Estrogenic Activity of Stilbene Derivatives

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We previously reported that *trans*-stilbene is metabolically activated to estrogenic compounds by a liver microsomal enzyme system. In this study, we demonstrated the structural requirement for estrogenic activity of various stilbene derivatives including proestrogens. High estrogenic activity in 4,4′-dihydroxystilbene, 4-amino-4′-hydroxystilbene, 4,4′-dihydroxy-α-methylstilbene, hexestrol, and diethylstilbestrol (DES), moderate activity in 4-hydroxystilbene, 4-aminostilbene, 4-hydroxyazobenzene, and 4-hydroxy-4′-nitrostilbene, low activity in 4-nitrostilbene, 4,4′-dihydroxydibenzyl, resveratrol, and 4-hydroxy-α-methylstyrene, and marginal activity in 4,4′-dimethoxystilbene and 4-hydroxymethylstilbene were observed in an estrogen reporter assay using the estrogen-responsive human breast cancer cell line MCF-7 and a binding assay with rat uterus estrogen receptor. In contrast, α-methylstilbene, 4,4′-dimethoxystilbene, 4-hydroxymethylstilbene, dibenzyl, tolan and azobenzene also exhibited estrogenic activities after incubation with liver microsomes of 3-methylcholanthrene- or phenobarbital-treated rats in the presence of NADPH. These results suggest that the structural requirements for the estrogenic activities of stilbene derivatives are a *p*-hydroxyl group in the A-phenyl ring, vinyl linkage, and a B-phenyl ring for the maximal activity, and hydrophobicity of the linkage for higher activity as observed in DES. *p*-Nitro and amino groups in the A-phenyl ring are also effective for the estrogenic activity.

Key words —— stilbene derivative, estrogenic activity, structure-activity relationship, stilbene-proestrogen, human breast cancer cell MCF-7

INTRODUCTION

Stilbene derivatives, such as diethylstilbestrol (DES), euvestin, 4,4′-diaminostilbene and pinosylvin, have a variety of biological actions, including hormonal, hypocholesterolemic, sympathomimetic, antifungal, antiallergic, antibacterial, antimalarial and anticancer activities.[1–4] DES is the most famous stilbene derivative, which was used medically for prostate and breast cancer, and to prevent threatened abortions.[5–6] However, it was found to induce vaginal adenocarcinoma.[5,7] Stilbene derivatives are synthesized by stilbene synthase, which utilizes 3-malonyl-CoA and a starter CoA ester such as *p*-coumaroyl-CoA in plants.[8,9] Some hydroxystilbenes and their oligomeric derivatives play an important role as stilbenoid phytoalexin, and have been implicated in the process of induced resistance in some plants.[8] Resveratrol (4,3′,5′-trihydroxystilbene), which is found in grapes, peanuts and pineapples, and also grape products such as wine, exerts potent antioxidant and anti-inflammatory activities.[10] This hydroxylated stilbene inhibited tumor formation in mammary glands and skin in mice exposed to dimethylbenz[*a*]anthracene.[11] Some reports also showed that resveratrol was an antagonist of the aryl hydrocarbon receptor.[12–14]

Some man-made chemicals and naturally-occurring compounds in the environment have the potential to disrupt the endocrine system by mimicking endogenous estrogens, and are called xenoestrogens. These chemicals include chlorinated organics, such as the insecticides kepone, *o*,*p*′-dichlorodiphenyltrichloroethane (*o*,*p*′-DDT), dieldrin and methoxychlor, and some polychlorinated biphenyl congeners, and nonchlorinated compounds from the plastics and detergent industries, such as alkylphenols and bisphenol A.[15,16] Xenoestrogens contain a number of chemical classes that display a broad range of structural diversity.[17] This creates a particular need for knowledge of the structure-activity relationship

