rections, the overall plasma clearance of caffeine was virtually unaffected by acute aminophylline administration.

The need for establishing dosage guidelines for safe intravenous administration of theophylline has been stressed (22). Theophylline has a narrow therapeutic index of 10–20 μg/ml, and the risk of toxicity, including seizures and death, increases as serum concentrations exceed this range. In asthmatic patients receiving chronic theophylline therapy, routine monitoring of serum drug levels is recommended. Interactions of theophylline with other exogenous compounds may influence the therapeutic outcome of this drug. For example, methylxanthines such as caffeine and theobromine, present in various common beverages and foods, were shown to inhibit theophylline metabolism in humans (9). In the present study, the theophylline half-life in beagle dogs actually decreased after multiple doses of caffeine, probably due to enzyme induction by the latter. Nevertheless, plasma theophylline levels were elevated and sustained as a result of metabolic conversion of administered caffeine to theophylline.

These observations may suggest the need to reduce theophylline maintenance doses in asthmatics who are chronic coffee drinkers. Caution is also warranted in treatment with theophylline in breast-fed infants, who may be exposed to methylxanthines in the milk (23, 24). Recently it has become evident that theophylline is biotransformed to caffeine by *N*-methylation in the premature newborn infant (25–28), which further complicates theophylline–caffeine interactions. This metabolic pathway was not observed in adult humans (25) and dogs in the present study.

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Nonsteroidal Estrogens and Antiestrogens: Biological Activity of Cyclopropyl Analogs of Stilbene and Stilbenediol

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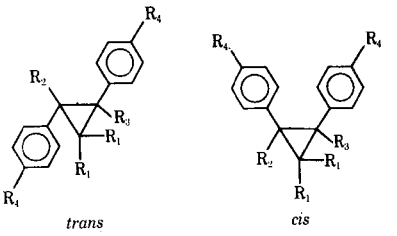
Abstract □ The estrogenic, antiestrogenic, and receptor binding activity of a series of cyclopropyl analogs of stilbene and stilbenediol were determined using the uterotropic assay in the mouse and the receptor binding assay with rat uterine cytosol. One compound, 1,1-dichloro-*cis*-2,3-diphenylcyclopropane (II), displayed antiestrogenic activity *in vivo* with a low affinity for the estrogen receptor *in vitro* and showed tumor remission activity on 7,12-dimethylbenz(*a*)anthracene-induced estrogen-dependent rat mammary tumors. Compounds VIII, IV, and V (in that order) exhibited the greatest estrogenic activity in the mouse and the greatest receptor binding activity *in vitro*. Compound VIII exhibited antifertility activity in the mouse.

Keyphrases □ Estrogenic activity—cyclopropyl analogs of stilbene and stilbenediol, mice □ Antiestrogenic activity—cyclopropyl analogs of stilbene and stilbenediol, mice □ Receptor binding activity—cyclopropyl analogs of stilbene and stilbenediol □ Stilbene and stilbenediol—cyclopropyl analogs, pharmacological activity in mice and rats

Various rigid ring systems have been used to lock functional groups into desired conformations to study receptor interactions (1, 2). The cyclopropane ring is a relatively new structure to be employed synthetically to produce a fixed stereochemical configuration with minimal steric interference in potential medicinal agents (3–5). Magarian and Benjamin (6) reported the preparation of a series of stilbene and stilbenediol derivatives (Table I) containing

Table I—Estrogenic, Antiestrogenic, and Receptor Binding Activity of the Cyclopropyl Analogs



Compound	Configuration	R_1	R_2	R_3	R_4	Relative Uterotropic Activity[a]	Antiestrogenic Activity[b]	Relative Receptor Binding Activity[c]
Estradiol						100	—[d]	100
I	*trans*	Cl	H	H	H	—[d]	—[d]	0.02
II	*cis*	Cl	H	H	H	—[d]	36 μg	0.0086
III	*trans*	H	C_2H_5	C_2H_5	OCH_3	—[d]	—[d]	0.4
IV	*trans*	Cl	C_2H_5	C_2H_5	OH	1.63	—[e]	48.6
V	*trans*	Cl	C_2H_5	C_2H_5	$OCOCH_3$	1.57	—[e]	3.6
VI	*trans*	Cl	H	H	OCH_3	—[d]	—[d]	0.0038
VII	*trans*	Cl	C_2H_5	C_2H_5	OCH_3	—[d]	—[d]	0.049
VIII	*trans*	H	C_2H_5	C_2H_5	OH	2.55	—[e]	9.1
IX	*cis*	H	H	H	H	—[d]	—[d]	0.0045
X	*trans*	H	H	H	H	—[d]	—[d]	0.0064

