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Potential Steroidal Antiestrogens

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A series of analogues of 17β-estradiol has been synthesized and the compounds have been tested, using sucrose density gradient analysis, for their ability to compete with [6,7-^{3}H]-17β-estradiol for the estrogen-receptor protein from mouse uterine homogenates. Active compounds were also tested for antiuterotrophic activity in immature rats and/or mice. 3,17β-Dihydroxy-6-phenylestra-1,3,5(10),6-tetraene (**14**) was the most active new compound in the in vitro test suppressing the binding of 17β-estradiol by 34 and 87%, respectively, at molar ratios of 1 and 3.16. It was significantly more potent than the intermediate 6-oxoestradiol (**4**) which produced a 52% inhibition of binding at a molar ratio of 3.16. The thiosemicarbazone of 6-oxoestradiol (**17**) and the derived 3,17β-dihydroxy-6-(2-imino-4-oxothiazolidinyl-1-imino)estra-1,3,5(10)-triene (**19**) produced, respectively, only 46 and 16% inhibition of binding at a molar ratio of 10. Introduction of a 1-methyl substituent into either 6-oxo or 6-phenyl compounds reduced affinity for the receptor significantly (compounds **5** and **15**) and conversion of the 3-OH into a β-dialkylaminoethoxy group virtually destroyed all binding activity (compounds **2**, **6**, **10**, and **11**). At a molar ratio of 10 compound 14 failed to suppress the uterine weight response of immature rats to 17β-estradiol, whereas compound **15**, at a molar ratio of 200, produced a significant increase in the uterine weight of immature rats but not of immature mice even at a molar ratio of 1000.

Hoover et al.[1] concluded from a survey of 45 853 cases of breast cancer that the risk of endometrial cancer is increased by treatment with hormones. During the period covered by this survey (1935–1971) the hormones used are believed to have been mainly nonsteroidal, e.g., diethylstilbestrol, which has also been linked with carcinoma of the genital tract in daughters of mothers treated with the drug during pregnancy[2,3] and in young women receiving the drug for ovarian agenesis.[4] Currently available antiestrogens are related to the nonsteroidal triphenylethylene group of estrogens and possess weak estrogenic activity,[5] a property which would appear to be highly undesirable if the drug is to be administered over a long period in the treatment of hormone-dependent carcinoma of the breast.

Among other classes of drugs, e.g., cholinomimetics, histamine, and sympathomimetics, the introduction of large nonpolar substituents into an agonist often leads to a loss of agonistic and the development of antagonistic properties.[6] In a search for new antiestrogens which lack estrogenic activity we have synthesized a number of novel ring A aromatic steroids in which the nucleus is substituted with phenyl, benzyl, and 2-imino-4-oxothiazolidinyl-1-imino groups. The effects of replacing the 3-hydroxyl by β-dialkylaminoethoxy and the introduction of the 1-methyl substituent in these modified steroids have also been studied.

The affinities of the new compounds for the estrogen receptor protein have been determined in vitro by measuring their ability to compete with tritium labeled 17β-estradiol, and compounds with significant binding activity have been tested in vivo for antiuterotrophic activity in rats and/or mice.

Chemistry. The required compounds were synthesized via the routes shown in Scheme I. Chromic acid oxidation of 17β-estradiol diacetate (**3**) followed by hydrolysis yielded the key intermediate 6-oxoestradiol[7] (**4**). The position of the carbonyl group was confirmed by the aryl ketone band (ν 1680 cm^{-1}) in the IR spectrum, the downfield shift of the C_4-H signal in the NMR spectrum to δ 7.41 due to the deshielding of the adjacent carbonyl group, and the similarity of the UV spectrum to that of the 1-methyl homologue **5**, which was synthesized from 3,17β-dihydroxy-1-methylestra-1,3,5(10),6-tetraene[8] (**1**) by the method of Pelc.[9]

β-Diethylaminoethyl chloride or its dimethyl analogue with compound **1** or ketones **4** or **5** in the presence of sodium methoxide yielded the dialkylaminoethyl ethers **2** and **6–9** as oils which were purified as their hydrochlorides. The salts were very hygroscopic and retained water of crystallization even after drying at raised temperature and reduced pressure (Table I).

