SEPARATION, STRUCTURAL ELUCIDATION AND ESTROGENIC ACTIVITY STUDIES OF THE STRUCTURAL ISOMERS OF 4-NONYLPHENOL BY GC-PFC COUPLED WITH MS AND NMR

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ABSTRACT

A commercial nonylphenol (NP) reagent was separated into 14 fractions, NP1 to NP14 using a gas chromatograph equipped with a preparative fraction collector (GC-PFC). The identification of the major components of 14 fractions was attempted by analysis of their nuclear magnetic resonance (NMR) spectra, and the estrogenicity of the isomers was tested in a recombinant yeast screen system. Eleven structural isomers of NP were characterised and identified as the following para- (position 4-) branch isomers of phenol:

NP1 1,3-dimethyl-1-propyl-butyl
NP2 1,1,3-trimethyl-hexyl
NP3 1,4-dimethyl-1-ethyl-pentyl
NP4 1,3-dimethyl-1-ethyl-pentyl
NP5 1,1,4-trimethyl-hexyl
NP6 1,3-dimethyl-1-ethyl-pentyl
NP7 1,1-dimethyl-2-ethyl-pentyl
NP8 1,2-dimethyl-1-ethyl-pentyl
NP9 1,2-dimethyl-1-propyl-butyl
NP10 1,2-dimethyl-3-hexyl
NP11 1,1,2-trimethyl-hexyl
NP12 1-ethyl-1-methyl-hexyl

The three isomers NP8, NP13 and NP14 still remain to be identified. NP7 (4-(1,1-dimethyl-2-ethyl-pentyl)-phenol) was found to exhibit the highest estrogenic activity, corresponding to 1.9×10⁻³ that of 17β-estradiol (E2). The relative composition of the NP isomers varied between the dissolved phase and suspended solids separated from a water sample in New York-New Jersey Harbour, USA. The proportions of the isomers in the NP mixture were different from the proportions in the environmental samples. In suspended solids, the NP7 was present in higher proportions than other isomers.

Key words: Nonylphenol isomers, GC-PFC, NMR, estrogenic activity, recombinant yeast screen assay.

INTRODUCTION

Alkylphenolic chemicals are used as detergents. Their derivatives, alkylphenol polyethoxylates, are the second largest group of nonionic surfactants in commercial production, and are widely used not only in detergents but also in paints, herbicides, pesticides, and other formulation products (White et al. 1994). Nonylphenol (NP) is manufactured by the alkylation of phenol with nonene isomers. Therefore, it is expected that commercial NP would be a mixture of 4-substituted monoalkylphenols with various isomeric and branched nonyl groups. Mixtures of nonene isomers are industrially synthesised from propylene (CH₃CH=CH₂) by trimerisation (Lee and Peart 1995).

Dodds and Lawson (1937) reported that propyl and propenyl phenols are weakly estrogenic substances. Later they found that a man-made estrogen, diethylstilbestrol (C₁₈H₂₀O₂), had almost the same activity as natural estrogen (Dodds and Lawson 1938). Routledge and Sumpter (1997) reported that the estrogenic activity for human estrogen receptor (hER) of alkylphenols when examined for structure-activity relationships depended on the number of carbon atoms in the alkyl chain, and that branching (tertiary > secondary = normal) of the alkyl group affects estrogenicity. An earlier work (Sekine et al. 2001) also reported that a NP mixture that interfered with surrounding wells during assay of estrogenic activity in recombinant yeast screen was estrogenic, but non-branched n-nonylphenol (n-NP) was not. The biological activities of chemicals are usually isomer-specific: for example, 17β-estradiol (E2) that is produced naturally in ovary and also synthesised artificially from estrone is strongly estrogenic, whereas its α-isomer is not estrogenic (Velle 1976, Grandbois et al. 2000). A recent work in this laboratory reported variation in estrogenic activity among fractions of a NP mixture obtained by high performance liquid chromatography (HPLC) (Kim et al. 2004).
Estrogenic isomers of nonylphenols

Bhatt et al. (1992) reported that the isomers of a NP mixture, separated using a cross-linked methyl silicon capillary column by gas chromatography coupled with mass spectroscopy (GC-MS) or Fourier transform-infrared (GC-FTIR), were used to unequivocally characterise some of the isomers of p-NP. From this work, the NP mixture was found semiquantitatively to have 90% para-isomers and 10% ortho-isomers by a 13C nuclear magnetic resonance (NMR). Wheeler et al. (1997) reported that high-resolution gas chromatographic analyses of p-NP at selected oven temperatures have resolved 22 para-isomers, where 11 isomers were characterised using GC-MS and GC-FTIR. Six fractions of a NP mixture were separated using a two-dimensional capillary gas chromatograph equipped with a preparative fraction collector (GC-PFC), and their different estrogenic activities in a mammalian cell line, MVLM (MCF-7) were determined (Yamashita et al. 1999).