[a] Activity expressed as a percentage of estradiol activity according to Bliss (10). [b] Dose of analog that would produce a 50% reduction in the uterotropic response to 0.04 μg of estradiol. [c] (Concentration of estradiol that displaced 70% [^{3}H]estradiol/concentration of analog that displaced 70% [^{3}H]estradiol) × 100. [d] No response. [e] Not tested.

the cyclopropyl moiety in place of the double bond to produce conformationally rigid, isomeric structures with substituent groups above and below the plane of the cyclopropane ring. This configuration is in contrast to the coplanar structure of the parent series, such as in the stilbenediol derivative *trans*-diethylstilbestrol, where resonance interactions and minimal steric interference tend to hold the two aromatic rings and connecting ethylene carbons in the same plane. It was anticipated that this new stereochemical conformation in the stilbene and stilbenediol series might produce antiestrogenic activity by preventing estrogens from expressing their full effects on estrogen target tissue and thus could be used to probe estrogen receptor events (6).

These compounds were studied to provide additional insight into the steric requirements of the ligand that binds to the estrogen receptor since the molecular shape of the ligand determines antiestrogenic activity (7). They also served as a basis for obtaining structural information for the design of new antiestrogens. The present study examined the cyclopropyl analogs not only for estrogenic, antiestrogenic, and receptor binding activity but also for antifertility and antineoplastic activities.

EXPERIMENTAL

Uterotropic Assay for Estrogenic and Antiestrogenic Activity—The assay for estrogenic activity was a modification (8) of the method of Rubin *et al.* (9). Immature Swiss–Webster mice, 10–14 g (~21 days old), were distributed randomly into groups of five or six mice each. Estradiol and the test compounds were dissolved separately in sesame oil and administered subcutaneously in a volume of 0.1 ml. Control animals were treated with the same volume of sesame oil alone.

All animals were treated daily for 3 consecutive days. On the 4th day, the animals were sacrificed; the uteri were dissected carefully, blotted lightly, and weighed to the nearest 0.1 mg. Body weights also were recorded. Estradiol was used in the dosage range of 0.01–0.04 μg (total dose) as the assay standard. Each cyclopropyl analog was examined over a dosage range of 1–25 μg (total dose).

The uterotropic assay also was used to evaluate the antiestrogenic activity of the test compounds that did not produce an estrogenic response in the previous assay. The antiestrogenic assay was conducted as described for estrogenic activity, except that each animal in the cyclopropyl analog treatment groups received a standard stimulating dose of estradiol (0.04 μg). The test compounds and estradiol were administered separately at different injection sites to minimize possible physical interaction or reduce absorption of either compound. Antiestrogenic activity was measured as a decrease in the estradiol-stimulated uterotropic response in groups that received both the test compound and estradiol as compared to a group treated with estradiol alone.

A line of best fit was plotted for each compound that produced an estrogenic or antiestrogenic response. Regression analysis was used to calculate each line. The slope of the response to each analog was compared to the slope of the estradiol response to determine parallelism. The relative uterotropic activity of each compound was expressed as a percentage of the estradiol activity according to the method of Bliss (10).

Histological Preparation and Examination—Mouse uterine tissue was fixed in 10% formaldehyde, embedded in paraffin, sectioned on a microtome, and stained with hematoxylin–eosin. Slides were examined with a compound light microscope, and measurements were made on each cross section at several levels along the uterine horn. These measurements included: (*a*) the total uterine horn diameter or thickness at two points, and (*b*) the endometrial thickness at two points as measured from the endometrial–myometrial border to the maximum invagination of the endometrium into the uterine horn lumen. Photomicrographs of uterine horn cross sections and epithelial linings were taken with a camera[1] attached to a compound light microscope.

