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Synthesis of Haptens for Development of Antibodies to Alkylphenols and Evaluation and Optimization of a Selected Antibody for ELISA Development

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The development of an enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies for a class of endocrine disrupting compounds, 4-nonylphenol, is described. The parent molecule was derivatized at the ortho position of the free phenolic hydroxyl group to obtain the hapten, NP1, and it was conjugated with keyhole limpet hemocyanin, which was used as an immunogen. Four antisera were generated and screened against three coating antigens. The most sensitive ELISA from the screening tests (antiserum NP03As, 1/1000, and coating antigen NP1-BSA, 1 μg/mL) was further optimized and characterized. The influence of various physicochemical factors (organic solvent, pH, ion strength) was investigated. Methanol as the additive organic solvent was found to be the best organic solvent for the ELISA, with optimal sensitivity observed at a concentration of 5%. The ELISA parameters were changed at more acidic or basic pH values, whereas higher ionic strengths strongly suppressed the I_{50} value and the maximum absorbance. The most sensitive ELISA for 4-nonylphenol exhibited an I_{50} value of 38.6 $\pm$ 5.5 μg/L, with a dynamic range from 12 to 350 μg/L, and the lower limit of detection was 7.7 $\pm$ 1.3 μg/L. The optimized ELISA displayed no significant cross-reaction against the parent compounds, nonylphenol ethoxylates, degradation products, carboxylates, and bisphenol A, except in 4-octylphenol.

KEYWORDS: Nonylphenol; octylphenol; alkylphenols; antibody; ELISA

INTRODUCTION

Alkylphenol polyethoxylates are a class of representative nonionic surfactants widely used in industrial applications such as textiles manufacturing, pulp and paper production, agricultural chemicals, paints, and in various household products. After use, alkylphenol polyethoxylates, which are mainly used in aqueous solutions, are discharged from the environment mainly via wastewater systems. Talmage (*1*) suggested that after secondary wastewater treatment, often more than 95% of the alkylphenol polyethoxylates is removed. However, they are omnipresent in the environment, and actually there are some reports about the investigation of the concentrations in water and sediment samples collected from rivers, lakes, and seas that were found at ppb levels (*2*−*7*). Nonylphenol ethoxylates, which are one of the most representative surfactants, are comprised of nonylphenols with an average of about 10 and a range of 1 to about 20 ethoxy units. Anaerobic biodegradation of nonylphenol ethoxylates during wastewater treatment or after discharge to the environment can result in nonylphenol diethoxylate and monoethoxylate with shorter ethoxy chains (*8*, *9*) and proceeds with subsequent deethoxylation to the eventual formation and accumulation of 4-nonylphenol. These nonylphenol ethoxylate degradation products are more hydrophobic and more toxic than parent compounds (*10*), and especially, nonylphenol is known to be a toxicant for water organisms (*11*). 4-Nonylphenol belongs to a class of xenoestrogens or endocrine disrupting compounds, which are believed to be due to their ability to mimic or antagonize the effects of endogenous hormones or to disrupt biosynthesis and metabolism of endogenous hormones and hormone receptors as described by Sonnenschein and Soto (*12*). So, the increasing public concern about estrogen-active compounds in the environment in the past decade has resulted in comprehensive investigations into the occurrence and estrogenic activity of these substances because they may appear in the feminization of male fish and as an observable parallel to 4-nonylphenol downstream from sewage treatment plants (*13*). The binding of nonylphenol to estrogen receptors results in the expression of several responses both in in vitro and in vivo systems such as the induction of vitellogenin (*14*) or the proliferation of human estrogen sensitive MCF-7 breast tumor cells (*15*), and the bioassays confirmed the estrogenic potential of nonylphenol, even in low concentrations. 4-Nonylphenol is also considered to be an omnipresent pollutant and is often detected in sediment (*3*−*5*, *7*, *16*), water including effluent water

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or influent water (*2*−*5*, *17*), sewage sludge (*18*), and biological samples (*2*, *19*) at the order of ppb levels. It seems that these investigations are useful to elaborate provisions for control of the discharge to environment and for regulation of the use.

These environmental monitoring for alkylphenols including 4-nonylphenol or 4-octylphenol (4-*t*-octylphenol) and the related compounds has been mainly performed with chromatographic techniques such as gas chromatography (GC) (*2*, *16*−*19*) or high-performance liquid chromatography (HPLC) (*3*−*7*). Of these two techniques, the former has mainly used GC equipped with mass spectrometry (GC/MS) (*2*, *16*−*19*) or tandem MS (GC/MS/MS) (*18*). However, since these compound groups have a wide range of polarity and volatility, and because the higher molecular weight alkylphenol polyethoxylates have a low volatility, generally GC analysis has restricted them with fewer ethoxy units or alkylphenols. Hence, HPLC analysis has been mainstream and has been used with MS (HPLC/MS) (*3*−*7*). Since these chromatographic techniques can achieve highly sensitive analysis with high accuracy and have a talent in the identification ability of unknown compounds, they would be really suitable for environmental monitoring in institutes. However, they are indispensable to sample cleanup such as classical processes, liquid−liquid partition, column chromatography (*2*, *18*, *19*), and solid-phase extraction (SPE) (*3*, *5*−*7*, *17*), and it is necessary to use a large quantity of organic solvents for management of many samples. So, since there is a limitation in handling of sample cleanup, it requires time and labor, and furthermore, there is a fear of health hazards of analysts or environmental pollution by a large use of toxic organic solvents. Hence, they are not very suitable for primary screening analysis.

Immunochemical techniques including immunoassays (enzyme-linked immunosorbent assays, ELISAs), which are based on specific antigen−antibody interactions, are suitable for analysis, especially in the sampling site as advocated by Hammock and Mumma (*20*). Since the methodology allows rapid, simple, sensitive, and cost-effective monitoring, many antibodies (monoclonal and polyclonal antibodies) against various environmental pollutants such as agrochemicals (*21*−*23*), dioxins (*24*), and a kind of endocrine disrupting compounds, bisphenol A (*25*, *26*), have been produced, and immunochemical analyses for residual or environmental monitoring in various matrixes have been developed. Recently, Fránek et al. (*27*) and Zeravik et al. (*28*) reported ELISAs for 4-nonylphenol based on monoclonal and polyclonal antibodies. However, their antibodies used in the ELISAs were raised against carboxylic derivatives (haptens) synthesized based on 4-*n*-nonylphenol (*27*, *28*). Really, nonylphenol is a complex mixture of branch isomers of nonyl group and ring isomers with approximately the following composition: 90−93% *p*-nonylphenol (4-nonylphenol) and 3−6% *o*-nonylphenol (2-nonylphenol) as described by Wheeler et al. (*29*) because technical nonylphenol is synthesized through Friedel−Crafts alkylation between phenol and commercial nonene, which is a complex mixture of a predominantly propylene trimer. So, we directed our attention to the structural problem of the previous reported haptens (*27*, *28*), and more practical haptens for the development of antibodies against 4-nonylphenol and/or 4-octylphenol were designed and synthesized. Additionally, ELISAs based on produced antibodies were optimized for application to the environmental monitoring, and finally, we assessed whether the established ELISAs were suitable for this purpose.

