Optical Resolution and Absolute Configuration of Branched 4-Nonylphenol Isomers and Their Estrogenic Activities

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(Received October 30, 2006; Accepted December 7, 2006; Published online December 15, 2006)

To determine the effects of optical isomerism on the estrogenic activity of 4-nonylphenol (NP) isomers, four optically active NP isomers, (3*R*)-4-(3-ethyl-2-methylhexan-2-yl)phenol, (3*S*)-4-(3-ethyl-2-methylhexan-2-yl)phenol, (4*R*)-4-(2,4-dimethylheptan-4-yl)phenol and (4*S*)-4-(2,4-dimethylheptan-4-yl)phenol, were prepared and separated using chiral HPLC. Their absolute configurations were elucidated by X-ray crystallographic analysis of their bromobenzoylated derivatives. The estrogenic activities (recombinant yeast screen assay) of the optically active NPs were similar to those of the corresponding racemates.

Key words —— 4-nonylphenol, optical resolution, estrogenic activity, X-ray crystallography

INTRODUCTION

4-Nonylphenol (NP) is a degradation product of 4-nonylphenol ethoxylate (NPEO), a nonionic surfactant used worldwide.[1)] The commercially available NP mixture is synthesized by Friedel-Crafts alkylation of phenol with nonene. Technically, nonene ("propylene trimer") is a mixture of C_9-olefins containing various degrees of branching. Thus, the resulting NP consists of a mixture of 4-substituted mono-alkylphenols with variously branched nonyl groups.[2,3)] Wheeler *et al*.[4)] identified 22 NP isomers using high-resolution mass spectrometry-gas chromatography with a 100 m capillary column. More recently, Ieda *et al*.[5)] tentatively identified 102 components in an NP mixture by comprehensive two-dimensional gas chromatography (GC × GC) combined with mass spectrometry.

NP is a very important environmentally relevant substance. It is a persistent, toxic, endocrine-disrupting chemical.[6–10)] Even today, there is worldwide scientific and public discussion on the potential consequences of long-term dietary exposure of humans to these compounds. In recent investigations, it has been shown that the structural features of different alkylphenols affect their estrogenic activities, and the estrogenic effect of an individual NP isomer is heavily dependent upon the structure of its sidechain.[10,11)] Consequently, in the field of NP research, it is absolutely necessary to consider NPs from an isomer-specific viewpoint.[12)]

Although a few groups have reported the synthesis of NP isomers,[13–17)] all of these NP isomers were optically inactive (racemic) compounds. In our previous studies, we described preparative fractionation of a commercial NP mixture using HPLC to afford fourteen NP isomers (Fig. 1).[18)] In addition, we had recently synthesized seven branched NP (structural) isomers (NP-C, NP-D, NP-E(G), NP-F, NP-I, NP-M and NP-N) *via* two different synthetic pathways. NP-I [4-(3-ethyl-2-methylhexan-2-yl)phenol, racemate] showed the highest estrogenic activity [50% effective concentration (EC_{50}) of NP-I is *ca* 1/300 to that of 17β-estradiol (E2)] than the others on the recombinant yeast screen assay system.[19)]

The initial step in the molecular mechanism of hormonal action of estrogens is a binding of the steroid to its receptor.[20)] Xenoestrogens require a

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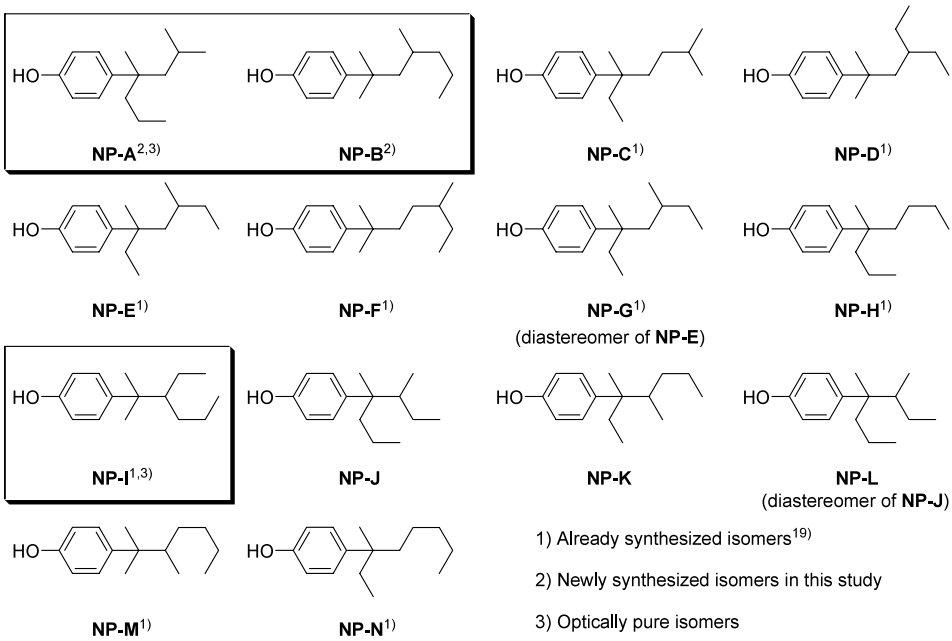


