Identification, Determination, and Biological Evaluation of a Novel Styrene Trimer Contained in Polystyrene Food Containers. 2

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An unidentified styrene trimer (ST) isolated from the acetonitrile extract of polystyrene (PS) food containers was characterized as (1*S**,6*R**,7*S**,8*S**,11*R**)-6,11-diphenyltricyclo[6,2,2,$0^{2,7}$]dodeca-2,9-diene. The content and migration of this compound in PS food containers were determined by GC-MS (SIM). Furthermore, an endocrine-disrupting effect was tested using in vitro and in vivo assays of the compound. In conclusion, it seems that the compound does not present the effect.

Keywords: *Styrene trimer; tricyclo[6,2,2,$0^{2,7}$]dodeca-2,9-diene; polystyrene; GC-MS (SIM) analysis; material and migration tests; biological evaluation*

INTRODUCTION

Today, we live in an environment where numerous types and large quantities of chemical substances exist, and their presence in our life is the inevitable price we pay for technological progress. Public awareness is increasing, and the public is questioning the safety of food packaging that is essential to food hygiene, specifically in regard to food wrappers and containers.

Styrene dimers (SD) and styrene trimers (ST), which we are researching, were listed as endocrine disrupters by Colborn et al. in the Wingspread Statement (*1*), although scientific data had not yet been published. Recently, Kawamura et al. reported that three SD [2,4-diphenyl-1-butene (NSD-01), *cis*-1,2-diphenylcyclobutane (NSD-08), and *trans*-1,2-diphenylcyclobutane (NSD-09)], two ST [2,4,6-triphenyl-1-hexene (NST-01) and 1-phenyl-4-(1-phenylethyl)tetralin (NST-03)], and two unknown compounds detected within the ST retention window were detected from an extract of a polystyrene (PS) food container (*2*).

In a series of studies on the safety of PS food containers (*3*), we have proved that one unknown compound was 1-phenyl-4-(2-phenylethyl)tetralin (NST-12) (*4*), and eluted SD and ST showed no endocrine-disrupting action by in vivo and in vitro assays, which were compared in detail by Andersen et al. and EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee) (*3*−*7*). However, another unknown compound (**1**) has not been identified or tested for bioassays. This compound was presumed to be 1,3,5-triphenylcyclohexane (**2**) on the basis of GC-MS data shown in Figure 1 (*2*). However, we confirmed the absence of **2** in the eluted compounds when we measured the GC-MS of **2** (two isomers) yielded by the reduction of 1,3,5-triphenylbenzene (*8*).

In this paper, we report the isolation, structure elucidation, determination, and biological evaluation of **1** (NST-13).

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MATERIALS AND METHODS

Materials. PS resin, which was a precursor of an end user product, was employed for the isolation.

Expanded PS (EPS), PS paper (PSP), and high-impact PS (HIPS) were analyzed to compare the differences of PS materials, because they were being used for polystyrene food containers.

Isolation. PS resin (10 kg) was refluxed with acetonitrile (20 L) for 20 h; the extract was then concentrated until dry under a reduced pressure to yield a colorless oil (102 g). This residue was chromatographed on silica gel (IR-60-40/63-W, Daiso Co.) for elution with *n*-hexane (two times). A fraction containing **1** was identified by GC-MS. The fraction (600 mg) was rechromatographed on a column of TKSgel ODS-80TSQA using acetonitrile/water (8:2) to get 67 mg of **1**.

Spectroscopy. NMR spectra excluding HMBC were measured in $CDCl_3$ or benzene-d_6 with a JEOL GSX-400 spectrometer. The HMBC spectrum was measured in benzene-d_6 with a JEOL A-400 spectrometer. Shifts (in parts per million) are relative to internal tetramethylsilane.

Analysis Conditions. A material test was conducted according to the method of Kawamura et al. (*9*). Migration tests were conducted according to Food Sanitation Law in Japan. SD (NSD-01, -08, and -09), ST (NST-01, -03, and -12), and **1** were employed as standards.

Material Test. Chopped PS (0.5 g) was extracted with cyclohexane/2-propanol (1:1, 10 mL) using an incubator (Taitec Parsonal-10, 37 °C, 17 h). This solution (50 μL) and 20 ppm of *p*-terphenyl (internal standard)/*n*-hexane (50 μL) were mixed and diluted with *n*-heptane to 5 mL.