In this study, the isomers in a NP mixture were separated by GC-PFC, the separated isomers were subsequently identified by NMR and the estrogenic activity of each of the isomers was tested by a recombinant yeast screen assay. In order to obtain authentic standards for bioassay and for structure confirmation of individual isomers, two synthesised isomers of NP were successfully separated and identified. The MS and NMR spectra of the two synthesised isomers were confirmed to be identical with those of the earlier separated isomers. The GC-PFC/NMR method was applied in the determination of NP in water samples collected from New York-New Jersey Harbor, USA, in both the dissolved phase and the suspended solids.

MATERIAL AND METHODS

Nonylphenols and 17β-estradiol

Three stock preparations of nonylphenol mixtures (CAS No. 84852-15-3) were obtained from three chemical companies. Two sources were local, the Tokyo Kasei Kogyo Co. (TKK) and Kanto Chemical Co. (Kanto), Japan; these NP preparations were used in the separation, identification and estrogenic activity studies described in the following sections. A NP preparation was also obtained from Aldrich Chemical Inc. (Aldrich), USA; this preparation was used to confirm that the chromatograms by GC-MS were similar to those obtained with local preparations. The TKK NP mixture is described as a mixture of para-substituted phenols with branched isomerism of the nonyl sidechain. The Aldrich NP mixture is described as a mixture of ring and chain isomers.

Two NP-isomers, 4-(1,1,4-trimethyl-hexyl)-phenol (NP5) and 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol (NP7), were synthesised by Uchiyama et al. (2005).

A 3000-mg/L solution of the NP mixture (TKK) was prepared in hexane for preliminary fractionation by HPLC before using GC-PFC.

17β-Estradiol (E2) (CAS No. 50-28-2) was purchased from TKK.

Environmental sampling

A water sample (8L) was collected from New York-New Jersey Harbor, USA.

Fractionation

Preliminary fractionation by HPLC

Preliminary studies on the separation of n-NP and NP by normal phase thin layer chromatography (MERCK, silica gel 60 F254) using hexane-ethyl acetate (10:1) as the mobile phase provided good results. Therefore, the first fractionation of the NP mixture was undertaken utilising HPLC (Hewlett-Packard (HP) 1100 with UV detector set at 254 nm) using a Shisheido silica column (length 250 mm × i.d. 4.6 mm) at flow rate of 1 mL/min at ambient temperature, eluted with hexane-ethyl acetate (30:1). The fractionation gave six fractions F1 to F6. Subsequently, fractions F3, F4 and F5 were used in the succeeding fractionation by GC-PFC.

Fractionation by gas chromatograph equipped with a preparative fraction collector (GC-PFC)

The three fractions, F3, F4 and F5 of the six fractions from the preliminary HPLC, were further separated by GC-PFC procedures. Substances in the three fractions were detected by GC-MS, and they were identified as NP isomers with selected ion monitor (SIM) at m/z 220 (Kim et al. 2004). Each fraction F3 to F5 was evaporated, dissolved in 3 mL hexane, and injected into the GC systems using a Gerstel large volume autosampler. More than 350 repeated injections into the GC of five µL of the hexane solution resulted in a total of more than 5 mg of each fraction of NP mixture fractionated using the solvent venting mode.

The GC-PFC analysis was performed using an HP 6890 series gas chromatograph with a flame ionisation detector (FID). The capillary column (C1) was 60 m DB-5 (i.d. 250 µm, film thickness 0.25 µm: J & W Scientific, USA) and the transfer capillary column (C2) was unfilled and deactivated fused silica capillary (i.d. 320 µm, length 870 mm: Agilent, USA). Helium was used as the carrier gas at a constant flow of 1.5 mL/min. The programmed temperature vaporisation-based large-volume injection (PTV-LVI) was set at 40°C for 10 s, and the temperature then raised to 280°C at 12°C/s and held for 10 min to evaporate the solvent. The temperature program for the column began at 70°C held for 1 min, raised at 30°C/min to 200 °C, then raised at 2°C/min to 220°C and 10°C/min to 280°C, which was maintained for 8 min. The detector temperature was set at 280°C. After passing through column C1, 99% of each of the injected fractions flowed into column C2 at a crosspiece and the residual 1% flowed into the FID cell. A glass capillary tube collector, chilled in a cooling box with ice, trapped each eluting fraction sample. The samples were collected by 1/100 second of the peak retention time using a switching device. After collection, each fraction was transferred into individual glass vials by rinsing each glass capillary tube with hexane. The collected samples were further purified by HPLC, confirmed by GC-MS prior to identification with NMR and analysed by GC-MS to check the purity. The purified samples were also used for assay of estrogenic activity.
Identification Methods

