# Novel Metabolic Pathways of *p-n*-Nonylphenol Catalyzed by Cytochrome P450 and Estrogen Receptor Binding Activity of New Metabolites

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Nonylphenol, which is used industrially as a surfactant, is an endocrine-disrupting chemical (EDC) which has estrogenic activity. The novel biotransformation of nonylphenol was investigated, based on our previously reported *ipso*-metabolism of *para*-substituted phenols by cytochrome P450 (P450). Three novel metabolites of nonylphenol, *i.e.*, nonylquinol, 4′-hydroxynonanophenone (CO-NP) as benzyl-oxidized nonylphenol, and hydroquinone, were detected in a rat liver microsome reaction mixture. On the other hand, production of 1-(4′-hydroxyphenyl)nonan-1-ol (OH-NP), namely benzyl-hydroxylated nonylphenol, was detected in a human liver microsome reaction mixture. The formation of all these metabolites was suppressed by the addition of P450 inhibitor. This showed that all nonylphenol metabolism was catalyzed by P450. To identify which P450 isoenzyme is involved in each reaction, fourteen human P450 (CYP) isozymes, CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and CYP4A11, were examined. CYP1A1, 1A2, and CYP2B6 effectively catalyzed the production of nonylquinol. CYP2B6 also catalyzed the benzyl-hydroxylation to give OH-NP. Hydroquinone was formed mainly from OH-NP, not *via* CO-NP. We examined the estrogenic activity of these new metabolites by estrogen receptor (ER)-binding reporter gene assay. Nonylquinol, OH-NP and hydroquinone have no ER-binding activity. However, CO-NP showed the same level of estrogen receptor binding activity as nonylphenol. Moreover, the amount of CO-NP formed was small. Therefore, the novel metabolic pathways led overall to metabolic inactivation, as concerns the estrogenic activity of nonylphenol through the ER.

**Key words** —— cytochrome P450, metabolism, endocrine disruptor, nonylphenol, estrogenic activity

## INTRODUCTION

Recently, there has been growing social concern that industrially and naturally occurring compounds in the environment may have adverse effects on the endocrine systems of humans and wildlife. These compounds are called endocrine disrupters or endocrine disrupting chemicals (EDCs). Many EDCs affect sex hormone systems, including estrogen, and it is already known that various compounds such as insecticides, plasticizers, and surfactants have estrogenic activity.

Nonylphenol, which has several functions, including those of surfactant, stabilizer, and antioxidant of plastics, is one of the EDCs which have estrogenic activity.

Revealing the metabolism and identifying the metabolites of toxic compounds such as EDCs are important research subjects, because many compounds are affecting biological activities through metabolism. Among the major reported metabolites of *n*-nonylphenol are glucuronide, sulfate-conjugated compound at the phenol group, aromatic-ring hydroxylated compounds (catechol compounds), aliphatic chain hydroxylated compound at the $\omega$-1 position, $\omega$-position oxidized compounds, and further $\beta$-oxidized aliphatic chain compounds (Fig. 1).[1,2]

We have previously demonstrated that the *ipso*-position metabolism reaction (Fig. 2) of *para*-substituted phenols is catalyzed by cytochrome P450 (P450). That is, when a substituent is a mem-

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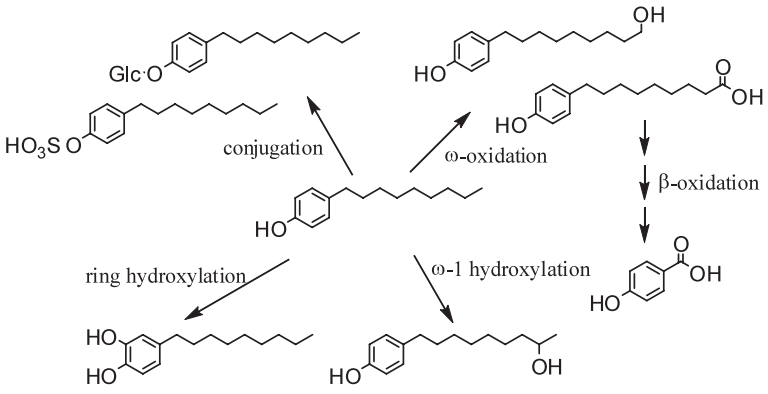