MATERIALS AND METHODS

Chemicals and Immunochemicals. Technical grade 4-nonylphenol, a mixture of compounds with branched side chains, was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Sigma-Aldrich, Inc. (St. Louis, MO). Environmental analysis-grade 4-nonylphenol (96.7% purity) and 4-*n*-nonylphenol (99.5% purity) were from Kanto Chemical Co., Inc. (Tokyo, Japan) and Dr. Ehrenstorfer GmbH (Augsburg, Germany), respectively. Environmental analysis-grade 4-octylphenol (97.5% purity) and 4-*n*-octylphenol (98.5% purity) were from Kanto Chemical Co., Inc. Technical grade 4-octylphenol (97% purity) was from Tokyo Kasei Kogyo Co., Ltd. and Sigma-Aldrich, Inc. Nonylphenol monoethoxylate (99.9% purity), nonylphenol diethoxylate (99.1% purity), nonylphenoxy acetic acid (98.1% purity), nonylphenol monoethoxylate acetic acid (99.6% purity), nonylphenol diethoxylate acetic acid (99.5% purity), octylphenol monoethoxylate (99.9% purity), octylphenol diethoxylate (99.9% purity), bisphenol A (99% purity), bis-(4-hydroxyphenyl)methane (bisphenol F, 98% purity), 4-*sec*-butylphenol (96% purity), 4-*t*-butylphenol (99% purity), 4-nonylphenol ethoxylate (technical grade, nonylphenol monoethoxylate and diethoxylate, 99.5% purity), Tergitol NP-9 (nonylphenol poly(ethylene glycol) ether), and 4-octylphenol ethoxylate (technical grade, octylphenol monoethoxylate and diethoxylate, 99.5% purity) were from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan), Sigma-Aldrich, Inc., and Dr. Ehrenstorfer GmbH, respectively. Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), goat anti-rabbit IgG peroxidase conjugate as the second antibody, Freund's complete and incomplete adjuvant, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 3,3′,5,5′-tetramethylbendizine (TMB), 2,4,6-trinitrobenzenesulfonic acid (TNBSA), and sodium dodecyl sulfate (SDS) were from Sigma-Aldrich, Inc. Tween 20, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol, and salts were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

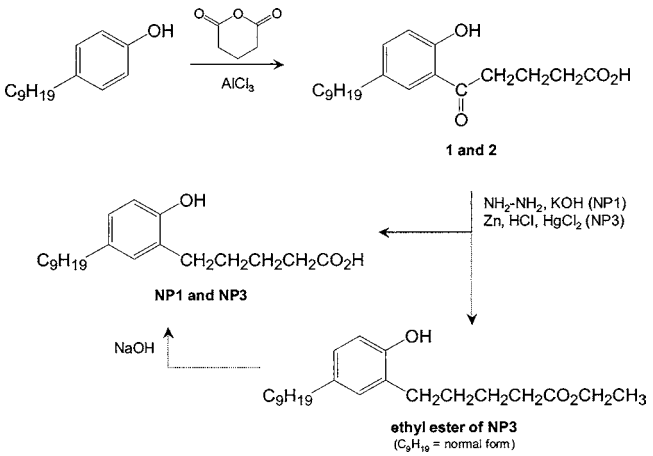

Figure 1. Synthetic pathways for haptens NP1 and NP3. C_9H_{19} group of NP1 and intermediate **1** is a mixture of branched side chains, and the group of NP3 and intermediate **2** is the normal form.

Instrument. ^{1}H nuclear magnetic resonance (NMR) spectra of synthesized compounds were obtained on a 500 MHz NMR spectrometer, DRX500 (Bruker, Germany), using tetramethylsilane as an internal standard. Infrared (IR) spectra were obtained on an FT-IR spectrometer, FREEXACT-II (Horiba, Kyoto, Japan). ELISA was performed on 96-well microtiter plates (Nunc-Immuno plate, MaxiSorp surface, Roskilde, Denmark) and spectrophotometrically read with a microplate reader, PerkinElmer Wallac ARVO HTS 1420 multilabel counter (Turku, Finland).

Hapten Synthesis and Verification. The synthetic pathways for haptens used for immunization and antigen coating are presented in **Figures 1** and **2**. The procedures for the synthesis of each hapten are as follows.

NP1, 5-(2-Hydroxy-5-nonylphenyl)pentanoic Acid. To a solution of 4-nonylphenol (4 g, 18.2 mmol) in 5 mL of nitrobenzene was added aluminum chloride (2.2 g, 16.5 mmol), and then the mixture was stirred for 15 min at room temperature. Next, glutaric anhydride (1.2 g, 10.5 mmol) was added to the previous solution at room temperature, and then the reaction mixture was stirred for 1.5 h at 100 °C. After the resulting mixture was cooled, 20 mL of water and 20 mL of ethyl acetate were added to it, and then the synthesized product was extracted

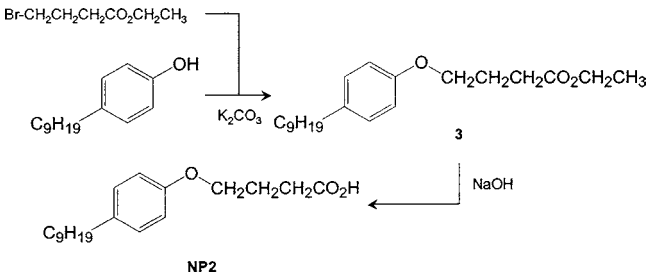

Figure 2. Synthetic pathway for hapten NP2. C_9H_{19} group of NP2 and intermediate **3** is a mixture of branched side chains.

to the ethyl acetate phase. The ethyl acetate phase was washed with water and was dried over anhydrous magnesium sulfate. The ethyl acetate was removed by a rotary evaporator under reduced pressure to give the crude product. The product was column chromatographed on silica gel using *n*-hexane/ethyl acetate (10% → 30% ethyl acetate in *n*-hexane) as an eluent to afford intermediate **1**, 4-(2-hydroxy-5-nonylbenzoyl)butyric acid (1 g, 60%) as a brown gum: ^{1}H NMR ($CDCl_3$): δ 12.17, 11.93 (ss, 1H, OH), 7.93−7.57 (m, 1H, Ar-1H), 7.29−6.74 (m, 2H, Ar-2H), 3.11−3.09 (m, 2H, CH_2), 2.57−2.55 (m, 2H, CH_2), 2.12−2.08 (m, 2H, CH_2), 1.9−0.7 (m, 19H, C_9H_{19}). IR (ν_{max}, Nujol) 1700, 1640.