GC-MS

GC-MS analysis was performed using a HP 6890 series gas chromatograph interfaced to a HP 5973 mass detector. The GC-MS operating conditions were as follows: Sample injection was made by a PTV-LVI and an autosampler onto a 30 m DB-5 column (i.d. 250 μm, film thickness 0.25 μm: J & W Scientific, USA). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The PTV injector was set at 40°C for 10 s, raised to 280°C at 12°C/s and held for 10 min to evaporate the solvent. The temperature program for the column began at 70°C held for 1 min, followed by a 10°C/min ramp-up to 280°C which was held for 8 min. After eluting from the GC column, the samples were carried through a 250°C transfer line into the ion source of the mass spectrometer held at 230°C. The electron energy was 70 eV. Identification of NP was carried out by monitoring at m/z 107 [M-C$_6$H$_5$]+, m/z 121 [M-C$_9$H$_7$]+, m/z 135 [M-C$_{10}$H$_7$]+, m/z 163 [M-C$_{11}$H$_7$]+ and m/z 220 [M]+ in selected ion monitoring (SIM) mode in full scan mode from m/z 50 to 600.

NMR

The $^1$H- and $^{13}$C- nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM (lambda-400 and lambda-500) NMR instrument in CDCl$_3$ containing tetramethylsilane as an internal standard. The $^1$H-and $^{13}$C-NMR spectral data were assigned by the aid of $^1$H-$^1$H correlated spectroscopy ($^1$H-$^1$H COSY), distortionless enhancement polarisation transfer spectroscopy (DEPT), $^1$H-detected multiple quantum coherence spectrum (HMQC) and $^1$H-detected heteronuclear multiple bond connectivity spectrum (HMBC).

Estrogenic activity

Each fraction was tested for estrogenic activity by the recombinant yeast screen assay (Routledge and Sumpter 1996). The yeast was kindly supplied by Dr Sumpter, Brunel University, UK. In this system, human estrogen receptor (hER) is expressed in a form capable of binding to estrogen-responsive sequence (ERE). The yeast cells also contain expression plasmids carrying the reporter gene, lacZ, which is regulated by the ERE. Activation of the receptor by binding of ligand causes expression of the reporter gene lacZ, which produces the enzyme β-galactosidase. The activity of the estrogen-inducible β-galactosidase was measured by the cleavage of chlorophenyl-red-β-galactopyranoside (CPRG) to form a coloured product. Fractionated NP was diluted with dimethyl sulfoxide (DMSO) and added to the yeast culture media which contained CPRG in wells of microtiter plates. Plates were incubated for four days at 28°C. Colour development was measured at 540 nm and 620 nm and the difference in the measurements was taken to represent the activity of β-galactosidase, which correlated well with the estrogenicity of standard E2.

The amount of colour developed was plotted against the molar concentrations of sample to give a dose-response curve. From this curve, the minimal effective concentration was calculated from half of the maximum effect. Under conditions in the laboratory, the minimal effective concentration varied by 20 to 50% depending on samples. Hence four to six independent experiments were carried out to calculate the mean value. The activities of the different fractions of the NP reagent were determined by comparison of the minimal effective concentration of each fraction relative to that of E2 (being included in all assay plates as the standard).

Analytical procedure for the environmental sample

A schematic illustration of the overall analytical method is given in Figure 1. Dissolved phase and suspended solid in water samples were separated by passing the 8-L water sample through a glass fibre filter GFF (4.5 μm) and the dissolved phase was further passed through an XAD-2 resin column (23 mm ID X 35 cm length + 4 cm 24/40 ground glass for column head-separation funnel, Supelco, Bellefonte, Philadelphia). The ions are not exchanged by this XAD-2 but materials only adsorbed. The XAD-2 resin was Soxhlet-extracted for 16 hours with a hexane-acetone mixture (1:1), the extract concentrated in a Kuderna-Danish (KD) tube, and the concentrated extract was redisolved in hexane. The suspended solid collected on GFF filters were Soxhlet-extracted for 16 hours with a hexane-acetone mixture (1:1), concentrated in a KD tube and the extracts redisolved in hexane. The resulting extracts were further concentrated by solid phase extraction (SPE) by passing them through a Sep-Pak silica cartridge to remove the residue, and 6 mL of a mixture of hexane and diethyl ether (8:2) was used to elute the NP. The SPE extracts were purified using HPLC on a Shisheido silica column (as described above) for identification of NP with the GC-MS.

