Nonylphenol Isomers Differ in Estrogenic Activity

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Nonylphenol belongs to the most investigated xenohormones acting at the estrogen receptor. Technical nonylphenol contains approximately 20 para-substituted isomers. Because of limitations in testing and quantifying all 20 isomers in the mixture, the linear form, 4n-NP, is often used as a reference substance, even though it is not present in the technical mixture. Here, we report on the synthesis and estrogenic potency of six nonylphenol isomers that occur at different proportions in technical nonylphenol mixtures. The relative potency of each isomer was determined by use of the MVLN transcriptional activation cell assay. As well, a subset of isomers was tested in the E-screen assay. One isomer, *p*353-NP, exhibited the same relative potency as the nonylphenol mixture, whereas the other isomers were found to be less potent. Two isomers, *p*22-NP and *p*262 NP, and the linear 4n-NP were found to be weak ER agonists with responses near the detection limit in the MVLN assay. Two isomers, *p*262-NP and 4n-NP, exhibited measurable activity in the E-screen. Our results demonstrate that defined *p*-NP isomers are most suitable for reflecting the estrogenic potency of technical NP mixtures. Among other applications, they should be used in the future to explain differences in estrogenic potency due to NPs as detected by various in vitro assays.

Introduction

Nonylphenol (*p*-NP) is used for the production of plastics, phenolic oximes, and nonylphenol ethoxylates in detergents and pesticide formulations and is ubiquitous in several environmental matrices. *p*-NP has been banned from use in the European Union since 2003 because of its high toxicity to invertebrates and estrogenic activity in vertebrates.

The potency of the estrogen receptor (ER) agonist *p*-NP has been demonstrated in vitro and in vivo in fish and mammals (*1*, *2*), by assessment of its direct interaction with the estrogen receptor (*3*, *4*). *p*-NP and 4-*tert*-octylphenol exhibit the greatest estrogenic potencies found for industrial chemicals, with relative potencies 10^3–10^6-fold less than that of 17β-estradiol (*1*, *3*).

Due to the synthetic methods used, technical *p*-NP is a mixture of approximately 20 para-substituted isomers with differently branched alkyl chains (*5*–*9*). 4n-NP, a non-branched isomer, exhibits different physicochemical properties than the branched isomers and is not found in the *p*-NP mixture (*10*). Nevertheless, 4n-NP is often used as a reference substance for *p*-NP because of its commercial availability (*11*, *12*). The degree to which this surrogate isomer has similar estrogenic potencies as the isomers in the technical mixtures will determine the accuracy of risk assessments based on this assumption.

Much research has been conducted to improve understanding of the interaction of xenoestrogens with the ER. A free phenolic hydroxyl group is essential for binding of 17β-estradiol (E2) to the ER and for alkylphenols, which exhibit greater binding affinity to the ER than phenol (*10*). Other studies of the structure-potency relationships of alkylphenols have found that the greatest relative potency was associated with a para-substituted side chain and that estrogenic potency and affinity was less for substances with more than one side chain (*10*, *13*). Furthermore, the potency was greatest for alkylphenols branched at the α-carbon and with a side chain length of eight to nine carbons (*13*). 4-*tert*-octylphenol was found to be the most potent alkylphenol, and it has been suggested that the low estrogenic activity of *p*-NP compared to that of 4-*tert*-octylphenol might be due to the isomeric heterogeneity of *p*-NP (*13*). Therefore, it is possible that *p*-NP contains isomers with greater or lesser estrogenic potency than 4-*tert*-octylphenol. Because the isomeric composition of *p*-NP differs among producers (*7*) and environmental degradation can lead to a shift in isomeric composition of *p*-NP (*14*), it is necessary to know the estrogenic potency of individual isomers, both for understanding interactions of alkylphenols with the ER and environmental risk assessment.

In the present study, *p*-NP isomers were synthesized, and their relative potencies were determined by use of the MVLN transcriptional activation cell assay. This assay, based on human breast cancer cells, is suitable for detecting estrogenic potency of single substances (*3*, *15*) and environmental samples (*15*, *16*). In addition, a more integrative breast cancer cell-based bioassay, the E-screen, was applied to confirm the ranking of estrogenic potency for selected *p*-NP isomers.