Migration Test. *n*-Heptane (EPS, 410 mL; PSP, 570 mL; HIPS, 600 mL) at 25 °C was added to PS containers, and this solution was permitted to stand for 1 h at 25 °C. A 5 mL aliquot was measured and concentrated by N_2 flow to ∼0.2 mL; 2 ppm of *p*-terphenyl/*n*-hexane (0.1 mL) was added to the residue and diluted with *n*-heptane to 1 mL.

GC-MS Conditions. The analysis was performed using a Hewlett-Packard HP-6870 GC, equipped with a DB-5ms (J&W Scientific, Inc.) capillary column (0.25 mm i.d. × 30 m, 0.25 μm film thickness) and a mass spectrometer JEOL Automass II as a detector. The carrier gas used was helium, at a flow rate of 1.3 mL/min. Column temperature was initially 100 °C and then was gradually increased to 300 °C at 20 °C/min and kept at 300 °C for 5 min. For GC-MS detection, an electron ionization system was used with an ionization of 70 eV in the selected ion monitoring (SIM) mode. Monitored ions were *m*/*z*

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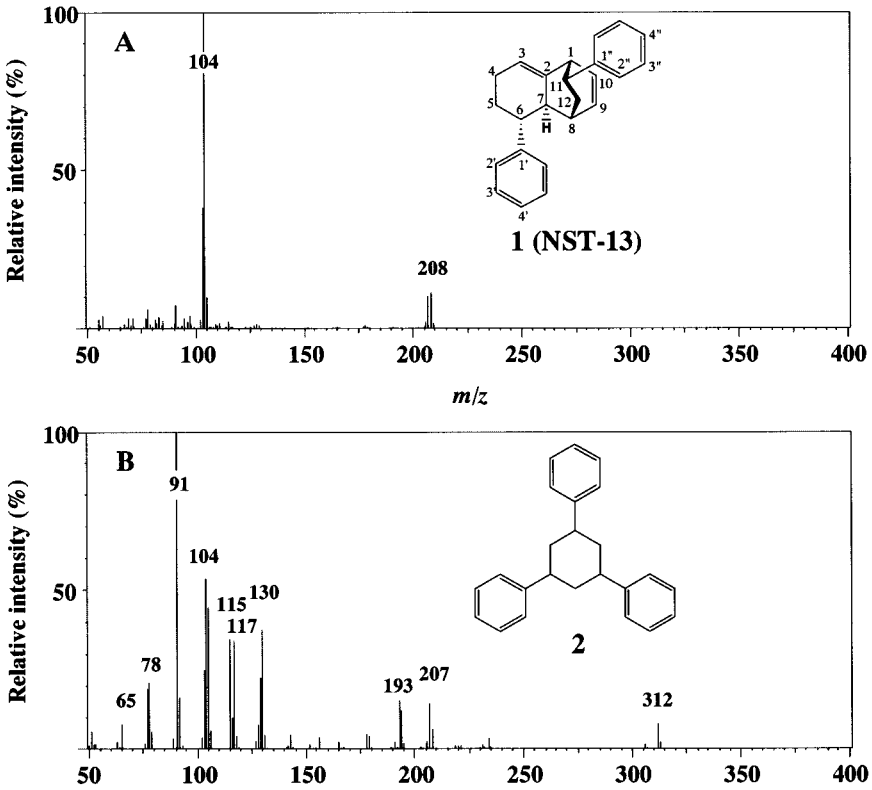

Figure 1. Chemical structures and mass spectra of **1** (NST-13) (A) and 1,3,5-triphenylcyclohexane (**2**) (B).

91 (NST-01, -03, and -12), 104 (**1**, NSD-08 and -09), and 230 (*p*-terphenyl). One microliter of the test samples was injected automatically in a splitless mode. Injector, interface, and ion source temperatures were set at 250, 250, and 230 °C, respectively.