Fig. 1. Chemical Structures of Branched NP Isomers in Commercial NP

certain structure essential for binding to an estrogen receptor, so each NP optical isomer might exhibit a different estrogenic activity. We report herein optical resolution of racemic NP-I using HPLC and elucidation of its absolute structure to determine the effect of optical isomerism on estrogenic activity. Furthermore, optically active NP isomers NP-As [(4*R*)- and (4*S*)-4-(2,4-dimethylheptan-4-yl)phenol] and racemic NP isomer NP-B [4-(2,4-dimethylheptan-2-yl)phenol] could newly be prepared by Friedel-Crafts alkylation. Six synthetic NP isomers [NP-I (racemate), (*R*)-NP-I, (*S*)-NP-I, (*R*)-NP-A, (*S*)-NP-A and NP-B (racemate)] had various estrogenic activities on recombinant yeast screen assay. This is the first report on the synthesis of chiral NP compounds and their estrogenic activities.

MATERIALS AND METHODS

General —— Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. ^{1}H and ^{13}C spectra were recorded on a JEOL (Tokyo, Japan) JNM-LA600 FT-NMR in $CDCl_3$ containing tetramethylsilane as an internal standard. EI-MS analyses were carried out with a JEOL JMS-GCMATE. Optical rotations were measured on a JASCO (Tokyo, Japan) P-1030 digital polarimeter at the sodium D line (589 nm). Column chromatography was carried out on Wakogel (Wako Pure Chemical Ind., Ltd., Osaka, Japan) C-200 (100–200 mesh). Analytical thin-layer chromatography (TLC) was carried out using Merck (Darmstadt, Germany) Kieselgel 60 F_{254} plates with visualization by ultraviolet light, anisaldehyde stain solution or phosphomolybdic acid stain solution. THF was distilled from sodium benzophenone ketyl.

Materials —— Lithium (Li), phenol and 1,8-diazabicyclo-[5.4.0]-undec-7-ene were obtained from Wako Pure Chemical Industries (Osaka, Japan). 3-Bromohexane (90% purity by GC), *n*-propylmagnesium bromide (2.0 M in THF), BF_3•Et_2O and 4-methyl-2-pentanone were purchased from Tokyo Chemical Industry (TCI) Co., Ltd. (Tokyo, Japan). 2-Bromobenzoyl chloride and 4-bromobenzoyl chloride were obtained from Acros Organics.

Synthesis of NP Isomers

3-Ethyl-2-Methyl-2-Hexanol —— To a stirred suspension of Li (134 mg, 19.1 mmol) in THF (10 ml), anhydrous acetone (404 μl, 5.50 mmol) and 3-bromohexane (1.15 ml, 8.16 mmol) in THF (10 ml) were added successively under argon atmosphere. The reaction mixture was ultrasonicated for 30 min at 0–5°C and then sat. NH_4Cl aq. (10 ml) was added to the reaction mixture, which was then extracted with ethyl acetate (2 × 40 ml). The combined organic layers were washed successively with water (3 × 20 ml) and brine (20 ml), and dried over anhydrous Na_2SO_4. After filtration, the fil-

trate was concentrated *in vacuo* and the residue was purified by silica gel column chromatography using *n*-hexane–ethyl acetate (9:1) to afford 3-ethyl-2-methyl-2-hexanol (290 mg, 37%).

4-(3-Ethyl-2-Methylhexan-2-yl)Phenol (NP-I) —— The title compound was synthesized by Vinken's procedure.[14] To a stirred solution of 3-ethyl-2-methyl-2-hexanol (203 mg, 1.41 mmol) and phenol (550 mg, 5.80 mmol) in petroleum ether (100 ml), BF_3•Et_2O (250 ml, 1.45 mmol) was added at room temperature under argon atmosphere. The reaction mixture was stirred at room temperature for 12 hr, and then crushed ice and water (100 ml) were added. The organic layer was washed with water (4 × 100 ml) to remove excess phenol, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by silica gel column chromatography eluted with *n*-hexane–ethyl acetate (15:1) to give NP-I (78 mg, 25%).