Quantitation

Quantitation was made according to an accepted procedure (Horig et al. 2004). Briefly, a surrogate substance, n-NP-$^{13}$C$_6$ was added as internal standard. NP isomers were determined using the relative response factor (RRF) by GC-MS. The GC-MS system conditions were as follows: Sample injection was made by a PTV-LVI and an autosampler onto a 30 m DB-5 column (i.d. 250 μm, film thickness 0.25 μm : J & W Scientific, USA). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The PTV injector was set at 40°C for 10 s and the temperature raised to 280°C at 12°C/s and held for 10 min to evaporate the solvent. The temperature program for the column began at 50°C held for 1 min, followed by a 10°C/min ramp-up to 250°C, which was held for 2 min. After eluting from the GC column, the samples were carried through a 250°C transfer line into the ion source of the mass spectrometer held at 230°C. The electron energy was 70 eV. Calibration curves for individual isomers were made. Five selected ions were used for calibration of the following isomers, NP1 to NP14; m/z 107, 121, 135, 149, 163, 177, 191 and 220 in a full scan mode from m/z 50 to 600. Recoveries of NP1 to NP14 were calculated at 73% to 109%.
RESULTS AND DISCUSSION

Structural elucidation of fractionated isomers of NP

Identification of isomers by MS

The selected ion mass chromatograms (m/z 220) obtained by GC-MS of the fractionated commercial NP (TKK and Kanto) after HPLC and GC-PFC are shown in Figure 2. As shown in Figure 2, the fractionated commercial NP reagent has 14 major components, NP1 to NP14. Consistent with results obtained by Bhatt et al. (1992) and Wheeler et al. (1997), three HPLC fractions, F3, F4 and F5 were obtained with commercial NP, and the NP isomers were identified by SIM at m/z 220. Three kinds of NP preparations, two from Japan and one from USA were compared in this way. The chromatographic fractionation of the NP reagent widely used in USA was found to be the same as those of the Japanese NP reagent.

When the NP HPLC fractions F3, F4 and F5 were further fractionated by GC-PFC (Figure 2a, b and c) respectively, 14 fractions corresponding to 14 NP isomers were identified. The NP-isomers, with corresponding peaks shown in Figure 2, are the following designated NP# (with the fraction source given in brackets): NP1 (F3), NP2 (F4), NP3 (F4), NP4 (F3), NP5 (F5), NP6 (F3), NP7 (F5), NP8 (F3), NP9 (F3), NP10 (F4), NP11 (F5), NP12 (F4), NP13 (F5) and NP14 (F4). Figure 2(d) combines the chromatograms for HPLC fractions F3, F4 and F5 to show the 14 NP isomers. The retention time of NP4 (base peak ion: m/z 135) in F3 coincided with that of NP5 (base peak ion: m/z 149) in F5, but their mass spectra were different. NP2, NP3, NP11 and NP12 were contained...
Figure 2. Single ion (m/z 220) mass chromatograms. a (HPLC-fraction 3), b (HPLC-fraction 4), c (HPLC-fraction 5) and d (a, b and c combined in one panel) of NP mixture by GC-MS. The three (a,b,c) of 5 fractions of preliminary separation by HPLC are shown. Ticks and numbers on peaks are labelled as NP isomers and retention time. Scale on the X-axis is time in minutes.
Figure 3. Structures of the compounds identified and separated from NP mixtures by GC-PFC. Asterisk (*) shows asymmetric carbon. The chemical structures of NP 8, 13 and 14 are not determined yet.

Figure 4. $^1$H (500 MHz) and $^{13}$C (125 MHz)-NMR spectra, and the elucidated structure, of NP7. The upper chromatogram is the $^1$H and the lower is $^{13}$C.
Structural elucidation of isomers by NMR

The structural elucidation and the estimation of the purity of separated fractions were performed by analyses of their NMR spectra. Figure 3 gives the elucidated structures of 11 of the 14 isolated isomers. The three isomers, NP8, 13 and 14 still remain to be identified.

The MS spectrum of NP7, the most estrogenic compound (see section: Estrogenic activity of NP isomers, below), showed the fragment ion at m/z 135 as base peak, suggesting the presence of dimethyl groups at α-carbon to the phenol ring. The 1H-NMR spectrum of NP7 (Figure 4a) shows the signals due to a para-substituted phenol ring ([δ 6.75, 2H, d, J=8.8 Hz], (δ 7.19, 2H, d, J=8.8 Hz)], gem-dimethyl groups (δ 1.21, s, 6H) and two primary methyl groups (δ 0.79, 6H, br. t, J=7.2 Hz). The 1H-1H COSY, HMQCC and DEPT spectra (Figure 4b) suggest the presence of three methylene groups (δc 23.2, 33.8 and 24.5) and a methyne group (δc 50.9). The connectivity of these groups was investigated by HMBC. The HMBC spectrum of NP7 showed the cross peaks due to the long range correlations between the proton signal at δ 1.21 (gem-dimethyl at α-carbon to the phenol ring) and the carbon signals at δc 41.0 (C-1), 50.9 (C-2) and 143.1 (C-1’), and between the proton signal at δ 0.79 (CH × 2; H-2” and H-5) and the carbon signals at δc 24.5 (C-2), 31.3 (C-3) and 23.2 (C-4). Thus, the structure of the major component of NP7 was derived as 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol.

