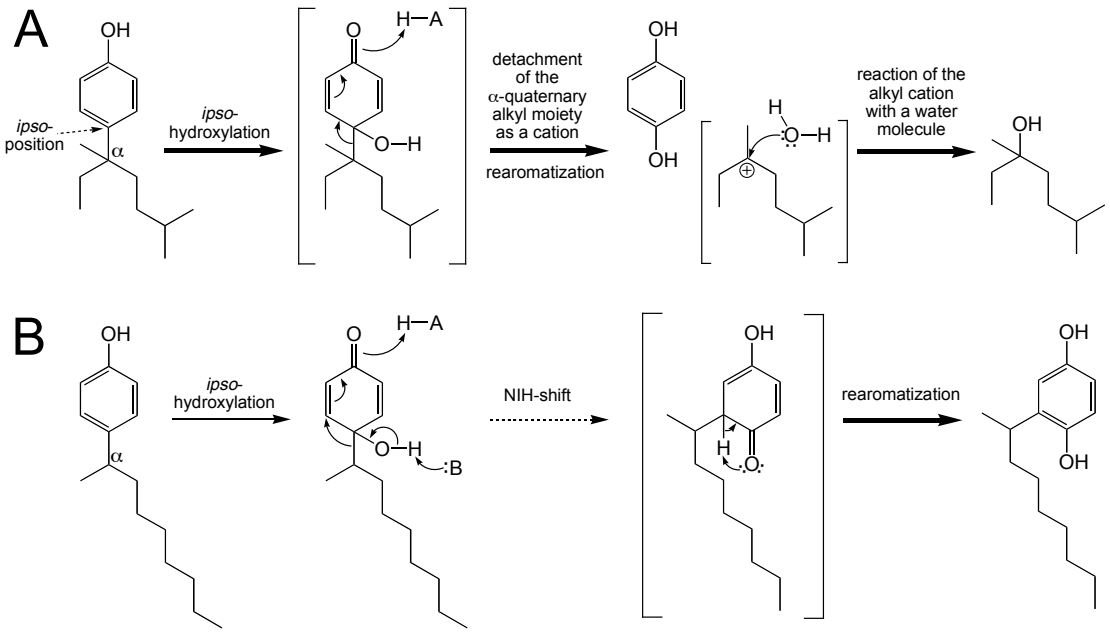

A
OH
ipso-
position
α
ipso-
hydroxylation
O
H—A
O—H
detachment
of the
α-quaternary
alkyl moiety
as a cation
rearomatization
OH
OH
H
O—H
reaction of the
alkyl cation
with a water
molecule
OH
B
OH
α
ipso-
hydroxylation
O
H—A
O—H
:B
NIH-shift
OH
H
O
rearomatization
OH
OH