Optical Resolution of NP-I —— Preparative scale HPLC [pump: Shimadzu (Kyoto, Japan) LC-10AD; UV detector: Shimadzu SPD-10A; column: Daicel Chemical Industries, Ltd. (Tokyo, Japan) Chiralcel OJ-H, length 250 mm × *i.d.* 10 mm; flow rate: 3 ml/min; detection: UV (254 nm)] using *n*-hexane–^{i}PrOH (9:1) gave chiral NP-I-1 {retention time: 7.9 min $[\alpha]_D$+14.5 (c = 1.0, MeOH)} and NP-I-2 {retention time: 10.1 min $[\alpha]_D$ − 14.3 (c = 1.0, MeOH)}.

2-Bromobenzoylation of NP-I-1 —— To a stirred solution of NP-I-1 (25 mg, 0.11 mmol) in CH_2Cl_2 (1 ml) was added successively 1,8-diazabicyclo-[5.4.0]-undec-7-ene (35 mg, 0.23 mmol) and 2-bromobenzoyl chloride (30 mg, 0.14 mmol) at 0°C under argon atmosphere. The reaction mixture was warmed to room temperature and stirred for 30 min. The reaction mixture was partitioned between CH_2Cl_2 (5 ml) and H_2O (3 ml). The organic layer was washed with brine (2 × 3 ml) and dried over anhydrous Na_2SO_4. The crude material was purified by silica gel column chromatography using *n*-hexane–ethyl acetate (20:1) to give *O*-2-bromobenzoyl-NP-I-1 (41 mg, 90%). This was crystallized from ^{i}PrOH and H_2O to give colorless needles and used for X-ray crystallographic analysis. mp 81–82°C (^{i}PrOH–H_2O); $[\alpha]_D$ + 9.9 (c = 0.94, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.80 (2 × 3H, t, J = 7.2 Hz), 1.00–1.06 (2H, m), 1.11–1.16 (1H, m), 1.24 (2 × 3H, s), 1.28–1.40 (4H, m), 7.15 (2H, d, J = 8.9 Hz), 7.38 (2H, d, J = 8.9 Hz), 7.38 (1H, dt, J = 7.6, 1.1 Hz), 7.43 (1H, dt, J = 7.6, 1.7 Hz), 7.72 (1H, dd, J = 7.6, 1.1 Hz), 8.00 (1H, dd, J = 7.6, 1.7 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 14.4 (CH_3), 14.6 (CH_3), 23.2 (CH_2), 24.4 (CH_2), 25.3 (CH_3), 25.9 (CH_3), 33.8 (CH_2), 41.5 (C), 50.7 (CH), 120.6 (2 × CH), 122.2 (C), 127.2 (2 × CH), 127.3 (CH), 131.5 (C), 131.8 (CH), 133.0 (CH), 134.6 (CH), 148.2 (C), 148.6 (C), 164.7 (C); EI-MS m/z: 402 $[M]^+$; HR-EI-MS m/z: 402.1198 ($[M]^+$, Calcd for $C_{22}H_{27}O_2Br$: 402.1194).

2-Bromobenzoylation of NP-I-2 —— 2-Bromobenzoylation of NP-I-2 was carried out by the same procedures as described above. *O*-2-bromobenzoyl-NP-I-2 (95% yield): Colorless needles, mp 78–79°C (^{i}PrOH–H_2O), $[\alpha]_D$ − 9.9 (c = 1.10, $CHCl_3$).

2,4-Dimethyl-4-Heptanol (1) —— A solution of 4-methyl-2-pentanone (4.0 g, 40.0 mmol) in THF (30 ml) was added dropwise to a suspension of *n*-propylmagnesium bromide (2.0 M in THF, 20.0 ml, 40.0 mmol) at room temperature. The mixture was heated at reflux for 1 hr. After cooling to 0°C, the reaction was quenched with sat. NH_4Cl aq. (60 ml). The mixture was poured into a separatory funnel by decantation to remove insoluble solid material and partitioned between ethyl acetate (50 ml) and H_2O (30 ml). The organic layer was washed with H_2O (30 ml) and brine (2 × 30 ml), and dried over anhydrous Na_2SO_4. Filtration and evaporation *in vacuo* furnished crude product (6.0 g), which was purified by silica gel column chromatography using *n*-hexane–ethyl acetate (10:1) to give *tert*-alcohol **1** (2.9 g, 50%) as a colorless oil. ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.92 (3H, t, J = 6.9 Hz), 0.959 (3H, d, J = 6.5 Hz), 0.964 (3H, d, J = 6.5 Hz), 1.17 (3H, s), 1.31–1.40 (2H, m), 1.38 (2H, dd, J = 6.2, 2.4 Hz), 1.41–1.48 (2H, m), 1.78 (1H, m); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 14.7 (CH_3), 17.2 (CH_2), 24.1 (CH), 24.7 (CH_3), 24.9 (CH_3), 27.3 (CH_3), 45.2 (CH_2), 50.4 (CH_2), 73.4 (C).