**Fig. 1.** Known Metabolic Pathways of Nonylphenol

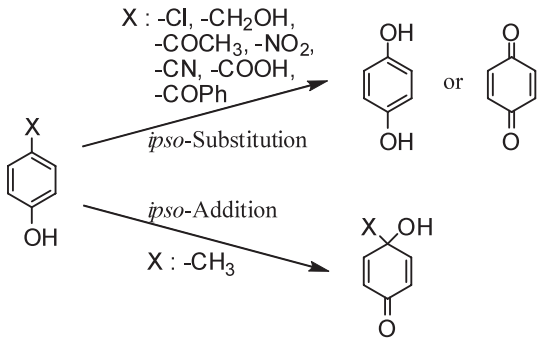


**Fig. 2.** The *Ipso*-position Reaction of *Para*-substituted Phenols Catalyzed by P450

ber of the alkyl group, *e.g.* a methyl group, a quinol metabolite is formed through an *ipso*-addition reaction. On the other hand, when a substituent is a halogen or a member of the hydroxymethyl, acetyl, nitro, cyano, carboxyl, or benzoyl group, a hydroquinone or benzoquinone is formed by an *ipso*-substitution reaction.[3–5] Further, we have demonstrated that estrogen as estrone and 17$\beta$-estradiol, each of which contains a *para*-alkylphenol moiety, is also metabolized through *ipso*-addition to the corresponding quinols by P450.[6] Estrogens which undergo *ipso*-addition lose their estrogen receptor (ER)-binding activity. As nonylphenol is also a *para*-substituted phenol, it is expected to give an *ipso*-addition metabolite of nonylphenol in a reaction catalyzed by P450. Moreover, after the oxidation of the benzyl position catalyzed by P450, it is also a possible factor in *ipso*-substitution metabolism (Fig. 3).

In this study, we examined the new metabolite formation of nonylphenol catalyzed by rat or human liver microsomes and human P450 (CYP), and we performed ER-binding assay of these new metabolites. *p-n*-Nonylphenol, which has a linear alkyl chain, was used in this study, although nonylphenol is usually a mixture of various isomers that have alkyl side chains represented by $-C_9H_{19}$.

## MATERIALS AND METHODS

**Chemicals** —— *p-n*-Nonylphenol, 1-iodononane, magnesium, *p*-benzoquinone, nicotinamide adenine dinucleotide phosphate ($NADP^+$), and sodium borohydride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase (G-6-P DHase) were obtained from Roche Diagnostics (Basel, Switzerland). Hydroquinone and *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 4′-Hydroxynonanophenone (CO-NP) and SKF-525A were obtained from Lancaster Synthesis (Lancaster, U.K.) and Daiichi Pure Chemicals (Tokyo, Japan), respectively.

**Synthesis of 4-hydroxy-4-nonyl-2,5-cyclohexadien-1-one (Nonylquinol)** —— In a round-bottom flask were placed *p*-benzoquinone (2160 mg, 20 mmol) and diethyl ether (200 ml) under nitrogen. The solution was cooled to $-30°C$, and the temperature was maintained throughout the reaction. Added to the benzoquinone solution was a diethyl ether solution of the Grignard reagent prepared from 1-iodononane (3300 mg, 13 mmol) and magnesium (292 mg, 12 mmol). The resulting mixture was stirred for 30 min. The reaction mixture was then poured through saturated aqueous ammonium chloride (400 ml) to quench the reaction and was extracted with ethyl acetate ($3 \times 200$ ml). The organic phase was washed with saturated

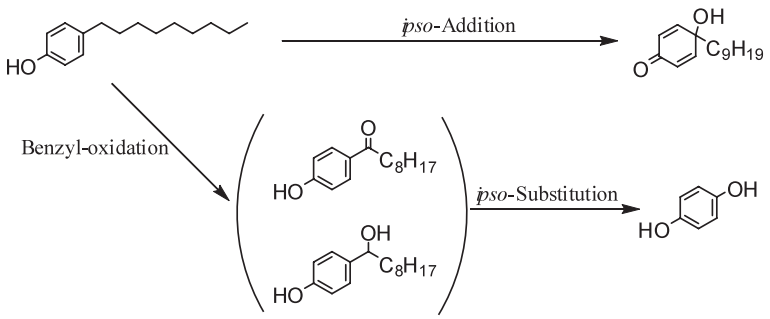


**Fig. 3.** Expected New Metabolic Pathways of Nonylphenol Catalyzed by P450

aqueous sodium chloride (2 × 200 ml) and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure. The product was purified by silica gel column chromatography eluted with hexane/ethyl acetate. After recrystallization from hexane, nonylquinol (99 mg, 0.42 mmol) was obtained. The nonylquinol was identified on the basis of $^1$H-NMR and mass spectra. m.p. 82–85°C; $^1$H-NMR (500 MHz, $CDCl_3$): $\delta$ 6.82 (d, $J$ = 10.0 Hz, 2 H), 6.19 (d, $J$ = 10.0 Hz, 2 H), 1.89 (s, 1 H, $D_2O$ exchangeable), 1.75 (m, 2 H), 1.25 (s, 14 H), 0.88 (t, $J$ = 6.5 Hz, 3 H); electron ionization mass spectrometry of the trimethylsilylated derivative: $m/z$ 308 $[M]^+$, 195 $[M\text{-}C_8H_{17}]^+$, 181 $[M\text{-}C_9H_{19}]^+$.