To a solution of intermediate **1** (1.3 g, 3.9 mmol) in 6 mL of tri(ethylene glycol) was added hydrazine monohydrate (450 mg, 9 mmol), and then the mixture were stirred for 14 h at room temperature. Next, potassium hydroxide (85%, 7.3 g, 0.11 mol) was added to the previous solution, and then the mixture was heated for 30 min at 120 °C to evaporate water with stirring. Moreover, the mixture was heated for 1 h at 210 °C and then was cooled to 70 °C. Afterwards, to the cooled mixture was added hydrazine monohydrate (450 mg, 9 mmol), the mixture was heated for 30 min at 130 °C to evaporate water with stirring, and then the mixture was stirred for 1.5 h at 195 °C. The resulting mixture was cooled to 70 °C, 20 mL of water was added to it, and then it was acidified to pH 5 with 5 M hydrochloric acid. Next, to the mixture was added 20 mL of ethyl acetate, and then it was extracted two times. The ethyl acetate phase was washed with water and was dried over anhydrous magnesium sulfate. The ethyl acetate was removed by a rotary evaporator under reduced pressure to give the crude product. It was column chromatographed on silica gel using *n*-hexane/ethyl acetate (7:3) as an eluent to afford NP1 (90 mg, 7%) as a brown gum: ^{1}H NMR ($CDCl_3$): δ 7.25−6.74 (m, 3H, Ar-3H), 2.5 (m, 2H, CH_2), 2.4 (m, 2H, CH_2), 1.85−0.65 (m, 23H, C_9H_{23}). IR (ν_{max}, Nujol) 3300, 1700.

NP2, 4-(4-Nonylphenoxy)butyric Acid. To a solution of 4-nonylphenol (1.6 g, 7.3 mmol) in 30 mL of ethanol was added ethyl 4-bromobutylate (1.6 g, 8.2 mmol) and potassium carbonate (1.2 g, 8.7 mmol), and then the mixture was refluxed for 2 h. After the resulting mixture was cooled and filtered, the filtrate was concentrated. To the residue was added 20 mL of water, and the aqueous phase was extracted with 20 mL of ethyl acetate (two times). The combined ethyl acetate phase was washed with water, 10% sodium hydroxide, water, 1 M hydrochloric acid, and water. The organic solution was dried over anhydrous magnesium sulfate and then concentrated under reduced pressure. The intermediate **3** was obtained as a crude product (2.3 g, 96%). To a solution of nonpurified intermediate **3** (2.3 g, 6.9 mmol) in 15 mL of ethanol was added sodium hydroxide (0.8 g, 20 mmol) dissolved in 10 mL of water, and then the mixture was stirred at ambient temperature for 1 h. After the evaporation of ethanol, the resulting aqueous solution was acidified (pH 4) with 1 M hydrochloric acid and was extracted with 10 mL of ethyl acetate (two times). The combined ethyl acetate phase was washed with water and evaporated under reduced pressure. The resulting gum was immediately flash chromatographed on silica gel eluting with *n*-hexane/ethyl acetate (4:1) to give 0.45 g (21%) of NP2 as a colorless gum. ^{1}H NMR ($CDCl_3$): δ 7.13−7.25 (m, 2H, Ar-2H), 6.74−6.82 (m, 2H, Ar-2H), 4.00 (t, 2H, CH_2), 2.60 (t, 2H, CH_2), 2.08−2.12 (m, 2H, CH_2), 0.6−1.7 (m, 19H, C_9H_{19}). IR (ν_{max}, Nujol) 1700.

NP3, 5-(2-Hydroxy-5-*n*-nonylphenyl)pentanoic Acid. Intermediate **2** was synthesized according to the same synthetic method for intermediate **1**. Yield was 42% (2.8 g) as a brown gum. ^{1}H NMR ($CDCl_3$): δ 12.13 (s, 1H, OH), 7.55 (d, 1H, Ar−H), 7.29−7.33 (m, 1H, Ar−H), 6.93 (d, 1H, Ar−H), 3.13 (t, 2H, CH_2), 2.54−2.59 (m, 2H, CH_2), 2.10−2.13 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.33−1.39 (m, 14H, C_7H_{14}), 0.90 (t, 3H, CH_3). IR (ν_{max}, Nujol) 2916, 2850, 1697, 1641.

After powdery zinc (2.5 g, 38.2 mmol) and mercury (II) chloride (48 mg, 0.18 mmol) were added to 3.6 mL of water, to the mixture was added 2.5 mL of water, 2.5 mL of concentrated hydrochloric acid, and intermediate **2** (2.5 g, 7.5 mmol) dissolved in 1.3 mL of ethanol. The mixture was refluxed for 8 h, and then the resulting mixture was cooled to ambient temperature. Afterwards, to the resulting mixture was added 20 mL of ethyl acetate, and then it was filtrated. The aqueous phase was further extracted with 20 mL of ethyl acetate, and the combined organic solution was washed with water. The organic phase was dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Flash chromatography of the resulting residue recovered 110 mg of NP3 as a white powder [^{1}H NMR ($CDCl_3$): δ 6.86−6.90 (m, 2H, Ar-2H), 6.65 (d, 1H, Ar−H), 2.60 (t, 2H, CH_2), 2.48−2.51 (m, 2H, CH_2), 2.39−2.42 (m, 2H, CH_2), 1.67−1.72 (m, 2H, CH_2), 1.26−1.29 (m, 12H, C_6H_{12}), 0.88 (t, 3H, CH_3). IR (ν_{max}, Nujol) 3350, 2912, 2846, 1722, 1709.] and 350 mg of ethyl ester of NP3 as an orange-colored gum [^{1}H NMR ($CDCl_3$): δ 6.86−6.90 (m, 1H, Ar-2H), 6.67 (d, 1H, Ar−H), 5.12 (s, 1H, OH), 4.11−4.15 (m, 2H, CH_2), 2.60 (t, 2H, CH_2), 2.49 (t, 2H, CH_2), 2.35 (t, 2H, CH_2), 1.69 (m, 2H, CH_2), 1.64 (m, 2H, CH_2), 1.55 (m, 2H, CH_2), 1.23−1.29 (m, 15H, C_7H_{15}), 0.88 (t, 3H, CH_3).]. The ethyl ester of NP3 was hydrolyzed with sodium hydroxide in water/ethanol, and then 170 mg of NP3 was obtained. Total yield was 25.5% (280 mg).