Biological Evaluation. *Estrogen Receptor (ER) Binding Assay*. Female 8-week-old Sprague-Dawley rats [180−210 g weight, Crj:CD (SD) IGS; Charles River Japan Inc.] were ovariectomized. After 2 days, rats were sacrificed. Uteri were excised and homogenized with a Polytron homogenizer in ice-cold TEDG buffer (10 mmol/L Tris-HCl, 1.5 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, pH 7.4) at a concentration of 1.0 g of tissue/5 mL of buffer. The resulting homogenate was centrifuged (105000*g*) for 60 min at 4 °C, and the supernatant is low-salt unoccupied cytosolic receptors (ER-rich cytosol). Twenty-five microliters of prepared cytosol receptor was incubated with 2 nmol/L of [2,4,6,7-^{3}H(N)] estradiol ([^{3}H]E_2; NEN Life Science Products, Inc., Boston, MA) and 100 μL of TEDG buffer containing test compounds or 17β-estradiol (E_2; Sigma Chemical Co., St. Louis, MO) in glass tubes for 18 h at 4 °C. Test compounds were dissolved in dimethyl sulfoxide (DMSO) (the final concentration of DMSO was 0.2%) and added TEDG buffer. After the incubation period, 0.5 mL of hydroxylapatite (HAP) slurry (made in 50 mmol/L Tris-HCl, pH 7.4) was added to each tube to separate the bound [^{3}H]E_2 from the free ligand. These tubes were incubated in an ice-cold water bath for 20 min and vortexed at 5-min intervals. Tubes were centrifuged (600*g*) at 4 °C for 3 min, and the resulting HAP pellet was washed with 2 mL of Tris buffer (50 mmol/L, pH 7.4) three times. After the washings, ice-cold ethanol was added to each tube to extract the radiolabeled E_2, and tubes were vortexed at 5-min intervals. Tubes were centrifuged (600*g*) at 4 °C for 10 min, and the resulting supernatant was decanted into vials containing 10 mL of scintillation cocktail. Radioactivity was measured on a liquid scintillation counter (Tri-Carb 2700TR, Packard Instrument Co., Meriden, CT). Nonspecific binding was assessed by the addition of 100 molar excesses of nonlabeled E_2. Data were plotted as percent of [^{3}H]E_2 bound versus molar concentration of test samples, and the affinity for ER was evaluated by the antagonistic ability of test compounds.

MCF-7 Cell Proliferation Assay (E-SCREEN). Human breast cancer cell line MCF-7 proliferation assay was carried out according to the methods of Soto et al. (*10*, *11*). MCF-7 cells (Dainippon Pharmaceutical Co., Ltd. Japan) were plated in 12-well plates (FALCON) at an initial concentration of 2.0×10^4 cells/well in DME medium (Gibco BRL, Life Technologies, Inc., Rockville, MD) supplemented with 5% FBS. Cells were allowed to attach for 24 h, and then the seeding medium was replaced with the experimental medium (5% charcoal dextran stripped human serum supplemented to phenol red free DME medium) with test compounds dissolved in ethanol (the final concentration of ethanol was 0.1%). The bioassay was terminated on day 6, and the cell number was counted. Data were described as the percent of the number of control vehicle treated cells.

Uterotrophic Assay. A uterotrophic assay was carried out according to the methods of Odum et al. (*12*). **1** was dissolved in corn oil (Nacalai Tesque Inc., Japan) and was administered to 21-day-old female rats [Crj:CD (SD)IGS; Charles River Japan Inc.] by subcutaneous injection at doses of 0.02, 0.2, or 2 mg/kg/day for 3 days.

RESULTS AND DISCUSSION

Structural Elucidation. Although the molecular weight of ST was not determined by EIMS, tests with CIMS measured in methane gas and HREIMS demonstrated that **1** had the same molecular formula as ST (CIMS, MH^+, *m/z* 313; HREIMS, M^+, *m/z* 312.1888, Δ + 1.0 mmu). A detailed analysis of ^{1}H NMR, ^{13}C NMR, DEPT, and ^{1}H−^{13}C COSY spectra showed that **1** contained 3 methylene,18 methine, and 3 quaternary carbons (Table 1). ^{1}H signals at δ 7.01−7.17 (10H, m) and ^{13}C methine signals at δ 125.7 (d), 126.1 (d), 128.4 × 2 (d), 128.0 × 2 (d), 127.8 × 2 (d), and 127.4 × 2 (d) were assigned to the two monosubstituted benzene units. The other six olefinic carbons were observed at δ 117.4 (d) (C3), 129.4 (d) (C10), 136.6 (d) (C9), 143.2 (s) (C2), 145.5 (s) (C1′), and 146.4 (s) (C1″). The ^{1}H−^{1}H