**Synthesis of 1-(4′-hydroxyphenyl)nonan-1-ol (OH-NP)** —— In a round-bottom flask were placed CO-NP (234 mg, 1 mmol) and ethanol (1 ml). Sodium borohydride (76 mg, 2 mmol) was added to the solution over a period of 5 min. The resulting mixture was stirred for 4 hr at room temperature. Then water (4 ml) was added to the reaction mixture, and extraction was done with diethyl ether (4 × 5 ml). The organic phase was washed with saturated aqueous sodium chloride (2 × 5 ml) and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure. The product was purified by silica gel column chromatography eluted with hexane/ethyl acetate. After recrystallization from dichloromethane, OH-NP (95 mg, 0.4 mmol) was obtained. OH-NP was identified on the basis of $^1$H-NMR and mass spectra. m.p. 89°C; $^1$H-NMR (500 MHz, $CDCl_3$): $\delta$ 7.22 (d, $J$ = 8.0 Hz, 2 H), 6.80 (d, $J$ = 8.0 Hz, 2 H), 4.87 (s, 1 H, $D_2O$ exchangeable), 4.59 (t, 1 H), 1.25 (m, 14 H), 0.87 (t, $J$ = 6.5 Hz, 3 H); electron ionization mass spectrometry: $m/z$ 236 $[M]^+$, 218 $[M\text{-}H_2O]^+$, 123 $[M\text{-}C_8H_{17}]^+$.

**Preparation of Rat Liver Microsomes** —— Wistar/ST rats (6 weeks old, 200–220 g each) were intraperitoneally injected with phenobarbital (60 mg/kg in saline) for 3 days and sacrificed 24 hr after the last injection. The liver microsomes were prepared in accordance with previously described procedures.[7]

The microsomal protein concentration was determined by the method of Lowry *et al.*[8] using bovine serum albumin as a standard, and the P450 content was measured as described by Omura and Sato.[9]

**Rat Liver Microsomal Incubation** —— The incubation mixture containing rat liver microsomes (4 mg protein), substrate (1 mM), KCl (60 mM), $MgCl_2$ (4 mM), G-6-P (4 mM), and G-6-P DHase (5 units) in 2.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) was preincubated for 3 min at 37°C. The reaction was initiated by adding $NADP^+$ (final 0.4 mM). After being incubated for 30 min at 37°C, the mixture was treated with 2 ml of ice-cold ethyl acetate to stop the reaction and to extract the products, and the organic phase was separated. The organic phase was dried over anhydrous $Na_2SO_4$ and concentrated by nitrogen flushing. The products formed were trimethylsilylated with BSTFA and pyridine. After the removal of the excess BSTFA by nitrogen flushing, the residue was dissolved in a small amount of acetone and analyzed by GC-MS (JEOL (Akishima, Japan) AUTOMASS SUN200, capillary column HP-1 0.32 mm × 30 m; J & W Scientific (Folsom, CA, U.S.A.)). The injection temperature was 260°C. The initial column temperature was 90°C for 3 min; then it was raised at intervals of 10°C/min to 250°C, followed by an isothermal hold at this temperature.

**Human Liver Microsomes and Cytochrome P450 (CYP)** —— Pooled human liver microsomes were obtained from Gentest Co. (Woburn, MA, U.S.A.). Recombinant CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and

CYP4A11 expressed in the microsomes of insect cells (BTI-TN-5B1-4) infected with a baculovirus containing CYP and NADPH-CYP reductase cDNA inserts and control microsomes (expressed with NADPH-CYP reductase and cytochrome $b_5$) were also obtained from Gentest.

**Incubation with Human Liver Microsomes or CYP Isozymes** —— Incubation with human liver microsomes or cDNA expressed CYP isozymes was performed similarly to the incubation with rat liver microsomes as described above, except that the incubation here was carried out in a final volume of 1 ml and the incubation mixture contained human liver microsomes (1 mg protein) or 0.1 μM CYP isozymes, 0.1 mM substrate, 60 mM KCl, 4 mM $MgCl_2$, 4 mM G-6-P, 5 units G-6-P DHase, and 0.4 mM $NADP^+$.

**ER-Binding Reporter Gene Assay** —— For estrogen responsive element (ERE)-luciferase reporter gene assay using MCF-7 cells, the culture medium was changed to phenol red-free MEM (Sigma Chemical Co. (St. Louis, MO, U.S.A.)) containing penicillin, streptomycin and dextran-charcoal-treated fetal bovine serum for 2–3 days. Transient transfections to MCF-7 cells were performed using Transfast (Promega Co., Madison, WI, U.S.A.), in accordance with the manufacturer's protocol. The transfections were done in 12-well plates at $1 \times 10^5$ cells/well with 1.9 μg of $p(ERE)_3$-SV40-Luc and 0.1 μg of pRL/CMV (Promega Co.) as an internal standard. 24 hr after the addition of the sample (final concentration, $10^{-4}$ to $10^{-8}$ M) dissolved in 10 μl of ethanol, the assay was performed with a Dual Luciferase assay kit (Promega Co.) in accordance with the manufacturer's protocol.