Reactions of these ketonic ethers **6–9** with phenylmagnesium bromide or benzylmagnesium chloride gave the required aminoalkyl ethers **10–13** and the related free phenols **14** or **15** were obtained by reacting phenylmagnesium bromide with phenolic ketones **4** and **5**, respectively. The double bond in the 6-benzyl-substituted compound **13** was assigned endocyclic from a comparison of its UV spectrum with that of the 6-phenyl compounds and by the presence of signals due to the methylene protons of benzyl and a single C_7-proton in the NMR spectrum. 6-Oxoestradiol diacetate (**4** diacetate) was converted into its thiosemicarbazone **16** which condensed with ethyl bromoacetate to form 3,17β-acetoxy-6-(2-imino-4-oxothiazolidinyl-1-imino)estra-1,3,5(10)-triene (**18**). The free phenolic thiosemicarbazone **17** with ethyl bromoacetate gave the corresponding dihydroxy derivative **19**. The NMR spectrum of the thiazolidinyl compound **18** was consistent with the assigned structure, the C_4-H signal being shifted downfield to δ 7.71 due to the deshielding of the exocyclic double bond at C_6. The UV spectra of diol **19** and diacetate **18** were very similar and consistent with the assignment of the analogous 6-thiazolidinylimino structure to the diol **19**.

Biological Results. The ability of the compounds to compete with [6,7-^{3}H]-17β-estradiol for the cytoplasmic estrogen-receptor protein isolated from mouse uterine

Table I. Recrystallization Solvents, Yields, and Analytical Data for Compounds Described in the Text

Compd	Mp, °C	Recrystn solvent	% yield	Formula	Analyses
2·HCl	246-248	EtOH-Et_2O	24	$C_{23}H_{34}NO_2Cl·H_2O$	C, H, N
6·HCl	228-230	EtOH-Et_2O	63	$C_{22}H_{32}NO_3Cl·H_2O$	C, H, N[b]
7·HCl	206-208	EtOH-Et_2O	72	$C_{24}H_{36}NO_3Cl$	C, H, N
8·HCl	184 dec	EtOH-Et_2O	74	$C_{23}H_{34}NO_3Cl·0.5H_2O$	C, H, N
9·HCl	131	THF-Et_2O	65	$C_{25}H_{38}NO_3Cl$	C, H, N
10·HCl	128-130	THF-Et_2O	39	$C_{30}H_{40}NO_2Cl·H_2O$	C, H, N[b]
11·HCl	137-138	THF-Et_2O	40	$C_{29}H_{38}NO_2Cl·1.5H_2O$	C, H, N[b]
12·HCl	95-96	THF-Et_2O	39	$C_{31}H_{42}NO_2Cl·1.5H_2O$	C, H, N[b]
13·HCl	56	THF-Et_2O	46	$C_{31}H_{42}NO_2Cl·1.5H_2O$	C, H, N[b]
14	210-212	Et_2O-petr ether[c]	16	$C_{24}H_{26}O_2$	C, H[b]
15	276-278	Et_2O-petr ether[c]	18	$C_{25}H_{28}O_2$	C, H[b]
16	a	EtOH	62	$C_{23}H_{29}N_3O_4S$	C, H, N, S
17	255-256	EtOH-C_6H_{14}	59	$C_{19}H_{25}N_3O_2S$	
18	274-275	EtOH-$CHCl_3$-C_6H_{14}	80	$C_{25}H_{29}N_3O_5S$	C, H, N[b]
19	266-267	EtOH-C_6H_{14}	49	$C_{21}H_{25}N_3O_3S$	C, H, N

[a] Sintered at 196 °C, melted at 210 °C, and resolidified and melted 245-246 °C. [b] Discrepancy between calculated and measured accurate mass for isolated free base > 1.6 millimass units. [c] Bp 40-60 °C.