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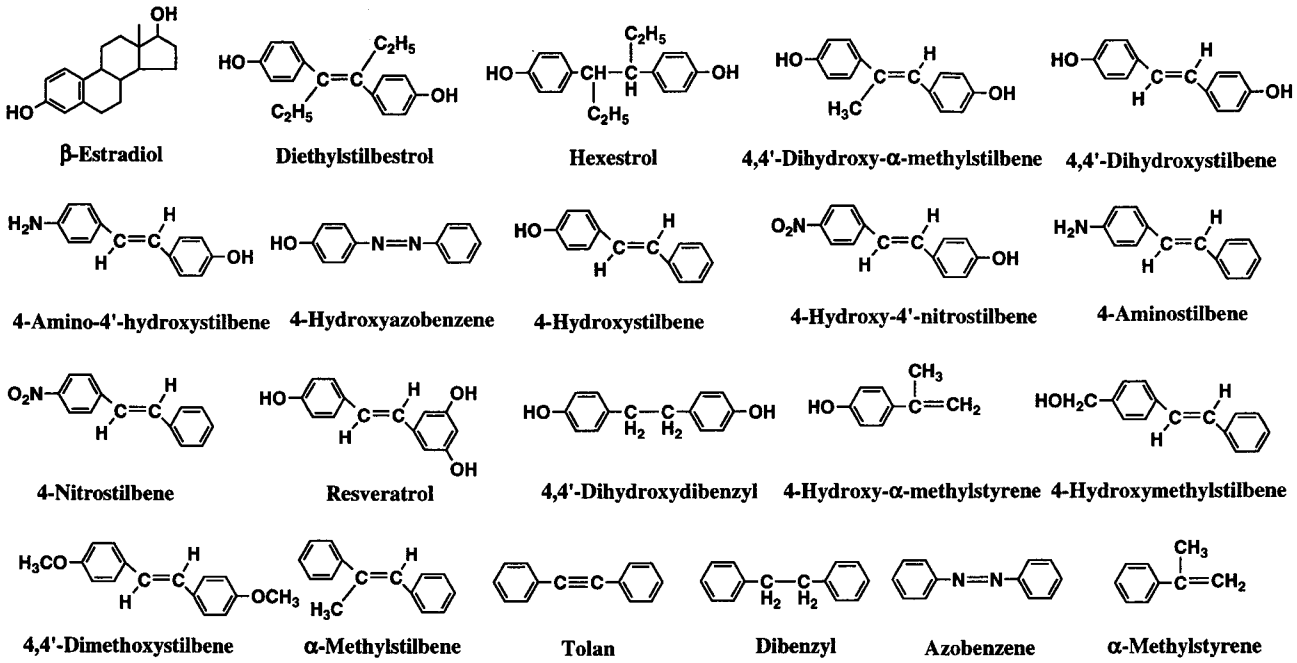


Fig. 1. Structures of Stilbene Derivatives and Related Compounds Tested for Estrogenic Activity

of estrogenicity. Recently, structure-activity relationship studies on estrogens have suggested some structural requirements.[18–24] Estrogens have been shown to have multiple sites of activity and exert biological actions. Many of the environmental estrogens are known to produce a wide variety of toxic effects in animals. They may play a role in the increasing incidence of breast cancer, testicular cancer, and other problems of the reproductive system in humans. Recently, we showed that stilbene, which is the parent compound of DES, and stilbene oxide were not estrogenic, but exhibited potent estrogenic activity after metabolic activation by a liver microsomal oxidation system.[25] In that report, we suggested that the estrogenic activity was due to the hydroxylated metabolites and that the metabolites were formed by cytochrome P450 1A1.[26]

In this report, the estrogenic activities of twenty kinds of stilbene derivatives including proestrogens, *i.e.*, 4-hydroxystilbene, 4,4′-dihydroxystilbene, 4-hydroxy-4′-nitrostilbene, 4-amino-4′-hydroxystilbene, 4-hydroxyazobenzene, 4,4′-dihydroxydibenzyl, resveratrol, DES, 4,4′-dihydroxy-α-methylstilbene, hexestrol, 4-nitrostilbene, 4-aminostilbene, α-methylstilbene, 4,4′-dimethoxystilbene, 4-hydroxymethylstilbene, 4-hydroxy-α-methylstyrene, tolan, dibenzyl, α-methylstyrene and azobenzene (Fig. 1) were examined in the presence or absence of a liver microsomal metabolic system in an estrogen reporter assay using the estrogen-responsive human breast cancer cell line MCF-7 and a binding assay with rat uterus estrogen receptor.