Conjugation of Carboxylic Acid Haptens to Carrier Proteins. The haptens were covalently coupled to the lysine groups of the carrier proteins such as KLH and BSA according to the activated ester method described by Karu et al. (*30*) with slight modifications. The reactions were conducted in 3 mL glass vials containing Teflon stir bars. Each hapten (0.25 mmol) was dissolved in 1.25 mL of dry DMF with equimolar NHS and a 10% molar excess of EDC. After 4 h of stirring at room temperature, the precipitated urea was removed by centrifugation, and the portions of activated esters were stored at −78 °C.

Proteins (50 mg each, KLH for immunogens, BSA for coating antigens) were dissolved in 5.0 mL of borate buffer (50 mM sodium borate·$10H_2O$, 0.9% NaCl, pH 9) in a 10 mL glass vials with Teflon stir bars. These solutions were allowed to stir vigorously, and 1.05 mL of DMF was added very slowly to bring the final DMF concentration to 20%. The resulting solutions were stirred at room temperature, and 250 μL of the activated ester supernate was added, a few microliters at a time, very slowly over a 20 min period. The solutions were gently stirred overnight at 4 °C and then exhaustively dialyzed against 3 L of 10 mM phosphate-buffered saline (PBS; 1.1 g/L Na_2HPO_4, 0.306 g/L KH_2PO_4, 0.9% NaCl, pH 7), which was changed four times a day for 2 days at 4 °C. All the protein conjugates were lyophilized at −40 °C, and unless otherwise indicated, working aliquots were stored at 4 °C in PBS at 1 mg/mL.

Determination of the Hapten Coupling Density. The extents of coupling of each hapten to the carrier protein were determined according to the TNBSA method described by Habeeb (*31*) with slight modifications. That is, 1 mL of 0.5 mg/mL protein solution or 0.5 mg/mL hapten−protein conjugate solution diluted with PBS, 1.0 mL of carbonate−bicarbonate buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, pH 9.6), and 1.0 mL of 0.1% TNBSA were reacted at 40 °C for 2 h. After that, 1.0 mL of 10% SDS was added to solubilize the protein and prevent its precipitation on the addition of 0.5 mL of 1 M HCl. After thorough mixing, the absorbance was measured at 335 nm.

Immunization. Female Japanese white rabbits (3−4 kg) were used for raising polyclonal antibodies. Routinely, 100 μg (protein equivalent) of NP1-KLH in 0.5 mL of PBS was thoroughly emulsified with an equal volume of Freund's adjuvant. The emulsion was intradermally injected at different sites on the neck and the back of the rabbit (about 10−20 sites per rabbit). Complete Freund's adjuvant was used in the initial immunization, and incomplete Freund's adjuvant was used for the subsequent boost injections. Boosts were given every 3 weeks in the same manner. On the seventh day after each boost, about 10 mL

of blood sample was drawn from the jugular vein of the ear to check the titer of the polyclonal antibody. The blood sample was allowed to coagulate for 2 h at room temperature, and then it was left to stand overnight in a refrigerator. Serum was separated from blood cells by centrifugation at 3000 rpm and was stored in conveniently sized aliquots at −78 °C. Boosts were given six times. Collected serum (1.8 mL) was diluted with PBS (6.4 mL), and then 4.5 mL of saturated ammonium sulfate solution was slowly added to diluted serum solution. After the mixture was stirred at room temperature for 30 min, it was centrifuged at 10 000 rpm at 4 °C for 30 min. The supernatant was removed, and then the pellet was dissolved into 2 mL of PBS. The solution was exhaustively dialyzed against 3 L of water, which was changed four times a day for 2 days at 4 °C, and finally dialyzed against 3 L of PBS.

Checkerboard Titration. A checkerboard titration was performed with each of the antisera collected from each rabbit. The checkerboard titration selected the combination of antiserum dilution and coating antigen concentration (hapten-BSA conjugate) that would provide the greatest sensitivity in ELISA. The optimized ELISA for 4-nonylphenol used a coating antigen concentration between 15.625 and 2000 ng/mL and an antiserum dilution between 1/250 and 1/8000. Titers of the antisera produced by four rabbits immunized with NP1-KLH were evaluated based on homologous and heterologous indirect ELISA.

Indirect ELISA, Competitive Indirect ELISA, and Cross-Reactivity of Selected Antibody. An indirect ELISA was conducted for the checkerboard titration. That is, 96-well microtiter plates were coated with a 100 μL/well coating antigen (hapten-BSA conjugate) in a coating buffer (50 mM carbonate−bicarbonate buffer, pH 9.6) and allowed to stand overnight at 4 °C. On the following day, the plates were washed five times with washing solution (PBS-T, PBS containing 0.05% Tween 20) by a Nunc-Immuno Wash 8 microplate washer and thoroughly tapped dry. Sites not coated with the coating antigen were blocked with a 200 μL/well blocking solution (3% skim milk in PBS). After incubation at 37 °C for 2 h, the plates were washed as described previously. To the wells was added a 100 μL/well anti-nonylphenol antiserum previously diluted with PBS, and the plates were incubated at room temperature for 1 h. After washing the plate, a 100 μL/well secondary antibody was diluted 1/3000 with PBS, and the plates were at room temperature for 1 h. The plate was washed, and a 100 μL/well substrate solution, which was prepared by the addition of 1 mL of 0.1 M sodium acetate, pH 5.5 (adjusted pH with 1 M citric acid), 200 μL of 1% TMB (DMSO solution), and 20 μL of 6% H_2O_2 to 18.78 mL of water, was added to each well. The enzymatic reaction was stopped after 20 min by adding 50 μL/well of 2 M H_2SO_4. The yellow plate was spectrophotometrically read in a single wavelength mode at 450 nm. The amount of the enzyme bound, as indicated by the change of colorless substrate to blue product, is directly related to the amount of the anti-nonylphenol antiserum bound to the plate-coating antigen.