Materials and Methods

Test Agents. Two commercially available technical *p*-NP mixtures with branched side chains were used, *p*-NP Fluka (85% purity, Fluka Germany) and *p*-NP Acros (99% purity, Aldrich Germany). Six isomers 4-(3′,5′-dimethyl-3′-heptyl)-phenol (*p*353-NP) or 4-NP₁₁₁ (*17*), 4-(3′,6′-dimethyl-3′-heptyl)-phenol (*p*363-NP) or 4-NP₁₁₂ (*17*), 4-(2′,5′-dimethyl-2′-heptyl)-phenol (*p*252-NP) or 4-NP₃₇ (*17*), 4-(2′,6′-dimethyl-2′-heptyl)phenol (*p*262-NP) or 4-NP₃₈ (*17*), 4-(3′-methyl-3′-octyl)phenol (*p*33-NP) or 4-NP₆₅ (*17*), and 4-(2′-methyl-2′-octyl)phenol (*p*22-NP) or 4-NP₉ (*17*), were synthesized as described previously (*7*, *18*). After synthesis, *p*-NP isomers

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were purified on a silica gel column with hexane:ethyl acetate (1:13) as the eluting solvent. Purities of the isomers were determined by GC/MS and were all greater than 99.9%. Stock solutions were prepared in ethanol and stored at −20 °C. p-NP isomers were stable at least for 2 years under this storage condition, as determined by GC/MS.

MVLN Cell Assay. MVLN cells are MCF-7 human breast carcinoma cells that have been stably transfected with an estrogen receptor controlled luciferase reporter gene (19). In this assay, expression of the exogenous reporter gene luciferase under control of the estrogen response element (ERE), vitellogenin-promoter from the African clawed frog (*Xenopus laevis*), is used as a sensitive measure of potency (19). Exposure to estrogen agonists leads to transcription of the reporter gene luciferase. Luciferase emits light in the presence of a reagent containing luciferin. The amount of light produced by the cells is directly proportional to the occupancy of the agonist on the ERE which is a function of the binding affinity and concentration of the agonist (15).

The culture medium used was hormone free Dulbecco's Modified Eagle Medium with Hams F-12 nutrient mixture (Sigma-Aldrich, Germany), 1 mM sodium pyruvate (Sigma-Aldrich, Germany), and 1 mg/L of insulin (Sigma-Aldrich, Germany) supplemented with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT). Detailed descriptions of cell culture conditions and chemical test procedures have been published previously (15, 16). Standard 96-well plates were seeded using a 250 μL suspension containing 7.5 × 10⁴ cells/mL in culture medium per well and incubated for 24 h. A test compound stock solution (2.5 μL) in ethanol was added to three replicates, and for each plate, a blank and an ethanol control were prepared. It has previously been shown that this ethanol concentration <1% has no effect in the assay (15, 16). Three 17β-estradiol concentrations (4.1−1000 pM) and one protein standard curve were also included in each test. Luciferase activity was measured after 72-h incubation with Spectrafluor Plus (Tecan, Austria) using LucLite cocktail (Packard Instruments). To test for cell toxicity, a fluorescamine based assay was used to measure the protein level (20). Each test compound was tested in at least three independent assays.

The relative potency and efficacy, the greatest activity measured in one replicate, of each individual NP isomer or the various technical mixtures were determined by use of methods that have been previously described (16). Subtraction of the blank luminescence mean value was used to produce relative luminesence units (RLU) for each well. The maximum response of E2 was set to 100%, and the RLU for each sample well was calculated as a percentage of this maximum induction of luciferase activity (RLU % E2 max). The limit of quantification of the assay was 7% of the E2 max, calculated as the 3-fold standard deviation of the solvent control. Sample with values less than half of the solvent control protein concentration were excluded from calculation of the dose response curve, because cytotoxicity would lead to less enzyme production and luminescence that might confound the detection of estrogenic activity. Effect levels (EC$_x$) were determined by fitting the best regression equations to the concentration response data in RLU % E2 max from 1.75 μM to 18 μM for each compound using Sigma Plot (version 8.0; SPSS Inc., Chicago, IL). The best description of the concentration−response for all NP compounds was a Box-Cox-Logit (21) regression and for estradiol the Hill regression (22). Because some isomer concentration−response curves did not reach or exceed the E2 EC$_{50}$, relative estrogenic potency (REP) was calculated by dividing the EC$_{20}$ of E2 by the EC$_{20}$ of p-NP. This calculation of the REP is suitable for comparison of different substances (23).

E-Screen Assay. MCF-7-cells were kindly provided by the Institute for Sanitary Engineering, Water Quality and Solid
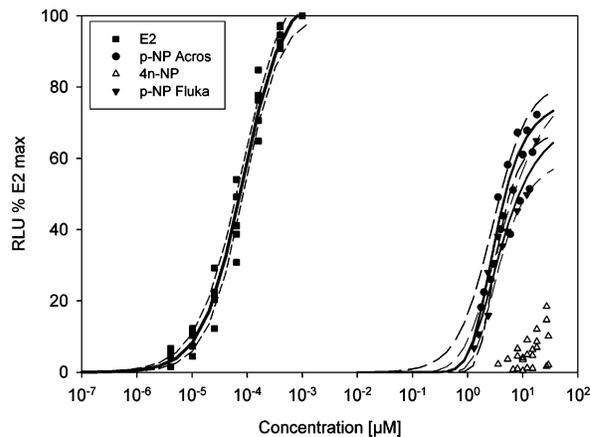
FIGURE 1. Concentration−response curves obtained with the MVLN assay for two technical nonylphenol mixtures and 4n-NP in comparison to estradiol. Symbols represent means of replicate measurements, and regression lines are presented with dotted lines representing 95% confidence levels.