(4*S*)- and (4*R*)-4-(2,4-Dimethylheptan-4-yl)-Phenol (NP-A-1 and NP-A-2) and 4-(2,4-Dimethylheptan-2-yl)Phenol (NP-B) —— To a stirred solution of phenol (1.31 g, 13.9 mmol) and 2,4-dimethyl-4-heptanol (500 mg, 3.47 mmol) in petroleum ether (200 ml), BF_3•Et_2O (493 mg, 0.44 ml, 3.47 mmol) was added dropwise at room temperature. The reaction mixture was stirred for 12 hr at the same temperature. The reaction was quenched by addition of H_2O (100 ml). The organic layer was washed with H_2O (4 × 100 ml) to remove excess phenol and dried over anhydrous Na_2SO_4. Filtration and evaporation *in vacuo* furnished crude product. Purification of the crude product by silica

gel column chromatography using *n*-hexane–ethyl acetate (20:1) gave a 1:1 mixture of NP-A and NP-B (0.84 g) as a colorless oil. They were separated by HPLC [Chiralpak AD-H, length 250 mm × *i.d.* 20 mm; flow rate: 6 ml/min; detection: UV (254 nm)] using *n*-hexane–^{i}PrOH (15:1) to give optically active NP-A-1 {retention time 14.1 min; $[\alpha]_D$ + 9.2 (c = 1.0, MeOH)}, NP-A-2 {retention time 15.3 min; $[\alpha]_D$ − 9.0 (c = 1.0, MeOH)} and optically inactive (racemic) NP-B (retention time 17.6 min) (product ratio: NP-A-1:NP-A-2:NP-B = 1:1:2).

Spectral Data for NP-A-1 —— ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.57 (3H, d, J = 6.5 Hz), 0.58 (3H, d, J = 6.5 Hz), 0.80 (3H, t, J = 7.2 Hz), 0.87–0.95 (1H, m), 1.11–1.20 (1H, m), 1.27 (3H, s), 1.42 (2H, dd, J = 13.7, 5.2 Hz), 1.43–1.50 (1H, m), 1.57–1.62 (2H, m), 6.75 (2H, d, J = 8.9 Hz), 7.14 (2H, d, J = 8.9 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 14.8 (CH_3), 17.3 (CH_2), 24.0 (CH_3), 24.5 (CH), 24.8 (CH_3), 25.3 (CH_3), 40.6 (C), 46.9 (CH_2), 52.8 (CH_2), 114.5 (CH), 127.7 (CH), 140.7 (C), 152.9 (C); EI-MS *m/z* (relative intensity): 220 ([M]$^+$, 8), 177 (23), 163 (49), 135 (5), 121 (100), 107 (46).

Spectral Data for NP-B —— ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.63 (3H, t, J = 6.9 Hz), 0.76 (3H, d, J = 7.6 Hz), 0.94–1.00 (1H, m), 1.04–1.10 (1H, m), 1.11–1.21 (2H, m), 1.27 (2 × 3H, s), 1.27–1.32 (1H, m), 1.42 (1H, dd, J = 13.7, 6.5 Hz), 1.58 (1H, dd, J = 13.7, 3.8 Hz), 6.75 (2H, d, J = 8.6 Hz), 7.20 (2H, d, J = 8.6 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 14.3 (CH_3), 19.9 (CH_2), 21.9 (CH_3), 29.2 (CH), 29.3 (CH_3), 30.0 (CH_3), 37.4 (C), 41.4 (CH_2), 52.0 (CH_2), 114.6 (CH), 127.2 (CH), 142.3 (C), 153.0 (C); EI-MS *m/z* (relative intensity): 220 ([M]$^+$, 5), 135 (100).121 (6), 107 (11).