Antifertility Assay—Adult (8-week-old) Swiss–Webster mice were used in the antifertility assay, which was a modification of the method of Wani *et al.* (11). The female mice were randomized into dosage groups containing eight mice per group. The test compounds were dissolved in sesame oil and administered by subcutaneous injection in a total volume of 0.1 ml. The control group received an equal volume of sesame oil.

The female mice were dosed daily for 23 consecutive days. Males of known fertility were caged with the females (one male per four females) from treatment Days 8 to 20. During this period, the females were checked for vaginal plugs; body weights were recorded weekly. The females were sacrificed on Day 27, and the uterine horns were examined for the number of fetuses and any gross malformation. In addition, the fetal weights were recorded.

Antineoplastic Assay—In this study, 50-day-old female Sprague–Dawley rats received a single oral dose of 10 mg of 7,12-dimethylbenz(*a*)anthracene dissolved in 1 ml of corn oil *via* a stomach tube. The animals were examined and palpated for tumors at weekly intervals until tumors were detected. Animals that displayed tumors of 1–3 cm in their largest diameter were included. The rats were distributed into experimental groups on the basis of the total tumor volume and number so that

[1] Polaroid Land Instrument ED-10.

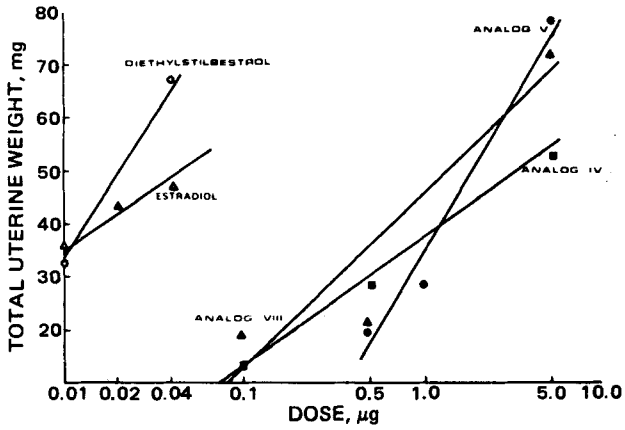


Figure 1—*Uterotropic activity of the active cyclopropyl analogs. Each point represents the mean uterine weight from five or six mice.*

each group contained approximately the same mean tumor volume and mean number at the beginning of treatment. Rats in the ovariectomized group had both ovaries removed 1 day prior to treatment.

Analog II was dissolved in olive oil at a concentration of 1.2 mg/ml. The treatment group received 0.6 mg of II (0.5 ml sc) three times per week, and the control group received an equal volume of olive oil. The dose of II was doubled after the 3rd week of therapy. Tumor size was determined twice weekly in all groups using a vernier caliper to measure the major and minor diameters of each tumor. Tumor volume was calculated based on an ellipsoid tumor shape ($V = 4/3\pi r_1^2 r_2$, where r_1 is the minor tumor radius) (12).

Receptor Binding Assay—A modification of the competitive receptor binding assay method of Korenman was used (13). Uteri were removed from female Sprague–Dawley rats weighing ~250 g. The uteri were cleaned of connective tissue and homogenized in 5 volumes (w/v) of ice-cold tromethamine buffer containing 0.02 *M* tromethamine hydrochloride, 0.0015 *M* disodium ethylenediaminetetraacetate, and 0.25 *M* sucrose; the pH was adjusted to 7.4. The tissue was homogenized using a motor-driven ground-glass tissue homogenizer placed in an ice water bath. The homogenate was centrifuged at 100,000×*g* for 1 hr at 4° using a swinging bucket rotor[2] on an ultracentrifuge[3].

The supernate (cytosol) was used immediately after preparation in the receptor binding assay. Incubations were conducted for 20 hr at 4° in a total volume of 0.5 ml of tromethamine buffer containing 100–150 μl of uterine cytosol, 0.01 μCi of 2,4,6,7-(*n*)-[^{3}H]17β-estradiol[4] (327 mCi/mg), and various concentrations of the test compounds. Each test compound was assayed at three concentrations over a range of 10^{-4}–10^{-6} *M* for the cyclopropyl analogs and of 10^{-7}–10^{-9} *M* for the estradiol standard. The test compounds were dissolved in ethanol; in all cases, the final ethanol concentration was <2% of the incubation medium.