A competitive indirect ELISA was used to assess the specificity (selectivity) of the antibody to free 4-nonylphenol and the cross-reactivities of structurally related analogues to the antibody. For competition, after equal volumes of standards in PBS containing 10% methanol and the anti-nonylphenol antiserum diluted with PBS were well mixed and were added to each well, the plates were incubated at room temperature for 1 h and washed. The following procedure was followed as described for the indirect ELISA. With the competitive ELISA format, analytes that do not react with the antibody would produce absorbance near 100%; conversely, analytes that do react with the antibody would decrease in percentage of absorbance.

Because the cross-reactivity is defined as the ability of structurally related analogues of the target analyte to bind to the specific antibody raised against the analyte, some other alkylphenols, nonylphenol ethoxylates, nonylphenoxy carboxylates, bisphenol A, and others were examined for selectivity of the ELISA by determining their respective I_{50} values in the competitive ELISA. Cross-reactivity values were calculated as the ratio of I_{50} of the 4-nonylphenol standard (Tokyo Kasei Kogyo Co., Ltd.) to I_{50} of the test analogues and was expressed as a percentage.

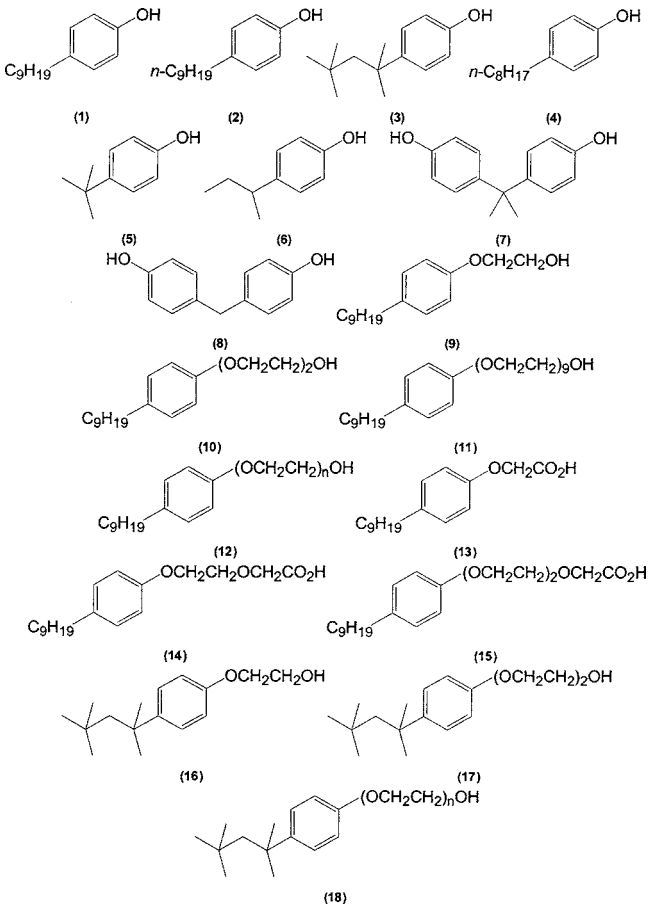

Figure 3. Chemical structures of 4-nonylphenol and its related compounds used for the cross-reactivity study.

RESULTS AND DISCUSSION

Hapten Design and Synthesis and Conjugation. In the case of development of antibodies against low molecular compounds such as 4-nonylphenol described in this paper, it is no exaggeration to say that the hapten design has the selectivity of the produced antibody in its hands. Generally, as antibodies are thought to best recognize the part of the hapten that is most distant from the conjugate linkage, a linking group opposite from the most characteristic groups of the molecule was desirable. When designing the hapten, we attempted to exploit the previously described general principle to develop polyclonal antibodies to selectively detect target 4-nonylphenol. 4-Nonylphenol has a variety of structurally related chemicals such as nonylphenol ethoxylates or nonylphenoxy carboxylates as given in **Figure 3** and is a very complex mixture of isomers, branched nonyl groups (*29*, *32*). So, when synthesizing the hapten, we should consider that commercial 4-nonylphenol or 4-nonylphenol resided and detected in environmental or biological matrixes is an aggregate of a large majority of structural isomers with branched alkyl chains, mainly para isomers. Technical 4-nonylphenol is a mixture of about 170 kinds of structural isomers, and Wheeler et al. (*29*) have achieved resolution of 22 kinds of isomers in commercial 4-nonylphenol (*p*-nonylphenol). The separated and identified isomers were categorized into five groups according to the isomer types: α-dimethyl form, α-ethyl-α-methyl form, α-methyl-α-propyl form, α,*β*-dimethyl form, and α-methyl form, respectively. On the other hand, a crucial structural difference between the target and the other related compounds is whether they have a free phenolic hydroxyl group in themselves as shown in **Figure 3**. Thus, our strategy for designing the hapten for immunization was to leave the only

unique portion distal from the carrier protein to improve antibody selectivity. So, we thought that the aliphatic spacer arm having four carbon atoms should be introduced into the ortho or meta position of the hydroxyl group. However, due to the bias of the electronic density between them, it was thought that it would be complicated to introduce it into the meta position. Thus, it was anticipated to elicit 4-nonylphenol-selective antibodies with an affinity for the whole molecule because the hapten NP1 containing an aromatic moiety is substituted with a spacer arm in the ortho position.

As shown in **Figure 1**, NP1 was synthesized via Friedel−Crafts acylation with glutaric anhydride, which can provide a four carbon spacer arm with a terminal carboxylic group for coupling to the carrier protein, to give the intermediate **1**. Reduction of the ketone group with the hydradine monohydrate and potassium hydroxide yielded NP1, which was a brown gum. However, since NP1 is a mixture of branched nonyl groups, it was difficult to clearly verify the structure with ^{1}H NMR. So, a single NP1 with a *n*-nonyl group (NP3) was synthesized via some different synthetic route from the route for NP1 shown in **Figure 1**, and we judged the final compound to be the objective NP1 based on the agreement of the R_f values with the objective NP1 and NP3, which was verified with ^{1}H NMR and the characteristics of the absorption with the IR spectrum.

As described by Szurdoki et al. (*33*), there are variety of carrier proteins for immunogens and coating antigens, and they recommended the use of KLH as a first choice for immunization because it is generally a very potent immunogen. So, we attempted to conjugate NP1 with KLH for an immunogen and NP1, NP2, and NP3 with BSA for coating antigens via the active ester method (*30*). The hapten density (the number of hapten molecules per molecule of carrier protein) of each conjugate was estimated with back-titration of the free ϵ-amino groups of lysine residues on carrier proteins before and after conjugation according to the method by Habeeb (*31*). From the available amino groups, 68.9% of NP1 was conjugated in the hapten-KLH conjugate, and 71.6% of NP1, 84.9% of NP2, and 60.2% of NP3 was conjugated in the hapten-BSA conjugates, respectively.