Waste Management (Stuttgart, Germany). The proliferation test using MCF-7 cells (E-screen assay) was carried out according to the method of ref 24 with some modifications. Briefly, 6000 cells were plated in 1 mL of experimental culture medium (without phenol red, containing charcoal-dextran treated fetal bovine serum [cd-FBS]) per well of 24-well tissue culture plates (TPP). After 24 h of cell attachment, experimental medium was removed and replaced by 1 mL of experimental medium containing 0.05% of p-NP or the different p-NP isomers (all in methanol; Merck) in five different dilutions. Each dilution was tested in two independent experiments in triplicate wells. Three different internal controls were included in each assay: positive control 10⁻¹⁰ M 17β-estradiol (E2) (Sigma) (concentration found to induce the maximal proliferative effect); a blank control consisting of experimental medium alone; a solvent control consisting of experimental medium with 0.05% methanol. Cells were counted after 7 days using the Alamar Blue (Biosource) assay as a measure of cell number (25). Briefly, plates were washed with 400 μL of PBS, followed by incubation in 400 μL of a 5% working solution of Alamar Blue prepared in PBS. After 60 min of incubation, fluorescence was measured at 530 and 595 nm using a Tecan fluorescent plate reader.

Fluorescence units were converted to cell number by means of a fluorescent units vs cell number linear correlation function. The proliferative effect (PE) was calculated as the ratio of the maximum cell proliferation achieved by either p-NP or the fractions to the cell number obtained in the solvent control. The estrogenic potency of a substance can be described by the relative proliferative effect (RPE). With the RPE it is possible to draw conclusions concerning the estrogenic potential of a tested substance in comparison to the maximal inducible proliferation by the reference substance 17β-estradiol. The RPE was calculated using the standard equation for the E-screen assay (24).

Results

Concentration−Response Relationships of Commercially Available Nonylphenol. A concentration−response relationship was developed for both technical p-NP mixtures, both mainly composed of branched isomers with different purities, up to 18 μM (Figure 1). At concentrations higher than 20 μM, the response decreased due to toxicity of the compounds on the cells, which was confirmed by decreasing protein levels (data not shown). The p-NP mixture from Acros exhibited slightly greater efficacy than that from Fluka, with a maximum response of 82% of that produced by E2 at 12−18 μM and

TABLE 1. Estrogenic Activity of Tested Compounds[a]

	r^2	efficacy [%]	EC$_{20}$ (CI) [μM]	EC$_{50}$ (CI) [μM]	REP $\times$ 10^{-5}
			MVLN Assay		
17β-estradiol	0.974	100	2.6×10^{-5} $(1.9-2.9 \times 10^{-5})$	8.3×10^{-5} $(6.4-8.5 \times 10^{-5})$	
p-NP Fluka	0.881	69.7	2.2 (1.8-2.6)	8.5 (6.9-12)	1.13 (1.05-1.66)
p-NP Acros	0.788	82	1.9 (1.2-2.5)	4.8 (3.9-5.8)	1.35 (1.18-1.58)
4n-NP	nd	20	nd	nd	nd
*p*353-NP	0.721	72	2.7 (1.8-3.3)	6.6 (5.2-9.5)	0.94 (0.86-1.05)
*p*363-NP	0.618	46	3.7 (2.6-4.6)	nd	0.69 (0.63-0.75)
*p*33-NP	0.704	51	4.2 (3.1-5.1)	nd	0.61 (0.57-0.62)
*p*252-NP	0.857	32	12.7 (7.2-nd)	nd	0.20 (nd-0.27)
*p*262-NP	nd	16.5	nd	nd	nd
*p*22-NP	nd	10.2	nd	nd	nd
			E-Screen Assay		
p-NP Acros	0.907	99	0.02 (−)	0.07 (−)	4.06 (−)
*p*353-NP	0.989	81	0.08 (0.05-0.11)	0.36 (0.28-0.47)	0.75 (0.57-0.96)
*p*363-NP	0.985	81	0.08 (0.04-0.13)	0.43 (0.32-0.56)	0.63 (0.48-0.84)
*p*262-NP	0.995	72	0.36 (0.30-0.41)	0.9 (0.84-0.95)	0.3 (0.28-0.32)