Table 1. NMR Data of 1 (NST-13) (400 MHz, in $CDCl_3$)

atom	δ C[a]	δ H[b] (J in Hz)	HMBC (C_6D_6)
1	47.3 (d)	3.22 (ddd, 1.2, 2.4, 6.1)	C2, C3, C7, C9, C10, C11, C12, C1″
2	143.2 (s)		
3	117.5 (d)	5.58 (dd, 3.2, 6.4)	C4, C5, C7
4	26.6 (t)	2.3, 2.4	
5	31.9 (t)	1.86, 1.98 (m)	C3, C4, C6, C7, C1′
6	44.1 (d)	2.62 (ddd, 3.2, 11.7, 14.4)	C4, C5, C7, C1′
7	47.8 (d)	2.09 (br d, 10.6)	
8	34.2 (d)	2.3	C2, C10, C11
9	136.6 (d)	6.44 (ddd, 1.2, 6.9, 8.1)	C1, C8
10	129.4 (d)	6.03 (ddd, 0.7, 6.8, 7.5)	C1, C2, C8
11	48.8 (d)	3.15 (ddd, 2.9, 4.2, 9.8)	C1, C2, C10, C12, C1″
12	27.9 (t)	2.21 (H_{12a}, ddd, 2.0, 9.8, 12.0) 1.39 (H_{12b}, br d, 12.9)	C7, C8, C9, C11, C1″
1′	145.5 (s)		
1″	146.4 (s)		
2′, 2″, 3′, 3″	127.4 × 2 (d) 127.8 × 2 (d) 128.0 × 2 (d) 128.4 × 2 (d)	7.35−7.14 (10H, m)	C1′, C1″
4′, 4″	125.7 × 2 (d) 126.1 × 2 (d)		

[a] s, singlet; d, doublet; t, triplet. [b] Underscored values are approximate chemical shifts.

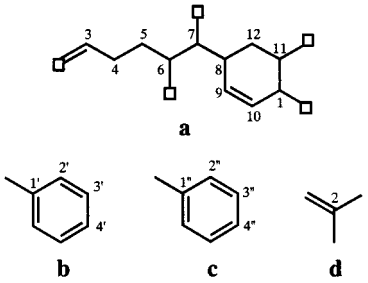

Figure 2. Four partial structures of **1** established by 2D-NMR (400 MHz) spectroscopy (□, quaternary carbons).

COSY spectrum of **1** gave four partial structures of **a**−**d** (Figure 2).

$^2J_{CH}$, $^3J_{CH}$ long-range coupling correlation in the HMBC spectrum of **1** was used to assemble the four partial units through three quaternary carbons (C2, C1′, and C1″). C1′/Ar-H, C1″/Ar-H cross-peaks suggested that C1′ and C1″ were located on the 1-postion of the monosubstituted benzene unit. The two benzyl methine carbons were assigned to C6 and C11 because their chemical shifts (C6, δ_C 44.1 and δ_H 2.62; C11, δ_C 48.8 and δ_H 3.15) were reasonable, and cross-peaks of H4/C1′, H5/C1′, H6/C1′, H11/C1″, and H12/C1″ were observed. The H1/C2, H8/C2, H10/C2, H11/C2, and H1/C3 cross-peaks suggested that there were connectivities from C1 and C7 to C2 (δ_C 143.2) and revealed that C1 of the **a** unit was connected to C3 of the **a** unit through a quaternary carbon (C2) of the **d** unit to form a tricyclo-[6,2,2,$0^{2,7}$]dodeca-2,9-diene skeleton. The presence of cyclohexene (C2−C7) was supported by typical coupling constants of protons ($^3J_{3H-4Ha}$ = 6.4 Hz, $^3J_{3H-4Hb}$ = 3.2 Hz, $^2J_{5Ha-5Hb}$ = 12 Hz, $^3J_{5Ha-6H}$ = 3 Hz, $^3J_{5Hb-6H}$ = 12 Hz, $^3J_{6H-7H}$ = 11 Hz). Eventually, the gross structure of **1** was completely elucidated.

The relative stereochemistry at C1, C6, C7, C8, and C11 was based on an analysis of $^3J_{H-H}$ coupling constants and NOE correlations from NOESY (mixing times of 1770 ms) data of **1** (Figure 3). The large coupling constant between H6 and H7 (11 Hz) suggested that H6 and H7 were 1,2-diaxial. The stereochemistry at C11 was determined by the NOESY cross-peak H12a/H11, whereas the stereochemistry at two bridgehead carbons, C1 and C8, was derived from the NOESY cross-peak H6/H12a.