## RESULTS

### Metabolism by Rat or Human Liver Microsomes

*Ipso*-addition of nonylphenol by rat or human liver microsomal P450: *p*-Nonylphenol was incubated with phenobarbital-treated rat liver microsomes or pooled human microsomes. Figures 4 and 5 show the GC-MS data of the trimethylsilylated derivatives of the nonylquinol synthetic stan-

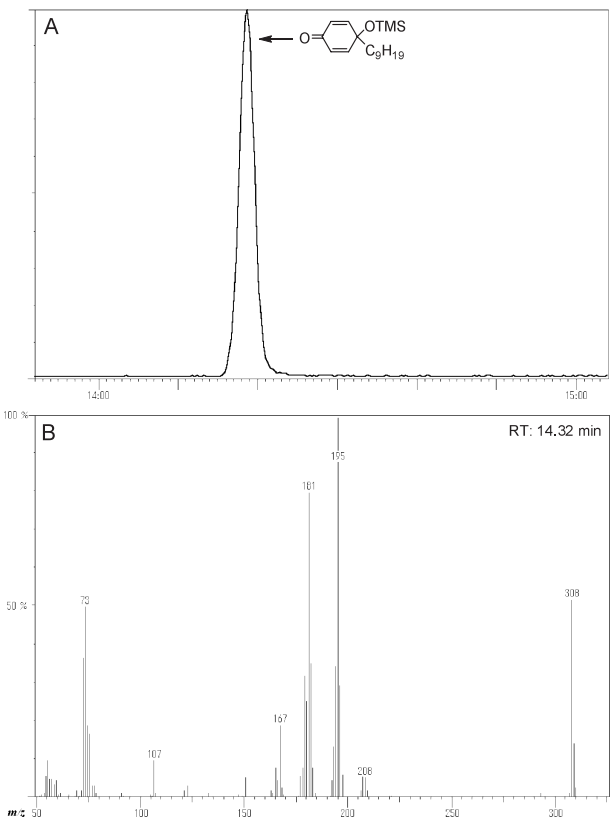


**Fig. 4.** GC-MS of Synthetic Standard Nonylquinol

A: Total ion chromatogram of synthetic standard nonylquinol; B: EI mass spectrum of synthetic standard nonylquinol.

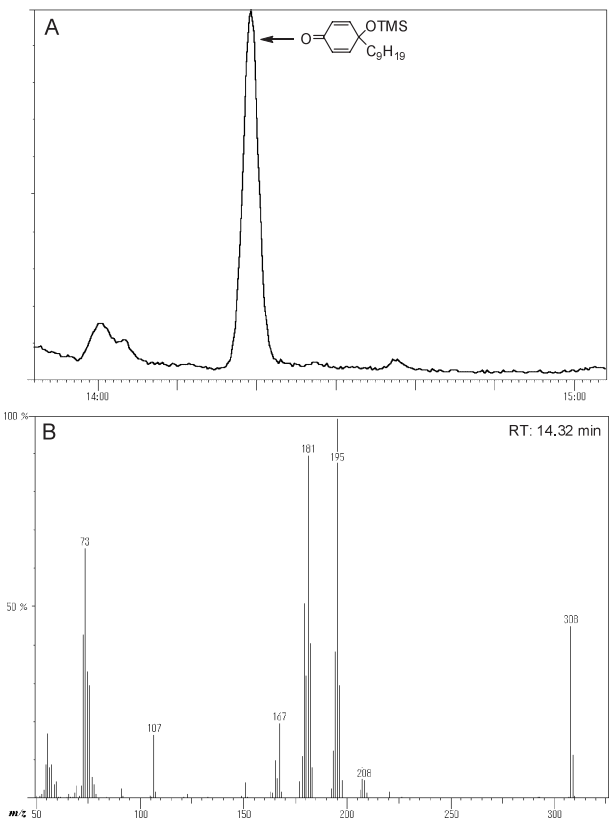


**Fig. 5.** GC-MS of Metabolite Extracts of Nonylphenol after Incubation with Rat Liver Microsomes and NADPH

A: Total ion chromatogram of metabolites extracted fraction; B: EI mass spectrum of metabolites extracted fraction at retention time 14.32 min.