Table II. Effect of Test Compounds at Varying Molar Ratios on the Binding of [6,7-^{3}H]-17β-Estradiol to Estrogen Receptor Protein in Ovariectomized Mouse Uterine Homogenates

Compd	Molar ratio to [6,7-^{3}H]-17β-estradiol	% radioactivity in 8S[a] peak rel to control
2	1000	103
4	3.16	38
5	1000	14
	100	38
6	1000	66
10	1000	101
11	1000	88
13	1000	89
14	10	3
	3.16	13
	1	66
15	1000	18
	100	87
17	10	54
19	10	84

[a] Percent = E8S/Etot × Ctot/C8S × 100 where E8S and Etot are respectively the counts per minute in the 8S peak and in all the fractions from the experimental sucrose gradient and C8S and Ctot are those from the control gradient.

Table III. Mean Uterine Weights (± SEM) of Uteri from Immature Mice (Tuck No. 1 Strain) or Rats (Wistar) Injected Subcutaneously on Three Successive Days with 17β-Estradiol Alone or with Test Compounds in Arachis Oil

Estradiol total dose, μg	Compd	Molar ratio[b]	Mean uterine wt (± SEM), mg
	Immature Mouse Experiments		
			13.7 ± 0.9
0.03			25.5 ± 1.9
0.03	14	10	28.6 ± 1.9
0.03	15	1000	32.9 ± 2.1
0.03	17	100	34.3 ± 3.0[a]
0.03	19	10	28.4 ± 2.0
	Immature Rat Experiments		
			40.1 ± 2.2
0.15			64.4 ± 3.5
0.15	14	10	74.1 ± 7.5
0.15	15	200	97.1 ± 12.3[a]
0.15	17	3.16	77.7 ± 9.5

[a] Significantly different from estradiol treated group, $p = 0.05$. [b] Maximum molar ratio of test compound relative to 17β-estradiol.

homogenates is shown in Table II. Of those compounds possessing an aminoethoxy substituent at C_3 only compound **6** exhibited appreciable suppression of estradiol binding at a molar ratio of 1000.

In the uterine weight assay 6-phenyl-$\Delta^{6,7}$-estradiol (**14**), which was the most active compound in the in vitro tests, did not suppress the responses of rats or mice to 17β-estradiol at a molar ratio of 10. The related 1-methyl-substituted compound **15** which was approximately 300 times less active than compound **14** in the in vitro competitive binding test increased significantly the uterine weight response of rats to 17β-estradiol at a molar ratio of 200 but produced no significant change in mice at a molar ratio of 1000 (Table III).

6-Oxoestradiol (**4**) was more active in the in vitro competitive binding assay than its thiosemicarbazone **17** and conversion into the thiazolidinone **19** resulted in a further decrease in binding activity.