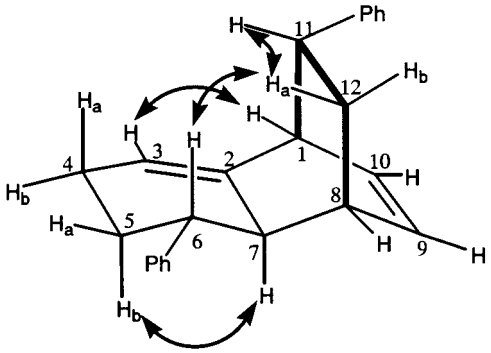

Figure 3. Relative configurations at C1, C6, C7, C8, and C11 of the tricyclo[6,2,2,$0^{2,7}$]dodeca-2,9-diene moiety of **1** (arrows, NOE correlations observed in the NOESY spectra).

Because this compound was isolated from an eluted mixture of PS and two double bonds were located at C2−C3 and C9−C10, it was considered that **1** was formed by two consecutive [4 + 2]π Diels−Alder cycloaddition reactions of three styrene monomers (SM). Two important ionic peaks of **1** in EI-MS, *m*/*z* 104 and 208, suggested the elimination of one and two styrene monomers from the molecular ion of **1** via retro-Diels−Alder reaction. It was assumed that **1** was synthesized by the formation mechanism shown in the scheme. The *exo*-cycloaddition of two SM gave **4**-*exo*, and then **1** was obtained by *endo*-addition of SM from the other side of the 6-position proton. **4**-*exo* and **4**-*endo* are considered to be intermediates to generate NST-03 (**5**) and NST-12 (**6**) eluted from PS (Scheme 1).

Analysis. In material and migration tests, **1** was not detected in EPS and PSP but was detected in HIPS (0.01 mg/g, 1.9 ppb). In both tests, the total amounts were larger in the order of EPS < PSP < HIPS, and the ratios of analytical values of respective compounds detected in all materials were almost equal to each other. (Tables 2 and 3)

ER Binding Assay. E_2 inhibited strong bonding of [^{3}H]estradiol for the ER in a dose-dependent manner, and the IC_{50} value was 2.78 nmol/L. In contrast, **1** did not inhibit bonding of [^{3}H]estradiol for the ER in a

Scheme 1. Proposed Mechanism of Formation of NST-03 (5), -12 (6), and -13 (1)

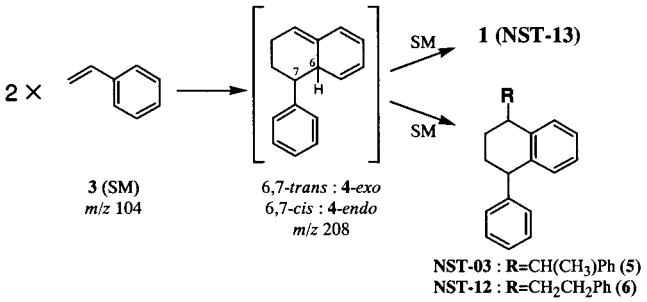

Table 2. Content of Styrene Dimers and Trimers from PS Material[a]

PS material	SD (mg/g) NSD-01	NSD-08	NSD-09	ST (mg/g) NST-01	NST-03	NST-12	NST-13	total (mg/g)
EPS	0.07	ND	0.01	0.22	0.09	ND	ND	0.39
PSP	0.21	ND	ND	0.64	0.11	ND	ND	0.96
HIPS	0.07	ND	0.03	0.55	0.72	0.05	0.01	1.43

[a] ND < 0.01 mg/g (n = 3).

Table 3. Migration of Styrene Dimers and Trimers from PS Containers[a]

PS container	SD (ppb) NSD-01	NSD-08	NSD-09	ST (ppb) NST-01	NST-03	NST-12	NST-13	total (ppb)
EPS	7.8	ND	ND	8.3	4.0	ND	ND	20.5
HIPS/PSP[b]	6.2	ND	ND	25.3	12.1	ND	ND	43.6
PSP/HIPS[b]	16.1	ND	6.6	117.1	129.3	8.3	1.9	279.3

[a] ND < 1 ppb (n = 3). [b] Outside/inside.

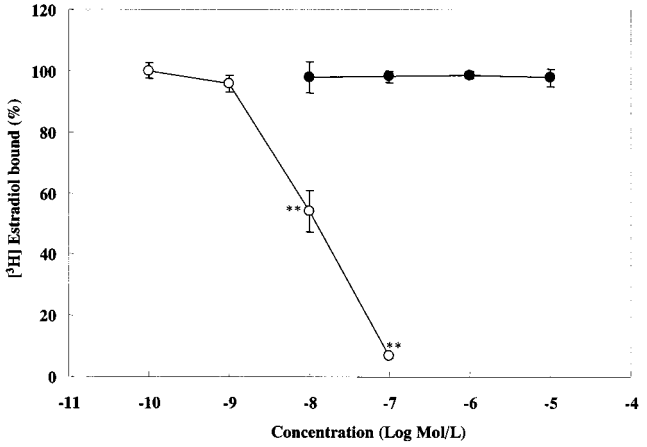

Figure 4. Estrogen receptor binding assay of styrene trimer (NST-13). Rat uterine cytosol was incubated with 2 nmol/L [^{3}H]estradiol (○) and NST-13 (●). After incubation for 18 h at 4 °C, the receptors were separated and the radioactivities were counted. Each value represents the mean ± SD of triplicate assays. **, significantly different from control, $p < 0.01$ (Dunnett test).