[a] r^2, coefficient of determination; efficacy, max. response % of E2 found in any replicate; CI, 95% confidence level; REP, relative estrogenic potency calculated by the EC$_{20}$ for the MVLN assay and the EC$_{50}$ for the E-screen; nd, not determined because maximum activity was below the calculated threshold.
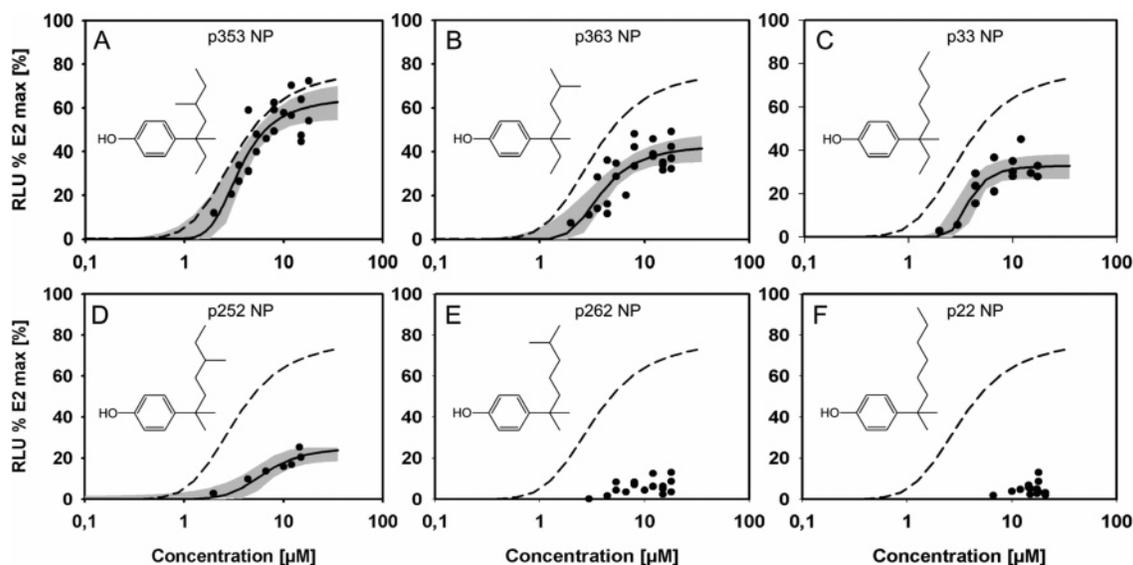

FIGURE 2. Concentration−response curves of six nonylphenol isomers for the MVLN assay. Symbols represent the mean of triplicates for each assay, and the solid best fit regression lines are surrounded by gray shaded areas representing the 95% confidence limit. The dashed line shows the response with the Acros *p*-NP. (A) *p*353-NP, (B) *p*363-NP, (C) *p*33-NP, (D) *p*252-NP, (E), *p*262-NP, (F) *p*22-NP.

69.7% at 18 μM, respectively (Figure 1). The calculated potency of the Fluka mixture was less, which suggests that this mixture contains, on average, less potent agonists (Table 1). In contrast, the linear 4*n*-NP isomer was found to be much less efficacious, reaching only 20% E2 max in one replicate at 27 μM and most of the measured activity was less than 7% of E2 max, which was the detection limit of the assay. Less luminescence, due to cytotoxicity, was produced in the presence of 4*n*-NP at concentrations greater than 40 μM (not shown).

Relative Estrogenic Potency of Nonylphenol Isomers. The six *p*-NP isomers tested in this study exhibited significant differences in their relative estrogenic potency in the MVLN assay. Complete concentration−response curves could be generated for only four of the isomers (Figure 2). The efficacies of *p*22-NP and *p*262-NP were generally less than the detection limit of the assay (Figure 2E,F). Therefore, effect concentrations and relative potencies could not be determined for these compounds (Table 1). The *p*353-NP isomer exhibited the greatest efficacy of all single isomers, with a maximal
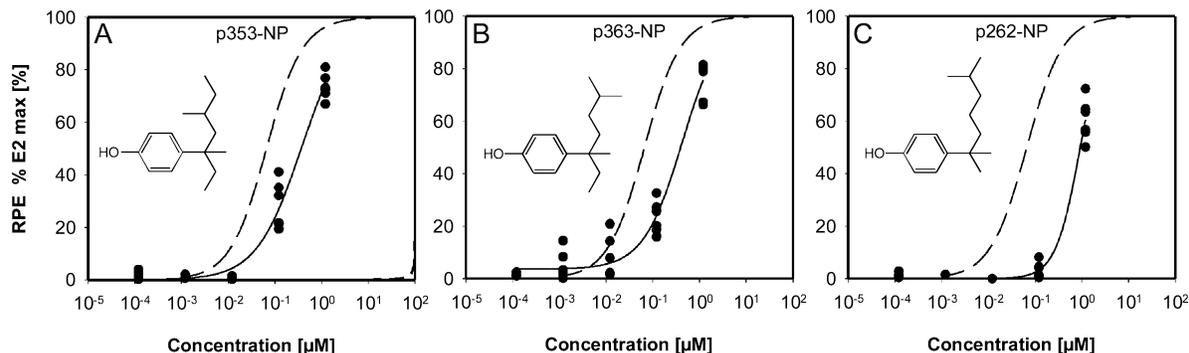
FIGURE 3. Concentration−response curves of three nonylphenol isomers for the E-screen assay. Symbols represent replicates of two assays. The dashed line shows the response with the Acros *p*-NP. (A) *p*353-NP, (B) *p*363-NP, (C) *p*262-NP.
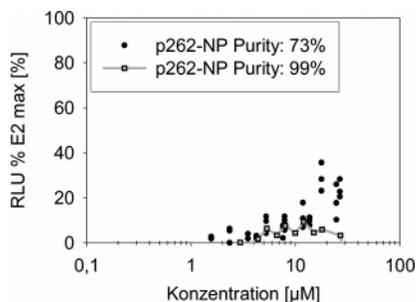