The chemical structures of NP3, 5, 7 and 11 in the F5 were similarly identified to be 4-(1,4-dimethyl-1-ethyl-pentyl)-phenol, 4-(1,1,4-trimethyl-hexyl)-phenol, 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol and 4-(1,2-trimethyl-hexyl)-phenol, respectively, using GC-MS and NMR spectra. Similarly, the other components fractionated from HPLC fractions F3 and F4 were identified as 4-(1,3-dimethyl-1-propyl-butyl)-phenol (NP1), 4-(1,1,3-trimethyl-hexyl)-phenol (NP2), 4-(1,3-dimethyl-1-ethyl-pentyl)-phenol (NP4), 4-(1,3-dimethyl-1-ethyl-pentyl)-phenol (NP6), 4-(1,2-dimethyl-1-ethyl-pentyl)-phenol (NP9), 4-(1,2-dimethyl-1-propyl-butyl)-phenol (NP10) and 4-(1-ethyl-1-methyl-hexyl)-phenol (NP12).

The major components of NP1, NP2, NP5, NP11 and NP12 were described in the previous work (Kim et al. 2004) and in the present work, those of NP3, NP4, NP6, NP7, NP9 and NP10 are shown in Table 1. The plane structures of NP4 and NP6 are the same, but the stereostructures of them are different because they are diastereomers of each other. The 2D-NMR spectra of NP4 and NP6 indicated that their plane structures are the same. If NP4 and NP6 are enantiomers each other, their spectral data should be the same and they can not be separated by usual GC or HPLC.

The assigned structures were confirmed by comparison to synthesised 4-(1,1,4-trimethyl-hexyl)-phenol (NP5) and 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol (NP7). As shown in Figure 3, all of the identified isomers possessed a tertiary α-carbon. The NP7 had the highest estrogenic activity (see Estrogenic activity of NP isomers, below).

The identified isomers in the present study together with those of previous studies (Bhatt et al. 1992, Wheeler et al. 1997) are shown in Table 2. The chemical structures of the identified isomers NP1, NP2, NP5, NP11, and NP12 corresponded to those of peaks A, B, F, M and N in our previous study (Kim et al. 2004), respectively. Two isomers NP3 and NP4 match with two of the peaks in Wheeler et al. (1997). In this study, unfortunately, we could not separate and identify the NP isomers which were reported by Bhatt et al. (1992). Lalah et al. (2003) synthesised 4-(1-ethyl-1,4,4-trimethyl-pentyl)-phenol, one of the minor isomers identified in previous study (Table 2).

Estrogenic activity of NP isomers

The relative estrogenic activities of the 12 NP isomers by recombinant yeast screen assay are shown in Figure 5. The NP7 exhibited the highest estrogenic activity, corresponding to 1.9×10^3 that of E2. The second highest estrogenic isomers are NP3 and NP12. The activity of NP11 was the lowest observed, while that of NP9 and NP10 were not tested. The extent of estrogenicity of the fractions was calculated as relative values to that of E2. The synthesised NP, 4-(1,1-dimethyl-heptyl)-phenol, in our preliminary study also exhibited estrogenic activity. The importance of tert-structure has already been pointed out: both the position (para > meta > ortho) and branching (tertiary > secondary > normal) of the alkyl group affect estrogenicity. Optimal estrogenic activity requires a single tertiary branched alkyl group composed of between six and eight carbons located at the para position on an otherwise unhindered phenol ring (Routledge and Sumpter 1997). In the 2-dimensional representation of NP7 (Figure 3) it appears that the chemical structure is not similar to that of bisphenol A. However, NP7 exhibited the highest estrogenic activity among the NP isomers that were isolated from the NP reagent in the present studies. Therefore, it is considered that the stereochemical conformation of NP7 could be similar to that of bisphenol A and fit to some extent in the estrogen receptor under the conditions of recombinant yeast screen assay or in a living body. The molecule is of course really 3-dimensional and the alkyl chain will adopt the lowest energy conformation. This will not be the planar conformation of the aromatic phenol ring of bisphenol A. The aromatic phenolic group of bisphenol A is very different from the aliphatic carbon chain of NP7, especially in terms of environmental partitioning and estrogenicity, which are the principal issues addressed in this paper. NP7 is no more like bisphenol A than any of the other isomers. Therefore, this statement regarding structural similarity between NP7 and bisphenol A is not clear so far.