**Table 1.** Formation of Novel Metabolites of Nonylphenol by Rat or Human Liver Microsome System (nmol/nmol P450)

| System | Nonyl quinol | OH-NP | CO-NP | Hydroquinone |
|---|---|---|---|---|
| Rat complete system | 3.21 ± 0.62 | < 0.01 | 0.12 ± 0.05 | 0.09 ± 0.01 |
| Rat – $NADP^+$ | < 0.01 | < 0.01 | 0.01 ± 0.01* | 0.01 ± 0.01* |
| Rat + SKF-525A | 0.52 ± 0.17* | < 0.01 | 0.01 ± 0.01* | < 0.01 |
| Human complete system | 1.68 ± 0.40 | 0.55 ± 0.16 | < 0.01 | < 0.01 |
| Human – $NADP^+$ | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| Human + SKF-525A | 0.52 ± 0.05* | 0.08 ± 0.03* | < 0.01 | < 0.01 |
| Complete – liver microsome | < 0.01 | < 0.01 | < 0.01 | < 0.01 |

Each value represents the mean of 6 samples ± S.D. The reaction mixture was incubated for 30 min at 37°C. The extracted products were analyzed by GC-MS after trimethylsililation. OH-NP: 1-(4′-hydroxyphenyl)nonan-1-ol, CO-NP: 4′-hydroxynonanophenone. Significant defferences compared with the complete system are indicated (* $p < 0.01$).

dard and the metabolite extracts, respectively. The peak was detected at a retention time of 14.32 min on the total ion chromatogram of the metabolites-extraction fraction. The retention time of this peak agreed with the synthetic standard, and the mass spectra of both peaks were identical. Therefore, it was determined that nonylquinol was contained in the extracted fraction.

When the microsomes or $NADP^+$ were omitted from the complete system, nonylquinol was not detected. Furthermore, when the P450 inhibitor, SKF-525A, was added (2 mM) to the incubation system, the amount of nonylquinol decreased (Table 1). These results indicate that nonylquinol formation, *i.e.* the *ipso*-addition of nonylphenol, was catalyzed by P450.

Benzyl-oxidation of nonylphenol: The metabolite-extraction fraction was analyzed by GC-MS after trimethylsilylation. In the case of rat liver microsomes, both CO-NP and hydroquinone were detected (Table 1). CO-NP was identified on the basis of its retention time and the *m*/*z* peak ratio of 306/221/208/193 in the GC-selected ion monitoring mode, compared with the authentic standard. Hydroquinone was also identified on the basis of its retention time and the *m*/*z* peak ratio of 254/239/223/112 in the GC-SIM mode, compared with the authentic standard. However, OH-NP was not detected.

In the case of human liver microsomes, OH-NP was detected in contrast to rat liver microsomes (Table 1). However, both CO-NP and hydroquinone were not detected. OH-NP was identified on the basis of its retention time and the *m*/*z* peak ratio of 291/267/193/179 in the GC-SIM mode, compared with the authentic standard.

When the microsomes or $NADP^+$ were omitted from the complete system, OH-NP, CO-NP and hydroquinone were not formed. Furthermore, when the P450 inhibitor, SKF-525A, was added to the incubation system, the amount of OH-NP, CO-NP or hydroquinone decreased (Table 1).

**Table 2.** Formation of Hydroquinone from Nonylphenol, OH-NP, or CO-NP by Rat Liver Microsome System (nmol/nmol P450)

| Substrate | Hydroquinone Formation |
|---|---|
| Nonylphenol | 0.09 ± 0.01 |
| OH-NP | 18.78 ± 1.58 |
| CO-NP | 1.00 ± 0.04 |

Each value represents the mean of 4 samples ± S.D. The reaction mixture containing nonylphenol, OH-NP, or CO-NP as a substrate was incubated for 30 min at 37°C. The amount of hydroquinone was analyzed by GC-MS after trimethylsililation.

*Ipso*-substitution of oxidized metabolites: OH-NP or CO-NP was also incubated with rat liver microsomes as a substrate. The amounts of hydroquinone formed from OH-NP and from CO-NP were both greater than the amount of hydroquinone formed from nonylphenol. In particular, the amount formed from OH-NP was about 200 times greater than that from nonylphenol (Table 2). When the microsomes or $NADP^+$ were omitted from the complete system, hydroquinone was not detected.

OH-NP metabolism by rat liver microsomes: A small amount (1 nmol; equivalent to the detected CO-NP formed from nonylphenol by rat liver microsomes) of OH-NP was incubated with the rat liver microsomes. The amount of substrate that remained after the incubation was quantified. The remaining OH-NP was 3.7% of the control in the process performed without $NADP^+$. The results suggest that most of the OH-NP disappeared as a result of metabolic reaction catalyzed by P450.

## Metabolism by the Isozymes of Human P450

Metabolism from nonylphenol to novel metabolites: Nonylphenol was incubated with human

P450 (CYP) isozymes, and the amounts of formed nonylquinol were determined. Table 3 shows nonylquinol formation during 30 min reactions. CYP1A2 and CYP2B6 showed the highest activity (the amounts of nonylquinol formed were 91.74 and 92.37 pmol/pmol P450, respectively). CYP1A1 (25.85 pmol/pmol P450) showed a higher activity than other isozymes.

The amounts of formed OH-NP, CO-NP and hydroquinone were also determined (Table 3). CYP2B6 showed the highest hydroquinone forming on the same level as rat liver microsomes. Although the amount of hydroquinone formation catalyzed by CYP2B6 (0.13 pmol/pmol P450) was much less than that of quinol formed by active CYPs, *i.e.* 1A1, 1A2, or 2B6, the formation of hydroquinone from nonylphenol catalyzed by CYP(s) was certainly similarly to that by rat liver microsome.