Discussion

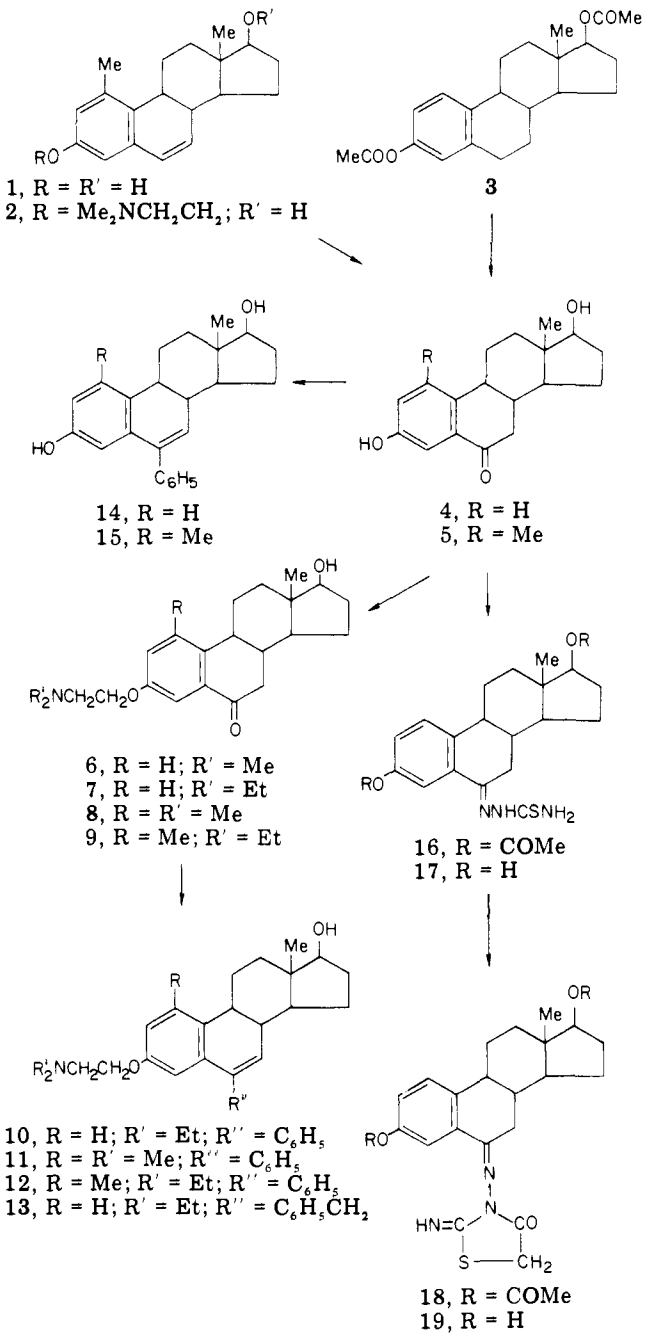
The 34% reduction in the radioactivity of the 8S peak in the sucrose gradient analysis produced by compound **14** at a molar ratio of 1 demonstrates clearly that the introduction of the phenyl group and its accompanying double bond into the 17β-estradiol molecule at C_6 does not hinder significantly the binding of the molecule to the estrogen-receptor protein of mouse uterus. However, the 6-phenyl substituent is unable to compensate for the disadvantageous effects of either a 1-methyl substituent (cf. compounds **4** and **5**, **14** and **15**, Table II) or aminoetherification of the 3-OH (compounds **10**, **11**, and **13**, Table II). Conversion of the 6-oxo group into its thiosemicarbazone derivative **17** leads to a lowering in binding activity which is further reduced by converting into the thiazolidine derivative **19**.

The failure of 6-phenyl-Δ^6-3,17β-estradiol (**14**), at a molar ratio of 10, to affect the uterine weight response to estradiol in immature mice and rats (Table III) was, in view of its high affinity for the receptor protein and the uterotrophic activity of the more weakly binding 1-Me derivative **15**, surprising. The lack of in vivo activity may be a consequence of metabolism to inactive products or to poor distribution and/or uptake by the target tissue.

Experimental Section

Melting points are uncorrected. Spectral data for representative compounds only are included. All other compounds yielded spectra consistent with the assigned structures. IR spectra were recorded on a Perkin-Elmer 257 grating spectrophotometer as Nujol mulls unless specified otherwise, and UV spectra were recorded on a Unicam SP800 spectrophotometer using EtOH as solvent. NMR spectra were recorded, except for compounds **14** and **18**, at the Physico-chemical Measurements Unit, Harwell, on a Varian HA-150D spectrometer using $CDCl_3$ as solvent and

Scheme I



compounds 14 and 18 in the Department of Organic Chemistry, University of Leeds, on a Brucker 90-mHz spectrospin.