The estrogenic activities of NP1, NP2, NP5, NP11 and NP12 and their corresponding A, B, F, M and N in our previous study are compared in Figure 6. The estrogenic activities of these isomers in the present study are higher than those of previous study, except for one isomer, NP11. It may be because impurities in A, B, F and N of HPLC fraction were better removed by GC-PFC fractionation in the present study.
Table 1. The special data and purity of separated NP isomers.

<table>
<thead>
<tr>
<th>Major component</th>
<th>$^1$H-NMR (500 MHz, CDCl$_3$)</th>
<th>$^1$C-NMR (125 MHz, CDCl$_3$)</th>
<th>GC/MS</th>
<th>GC Composition (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Base peak ion (m/z)</td>
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<tr>
<td>NP3 [4-(1,4-dimethyl-1-ethyl-penty-1)-phenol]</td>
<td>$\delta$ H: 0.65 (3H, t, $J = 7.5$ Hz), 0.80 (3H, d, $J = 6.6$ Hz), 0.80 (1H, m), 0.81 (3H, d, $J = 7.9$ Hz), 0.97 (1H, m), 1.20 (3H, s), 1.40 (1H, m), 1.47 (1H, m), 1.50 (1H, m), 1.65 (1H, m), 1.72 (1H, m), 4.56 (1H, m), 6.73 (2H, d, $J = 8.8$ Hz), 7.12 (2H, d, $J = 8.8$ Hz)</td>
<td>$\delta$ C: 8.8 (CH$_3$), 22.8 (CH$_2$ × 2), 23.7 (CH$_3$), 28.8 (CH), 33.3 (CH$_3$), 35.8 (CH$_2$), 40.4 (C), 40.7 (CH$_2$), 114.5 (CH × 2), 127.5 (CH × 2), 140.1 (C), 152.6 (C)</td>
<td>149</td>
<td>79</td>
</tr>
<tr>
<td>NP4 [4-(1,3-dimethyl-1-ethyl-penty-1)-phenol]</td>
<td>$\delta$ H: 0.64 (3H, t, $J = 6.6$ Hz), 0.66 (3H, t, $J = 7.0$ Hz), 0.76 (3H, d, $J = 6.6$ Hz), 0.91 (1H, m), 1.05 (1H, m), 1.25 (3H, s), 1.26 (1H, m), 1.32 (1H, m), 1.51 (1H, m), 1.66 (1H, m), 1.71 (1H, m), 4.69 (1H, brs), 6.75 (2H, d, $J = 8.8$ Hz), 7.14 (2H, d, $J = 8.8$ Hz)</td>
<td>$\delta$ C: 8.8 (CH$_3$), 11.2 (CH$_3$), 21.7 (CH$_3$), 23.6 (CH$_3$), 30.6 (CH), 31.3 (CH$_3$), 36.1 (CH$_2$), 40.9 (C), 50.7 (CH$_3$), 114.6 (CH × 2), 127.9 (CH × 2), 140.3 (C), 152.9 (C)</td>
<td>149</td>
<td>80</td>
</tr>
<tr>
<td>NP6 [4-(1,3-dimethyl-1-ethyl-penty-1)-phenol]</td>
<td>$\delta$ H: 0.49 (3H, d, $J = 6.7$ Hz), 0.63 (3H, t, $J = 7.3$ Hz), 0.78 (3H, t, $J = 7.0$ Hz), 1.06 (1H, m), 1.20 (1H, m), 1.24 (3H, s), 1.25 (2H, m), 1.48 (1H, m), 1.50 (1H, m), 1.70 (1H, m), 4.55 (1H, brs), 6.74 (2H, d, $J = 8.8$ Hz), 7.14 (2H, d, $J = 8.8$ Hz)</td>
<td>$\delta$ C: 8.5 (CH$_3$), 11.4 (CH$_3$), 21.3 (CH$_3$), 23.0 (CH$_3$), 30.8 (CH), 31.8 (CH$_3$), 36.5 (CH$_2$), 40.7 (C), 50.8 (CH$_3$), 114.6 (CH × 2), 127.9 (CH × 2), 140.3 (C), 153.0 (C)</td>
<td>149</td>
<td>87</td>
</tr>
<tr>
<td>NP7 [4-(1,1-dimethyl-2-ethyl-penty-1)-phenol]</td>
<td>$\delta$ H: 0.79 (6H, br, CH$_2$ × 2), 1.00 (2H, m), 1.08 (1H, m), 1.21 (6H, brs, CH$_2$ × 2), 1.29 (1H, m), 1.30 (1H, m), 1.32 (1H, m), 1.35 (1H, m), 4.54 (1H, brs), 6.75 (2H, d, $J = 8.8$ Hz), 7.19 (2H, d, $J = 8.8$ Hz)</td>
<td>$\delta$ C: 14.4 (CH$_2$), 14.6 (CH$_2$), 23.2 (CH$_2$), 24.5 (CH$_2$), 25.5 (CH$_2$), 25.9 (CH$_3$), 33.8 (CH$_3$), 41.0 (C), 50.9 (CH), 114.5 (CH × 2), 127.3 (CH × 2), 143.1 (C), 152.9 (C)</td>
<td>135</td>
<td>76</td>
</tr>
<tr>
<td>NP9 [4-(1,2-dimethyl-1-ethyl-penty-1)-phenol]</td>
<td>$\delta$ H: 0.55 (3H, t, $J = 7.3$ Hz), 0.72 (3H, t, $J = 7.3$ Hz), 0.80 (1H, m), 0.87 (3H, d, $J = 7.3$ Hz), 1.01 (2H, m), 1.12 (3H, s), 1.31 (1H, m), 1.56-1.69 (3H, m), 4.54 (1H, brs), 6.75 (2H, d, $J = 8.8$ Hz), 7.11 (2H, d, $J = 8.8$ Hz)</td>
<td>$\delta$ C: 8.8 (CH$_3$), 14.0 (CH$_2$), 14.2 (CH$_2$), 17.8 (CH$_3$), 21.4 (CH$_2$), 32.9 (CH$_3$), 34.3 (CH$_2$), 42.8 (CH), 43.9 (C), 114.5 (CH × 2), 128.2 (CH × 2), 140.1 (C), 152.8 (C)</td>
<td>149</td>
<td>93</td>
</tr>
<tr>
<td>NP10 [4-(1,2-dimethyl-1-propyl-buty-1)-phenol]</td>
<td>$\delta$ H: 0.57 (3H, d, $J = 6.7$ Hz), 0.80 (3H, t, $J = 7.5$ Hz), 0.73-0.80 (1H, m), 0.86-0.90 (1H, m), 0.88 (3H, t, $J = 7.2$ Hz), 1.05-1.10 (1H, m), 1.14 (3H, s), 1.49 (1H, m), 1.58-1.62 (2H, m), 1.62-1.68 (1H, m), 4.65 (1H, brs), 6.75 (2H, d, $J = 8.8$ Hz), 7.10 (2H, d, $J = 8.8$ Hz)</td>
<td>$\delta$ C: 13.2 (CH$_3$), 14.0 (CH$_2$), 14.9 (CH$_2$), 17.7 (CH$_2$), 18.8 (CH$_3$), 24.0 (CH$_2$), 42.5 (CH$_2$), 41.7 (C), 45.9 (CH), 114.6 (CH × 2), 127.9 (CH × 2), 140.6 (C), 152.8 (C)</td>
<td>107</td>
<td>95</td>
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</table>
Table 2. Identified NP isomers from commercial NP mixture.