MATERIALS AND METHODS

Chemicals —— Dibenzyl, tolan, azobenzene, 4-hydroxyazobenzene, DES, 4-hydroxy-α-methylstyrene, and hexestrol (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan), 4-nitrostilbene, 4-aminostilbene, 4-hydroxymethylstilbene, 4,4′-dihydroxydibenzyl, α-methylstilbene, α-methylstyrene and 4,4′-dihydroxy-α-methylstilbene (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), 17β-estradiol (Sigma Chemical Co., St. Louis, MO, U.S.A.), 4-hydroxystilbene, 4-amino-4′-hydroxystilbene and 4-hydroxy-4′-nitrostilbene (Lancaster Synthesis Ltd., Lancashire, U.K.), and resveratrol (Calbiochem-novabiochem Co., La Jolla, CA, U.S.A.) were used. 4,4′-Dimethoxystilbene was synthesized from *p*-methoxybenzaldehyde by the method of Ali *et al.*[3] 4,4′-Dihydroxystilbene was synthesized from 4,4′-dimethoxystilbene using a previously reported method.[25]

Animals —— Male Sprague-Dawley rats (210–230 g, 6 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed at 22°C with a 12-hr light/dark cycle, with free access

to tap water and a standard pellet diet MM-3 (Funabashi Farm, Funabashi, Japan). In some experiments, rats were given intraperitoneal administration of phenobarbital (80 mg/kg) or 3-methylcholanthrene (25 mg/kg) daily for three days before use.

Preparation of Liver Microsomes —— The livers were excised from exsanguinated rats and immediately perfused with 1.15% KCl. The livers were homogenized in four volumes of the KCl solution using a Potter-Elvehjem homogenizer. The microsomal fraction was obtained from the homogenate by successive centrifugation at $9000 \times g$ for 20 min and $105000 \times g$ for 60 min. The fraction was washed by resuspension in the KCl solution and resedimentation. The pellets of microsomes were resuspended in the solution to make 1 ml equivalent to 1 g of liver.

Cell Culture —— MCF-7 cell lines were maintained in MEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (Life Technologies, Rockville, MD, U.S.A.).

Assay of Estrogenic Activities of Styrene Derivatives in MCF-7 Cells —— Assay of estrogens was performed according to a previously reported method.[25] Briefly, for the estrogen responsive element (ERE)-luciferase reporter assay using MCF-7 cells, transient transfections in MCF-7 cells were performed using Transfast™ (Promega Co., Madison, WI, U.S.A.), in 12-well plates at 1×10^5 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. Twenty-four hours after addition of the sample, the assay was performed with a Dual Luciferase assay kit™ (Promega Co.). For the assay of metabolites produced from proestrogens, substrates (0.1 μmol) were incubated with 0.1 ml of rat liver microsomes (2 mg protein) in the presence of 1 μmol of NADPH for 30 min in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer. After incubation, the mixture was extracted with 5 ml of ethyl acetate and evaporated to dryness. The residue was dissolved with 1 ml of ethanol and an aliquot was used for the estrogenic activity assay. The total concentration of the substrate and its metabolites was calculated from the original amount of the substrate.

Competitive Binding Assay to Estrogen Receptor —— Rat uterus was homogenized with TEGDMo buffer (1 mM disodium EDTA, 1 mM dithiothreitol, 10 mM sodium molybdate, 10%(v/v) glycerol and 10 mM Tris–HCl, pH 7.4). The cytosolic fraction was obtained from the homogenate by centrifugation at $105000 \times g$ for 50 min. For the assay, 0.1 ml (2 mg protein) of the cytosolic fraction was added to the same volume of TEGDMo buffer containing 5 nM ^{3}H-E2 (17.7 KBq/ml) and various concentrations of test compounds. After incubation at 30°C for 40 min, 0.05 ml of a 1.5% charcoal and 0.15% dextran T70 mixture in TEG (1 mM disodium EDTA, 10%(v/v) glycerol and 10 mM Tris–HCl, pH 7.4) was added. After incubation for 10 min at 4°C with occasional vortexing, the suspension was centrifuged at $1000 \times g$ for 10 min. A 0.1 ml aliquot of the supernatant was transferred to another tube, and 1 ml of scintillator was added. The radioactivity in each tube was counted with a Wallac Micro-Beta Scintillation Counter (Perkin-Elmer Life Sciences).