4-Bromobenzoylation of NP-A-1 —— To a stirred solution of NP-A-1 (5.0 mg, 22.7 μmol) in CH_2Cl_2 (1 ml) was added successively 1,8-diazabicyclo-[5.4.0]-undec-7-ene (6.9 mg, 45.5 μmol) and 4-bromobenzoyl chloride (6.0 mg, 27.2 μmol) at 0°C under argon atmosphere. The reaction mixture was warmed to room temperature and stirred for 1 hr. The reaction mixture was partitioned between CH_2Cl_2 (5 ml) and H_2O (3 ml). The organic layer was washed with brine (2 × 3 ml) and dried over anhydrous Na_2SO_4. The crude material was purified by silica gel column chromatography using *n*-hexane–ethyl acetate (15:1) to give *O*-4-bromobenzoyl-NP-A-1 (8.3 mg, 91%), which was crystallized from ^{i}PrOH and H_2O to give colorless needles. The crystal was used for X-ray crystallographic analysis. mp 72–73°C (^{i}PrOH–H_2O); $[\alpha]_D$ + 5.4 (c = 0.89, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: 0.60 (3H, d, J = 6.5 Hz), 0.81 (3H, d, J = 6.5 Hz), 0.82 (3H, t, J = 7.2 Hz), 0.90–0.97 (1H, m), 1.14–1.22 (1H, m), 1.33 (3H, s), 1.46–1.53 (3H, m), 1.63–1.68 (2H, m), 7.12 (2H, d, J = 8.9 Hz), 7.34 (2H, d, J = 8.9 Hz), 7.65 (2H, d, J = 8.6 Hz), 8.05 (2H, d, J = 8.6 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: 14.8 (CH_3), 17.3 (CH_2), 24.0 (CH_3), 24.5 (CH), 24.8 (CH_3), 25.3 (CH_3), 41.1 (C), 46.8 (CH_2), 52.7 (CH_2), 120.6 (2 × CH), 127.6 (2 × CH), 128.7 (2 × C), 131.6 (2 × CH), 131.9 (2 × CH), 146.3 (C), 148.3 (C), 164.5 (C); EI-MS *m/z*: 402 [M$^+$]; HR-EI-MS *m/z*: 402.1194 ([M$^+$], Calcd for $C_{22}H_{27}O_2Br$: 402.1194).

4-Bromobenzoylation of NP-A-2 —— 4-Bromobenzoylation of NP-A-2 was carried out by the same procedures as described above.

O-4-bromobenzoyl-NP-A-2 (91% yield): Colorless needles, mp 72–73°C (^{i}PrOH–H_2O), $[\alpha]_D$ −5.7 (c = 1.00, $CHCl_3$).

X-Ray crystal data for *O*-2-bromobenzoyl-NP-I-1: $C_{22}H_{27}BrO_2$, MW = 403.35, $P2_1/c$, a = 7.856(5) Å, b = 27.04(16) Å, c = 10.02(6) Å, β = 111.79(3)°, V = 1976.4(2) Å^3, Z = 4, D_{calc} = 1.356 g/cm^3, Mo$K\alpha$ (γ = 0.71073 Å), μ = 2.092 mm^{-1}, F(000) = 840. A total of 20937 reflections were collected at T = 90 K. A prismatic crystal of dimensions 0.35 × 0.05 × 0.04 mm^3 was used to collect data from θ = 1.5 to θ = 27.5°. The structure was solved by direct methods and Fourier techniques and refined by full matrix least squares on F^2 to $R1$ = 0.0328, $WR2$ = 0.0596 using 8921 independent reflections (R_{int} = 0.042) with I > 2σ(I) and 483 parameters. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were positioned on stereochemical grounds and refined with the riding model. A final difference electron density map showed largest peak and hole of 0.534 and −0.469 e/Å^3 respectively.

X-ray crystal data for NP-A-1: $C_{22}H_{27}BrO_2$, MW = 403.35, $P2_1/c$, a = 9.213(5) Å, b = 21.45(16) Å, c = 10.20(6) Å, β = 90.02(5)°, V = 2016.0(5) Å^3, Z = 4, D_{calc} = 1.329 g/cm^3, Mo$K\alpha$ (γ = 0.71073 Å), μ = 2.051 mm^{-1}, F(000) = 840. A total of 26895 reflections were collected at T = 90 K. A prismatic crystal of dimensions 0.15 × 0.09 × 0.07 mm^3 was used to collect data from θ = 1.9 to θ = 30.04°. The structure was solved by direct methods and Fourier techniques and refined by full matrix least squares on F^2 to $R1$ = 0.0331, $WR2$ = 0.0544 using 9315 independent reflections (R_{int} = 0.042) with I > 2σ(I)

and 483 parameters. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were positioned on stereochemical grounds and refined with the riding model. A final difference electron density map showed largest peak and hole of 0.593 and -1.263 e/Å^3 respectively.