Screening and Selection of Antisera. The antisera of four rabbits immunized with NP1-KLH were screened against three different coating antigens using indirect noncompetitive ELISA. The objective was to find the coating antigen that has the highest affinity with tested antibody but could still be replaced by a target analyte. The titers of all the crude antisera obtained after the sixth booster injection showed comparatively high titers to their homologous assay, in which the same hapten was used in the coating antigen and immunogen. On the other hand, all antisera showed moderate titers against the heterologous coating antigen, NP2-BSA, but had little affinity against NP3-BSA. Although the antibody titers for all rabbits surely increased with repeated immunizations, no significant boost in the immune response was observed among the rabbits. So, since the titers of the crude antisera appeared to be low, the immunoglobulin fraction was purified with ammonium sulfate precipitation. The purification resulted in an approximately 2-fold concentration of the antibody (data not shown). On the basis of the titer curve, the purified NP03As diluted 1/1000 in each well and 1 μg/mL NP1-BSA and NP2-BSA as immobilized concentrations were chosen as the optimal working dilutions for further development.

Competitive inhibition experiments against 4-nonylphenol (Tokyo Kasei Kogyo Co., Ltd.) were performed based on the previously mentioned concentrations of four kinds of antisera and two kinds of coating antigens, and the combinations showing the lowest I_{50} values were selected as the optimal conditions for further developments. As given in **Table 1**, the I_{50} values ranged from 59 to 710 μg/L in the homologous and heterologous ELISA conditions tested. The homologous ELISA for NP03As with coating antigen NP1-BSA showed the lowest I_{50} value (59 μg/L), which is at least two times better than the heterologous ELISA using NP2-BSA. Although we expected that the ELISA sensitivity would be improved by using hapten heterology based on NP2-BSA as a coating antigen, the homologous ELISA that combines NP03As with NP1-BSA showed the greatest sensitivity in tested combinations. Hence, only this homologous ELISA condition was selected for further assay development and assay optimization.

Table 1. Screening and Selection of a Combination of Antisera and Coating Antigens with Indirect Competitive ELISA Against 4-Nonylphenol[a]

antiserum	coating antigen	I_{50} (μg/L)
NP01As	NP1-BSA	480
	NP2-BSA	710
NP02As	NP1-BSA	200
	NP2-BSA	680
NP03As	NP1-BSA	59
	NP2-BSA	125
NP04As	NP1-BSA	83
	NP2-BSA	105

[a] The technical 4-nonylphenol obtained from Tokyo Kasei Kogyo Co., Ltd. was prepared in 10 mM PBS containing 10% methanol. The final concentration of methanol was 5%.

ELISA Optimization. 4-Nonylphenol is considered to be a hydrophobic compound due to a slight solubility in water and adsorption activity to solids as suggested by Brix et al. (*34*). For example, since pyrethroid insecticides are highly hydrophobic, and may adhere to glass and plastic surfaces, the ELISAs for esfenvalerate (*21*), permethrin (*22*), or deltamethrin (*23*) developed by Hammock and co-workers required higher concentrations of organic solvent (methanol) to reduce such losses by solution of adsorbed material. Moreover, the organic solvents are often added to the running buffer to improve the solubility of the target analyte. First, the influences of organic solvents (methanol and DMSO) on the ELISA performance were assessed by preparing 4-nonylphenol in PBS containing various amounts. Methanol and DMSO significantly influence the sensitivity (I_{50} value) and the absorbance (A_{max} value) of the ELISA as shown in **Figure 4**. The I_{50} value of the ELISA varied depending on the difference concentration of the organic solvents. The lowest I_{50} values were found at 5% methanol (47 μg/L) and 5% DMSO (45 μg/L), which are about two times lower than that at 30% DMSO (100 μg/L). Although the absorbance values in the developed ELISAs for some pyrethroid insecticides were enhanced with higher methanol concentrations, those in our ELISA were suppressed in higher concentrations of both solvents up to 20%, especially as the influence of DMSO on maximum absorbance was significant. A very high concentration of the organic solvents will affect and retard the interaction between antibody and antigen and may denature protein reagents such as antibodies. On the other hand, since methanol is often used as an elution solvent from C_{18} SPE cartridges that are applied to concentrations of 4-nonylphenol or the related compounds in water samples, methanol was selected as a most suitable organic solvent for the ELISA, and on the basis of the I_{50} values and the A_{max} values for 4-nonylphenol standard curves, 5% methanol as the final concentration in each well was used in subsequent experiments. If the developed ELISA is applied to actual aqueous environ-

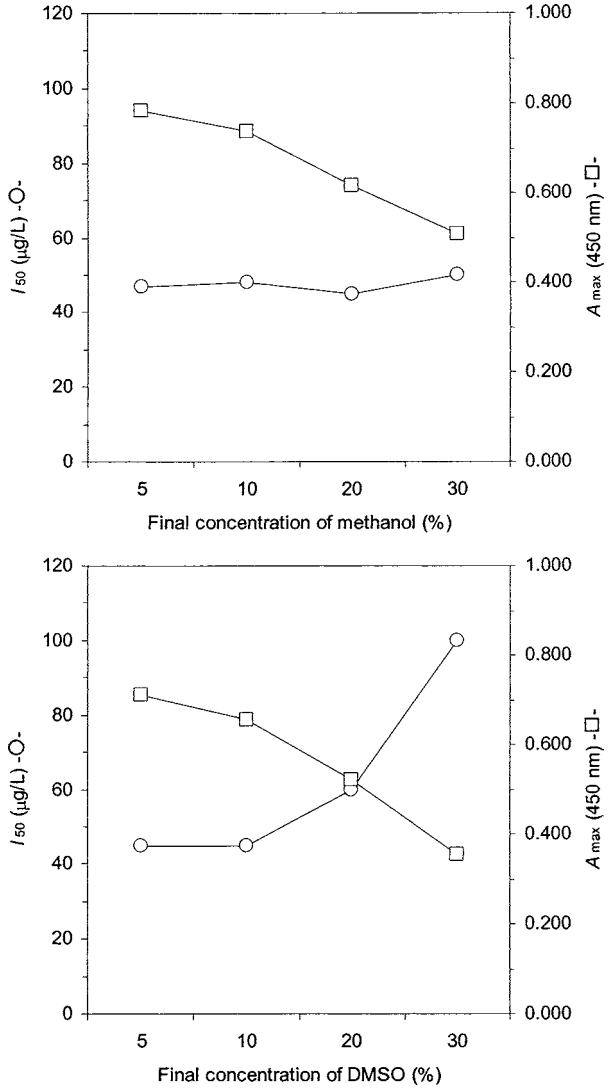

Figure 4. Influences of methanol and DMSO concentrations in the ELISA on the I_{50} values (○) and A_{max} values (□) of the 4-nonylphenol competitive standard curves. Data were obtained by averaging two standard curves, each run in triplicate.