RESULTS

Estrogenic Activity of Stilbene Derivatives Using ERE-Luciferase Reporter Assay in MCF-7 Cells

The estrogenic activities of some stilbene derivatives and the related compounds were examined using ERE-luciferase reporter assay in MCF-7 cells. 4,4′-Dihydroxystilbene, 4-amino-4′-hydroxystilbene, 4,4′-dihydroxy-α-methylstilbene, and hexestrol exhibited significant estrogenic activities on the cells in the range of 10^{-7}–10^{-5} M. However, these activities were one to two orders lower than that of DES. The activities of 4-hydroxystilbene, 4-aminostilbene, and 4-hydroxy-4′-nitrostilbene were also observed in the range of 10^{-6}–10^{-4} M. Resveratrol and 4-nitrostilbene exhibited minor estrogenic activity. In contrast, these estrogenic compounds were cytotoxic in MCF-7 cells at higher concentrations. Then, the estrogenic activities of these compounds decreased at higher concentrations (Fig. 2). α-Methylstilbene, α-methylstyrene, azobenzene, tolan, and dibenzyl did not demonstrate estrogenic activity in this assay (data not shown).

The estrogenic activities of 4-substituted stilbenes are shown comparing the EC50 values (Table 1). Among the 4-hydroxylated derivatives, DES had the lowest EC50 value, followed by hexestrol, 4,4′-dihydroxy-α-methylstilbene, 4,4′-dihydroxystilbene, and 4-amino-4′-hydroxystilbene. 4-Aminostilbene and 4-nitrostilbene also exhibited activity to some extent. In contrast, 4′-hydroxyl or 4-amino substituents enhanced the activity of 4-hydroxystilbene. However, substitution of the 3′,5′-dihydroxyl groups decreased the estrogenic activity

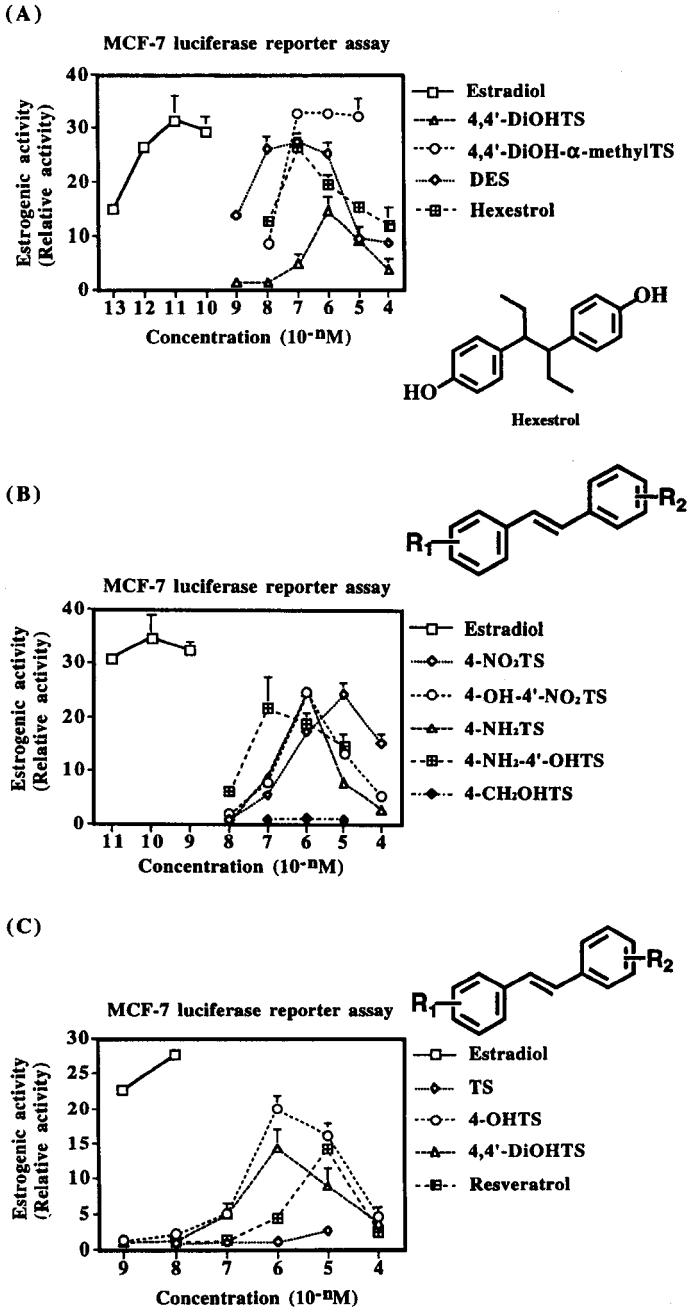


Fig. 2. Estrogenic Activity of Stilbene Derivatives Using ERE-Luciferase Reporter Assay in MCF-7 Cells