FIGURE 4. *p*262-NP tested in two different purities. *p*262-NP after synthesis with a purity of 73% (black dots) and after purification (gray squares).

response at 8 μM which was 71% of that caused by 1000 pM E2. The response was in the same range as that of the technical *p*-NP mixtures (Figures 2A and 1). *p*363-NP, *p*33-NP, and *p*252-NP exhibited efficacies that were only 46%, 51%, and 32% of that of E2, respectively (Figure 2B−D), and had relative potencies that were lower than those of *p*353-NP and the technical mixture.

In the E-screen assay, the tested isomers showed the same ranking (Figure 3), with *p*353-NP being the most potent followed by *p*363-NP and *p*262-NP. The REP found for *p*353-NP and *p*363-NP were equivalent in both assays (Table 1). In contrast to the MLVN assay, however, a significant estrogenic response was observed for the *p*262-NP isomer at the greatest concentration tested. Overall, the E-screen assay responded about 10-fold more sensitive with regard to *p*-NP isomer and E2 concentrations than the MLVN assay.

To investigate the influence of the purity for the test substance, three isomers were tested without purification after synthesis. Only slight differences in the relative potency were observed for *p*353-NP (purity 92%) and *p*363-NP (purity 89%) compared to the purified compounds (not shown). For *p*262-NP (purity 73%) an estrogenic potency was found with an efficacy of 40%, whereas no luciferase activity was detected for the purified isomer (Figure 4.).

Discussion

Nonylphenol Isomers Show Different Estrogenic Potencies.
The tested nonylphenol isomers varied in their estrogenic potency and efficacy. *p*353-NP exhibited the same relative potency as the technical *p*-NP mixture, whereas *p*262-NP and *p*22-NP caused no measurable luciferase activity in the MVLN bioassay (Figure 2). Cytotoxicity of these isomers was observed at concentrations greater than 18 μM with no great differences among isomers (data not shown). In the E-screen assay the same trends in estrogenic potency were observed, although *p*262-NP exhibited an estrogenic response at the greatest concentration tested. All isomers exhibited different potencies and efficacy in the MVLN as well as in the E-screen

assay, clearly indicating differences in the potency at the ER. These differences may be due to differences in receptor affinity, receptor activation, or activation/deactivation of nonreceptor-mediated pathways of gene activation.

Three other studies of the estrogenic potency of *p*-NP isomers are published (*9*, *14*, *26*). Kim et al. investigated fractions of technical *p*-NP mixtures (Tokyo Kasei Kogyo) for their relative potencies by use of the YES-assay (*9*, *14*). The predominant compound in each HPLC fraction was determined by NMR. The purity of the fractions ranged from 53 to 93%. The results of both studies revealed inconsistencies in the ranking of the isomers as judged by estrogenic potency and the REPs:

$$\underset{(4 \times 10^{-4})}{p252\text{-NP}} > \underset{(8 \times 10^{-5})}{p22\text{-NP}} > \underset{(6 \times 10^{-5})}{p33\text{-NP}} = \underset{(6 \times 10^{-5})}{p232\text{-NP}} > \\ \underset{(4 \times 10^{-5})}{p242\text{-NP}} \text{ (ref. 9)}$$

$$\underset{(1 \times 10^{-3})}{p33\text{-NP}} > \underset{(9 \times 10^{-4})}{p252\text{-NP}} > \underset{(8 \times 10^{-4})}{p242\text{-NP}} > \underset{(7 \times 10^{-5})}{p232\text{-NP}} \text{ (ref. 14)}$$

These deviations in the estrogenic potency may be due to the low purity of the tested compounds as suggested by the authors themselves (*9*) and others (*27*). We also found that the relative potencies of the *p*-NP compounds and the technical mixtures were influenced by their purity. *p*262-NP with a purity of 73% had a relatively small estrogenic activity that produced a maximum efficacy of 29% of the E2 maximum in the MVLN assay, whereas no estrogenic activity could be demonstrated for the pure *p*262-NP (Figure 4). The impurity contained more potent estrogenic compounds, which could lead to misinterpretation of the estrogenic potency for *p*262-NP. This could also be true for the results of Kim et al. (*9*, *14*) with much lower purities down to 53%. Despite the role of impurities, pure *p*262-NP produced an estrogenic response at the greatest concentration tested in the E-screen assay, thus illustrating the importance of combining bioassays of differing complexity to ensure a comprehensive evaluation of estrogenic potency. Nevertheless, to exclude possible effects due to impurities, it is important to purify compounds to the greatest possible extent before using them for determining relative ER potencies.