OH-NP formation was not confirmed in the rat liver microsome system, whereas OH-NP was formed in significant amounts by CYP2B6 from nonylphenol (39.62 pmol/pmol P450). The metabolism of nonylphenol to OH-NP catalyzed by CYP2B6 was confirmed by comparison of the GC-selected ion monitoring (SIM) data with the synthetic standard. However, with other isozymes or the control, OH-NP was not detectable. The amount of CO-NP formed from nonylphenol was considerably smaller than the amount of nonylquinol formed from nonylphenol. CO-NP formation was mainly catalyzed by CYP2B6 (1.53 pmol/pmol P450).

Metabolism from intermediary metabolites of nonylphenol: OH-NP or CO-NP was incubated with CYP isozymes, and the amount of formed CO-NP or hydroquinone was determined. As a result, it was confirmed that OH-NP was metabolized to CO-NP by CYP (Fig. 6). CYP3A5 showed the highest CO-NP forming activity, the

**Table 3.** Formation of Novel Metabolites from Nonylphenol by Human P450 Isozymes (pmol/pmol P450)

| Isozyme | Nonylquinol | OH-NP | CO-NP | Hydroquinone |
|---|---|---|---|---|
| Control | 2.54 | < 0.01 | 0.03 | < 0.01 |
| CYP1A1 | 25.85 | < 0.01 | 0.03 | 0.05 |
| CYP1A2 | 91.74 | < 0.01 | 0.14 | 0.03 |
| CYP2A6 | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| CYP2B6 | 92.37 | 39.62 | 1.53 | 0.13 |
| CYP2C8 | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| CYP2C9 | 1.98 | < 0.01 | 0.07 | < 0.01 |
| CYP2C18 | 0.73 | 0.54 | < 0.01 | < 0.01 |
| CYP2C19 | 0.99 | < 0.01 | < 0.01 | < 0.01 |
| CYP2D6 | 0.56 | < 0.01 | < 0.01 | < 0.01 |
| CYP2E1 | 1.98 | < 0.01 | 0.04 | < 0.01 |
| CYP3A4 | 6.78 | < 0.01 | 0.04 | 0.01 |
| CYP3A5 | 0.92 | < 0.01 | < 0.01 | < 0.01 |
| CYP3A7 | 0.19 | 0.46 | 0.08 | < 0.01 |
| CYP4A11 | 0.19 | < 0.01 | < 0.01 | < 0.01 |

Each value represents the mean of 2 samples. The product formation was analyzed by GC-MS after incubation for 30 min at 37°C. Control means microsomes expressed with NADPH-CYP reductase and cytochrome $b_5$.

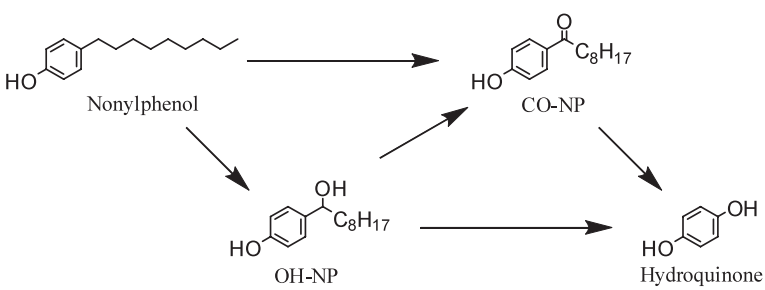


**Fig. 6.** Metabolic Pathway from Nonylphenol to Hydroquinone Catalyzed by P450

**Table 4.** Formation of Novel Metabolites of Nonylphenol from Intermediary Metabolites by Human P450 Isozymes (pmol/pmol P450)

| Isozyme | CO-NP from OH-NP | HQ from OH-NP | HQ from CO-NP |
|---|---|---|---|
| Control | 1.56 | < 0.01 | < 0.01 |
| CYP1A1 | 1.54 | 3.14 | 0.34 |
| CYP1A2 | 0.54 | 68.76 | 1.91 |
| CYP2A6 | 1.11 | < 0.01 | < 0.01 |
| CYP2B6 | 7.61 | 1.45 | 0.25 |
| CYP2C8 | 2.87 | < 0.01 | < 0.01 |
| CYP2C9 | 1.95 | 0.04 | < 0.01 |
| CYP2C18 | 2.02 | < 0.01 | < 0.01 |
| CYP2C19 | 2.10 | 21.43 | < 0.01 |
| CYP2D6 | 0.84 | 4.36 | < 0.01 |
| CYP2E1 | 1.77 | < 0.01 | < 0.01 |
| CYP3A4 | 5.33 | 21.40 | 0.07 |
| CYP3A5 | 14.69 | 111.30 | < 0.01 |
| CYP3A7 | 3.61 | 6.48 | < 0.01 |
| CYP4A11 | 1.13 | 0.78 | 0.62 |