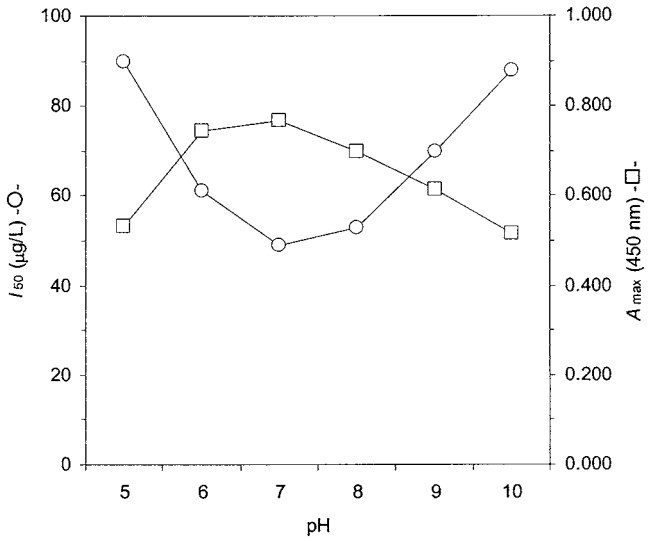

Figure 5. Influences of the pH of the running buffer in the ELISA on the I_{50} values (○) and A_{max} values (□) of the 4-nonylphenol competitive standard curves. Data were obtained by averaging two standard curves, each run in triplicate.

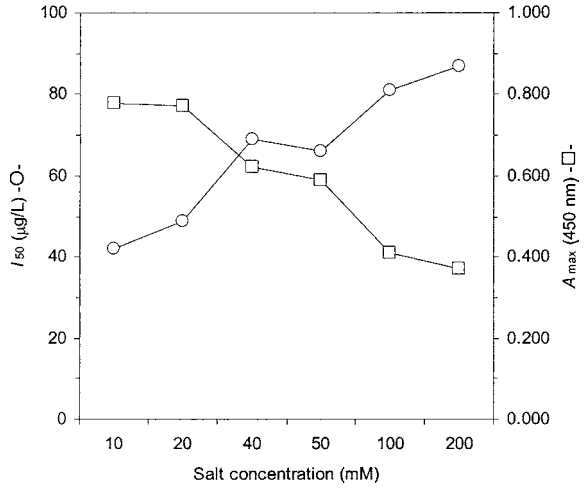

Figure 6. Influences of the salt (phosphate ion) concentration of the running buffer in the ELISA on the I_{50} values (○) and A_{max} values (□) of the 4-nonylphenol competitive standard curves. Each tested running buffer contains 0.9% NaCl. Data were obtained by averaging two standard curves, each run in triplicate.

mental samples, it should grasp the potential influences of pH and ionic strength.

To study the influence of pH on the assay performance, competitive curves were obtained by preparing standards in PBS at different pH values covering the range of 5−10. The variation of the curve parameters (I_{50} value and A_{max} value) as a function of pH is shown in **Figure 5**. The recognition of NP1-BSA was represented by the response in the absence of analyte, and the A_{max} value showed a maximum at pH 6−7. On the other hand, the assay sensitivity (I_{50} value) increased at a more acidic or basic pH value (<6 or >8). From the curves plotted in the graph shown in **Figure 5**, it seems that a neutral pH (7) could be a reasonable choice for the running buffer of the competition steps.

Because the ionic strength of the running buffer containing the antibody can affect antibody binding, the ionic strength of the running buffer was varied by increasing the concentration of the phosphate ion from 10 to 200 mM (**Figure 6**). A higher salt concentration in the ELISA resulted in lower optical densities (A_{max} values) and higher I_{50} values. The A_{max} values at salt concentrations between 40 and 50 mM and 100 and 200 mM decreased by approximately 25 and 50%, respectively, from the A_{max} value at a salt concentration 10 mM PBS. Thus, the maintenance of a minimal ionic strength appeared to be important.

The optimized ELISA for 4-nonylphenol was established by utilizing 100 μg/mL NP1-BSA as a coating antigen, NP03As at a dilution of 1/1000 in each well, 4-nonylphenol in running buffer PBS containing 5% methanol (as final concentration in each well), pH value of 7, and concentration of PBS of 10 mM, respectively. The standard curve for technical grade 4-nonylphenol obtained from Tokyo Kasei Kogyo Co., Ltd. under the conditions is shown in **Figure 7**. The analytical characteristics of the optimized ELISA were estimated from the standard curve, which was obtained by averaging five curves, each run in triplicate. The I_{50} value of the ELISA was 38.6 ± 5.5 μg/L. The limit of detection, estimated as the concentration of the analyte 4-nonylphenol giving a 10% inhibition (I_{10} value) of the maximum absorbance (A_{max}), and the dynamic range for the ELISA, calculated as the concentration of the analyte providing a 20−80% inhibition (I_{20}−I_{80} values) of the maximum

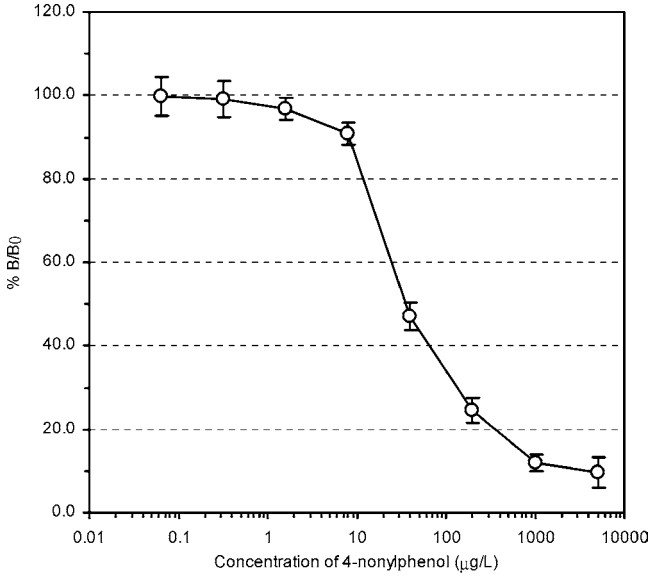

Figure 7. Standard curve for the ELISA, using coating antigen NP1-BSA (1 μg/mL) and antiserum NP03As (diluted 1/1000, final dilution in the well). The standard curve represents the average of five curves. Error bars represent standard deviation.

signal (35), were 7.7 ± 1.3 and 12−350 μg/L, respectively. Accordingly, the sensitivity of the developed ELISA markedly improved as compared with one of the indirect competitive ELISA developed by Fránek et al. (*27*) and was somewhat higher than the already developed direct competitive ELISA in which the I_{50} value was around 40 μg/L (*28*).