The estrogenic potency of differently branched *p*-NP isomers was compared to another study in which a yeast two-hybrid assay, using coactivator recruitment of the hERα, was used to determine relative potencies (*26*). From the synthesized 22 isomers only 9 were found in the technical *p*-NP (Kishida Osaka, Japan), including *p*22-NP, *p*262-NP, *p*22-NP, and *p*33-NP, all showing lesser estrogenic potency than the technical mixture. Comparisons among the results of the different studies available in the literature that have investigated the estrogenic potency of *p*-NP isomers does not yet yield a consistent pattern. The REP of the isomers varied over 1 order of magnitude, but, more importantly, the
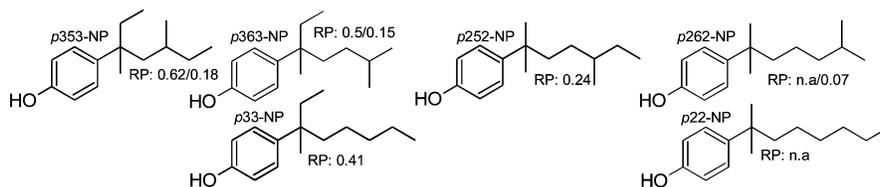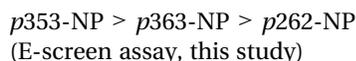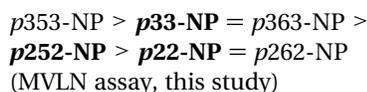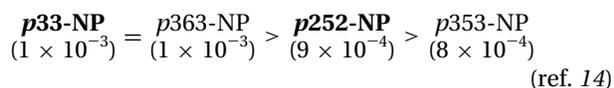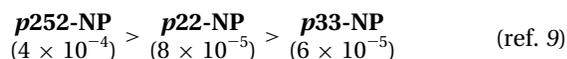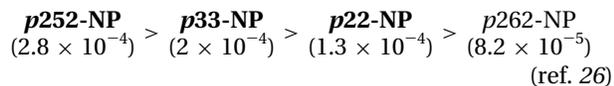
FIGURE 5. *p*-NP isomers investigated in our study, sorted by their relative potency (RP, MVLN/E-screen) from left to right.

ranking of the isomers are not consistent between the studies.

$$\underset{(2.8 \times 10^{-4})}{\textbf{\textit{p}252-NP}} > \underset{(2 \times 10^{-4})}{\textbf{\textit{p}33-NP}} > \underset{(1.3 \times 10^{-4})}{\textbf{\textit{p}22-NP}} > \underset{(8.2 \times 10^{-5})}{\textit{p}262\text{-NP}}$$

(ref. *26*)

$$\underset{(4 \times 10^{-4})}{\textbf{\textit{p}252-NP}} > \underset{(8 \times 10^{-5})}{\textbf{\textit{p}22-NP}} > \underset{(6 \times 10^{-5})}{\textbf{\textit{p}33-NP}}$$

(ref. *9*)

$$\underset{(1 \times 10^{-3})}{\textbf{\textit{p}33-NP}} = \underset{(1 \times 10^{-3})}{\textit{p}363\text{-NP}} > \underset{(9 \times 10^{-4})}{\textbf{\textit{p}252-NP}} > \underset{(8 \times 10^{-4})}{\textit{p}353\text{-NP}}$$

(ref. *14*)

*p*353-NP > **_p_33-NP** = *p*363-NP >
_p_252-NP > **_p_22-NP** = *p*262-NP
(MVLN assay, this study)

*p*353-NP > *p*363-NP > *p*262-NP
(E-screen assay, this study)

The reasons for these different findings are not clear. All studies are based on interactions with the hERα, which implies that differences in the receptor per se cannot explain the differences observed. Our results are the first to demonstrate concentration−response curves and statistical data to provide an impression of the robustness of the data sets. As stated above, impurities may account for the inconsistencies observed in the results of the studies conducted by Kim et al., but this is not the case for the results of our study (purity >99%) and that of Shioji et al. (purity >97%).