Each value represents the mean of 2 samples. The product formation was analyzed by GC-MS after incubation for 30 min at 37°C. Control means microsomes expressed with NADPH-CYP reductase and cytochrome $b_5$. HQ: Hydroquinone.

amount of CO-NP formed during the 30 min reaction was 14.69 pmol/pmol P450 (Table 4). CYP2B6 and CYP3A4 also showed a relatively high activity (7.61 and 5.33 pmol/pmol P450, respectively). These amounts exceeded those of formation from nonylphenol. Only a small amount of CO-NP was formed with the control or other isozymes. This shows that the alcohol in the benzyl position is easily oxidized (air oxidation, *etc.*) to ketone.

In the case of the metabolism from OH-NP to hydroquinone, CYP3A5 again showed the highest catalytic activity, and the amount of hydroquinone formed during the 30 min reaction was 111.30 pmol/pmol P450. CYP1A2, CYP2C19 and CYP3A4 also showed high catalytic activity in the formation of hydroquinone from OH-NP. These amounts were very large compared to those of other metabolic reactions. The metabolism from CO-NP to hydroquinone was mainly catalyzed by CYP1A2 (1.91 pmol/pmol P450). Although the amount of hydroquinone formed from CO-NP was relatively large compared to that from nonylphenol, it was small compared to the amount of hydroquinone formed from OH-NP or to the amounts of other metabolites *i.e.* nonylquinol or OH-NP from nonylphenol.

### ER-binding Assay

The results of the ER-binding reporter gene assay of nonylphenol, CO-NP, OH-NP, and nonylquinol are shown in Fig. 7. Nonylquinol and

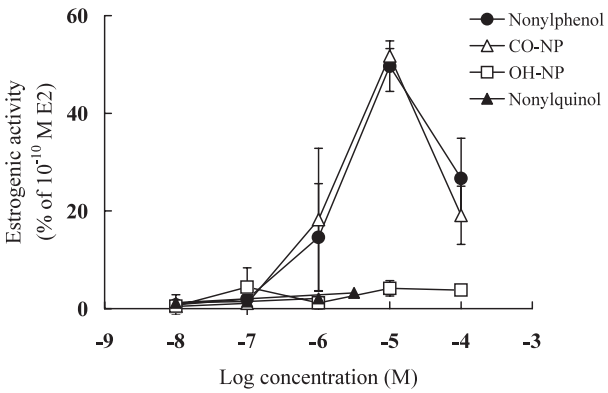


**Fig. 7.** Estrogenic Activity of Nonylphenol and New Metabolites of Nonylphenol

Each bar represents the mean ± S.D. ERE-luciferase reporter gene assay was performed in the presence of nonylphenol, CO-NP, OH-NP, and nonylquinol using MCF-7 cells. E2: estradiol.

OH-NP showed no ER-binding activity, and it has already been reported that hydroquinone has no ER-binding activity.[10] Although CO-NP showed some ER-binding activity, the activity was at the same level as that of nonylphenol. That is, the metabolism of nonylphenol to CO-NP was not metabolic activation. The decline in the estrogenic activity at high concentrations of nonylphenol and CO-NP was caused by the cytotoxicity of these compounds.

## DISCUSSION

*p*-Nonylphenol wastransformed into nonyl-

quinol during the incubation with rat or human liver microsomes, and the formation of nonylquinol depended on both the microsomes and NADPH. When the P450 inhibitor, SKF-525A, was added to the incubation system, the amount of nonylquinol formed decreased. The above results indicate that *p*-nonylphenol was metabolized by *ipso*-addition to nonylquinol by rat or human liver microsomal P450. This compound has been reported to be a metabolite of nonylphenol in bacteria,[11] but has not been reported to be a metabolite in humans or animals.

*p*-Nonylphenol was also transformed into CO-NP and hydroquinone during the incubation with rat liver microsomes. The formation of those metabolites depends on both the microsomes and NADPH. When SKF-525A was added to the incubation system, the amounts of those metabolites decreased.

The novel metabolic pathway of *p*-nonylphenol revealed in this study is clearly different from the already reported metabolic pathway of *p*-nonylphenol catalyzed by P450, as far as the products and mechanism of the metabolism are concerned. In this study, we assumed, based on our previous data, that novel metabolites were formed from nonylphenol, and we detected these metabolites efficiently by using a synthetic standard.

OH-NP was not detected as a metabolite of nonylphenol in the reaction catalyzed by rat liver microsomes. Most of the OH-NP disappeared during the metabolic reaction, when a small quantity of OH-NP was incubated as a substrate in the rat liver microsome/NADPH system. This result indicates that even if OH-NP was formed, most of the OH-NP would have been metabolized.