<table>
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<tr>
<td>NP8*1</td>
<td>4-(1,3-dimethyl-1-propyl-butyl)-phenol</td>
<td>o (Peak A)</td>
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<tr>
<td>NP5*1</td>
<td>4-(1,3-trimethyl-hexyl)-phenol</td>
<td>o (Peak B)</td>
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<tr>
<td>NP5*1</td>
<td>4-(1,4-dimethyl-1-ethyl-pentyl)-phenol</td>
<td>o (Peak F)</td>
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<td>o (Peak 8)</td>
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<tr>
<td>NP6*1</td>
<td>4-(1,3-dimethyl-1-ethyl-pentyl)-phenol</td>
<td>o (Peak M)</td>
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</tr>
<tr>
<td>NP9*1</td>
<td>4-(1,1,4-trimethyl-hexyl)-phenol</td>
<td>o (Peak N)</td>
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<tr>
<td>NP10*1</td>
<td>4-(1,2-dimethyl-1-propyl-butyl)-phenol</td>
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<tr>
<td>NP1*2</td>
<td>4-(1,1,2-trimethyl-hexyl)-phenol</td>
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<tr>
<td>NP12*1</td>
<td>4-(1-ethyl-1-methyl-hexyl)-phenol</td>
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<tr>
<td>others*2</td>
<td>4-(1,1-dimethyl-3-ethyl-pentyl)-phenol</td>
<td>(Peak D)</td>
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<td></td>
<td>4-(1-methyl-1-propyl-pentyl)-phenol</td>
<td>(Peak H)</td>
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<tr>
<td></td>
<td>4-(1,2-dimethyl-1-propyl-butyl)-phenol</td>
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</tr>
<tr>
<td></td>
<td>4-(1-ethyl-1,4,4-trimethyl-pentyl)-phenol</td>
<td>(Peak L)</td>
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</table>