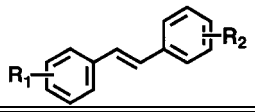
(A) Estrogenic activities of DES, hexestrol, 4,4′-dihydroxystilbene (4,4′-DiOHTS) and 4,4′-dihydroxy-α-methylstilbene (4,4′-DiOH-α-methylTS). (B) Estrogenic activities of 4-nitrostilbene (4-NO_2TS), 4-hydroxy-4′-nitrostilbene (4-OH-4′-NO_2TS), 4-aminostilbene (4-NH_2TS), 4-amino-4′-hydroxystilbene (4-NH_2–4′-OHTS), and 4-hydroxymethylstilbene (4-CH_2OHTS). (C) Estrogenic activities of trans-stilbene (TS), 4-hydroxystilbene (4-OHTS), 4,4′-dihydroxystilbene (4,4′-DiOHTS), and resveratrol. Each bar represents the mean ± S.D. of four experiments. Estrogenic activity is expressed as a relative activity with respect to the control experiments to which no chemical was added.

of 4-hydroxystilbene. Dimethoxystilbene and 4-hydroxymethylstilbene showed marginal estrogenic activity. Styrene derivatives, which lacked the B-phenyl group of stilbenes, showed lower activity compared to stilbenes. These facts suggest that a 4-substituent (hydroxyl, amino or nitro) of the A-ring of stilbenes is essential, while a vinyl linkage and substituted B-phenyl ring modulate the estrogenic activity.

Competitive Binding Assay of Stilbene Derivatives for Estrogen Receptor

The affinities of stilbene derivatives to estrogen receptor of rat uterus were examined. Some deriva-

Table 1. EC_{50} for MCF-7 Luciferase Reporter Assay and IC_{50} for ER Binding Assay



R1	R2	MCF-7 EC_{50} (M)	Binding assay IC_{50} (M)
Estradiol		1.3×10^{-12}	1.6×10^{-10}
H	H	$> 10^{-4}$	$> 10^{-3}$
4-OH	H	1.0×10^{-7}	1.3×10^{-4}
4-OH	4′-OH	7.7×10^{-8}	6.7×10^{-6}
3,5-OH	4′-OH	9.1×10^{-7}	6.3×10^{-4}
4-NO_2	H	8.5×10^{-7}	1.5×10^{-3}
4-NO_2	4′-OH	1.3×10^{-7}	1.9×10^{-2}
4-NH_2	H	2.3×10^{-7}	3.8×10^{-5}
4-NH_2	4′-OH	9.3×10^{-8}	1.5×10^{-5}
4-CH_2OH	H	$> 10^{-4}$	4.1×10^{-4}
DES		6.3×10^{-10}	3.2×10^{-10}
4,4′-Dihydroxy-α-methylstilbene		2.1×10^{-8}	2.0×10^{-6}
Hexestrol		1.1×10^{-8}	1.1×10^{-9}
4,4′-Dihydroxydibenzyl		1.0×10^{-6}	—
4-Hydroxy-α-methylstyrene		2.3×10^{-6}	—
4-Hydroxyazobenzene		1.1×10^{-7}	—

tives competitively inhibited the binding of ^{3}H-E2 (1×10^{-10} M) to estrogen receptor in the range of 1×10^{-9}–1×10^{-3} M (Fig. 3). The highest activity was observed in DES, followed by hexestrol, 4,4′-dihydroxy-α-methylstilbene and 4,4′-dihydroxystilbene. 4,4′-Dihydroxy derivatives showed a higher affinity to estrogen receptor compared to monohydroxy derivatives. High affinity was also observed for 4-aminostilbene and 4-amino-4′-hydroxystilbene (Fig. 3). These experiments support the above results of estrogenic activities in reporter assay using MCF-7 cells. The IC50 values of these activities are also listed in Table 1.