FIGURE 1

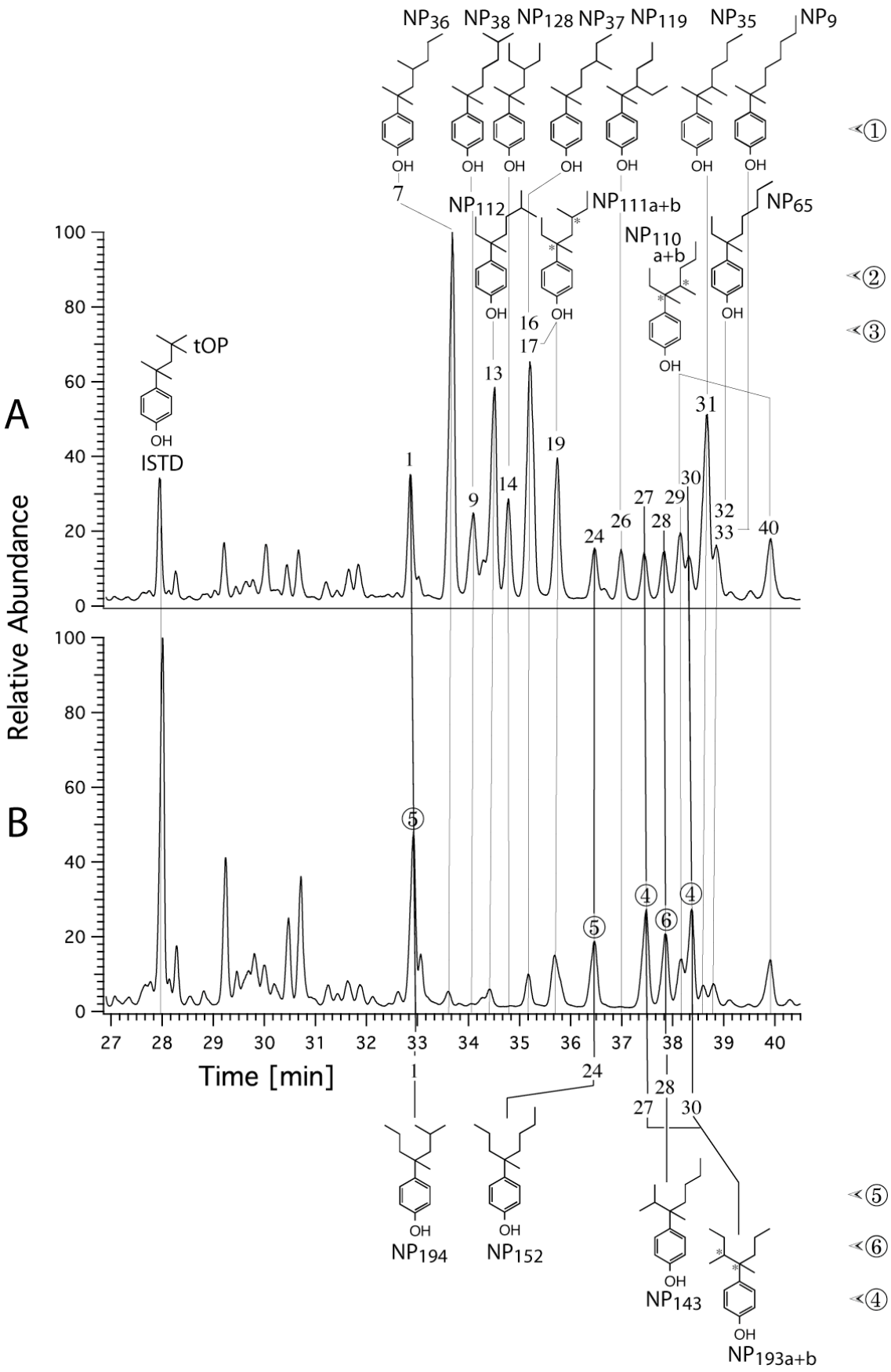

NP36 NP38 NP128 NP37 NP119 NP35 NP9
①
7
NP112
NP111a+b
NP65
NP110 a+b
②
③
16
17
tOP
ISTD
A
B
Relative Abundance
100
80
60
40
20
0
1
13
9
14
19
24
26
27
28
29
30
31
32
33
40
⑤
④
⑥
④
27 28 29 30 31 32 33 34 35 36 37 38 39 40
Time [min]
1
24
28
27
30
NP194
NP152
NP143
NP193a+b
⑤
⑥
④

FIGURE 2

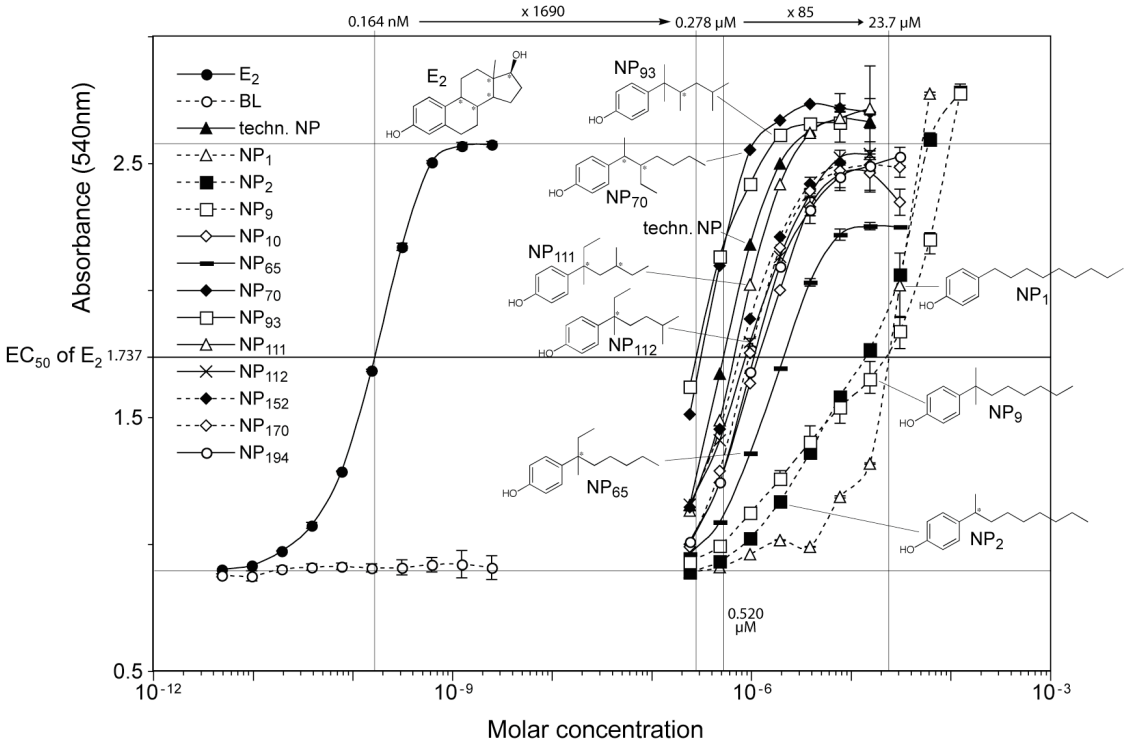

0.164 nM
x 1690
0.278 µM
x 85
23.7 µM
E2
BL
techn. NP
NP1
NP2
NP9
NP10
NP65
NP70
NP93
NP111
NP112
NP152
NP170
NP194
EC50 of E2 1.737
Absorbance (540nm)
2.5
1.5
0.5
techn. NP
0.520 µM
10-12
10-9
10-6
10-3
Molar concentration

FIGURE 3

"Brief" for Table of content:

Degradation of endocrine disrupting nonylphenols by microbial *ipso*-substitution markedly reshapes isomer distribution patterns and thus might alter the specific estrogenicity of aging material.