4-(1,2,5-trimethyl-hexyl)-phenol (Peak 8)
4-(1,3,4-trimethyl-hexyl)-phenol (Peak 10)
4-(1,2-dimethyl-heptyl)-phenol (Peak 12)
4-(1,2-methylpropyl)-pentyl-phenol (Peak 14)
4-(2,3-dimethyl-1-ethyl-pentyl)-phenol (Peak 16)
4-(1,3,5-trimethyl-hexyl)-phenol (Peak 17)
4-(1-isopropyl-hexyl)-phenol (Peak 18)
4-(1,3-dimethyl-heptyl)-phenol (Peak 19)
4-(1,4-dimethyl-1-ethyl-pentyl)-phenol (Peak 20)
4-[1-(2-methylpropyl)-1-methyl-butyl]-phenol (Peak 22)
4-(1,3-dimethyl-1-isopropyl-butyl)-phenol (Peak 1)
4-(1-ethyl-1,3,3-trimethyl-butyl)-phenol (Peak 4B)
4-(1,4-dimethyl-2-ethyl-pentyl)-phenol (Peak 11)
4-(1,2,4-trimethyl-hexyl)-phenol (Peak 12C)
4-(2-ethyl-1-methyl-hexyl)-phenol (Peak 13)
4-(1,2,5-trimethyl-hexyl)-phenol (Peak 15B)
4-(1-ethyl-1-methyl-hexyl)-phenol (Peak 16)

*1 The structures of isomers were determined by GC-MS and GC-FTIR.
*2 Unpublished
#1: Kanto, #2: TKK
Isomer-specific analysis of NP in environmental samples

By application of the isomer-specific analysis to river water taken from USA, the isomers were found in the dissolved phase and suspended solids, and their isomeric distributions were compared to that of the Japanese NP reagent.

The isomeric distributions of NP in the dissolved phase and the suspended solids are shown in Figure 7. The proportions of the isomers in the purchased NP mixture were different from the environmental NP sample. The NP14 was present in greater proportions in the dissolved phase and the suspended solids, while the NP2 and 3 were present in smaller proportions. The proportion of NP7 was higher in the suspended solids than in the dissolved phase, and in the suspended solids, NP7 was present in greater proportion than the other isomers. It seems to be environmentally important that estrogenic activity of the NP7 was the highest in the 14 kinds of the NP isomer as shown in Figure 7.

Our on-going study includes developing a method for quantitative structure-activity relationships for detailed tertiary structures and estrogenic activities of individual components in NP mixtures.

CONCLUSIONS

Fourteen fractions, NP1 to NP14 from NP reagent were isolated using GC-PFC, identified by GC-MS and NMR, and the estrogenic activity determined. The chemical structures of 11 isomers of NP1 to NP14 were confirmed as 4-(1,3-dimethyl-1-propyl-buty1)-phenol for NP1, 4-(1,1,3-trimethyl-hexyl)-phenol for NP2, 4-(1,4-dimethyl-1-ethyl-pentyl)-phenol for NP3, 4-(1,3-dimethyl-1-ethyl-pentyl)-phenol for NP4, 4-(1,1,4-trimethyl-hexyl)-phenol for NP5, 4-(1,3-dimethyl-1-ethyl-pentyl)-phenol for NP6, 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol for NP7, 4-(1,2-dimethyl-1-ethyl-pentyl)-phenol for NP9, 4-(1,2-dimethyl-1-propyl-buty1)-phenol for NP10, 4-(1,1,2-trimethyl-hexyl)-phenol for NP11 and 4-(1-ethyl-1-methyl-hexyl)-phenol for NP12 using GC-MS and NMR spectra. The NP7 was found to exhibit the highest estrogenic activity, corresponding to $1.9\times10^{-3}$ that of E2 in a recombinant yeast screen system.

Isomer-specific quantification of individual NP isomers based on a (the?) relative response factor (RRF) was developed with GC-MS. By application of the isomer-specific analysis to our environmental water taken from New York-New Jersey Harbor, USA, the isomers were found in the dissolved phase and suspended solids of the water. Their isomeric distributions were compared to that of a commercial Japanese NP which was similar to that used in USA. The proportions of the isomers in purchased NP samples and environmental NP samples were different. The NP14 was present in greater proportion in both the dissolved phase and the suspended solids, while the NP2 and 3 were present in smaller amounts.
The proportion of NP7 was higher in the suspended solids than in the dissolved phase. The estrogenic activity of the NP7 was the highest of the 14 kinds of NP isomers. Further work is needed to clarify theoretically the relationship between the branching structure of NPs and their estrogenic activity, including possible similarity between NP7 and bisphenol A. An on-going study includes developing a method for quantitative structure-activity relationship (QSAR) of individual components in the technical NP for their estrogenic activity, especially in relation to detailed tertiary structures. In order to obtain authentic standards for bioassay of individual isomers, synthetic studies of NPs with variously branched side chains are now in progress.

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REFERENCES


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