Estrogenic Activity of Stilbene Derivatives with a Microsomal Activation System

4,4′-Dimethoxystilbene, α-methylstilbene, azobenzene, dibenzyl, tolan, and 4-hydroxymethylstilbene showed negative or marginal effects in the estrogen screening test with MCF-7 cells as shown above. Next, the estrogenic activity of these compounds in the presence of a rat liver microsomal oxidation system was examined. When α-methylstilbene, azobenzene, dibenzyl and tolan were incubated with liver microsomes of 3-methylcholanthrene- or phenobarbital-treated rats in the presence of NADPH, the extract of the incubation mixture exhibited an estrogenic effect on the cells in the range of 10^{-6}–10^{-5} M, as in the case of stilbene described in our previous report[25] (Fig. 4). 4,4′-Dimethoxystilbene and 4-hydroxymethylstilbene were also metabolically activated to estrogenic compounds. However, the effects of these compounds were lower (data not shown). These facts suggest that 4,4′-dimethoxystilbene, α-methylstilbene, azobenzene, dibenzyl, tolan and 4-hydroxymethylstilbene were converted to the active metabolites, 4-hydroxyl derivatives or 4,4′-dihydroxyl derivatives, by the rat liver microsomes.

DISCUSSION

In this study, we demonstrated a relationship between the estrogenic activity and structure of stilbene derivatives using an estrogen reporter assay with MCF-7 cells and by binding assay with rat uterus estrogen receptor. A phenolic hydroxyl group at 4-position of the compounds is essential for the estrogenic activity of stilbene derivatives, as has been reported for other estrogens.[18–24] 4-Nitro and 4-amino groups also contribute to the activity. These groups may have affinity with estrogen receptors. α-Methyl-4-hydroxystyrene showed the lowest ac-

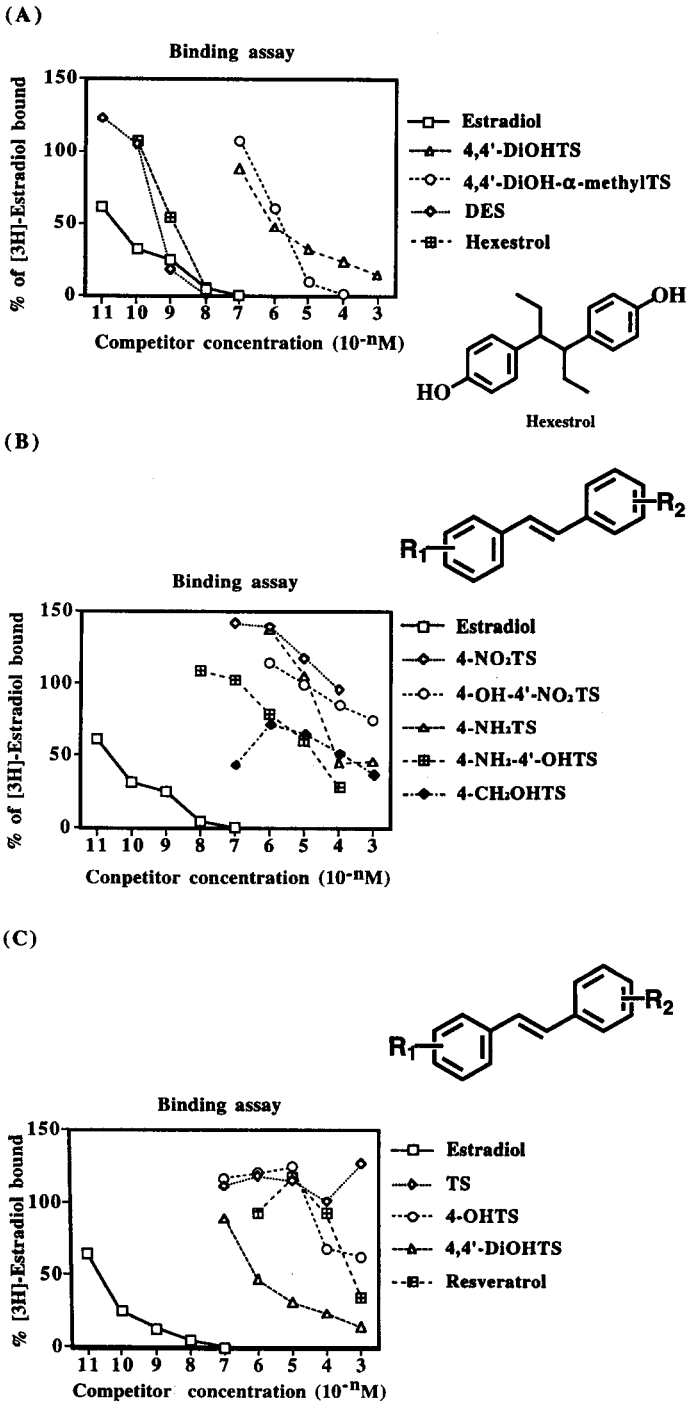


Fig. 3. Estrogen Receptor Binding Assay of Stilbene Derivatives Using Rat Uterus Cytosol