concentration of 10^{-8}−10^{-5} and did not exhibit estrogen bonding activity (Figure 4).

MCF-7 Cell Proliferation Assay (E-SCREEN). MCF-7 cells proliferated 4-fold over the control by the addition of 0.01−30 pmol/L of E_2. In contrast, **1** did not induce statistically significant proliferation of MCF-7 cells (Figure 5).

Uterotrophic Assay. E_2 (40 μg/kg) induced a significant increase in uterine weight in comparison to the control. In contrast, **1** (0.02−2 mg/kg) did not induce a significant increase in uterine weight (Figure 6).

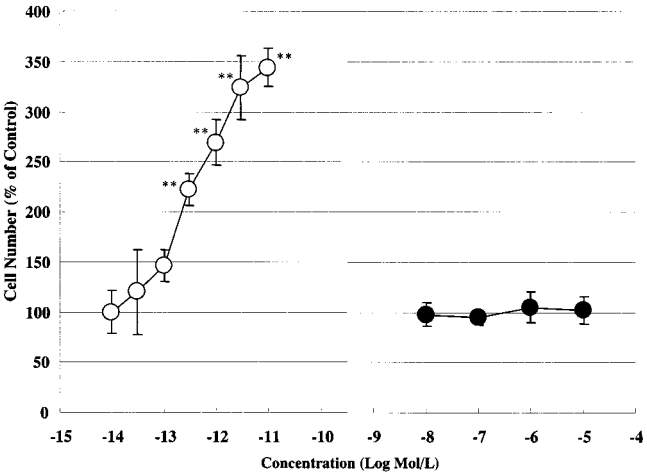

Figure 5. Effect of styrene trimer (NST-13) on proliferation of MCF-7 cells: estradiol (○); NST-13 (●). The cells were treated with test compounds for 6 days, and the cell number was counted. Each value represents the mean ± SD (n = 3 wells). **, significantly different from control, $p < 0.01$ (Dunnett test).

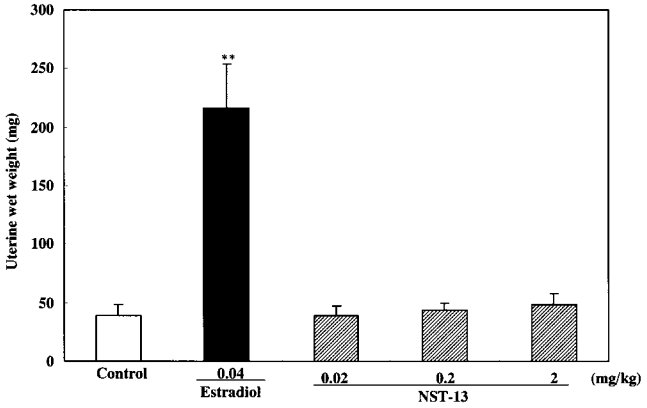

Figure 6. Effects of styrene trimer (NST-13) on uterine wet weight in immature rats. Immature rats were treated subcutaneously with NST-13 dissolved in corn oil for 3 days. After 24 h of final treatment, uteri were removed and weighed. **, $p < 0.01$ (vs control). Each value represents the mean ± SD (n = 5). **, significantly different from control, $p < 0.01$ (Dunnett test).

Conclusion. It was discovered with MS and NMR analysis that the unknown compound (**1**) in an extract of PS is (1*S**,6*R**,7*S**,8*S**,11*R**)-6,11-diphenyltricyclo-[6,2,2,$0^{2,7}$]dodeca-2,9-diene (NST-13). In material and migration tests, although **1** was undetected from EPS and PSP, a trace amount of **1** was detected from HIPS. Additionally, the ER bonding assay, MCF-7 cell proliferation assay (E-SCREEN), and 3-day uterotrophic assay applied to **1** did not show an endocrine-disrupting effect.

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