Assay differences can also contribute to this inconsistent pattern. Reporter gene assays, using gene translation as endpoint, were used in Kim et al. and our study, whereas Shioji et al. used coactivator recruitment as an endpoint. In the study of Shioji et al., a relatively great REP value of 1.8 × 10⁻³ was reported for *p*-NP, which were found, to be 10− 100-fold greater than those reported in the literature. For one isomer, which shows 153-fold greater potency than the technical mixture, a REP of 0.27 was found. This REP is in the range of estradiol derivates (estrone, estriol, or diethylstilbestrol) with very high estrogenic activity (*3*). The relative binding affinities for *p*-NP and 4n-NP were found to be in the range of 10⁻³ (*10*); therefore, it seems unreliable that the REP of a substance is higher than its relative binding affinity. In contrast to the above-mentioned estradiol derivates, all alkylphenols do not possess an OH group at the position of the D-ring of estradiol, an important structural feature for binding to, and activation of, the ER. In the E-screen, cell proliferation is used as the final outcome, representing the most integrative cellular response. The studies of Kim et al. and Shioji et al. used artificial yeast systems, whereas our study was conducted in human cells (MCF-7). It is well-known that the cellular context plays a major role in ER activation (*28*). The REP for *p*353-NP and *p*363-NP are nearly the same between both assays in our study but not for *p*262-NP and *p*-NP. Taken together, a critical evaluation of the responses provided by each of the assays is an important future task, but the use of combinations of available estrogenresponse assays, as presented here, appears to be best.

It is possible that different enantiomeres or racemates were tested which may lead to unequal receptor activation.

Most *p*-NP isomers are stereoisomers, and some are diastereomers, e.g. *p*353-NP. At least *p*353-NP is present in the technical mixture as a diastereomer, resulting in two peaks by GC analysis (*5, 7*). Similar information is not yet available for any of the other isomers investigated.

Structural Considerations for the Activity of Nonylphenol Isomers. It has been suggested that a tertiary branched α-carbon is an important structural feature for estrogenic potency of alkylphenols (*13*). In contrast, other researchers have pointed out that the bulkiness of the nonyl group is the most important feature for estrogenic potency (*26*). From their study, the length of the main alkyl chain and branching at the β-carbon and/or γ-carbon are more important for the estrogenic potency of alkylphenols than the branching at the α-carbon.

In our study the length of the side chain at the α-carbon seems to be another important feature for the estrogenic potency of alkylphenols, either directly or by shortening the length of the alkyl chain. The three isomers with an ethyl side chain at the α-carbon (*p*353-NP, *p*363-NP, *p*33-NP) all showed clear estrogenic potency (Figures 5 and 2A−C), whereas isomers with a methyl side chain at this position (*p*252-NP, *p*22-NP, *p*262-NP) showed only little or no estrogenic activity (Figures 5 and 2D−F). Isomers which were also branched in the alkyl side chain (*p*353-NP/*p*252-NP) exhibited greater estrogenic potency and efficacy than nonbranched isomers (*p*33-NP/*p*22-NP) or isomers branched at the end of the side chain (*p*363-NP/*p*262-NP). This feature also seems to be important for the structure−activity relationship of alkylphenols.

These results are similar to those presented by Shioji et al. (*26*), as shown in Figure 6. They pointed out that the length of the main chain is an important factor for the estrogenic potency of alkylphenols (Figure 6, left). Therefore, selected isomers from their study were rearranged by the same length of the main chain. By rearranging the isomers, it is obvious that isomers with methyl-ethyl or butyl branching at the α-carbon show the highest estrogenic potency (Figure 6, right) and that isomers branched at the middle of the main chain, as *p*353-NP and *p*252-NP, show higher estrogenic potency than isomers without branching or branched at the end of the main chain (Figure 6, middle).

Estrogenic Potencies of Commercially Available *p*-NP. The isomers investigated in this study are present in different relative proportions in the two technical mixtures, with *p*353-NP being the most prominent isomer in both mixtures representing about 20% of the mass (*7*). Both *p*-NP mixtures, which were composed primarily of branched nonylphenol isomers with different purities and isomeric distributions, exhibit measurable estrogenic potency. Due to the cytotoxic effects of *p*-NP, it was not possible to achieve complete efficacy (100% relative to E2) in the MVLN reporter gene cell assay. The calculated relative E2 potencies of the technical nonylpenol mixtures were 1.35 × 10⁻⁵ and 1.13 × 10⁻⁵ for the Acros and Fluka *p*-NP mixtures, respectively, in the MVLN assay and 4.06 × 10⁻⁵ for the Acros technical mixture of *p*-NP in the E-screen assay (Table 1). This is within the range of relative potencies previously reported for these compounds for the MVLN assay (*3, 29*) and other test systems (*9, 13*). For 4n-NP some authors found similar potency relative to E2 in the range of 10⁻⁴−10⁻⁷ (*9, 10*), whereas limited E2 relative
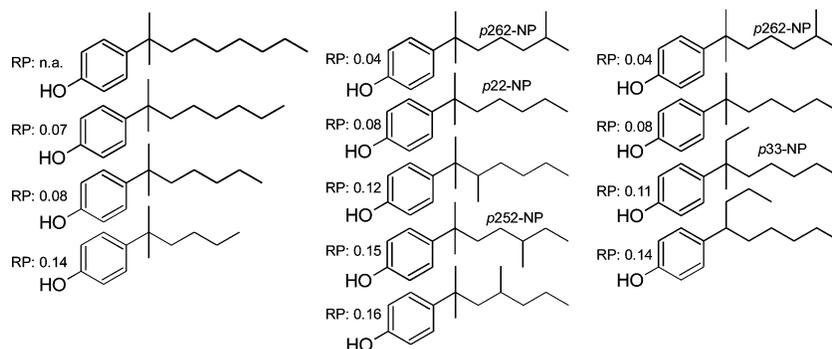
FIGURE 6. Rearranged structure–activity relationship, from the data of Shioji et al., for isomers with similar structure investigated in our study. Relative potency (RP) for each isomer compared to the technical mixture (Kishida, Japan) is given, as described in ref *26*.