(A) Binding affinities of DES, 4,4′-DiOHTS, and 4,4′-DiOH-α-methylTS. (B) Binding affinities of 4-NO_2TS, 4-OH-4′-NO_2TS, 4-NH_2TS, 4-NH_2–4′-OHTS, and 4-CH_2OHTS. (C) Binding affinities of TS, 4-OHTS, 4,4′-DiOHTS, and resveratrol. Each value represents the mean of three experiments. Data indicate percent values of the radioactivity of ^{3}H-17β-estradiol bound in the control experiments to which no chemical was added.

tivity among the 4-hydroxyl-containing compounds tested in this study. A lipophilic moiety attached to a vinyl group such as a phenyl group is necessary for the maximal activity of stilbene derivatives. Furthermore, a 4′-hydroxyl group may enhance the estrogenic activity of 4-hydroxystilbenes. Unexpectedly, the activity of resveratrol was lower than 4-hydroxystilbene. The cause may be due to the steric hindrance of 3′,5′-dihydroxyl groups in the B-ring for the estrogen receptor. 4-Hydroxyazobenzene and

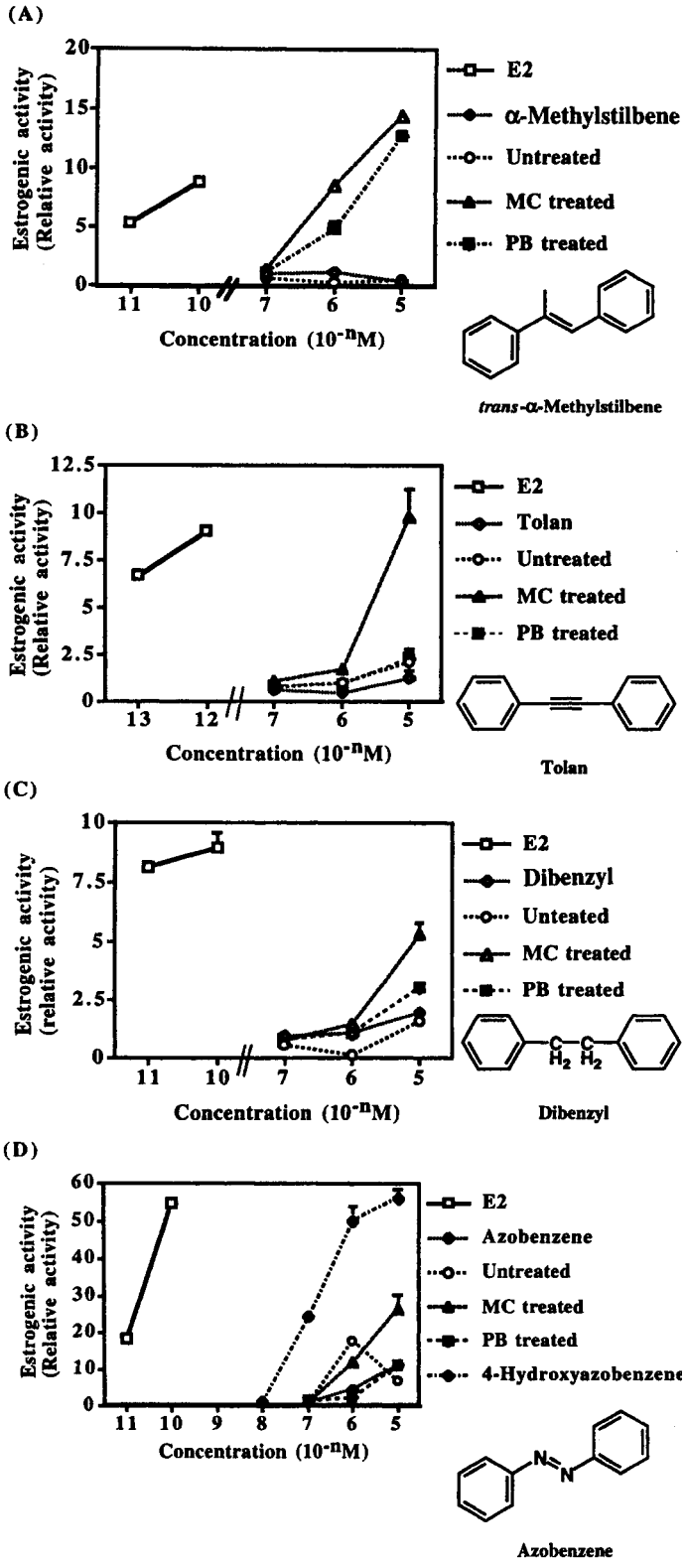


Fig. 4. Estrogenic Activity of Stilbene Derivatives with Rat Liver Microsomal Enzyme System Using ERE-Luciferase Reporter Assay in MCF-7 Cells

(A) Estrogenic activity of α-methylstilbene after metabolic activation. (B) Estrogenic activity of tolan after metabolic activation. (C) Estrogenic activity of dibenzyl after metabolic activation. (D) Estrogenic activity of azobenzene after metabolic activation. Each bar represents the mean ± S.D. of four experiments. Estrogenic activity is expressed as a relative activity with respect to the control experiments to which no chemical was added. A chemical was incubated with liver microsomes in the presence of NADPH, and the extract of the incubation mixture was subjected to the screening test. PB, phenobarbital; MC, 3-methylcholanthrene; E2, 17β-estradiol.

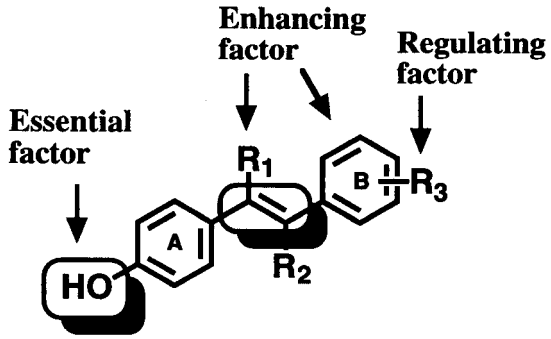


Fig. 5. Structural Requirement of Stilbene Derivatives for Estrogenic Activity