At the end of incubation, the cytosol-bound [^{3}H]estradiol was separated from the unbound [^{3}H]estradiol by the addition of 0.5 ml of dextran-coated charcoal solution (tromethamine buffer containing 0.05% dextran 70 and 0.5% activated charcoal[5]). The assay tubes were vortexed and centrifuged at 500×*g* for 15 min. The [^{3}H]estradiol concentration of a 0.5-ml aliquot of the supernate was determined by liquid scintillation spectrometry using an aqueous compatible scintillation medium[6]. Counting times were adjusted automatically to obtain a counting error of <1% using a liquid scintillation counter[7]. The [^{3}H]estradiol displacement for each test compound was determined by linear regression analysis and plotted graphically. The relative receptor binding activity of each analog was determined using the following ratio: (concentration of unlabeled estradiol producing 70% displacement of [^{3}H]estradiol/concentration of cyclopropyl analog producing 70% displacement of [^{3}H]estradiol) × 100.

RESULTS

Each compound was tested initially in the uterotropic assay. Compounds IV, V, and VIII produced uterotropic response curves that were

[2] Beckman model SW 27.1.
[3] Beckman model L5-65.
[4] Amersham Corp.
[5] Norite A, Sigma Chemical Co.
[6] Aquasol-2, New England Nuclear.
[7] Beckman model LS-100C.

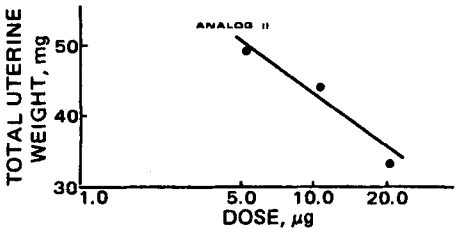


Figure 2—*Antiestrogenic activity of II. Each point represents the mean uterine weight from five or six mice.*

parallel to the estradiol curve (Fig. 1). The relative estrogenic activity of these analogs was VIII > IV > V. These compounds produced 1.5–2.5% of the uterotropic response of estradiol on a molecular weight basis (Table I). Diethylstilbestrol, a synthetic nonsteroidal estrogen containing the stilbenediol moiety, was equipotent to estradiol in the uterotropic assay (Fig. 1). Cyclopropyl analogs that displayed no estrogenic activity in the uterotropic assay were tested further for antiestrogenic activity. Only II produced an antiestrogenic response (Fig. 2 and Table I).

Since the uterotropic response is a nonspecific measure of estrogenic activity, the uteri were examined histologically to confirm the estrogenic nature of the uterotropic response. Estradiol and the cyclopropyl analogs (IV and VIII) significantly ($p < 0.001$) increased uterine diameter and endometrial thickness, which represented a specific estrogenic response (Table II).

The cyclopropyl analogs were tested for receptor binding activity and compared to the estradiol standard. All of the analogs were capable of displacing [^{3}H]estradiol from the estrogen receptor. However, IV, VIII, and V (in that order) displayed the greatest binding activity, from 4 to 50% of the receptor binding activity produced by estradiol on a molecular weight basis (Fig. 3 and Table I).

The estrogen (VIII) and the antiestrogen (II) were tested for antifertility activity using estradiol as a standard antifertility agent. Both estradiol and VIII exhibited good antifertility activity (Table III). The antiestrogen (II) produced no antifertility effect; however, one female in this treatment group had pups that were markedly underdeveloped, with a total fetal weight of 0.9 g/10 pups as compared to the group mean of 7.5 g/10.3 pups.