Generally, the residual levels of alkylphenols or various agrochemicals in environmental matrixes, mainly river or lake water, are considerably low as compared with other matrixes, for example, food or biological matrixes. Hence, especially, it can demand sufficient sensitivity to be parallel to the actual concentration levels, usually ppb or ppt levels from analytical methods including instrumental analytical methods or immunochemical methods presented in this paper for environmental monitoring of their compounds. It seems that the developed ELISA will require some progress in the sensitivity from this point of view. Moreover, it can be essential to investigate the application to real samples and validation with instrumental analytical methods to make the ELISA a practical monitoring tool for alkylphenol analysis. At any rate, further studies with spiked and real samples are in progress, and we will plan to accomplish the development of further practical ELISA for alkylphenols including 4-nonylphenol.

Cross-Reactivity. The selectivity of the ELISA based on NP03As was assessed by using various 4-nonylphenols obtained from different manufacturers and 4-nonylphenol-related compounds such as 4-octylphenol, ethoxylates, and carboxylates of both alkylphenols and other phenol derivatives (**Figure 3**). The results of the cross-reactivity study are presented in **Table 2**. For the determination of cross-reactivity, the I_{50} value of 4-nonylphenol obtained from Tokyo Kasei Kogyo Co., Ltd. was assigned a value of 100%, and the cross-reactivities for other compounds are reported according to their I_{50} values relative to this value. The I_{50} value of 4-nonylphenol (Tokyo Kasei Kogyo Co., Ltd.) was 43 μg/L. On the other hand, the I_{50} values of other 4-nonylphenol standards (Sigma-Aldrich, Inc. and Kanto Chemical Co., Inc.) were 49 μg/L (cross-reactivity = 87.8%) and 51 μg/L (cross-reactivity = 84.3%), respectively. These results indicate that there are hardly any significant differences in the sensitivity among the manufacturers. Interestingly,

Table 2. Cross-Reactivity of Several Structurally Related Analogues to the Antiserum NP03As in the Optimized ELISA[a]

analogue	I_{50} (μg/L)	cross-reactivity (%)[b]
4-nonylphenol (Tokyo Kasei Kogyo) **(1)**	43	100
4-nonylphenol (Sigma-Aldrich) **(1)**	49	87.8
4-nonylphenol (Kanto Chemical) **(1)**	51	84.3
4-*n*-nonylphenol **(2)**		ni[c]
4-octylphenol (Tokyo Kasei Kogyo) **(3)**	56	76.8
4-octylphenol (Sigma-Aldrich) **(3)**	52	82.7
4-octylphenol (Kanto Chemical) **(3)**	64	67.2
4-*n*-octylphenol **(4)**		ni
4-*t*-butylphenol **(5)**	188	22.9
4-*sec*-butylphenol **(6)**	820	5.2
bisphenol A **(7)**		ni
bisphenol F **(8)**		ni
nonylphenol monoethoxylate **(9)**		ni
nonylphenol diethoxylate **(10)**		ni
Tergitol NP-9 **(11)**		ni
nonylphenol ethoxylate (n = 1–2) **(12)**		ni
nonylphenoxy acetic acid **(13)**		ni
nonylphenol monoethoxylate acetic acid **(14)**		ni
nonylphenol diethoxylate acetic acid **(15)**		ni
octylphenol monoethoxylate **(16)**		ni
octylphenol diethoxylate **(17)**		ni
octylphenol ethoxylate (n = 1–2) **(18)**		ni

[a] The optimized ELISA consisted of antiserum NP03As (1/1000 as final dilution) and coating antigen NP1-BSA (1 μg/mL). All other ELISA conditions were as described under Materials and Methods. [b] Cross-reactivity was estimated as follows: (I_{50} value of 4-nonylphenol (Tokyo Kasei Kogyo)/I_{50} value of other analogue tested) × 100. [c] ni, no inhibition at the highest concentration tested (1000 μg/L).

NP03As cross-reacted to three kinds of 4-octylphenol standards (cross-reactivity >67%). Since it has α-dimethyl form as shown in the chemical structure, and numerous isomers of 4-nonylphenol mainly occupy the form as suggested by Wheeler et al. (*29*), it was thought that NP03As raised from NP1 that was synthesized by using technical 4-nonylphenol as a starting material showed a cross-reaction against them. Probably, it can be different to produce an entirely and highly specific antibody against only 4-nonylphenol. Actually, two of five antibodies developed by Zeravik et al. (*28*) also cross-reacted to 4-octylphenol. Since one of four polyclonal antibodies showing the most high sensitivity recognized not only 4-nonylphenol but also 4-octylphenol, the ELISA based on the selected antibody can be effective in monitoring both alkylphenols in which they are estimated as the total amounts. Because of the cross-reactivity against the other alkylphenols having shorter alkyl chains, two kinds of 4-butylphenol of the antibody clearly reduced to 22.9% (4-*t*-butylphenol) and 5.2% (4-*sec*-butylphenol). Although 4-*t*-butylphenol having an α-dimethyl form resulted in a somewhat higher cross-reactivity, the nonyl or octyl group having a longer alkyl chain and sterically more complex structure due to ramification of the side chains can take an important part in the immunological interaction. The cross-reactivities of NPEOs and nonylphenoxy carboxylates, parent compounds of 4-nonylphenol and 4-octylphenol, and their degradation products, were negligible. These findings indicate that the modification of the free phenolic hydroxyl group drastically hinders the antibody binding to the compounds, which support the strong participation of the functional group in the analyte−antibody interaction. Since bisphenol A and bisphenol F were also very poorly recognized by the antibody, probably, the results were also due to the steric disharmony being caused by two aromatic moieties in the interaction.

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