Estrogenic Activity —— Estrogenic activity of synthetic NPs was tested by the recombinant yeast screen assay.[1)] The yeast was kindly supplied by Dr. Sumpter, Brunel University, U.K. 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 ligand binding causes expression of the reporter gene *lacZ* that produces the enzyme β-galactosidase. The activity of the estrogen-inducible β-galactosidase was measured by the coloration of chlorophenyl-red-β-galactopyranoside (CPRG). Synthesized NP was diluted with dimethyl sulfoxide (DMSO) and added to the yeast culture media containing CPRG in wells of microtiter plates. Plates were incubated for four days at 28°C. Color development was measured at 540 and 620 nm and the difference in the measurements was assumed to represent the activity of β-galactosidase. This correlated well with the estrogenicity of the E2 standard. The amount of color development was plotted against the molar concentrations of sample to give a dose–response curve. From this curve, the results were evaluated by relative activity, calculated by dividing the EC_{50} of E2 by the EC_{50} of NPs. We carried out 4–6 independent experiments and calculated the mean value. For comparison of the activities between the NP samples, the EC_{50} of each sample was compared with that of E2, which was included in all the assay plates as the standard.

RESULTS AND DISCUSSION

Optical resolution of racemic NP-I was achieved by HPLC equipped with a chiral column (CHIRALCEL OJ-H) using *n*-hexane–^{i}PrOH (9:1) to give NP-I-1 $[\alpha]_D$ + 14.5 ($c = 1.0$, MeOH) and NP-I-2 $[\alpha]_D$ −14.3 ($c = 1.0$, MeOH) (Fig. 2). Other chiral columns, *i.e.* CHIRALPAK AD-H, AS-H and CHIRALCEL OD-H, were ineffective in optically resolving NP-I (data not shown). After the optical resolution of racemic NP-I, the faster eluting NP-I-1 was 2-bromobenzoylated for X-ray analysis. Crystallization of 2-bromobenzoylated NP-I-1 from ^{i}PrOH and H_2O gave single crystals {mp 81–82°C, $[\alpha]_D$ + 9.9 (c = 0.94, $CHCl_3$)}. X-ray crystallographic analysis revealed the stereogenic center to be the *R*-configuration (Fig. 2). In the same manner, NP-I-2 was converted to 2-bromobenzoate and crystallized from ^{i}PrOH and H_2O. The benzoate obtained showed the same spectral data (^{1}H-NMR, ^{13}C-NMR, MS) except for the different sense of the optical rotation {$[\alpha]_D$ − 9.9 (c = 1.10, $CHCl_3$)} compared to its enantiomer (NP-I-1), therefore the stereogenic center of NP-I-2 is the *S*-configuration.

The synthesis of NP-A by Friedel-Crafts alkylation was also attempted. Nonylalcohol **1**

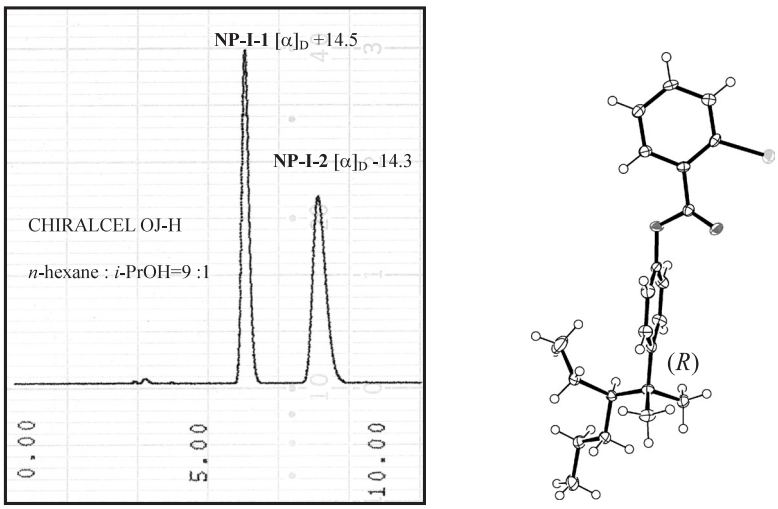
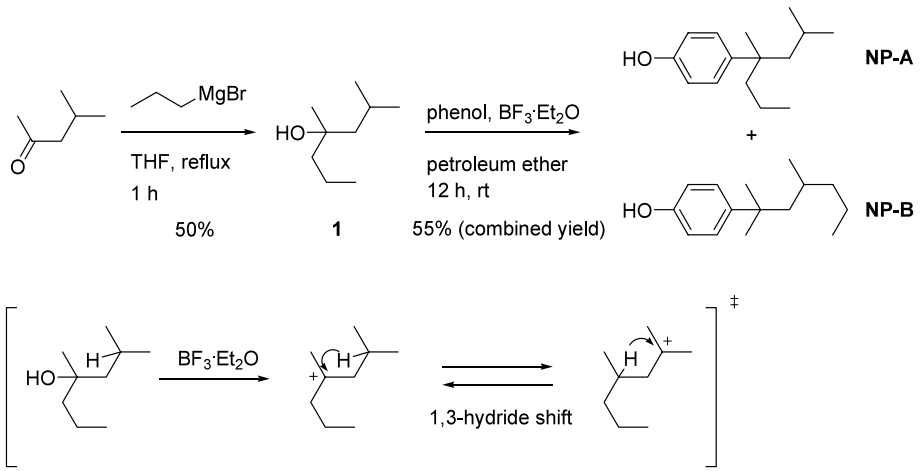