4-hydroxydibenzyl also showed the estrogenic activity. However, the activity of 4-hydroxydibenzyl was lower than those of 4-hydroxystilbene and 4-hydroxyazobenezene. Thus, a vinyl or azo linkage of two phenyl groups is a necessary element for maximal activity of stilbene estrogens. DES and 4,4′-dihydroxy-α-methylstilbene showed much higher activity than that of 4,4′-dihydroxystilbene. Substitution of the lipophilic group attached to the vinyl linkage may markedly enhance the estrogenic activity (Fig. 5). In contrast, we demonstrated that 4-hydroxyazobenzene is moderately estrogenic, as shown in Fig. 4. Azobenzene also exhibited the estrogenic activity after metabolic activation by the rat liver microsomes. Tar dyes, which are often used as food additives, may be estrogenic with or without metabolic activation.

Recently, we also reported the metabolic activation of proestrogenic styrene oligomers and 2-nitrofluorene to estrogens by a liver microsomal enzyme system in addition to stilbene and stilbene oxide.[25,27,28] In these reports, we showed that hydroxylated metabolites, which are formed from parent chemicals by the microsomal cytochrome P450 system, exhibited significant estrogenic activity. In this study, we have demonstrated that some stilbenes and their related compounds are converted to active estrogens by liver microsomal enzymes. It is also necessary to consider the activity of the metabolites produced from the parent compounds for the assessment of the toxicity of stilbene derivatives. In the microsomal system used in this study, tolan and azobenzene were mainly activated by cytochrome P450 1A, while α-methylstilbene and dibenzyl were activated by cytochrome P450 1A and 2B. The *in vivo* estrogenic activity of stilbene and dibenzyl has been reported in the literature[29,30]: the weight of the ovary in ovariectomized rats dosed with these compounds was increased compared to that in rats dosed with vehicle alone. It is thus possible that metabolic activation of the stilbene-related compounds used in this study, which are often used as industrial intermediates, to the active estrogens occurs in mammalian species.

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