potency was observed in the MVLN assay in this study and in other publications (*29*, *30*).

The differences in estrogenic potency between the two mixtures can be explained mostly by the lower purity of the mixture from Fluka. Purity cannot explain the lower efficacy of 69.7% of that of E2, which was observed for the Fluka mixture as compared to the 82% observed for the technical mixture from Acros. One possible explanation for this lesser efficacy might be a greater proportion of less potent isomers, such as *p*22-NP, *p*262-NP, and *p*252-NP that were present in the mixture from Fluka (*7*).

Because all investigated isomers exhibit equal or lower estrogenic activity than *p*-NP, it is obvious that *p*-NP contains some isomers with greater ER activity than those that we synthesized and tested in the present study. Only 51% mass of the isomers in *p*-NP from Fluka and 37% mass of *p*-NP from Acros were investigated for their estrogenic potency. The isomers with low potency only contribute to 7% and 3% to the technical mixture (*7*), so their influence on total estrogenic potency will be low. *p*353-NP, showing the same estrogenic potency as *p*-NP, contributes to 20% mass in both *p*-NP mixtures. The mass proportions of *p*-NP together with slightly greater estrogenic potency of the not tested isomers may explain the estrogenic potency of *p*-NP. The remaining isomers are more branched than the ones investigated (*5*). The greatest estrogenic potency was exhibited by the most branched isomer (*p*353-NP) which has been suggested from structure–activity relationships that bulkiness of isomers increase estrogenic potency (*26*). Therefore it may be comprehensible that most of the remaining isomers show greater potency than the ones tested. In addition it has to be kept in mind that some isomers in *p*-NP are chiral (*7*), which can also lead to differences in the estrogenic potency. All chiral isomers were tested as racematic mixtures, thus the influence of chirality could not be investigated here or in any previous study (*9*, *14*, *26*). It is unclear if the proportion of enantiomeres tested were the same as in the technical mixture. The results for *p*-NP isomers are rather inconsistent; we, therefore, think that it is not reliable at the moment to identify the most active isomer or to calculate the activity of technical mixtures from the activity of the isomers. Systematic research on alkylphenol isomers in different assays may help to collect the data to overcome this current short coming. Nevertheless the isomers exhibit different estrogenic potencies, and the relative amount of the isomers is different in varieties of *p*-NP mixtures (*7*). This can alter the estrogenic activity of *p*-NP and may explain, in addition to assay differences, the great variety found in the literature for the estrogenic potency of *p*-NP.

Implications for Testing Estrogenic Potency. Over 300 publications dealing with the estrogenic activity of nonylphenol have been published by now, mostly not taking into account that *p*-NP is not a pure substance but a mixture. Often the *p*-NP mixture used is not characterized, and the

results are compared to results from other mixtures; some authors did even not distinguish between 4n-NP and *p*-NP.

Because *p*-NP becomes a standard compound for xenoestrogens, it is important to consider the relative potencies of the individual isomers not only for environmental risk assessment of *p*-NP but also for determination of the interactions of alkylphenols with the estrogen receptor. This study shows that the use of mixtures or impure substances for the investigation of a special mode of action, like estrogenic activity, can lead to misinterpretations and unsatisfying conclusions. It was clearly shown that the nonylphenol isomers have relevant differences in their potency and efficacy, which means they have varying degrees of partial agonism at the estrogen receptor. Isomeric composition of *p*-NP differs between different producers (*7*) and shifts due to environmental degradation (*14*). It is therefore not acceptable to pool the isomers, or use a nonvalidated reference like 4n-NP, to build up models, structure–activity relationships, or risk assessments for estrogenic potency. We suggest that, if needed, *p*353-NP would be a better reference isomer for *p*-NP than 4n-NP. It is the most prominent isomer in all mixtures tested, has the same physicochemical properties as *p*-NP, and shows nearly the same estrogenic activity. The results from different studies, discussed above, suggest that defined *p*-NP isomers may be good test substances for the detection of assay differences. They show the same physicochemical properties and toxicity but different estrogenic potency.

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