Fig. 2. HPLC Analysis of Synthesized Racemic NP-I with Chiralcel OJ-H (Length 250 mm × *i.d.* 4.6 mm; Flow Rate: 1 ml/min) and X-ray Crystal Structure of 2-Bromobenzoylated NP-I-1



Scheme 1. Synthesis of NP-A and NP-B

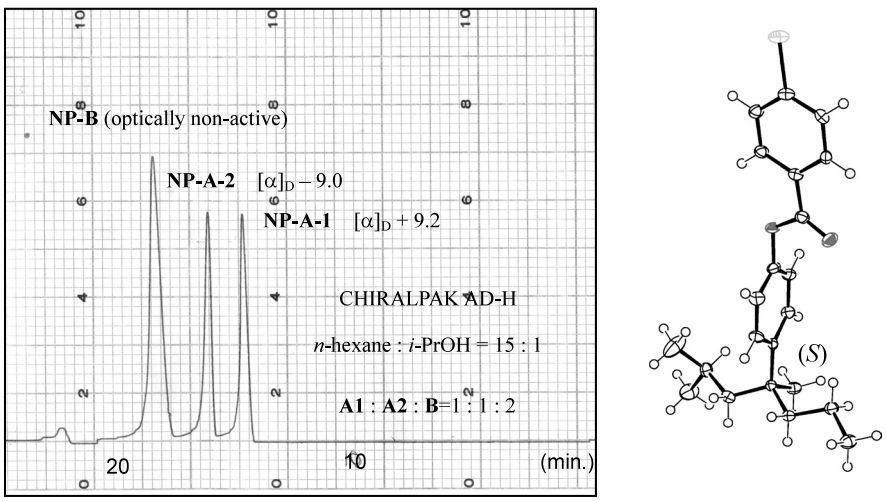


Fig. 3. HPLC Analysis of Synthetic Mixture of NP-A and NP-B with Chiralpak AD-H (Length 250 mm × *i.d.* 20.0 mm; Flow Rate: 6 ml/min) and X-ray Crystal Structure of 4-Bromobenzoylated NP-A-1

was prepared by Grignard reaction of 4-methyl-2-pentanone and *n*-propylmagnesium bromide in THF. Interestingly, alkylation of phenol with 2,4-dimethyl-4-heptanol **(1)** in the presence of BF_3•Et_2O as a Lewis acid in petroleum ether afforded a mixture of NP-A and NP-B, which was characterized by NMR spectroscopy (Scheme 1). Separation of the mixture was achieved by preparative scale HPLC equipped with a chiral column [Chiralpak AD-H, *n*-hexane–^{i}PrOH (15:1)] to give optically active NP-A-1 $[\alpha]_D$+9.2 (c = 1.0, MeOH), NP-A-2 $[\alpha]_D$ − 9.0 (c = 1.0, MeOH) and NP-B (optically inactive) (product ratio: NP-A-1:NP-A-2:NP-B = 1:1:2) (Fig. 3). Treatment of NP-A-1 with BF_3•Et_2O in petroleum ether gave only the recovered NP-A-1. Therefore, it is hypothesized that 1,3-hydride rearrangement *via* a tertiary carbocation intermediate occurred during the alkylation reaction (Scheme 1).