OH-NP and CO-NP were transformed into hydroquinone during the incubation with rat liver microsomes, and the formation of hydroquinone depended on both the microsomes and NADPH. As Ohe has shown,[5] in the *ipso*-metabolism reaction, the alkyl substituent is not eliminated and serves the *ipso*-addition product, and benzyl alcohol and benzophenone type phenol derivatives give hydroquinone by *ipso*-substitution. Therefore, it is hard to think that nonylphenol could have been directly metabolized to hydroquinone. The above result indicates that the hydroquinone produced during the metabolism of nonylphenol is a secondary metabolite *via* the *ipso*-substitution of OH-NP or CO-NP. Direct *ipso*-substitution of OH-NP to hydroquinone may be the main pathway, and not hydroquinone formation *via* CO-NP, because the amount of hydroquinone formed from OH-NP was larger than that formed from CO-NP (Table 2).

OH-NP was formed during the incubation with human liver microsomes, but CO-NP and hydroquinone were not detected. This result is very different from the case of rat liver microsomes. It indicates that the catalytic activity from OH-NP to CO-NP or hydroquinone of human liver microsomes was lower than that of rat liver microsomes.

Human P450 (CYP) also catalyzed the formation of nonylquinol, CO-NP and hydroquinone from nonylphenol. The CYP isozymes that catalyze nonylquinol formation were identified as mainly CYP1A2 and CYP2B6, followed by CYP1A1 (Table 3). The metabolism of estrone and 17$\beta$-estradiol into their corresponding quinols was catalyzed by CYP1A1 and CYP2B6, but not by CYP1A2.[6]

In the rat liver microsome experiments, OH-NP was not identified as a metabolite of nonylphenol. But CYP2B6 catalyzed the benzyl-hydroxylation of nonylphenol to give OH-NP. CYP2B6 also gave significant amounts of CO-NP and hydroquinone, but the amount of both metabolites was much lower than that of nonylquinol and OH-NP. As with the rat liver microsome reaction, the amount of hydroquinone formed from OH-NP was larger than that from CO-NP. Therefore, it is reasonable to assume that direct *ipso*-substitution of OH-NP to hydroquinone was the main pathway and not hydroquinone formation *via* CO-NP. The occurrence of this direct *ipso*-substitution of OH-NP was confirmed in the CYP1A2, CYP2C19, CYP3A4 and CYP3A5 reactions.

Although CYP1A2 and CYP3A5 showed high catalytic activity in the formation of hydroquinone from OH-NP, very small amounts of hydroquinone formed from nonylphenol were identified in the reactions with the two isozymes (Tables 3 and 4). These results suggest that CYP1A2 and CYP3A5 did not catalyze the formation of OH-NP from nonylphenol. Indeed, OH-NP was not detected in the CYP1A2 or CYP3A5 incubation mixture (Table 3).

Nonylquinol, OH-NP and hydroquinone have no ER-binding activity. Although CO-NP showed some ER-binding activity, its intensity did not exceed that of nonylphenol. Since the amount of CO-NP formed was small compared to that of nonylquinol, we believe that these novel metabolic pathways have almost no influence on estrogenic activity of nonylphenol.

However, hydroquinone, benzoquinone as an oxidized compound of hydroquinone, and quinol

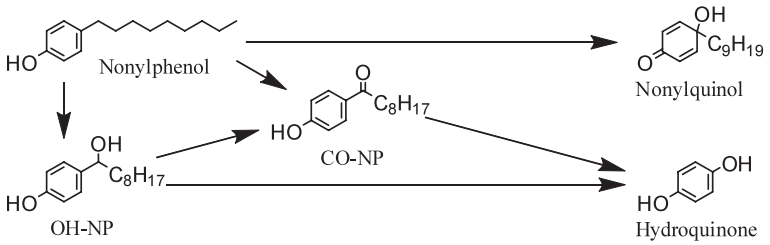


**Fig. 8.** Novel Metabolic Pathways of Nonylphenol

exhibited various types of toxicity, *e.g.* covalent binding with DNA, RNA, and proteins, including enzymes,[12–14] and reactive oxygen production through redox cycling.[12, 15] The toxicities of CO-NP as a novel metabolite should be investigated.

In conclusion, nonylphenol was transformed into nonylquinol as an *ipso*-addition metabolite, CO-NP and OH-NP as benzyl-oxidized metabolites, and hydroquinone as an *ipso*-substitution metabolite of OH-NP in reactions catalyzed by rat liver microsomal P450 and human P450, especially by CYP2B6. Hydroquinone was mainly formed directly from OH-NP, not *via* CO-NP.

In this study, we revealed some novel metabolic pathways catalyzed by P450, as shown in Fig. 8. These novel pathways are mainly catalyzed by CYP2B6. The metabolites have either no ER-binding activity or ER-binding activity which does not exceed that of nonylphenol. Therefore, it is reasonable to assume that the novel metabolic pathways examined in this study led to metabolic inactivation, as concerns the estrogenic activity of nonylphenol through the estrogen receptor.

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