The antitumor activity of the antiestrogen (II) was examined using the 7,12-dimethylbenz(*a*)anthracene-induced mammary tumor assay in the rat. The tumor data were expressed as both the tumor volume per animal (Fig. 4) and the tumor number per animal (Fig. 5). Ovariectomy produced a slightly greater reduction in tumor volume during the first 3 weeks of treatment, while tumor volume in the treated group remained essentially unchanged. Castration has been reported to cause regression of 7,12-dimethylbenz(*a*)anthracene-induced tumors (14) and was employed in the present study to verify the estrogen-dependent nature of the tumors in the rats. The number of tumors per animal increased in all three groups

Table II—Histological Examination of Uterine Tissue

Compound	Dose, μg	Uterine Diameter (Mean ± *SD*), mm	Endometrial Thickness (Mean ± *SD*), mm
Control	0.0	0.56 ± 0.15	0.16 ± 0.04
Estradiol	0.04	1.42 ± 0.24[a]	0.49 ± 0.15[a]
IV	5.0	1.53 ± 0.19[a]	0.48 ± 0.12[a]
VIII	5.0	1.30 ± 0.15[a]	0.40 ± 0.07[a]

[a] Significantly different from control ($p < 0.001$).

Table III—Antifertility Activity of Active Estrogenic and Antiestrogenic Cyclopropyl Analogs

Compound	Daily Dose, μg	Animals per Pregnant Group	Implants per Mouse (Mean ± *SD*)	Fetal Weight per Group (Mean ± *SD*), g
Control	0.0	6/8	11.2 ± 3.25	7.5 ± 3.33
Estradiol	0.033	0/8	0	—[a]
II	16.7	6/8	10.3 ± 1.86	7.5 ± 5.07
VIII	4.2	0/8	0	—[a]

[a] No response.

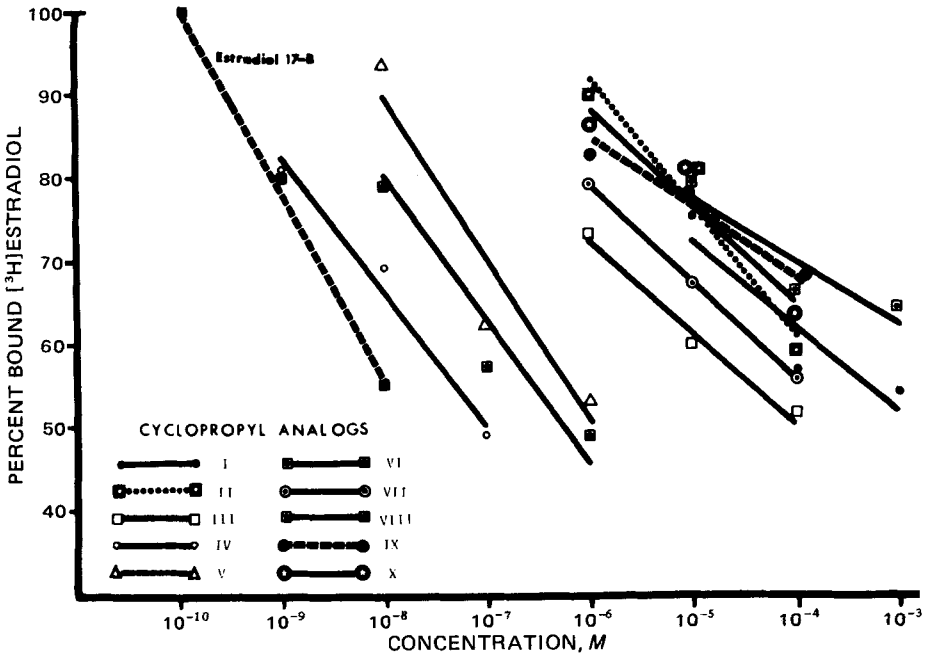


Figure 3—*Uterine receptor binding activity of the cyclopropyl analogs. Each point represents the mean of two or three separate determinations.*

during the treatment period; however, both ovariectomy and II reduced tumor incidence to approximately the same extent as compared to the control group. The total weight gain during treatment in the control and treated groups was not significantly different, suggesting that the antitumor activity of II was not the result of a general toxic effect of the compound. At the end of treatment, all animals were sacrificed, and the tumors were verified histologically to be adenocarcinomas.