After 4-bromobenzoylation of NP-A-1 (2-bromobenzoylated NP-A-1 was not crystallized), the crude crystals were recrystallized from ^{i}PrOH and H_2O to give single crystals {mp 72–73°C, $[\alpha]_D$ + 5.4 (c = 0.89, $CHCl_3$)}. X-ray crystallographic analysis revealed the stereogenic center of NP-A-1 to be the *S*-configuration (Fig. 3). In the same manner, the NP-A-2 was converted to 4-bromobenzoate and crystallized from ^{i}PrOH and H_2O. The resulting benzoate had a different sense of the optical rotation {$[\alpha]_D$ − 5.7 (c = 1.00, $CHCl_3$)} compared to its enantiomer (NP-A-1), therefore the stereogenic center of NP-A-2 is the *R*-configuration. The absolute structures of the four prepared chiral NPs are shown in Fig. 4.

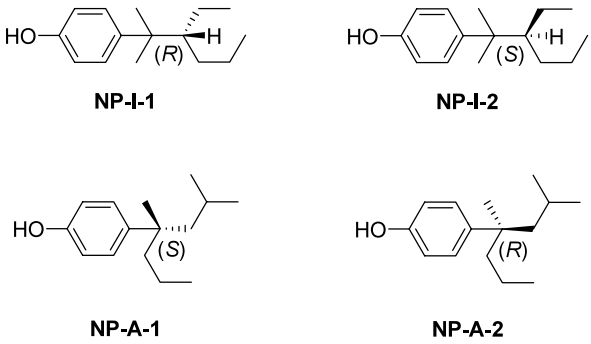


Fig. 4. Absolute Stereochemistry of Fractionalized NP-A and NP-I

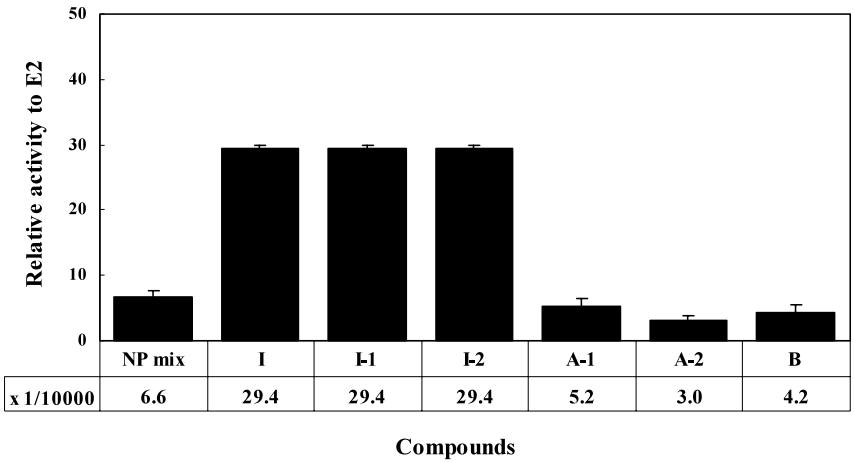


Fig. 5. Relative Estrogenic Activities (EC_{50} of E2/EC_{50} of NP Isomer) of NP Isomers to E2

Estrogenic activities (recombinant yeast screen assay) of the six synthetic NP isomers, NP-I (racemate), NP-I-1 [= (*R*)-NP-I], NP-I-2 [= (*S*)-NP-I], NP-A-1 [= (*S*)-NP-A], NP-A-2 [= (*R*)-NP-A] and NP-B, are shown in Fig. 5. The estrogenicity of each column was calculated as relative to that of E2. An optical isomeric effect of NP-I on estrogenic activity was not observed in this assay. Optically active NP-As and NP-B showed nearly the same activity as the technical NP mixture as shown in Fig. 5.

The initial step in the molecular mechanism of hormonal action of estrogens is a binding of the steroid to its receptor. The three-dimensional structure of the ligand-binding domain of the estrogen receptor complexed with E2 has been determined by X-ray crystallographic analysis. This structure reveals a relatively large ligand-binding cavity (450 Å), about twice the molecular volume of estradiol.[21] The large ligand-binding cavity of the estrogen receptor could be capable of recognizing both enantiomers of NP due to its relatively small size relative to E2.

In summary, we prepared six NP isomers, including four optically active NPs, and measured their estrogenic activity on recombinant yeast screen assay. An optical isomeric effect of the prepared NP isomers on estrogenic activity was not observed in this assay. Further studies are needed to clarify the relationship between structural, optical isomers of NPs and their estrogenic activities.

Acknowledgements We are extremely grateful to Dr. Sumpter, Brunel University, U.K. for supplying recombinant yeast cells. This study was supported in part by a grant for Effective Promotion of Joint Research with Industry, Academia, and Government, the Special Coordination Funds for Promoting Science and Technology, a grant to promote multi-disciplinary research projects, and a grant for Scientific Research (T.U. 17510049) from the Ministry of Education, Culture, Sports, Science, and Technology.

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