DISCUSSION

The objective of this study was to compare the pharmacological effects of the various stilbene and stilbenediol analogs in which the cyclopropyl moiety locked functional groups into a fixed stereochemical configuration to examine structure–activity relationships. Analogs included 1,1-dichloro-*cis*- and *trans*-2,3-diarylcyclopropanes and *trans*-2,3-diarylcyclopropanes along with *p*-hydroxyphenyl, *p*-acetoxyphenyl, and *p*-methoxyphenyl substituents and combinations with 2,3-diethyl substituents. Other compounds include reduced analogs (without the *gem*-dichloro groups) of *cis*- and *trans*-1,2-dicyclopropanes and *trans*-1,2-diarylcyclopropanes with *p*-methoxyphenyl and *p*-hydroxyphenyl groups in combination with 2,3-diethyl substituents.

The cyclopropyl moiety in place of the double bond in stilbene and stilbenediol produces a conformationally rigid, planar structure with group attachments above and below the cyclopropyl ring, as compared to the coplanar configuration of stilbene and stilbenediol. The present study indicates that the cyclopropyl analogs retain the ability to bind to the estrogen receptor and produce an estrogenic response; however, estrogenic activity is significantly reduced. Further estrogenic and receptor binding activity is modified by various functional group attachments and conformation of the cyclopropyl derivative. Accordingly, the phenyl rings in the cyclopropyl analogs are not in the same plane, and the hydrophobic attachment to the receptor is not expected to be as great as in the olefin analogs, thereby reducing binding affinity between the ligand and the receptor.

An examination of Table I reveals that the test compounds that produce estrogenic activity *in vivo* contain either *trans*-hydroxyphenyl groups (IV and VIII) or *trans*-acetoxyphenyl groups in combination with *trans*-ethyl groups (V). The cyclopropyl derivative of diethylstilbestrol (VIII) was the most potent estrogenic analog *in vivo;* IV, the *gem*-dichloro derivative, and V, the *trans*-acetoxyphenyl-*gem*-dichloro derivative, were approximately equipotent and less active than VIII. The *gem*-dichloro moiety thus appears to decrease estrogenic activity *in vivo*. The acetate group was reported to be hydrolyzed to the hydroxyl group in the body (11). Consequently, V may be metabolized to IV *in vivo*. However, both the hydroxyl and acetate groups are capable of hydrogen bonding, which is known to be important for significant receptor interaction and initiation of the estrogenic response (15).

Only the *gem*-dichloro cyclopropyl analog of *cis*-stilbene (II) displayed

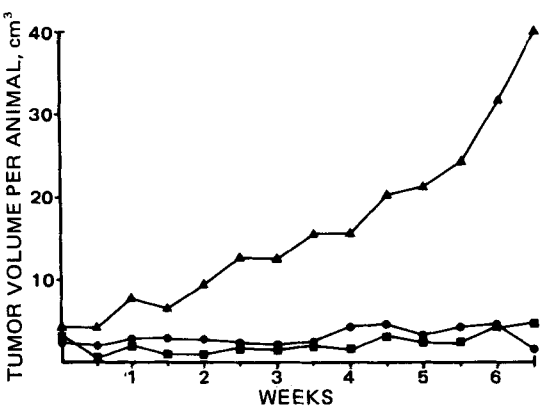


Figure 4—*Influence of II and ovariectomy on 7,12-dimethylbenz(a)anthracene-induced mammary tumor volume. The control group contained four rats, the II-treated group contained five rats, and the ovariectomized group contained three rats. Each point represents the mean tumor volume per animal. Key:* ▲, *control;* ●, *treated; and* ■, *ovariectomized.*

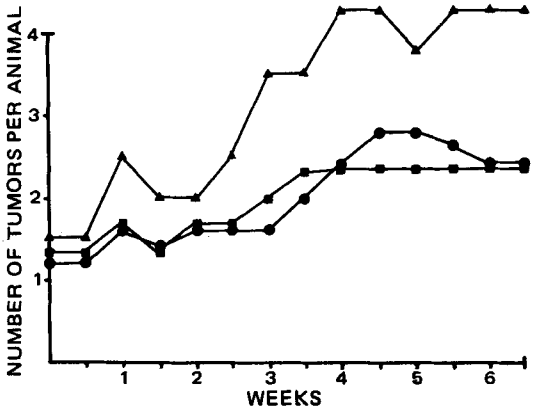


Figure 5—*Influence of II and ovariectomy on 7,12-dimethylbenz(a)anthracene-induced mammary tumor number per animal. The control group contained four rats, the II-treated group contained five rats, and the ovariectomized group contained three rats. Each point represents the mean tumor number per animal. Key:* ▲, *control;* ●, *treated; and* ■, *ovariectomized.*

significant antiestrogenic activity *in vivo*. Thus, the *cis*-configuration and *gem*-dichloro substitution appear to be important for antiestrogenic activity in the cyclopropyl series since neither the *trans*-isomer (I) nor the reduced compound (IX) was antiestrogenic in this study.

An examination of receptor binding activity in Fig. 3 and Table I indicates that various structural modifications significantly alter estrogen binding activity. The structure–activity relationships revealed by this series of cyclopropyl analogs are:

1. Replacement of hydroxyl groups with *O*-methyl groups decreases receptor binding activity (compare III with VIII and IV with VII). The importance of free hydroxyl groups for the production of estrogenic activity was demonstrated with other stilbenediol derivatives (16, 17).
2. The absence of *trans*-ethyl groups decreases receptor binding activity (compare VI with VII).
3. The *trans*-configuration produces a slightly greater binding affinity than the *cis*-configuration (compare I with II and IX with X).
4. The *gem*-dichloro substitution generally increases receptor binding activity (compare I with X, IV with VIII, and II with IX). However, the *gem*-dichloro substitution together with *p*-methoxy substitution decreases binding affinity (compare III with VII), perhaps due to a steric interaction of these bulky groups that interfere with access to the receptor site.

Receptor binding activity paralleled *in vivo* estrogenic activity in this series of compounds, except VIII, which displayed the most potent estrogenic activity *in vivo* and the second greatest receptor affinity *in vitro*. This result may be explained by the fact that receptor binding and the initiation of an estrogenic response are two separate events and have been dissociated in the *in vitro* receptor binding assay due to the isolation of the receptor complex. Korenman (13) demonstrated previously that the relative binding affinity of steroidal estrogens paralleled uterotropic activity.

It is difficult to make structure–activity conclusions based on this limited series of analogs. Other members of the cyclopropyl series are being synthesized and evaluated for biological activity. However, the information obtained from this study will guide the design of other members of this series. It appears from the results that the *gem*-dichloro analogs with *cis*-phenyl rings hold the greatest promise for antiestrogenic activity.

This study also indicates that the cyclopropyl analogs of stilbene and stilbenediol may become useful in the treatment of estrogen-dependent tumors and as potential antifertility agents. Antifertility agents with fewer side effects and more effective antineoplastic therapy for breast and uterine cancer clearly are needed. However, synthesis of the complete series of cyclopropyl analogs and thorough evaluation of these compounds will be required. Thus, the design, development, and biological evaluation of these potential therapeutic agents are in progress.

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First-Pass Metabolism of Ethinyl Estradiol in Dogs and Rats

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Abstract □ The contraceptive steroid ethinyl estradiol was extensively metabolized when given orally in solution to dogs. It was thought at first that metabolism occurred exclusively in the liver. However, use of standard equations to predict the oral bioavailability of drugs known to be metabolized by hepatic first pass resulted in significantly higher values than those obtained experimentally. To rationalize the data and to determine whether ethinyl estradiol also is metabolized in the gut wall during absorption, metabolism in rats was studied. The drug was administered in solution intraduodenally, intraportally, and intravenously as a bolus and by first-order infusion. The results indicate that, in rats, 40% of the drug is metabolized by the gut wall and 79% of the drug in the portal blood is metabolized by the liver after intraduodenal administration.

Keyphrases □ Ethinyl estradiol—first-pass metabolism in dogs and rats, gut wall metabolism in rats □ Bioavailability—ethinyl estradiol, first-pass metabolism in rats and dogs, gut wall metabolism in rats □ Metabolism—ethinyl estradiol in rats and dogs, first-pass metabolism and possible gut wall metabolism

The natural contraceptive steroids such as progesterone and estradiol are not effective orally due to their extensive metabolism in the GI tract during absorption and to hepatic first-pass metabolism (1, 2). Although introduction of the ethinyl group at the 17α-position renders the compound stable toward metabolic attack by 17α-hydroxylase, ethinyl estradiol, like estradiol, contains a phenolic hydroxyl group at the 3-position that is subject to sulfate and

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