Chemical shifts are reported in parts per million (δ) downfield relative to Me_4Si as internal standard, and coupling constants are approximate (±0.5 Hz) and their sign has not been determined. Accurate mass determinations were made at the Physico-chemical Measurements Unit, Harwell, on an AEI MS 902 spectrometer. TLC on silica gel (Merck) was used for identification purposes and homogeneity tests.

Aminoethylation of 1-Methylestra-1,3,5(10),6-tetraene and 6-Oxoestradiols (4 and 5). General Procedure. To the required estradiol (2 mmol) and NaOMe (2 mmol) in dry ethanol (25 mL) was added anhydrous C_6H_6 to produce a slight permanent turbidity followed, dropwise, by the required β-dialkylaminoethyl chloride (4 mmol) in dry Et_2O (40 mL). The Et_2O was distilled and the residue heated under reflux until colorless (2–4 h). The NaCl precipitate was filtered off and washed (EtOH) and the combined filtrate and washings were distilled. Addition of a cold saturated solution of HCl gas in anhydrous Et_2O to the oily residue in anhydrous Et_2O gave the required amine hydrochloride (see Table I for recrystallization solvents and melting points). Spectral data for compound **6**: λ_{max} (EtOH) 220 nm (ϵ 25574), 252 (9422), 319 (3269); $\bar{\nu}$ (liquid film) 2940, 2870, 2830, 2780 (Me_2NR), 1680 cm^{-1} (C=O); NMR δ ($CDCl_3$) 0.8 (s, 3 H, C_{18}-Me), 2.54 (s, 6 H, NMe_2), 2.98 (t, 2 H, J = 6 Hz, NCH_2), 3.73 (m, 1 H, C_{17a}-H), 4.25 (t, 2 H, J = 6 Hz, OCH_2), 7.11 (2 d, 1 H, J = 9 Hz, J = 3 Hz, C_2-H), 7.34 (d, 1 H, J = 9 Hz, C_1-H), 7.53 (d, 1 H, J = 3 Hz, C_4-H); m/e measured 357.2315, calculated 357.2304.

Reaction of 3-(β-Dialkylaminoethyl) Ethers of 6-Oxoestradiols (6 and 7) and 1-Methyl-6-oxoestradiols (8 and 9) with Grignard Reagents. General Procedure. To a cooled solution of amino ketone **6**, **7**, **8**, or **9** (1 mmol, released from its HCl salt by NH_4OH) in anhydrous THF (40 mL) was added with stirring the required Grignard reagent (5.5 mmol) in anhydrous ether. The mixture was stirred at room temperature for 4 h, the Et_2O distilled, and the residue heated under reflux on a water bath for 6 h. The cooled mixture was decomposed with HCl (10%, 40 mL) and extracted with ethyl acetate (2 × 40 mL), the extract washed with water until free from THF and dried (Na_2SO_4), and the solvent distilled. Addition of a cold saturated solution of dry HCl in Et_2O to the residue in anhydrous Et_2O or C_6H_6 gave the required products **10**, **11**, **12**, and **13** as their hydrochlorides. Spectral data for compound **11**: λ_{max} (EtOH) 225 nm (ϵ 27837), 272 (6397), 281 (5533), 303 (1901), 315 (1556); $\bar{\nu}$ (hydrochloride in Nujol) two broad bands 2450–2680 ($R_3{}^+NH$), 767, 700 cm^{-1} (C_6H_5); NMR δ ($CHCl_3$) 0.81 (s, 3 H, C_{18}-Me), 2.23 (s, 6 H, NMe_2), 2.57 (s) overlapping 2.59 (t, 5 H, J = 6 Hz, C_1-Me and NCH_2), 3.70 (m, 1 H, C_{17a}-H), 3.87 (t, 2 H, J = 6 Hz, OCH_2), 5.93 (d, 1 H, J = 2 Hz, C_7-H), 6.42 (d, 1 H, J = 3 Hz, C_2-H), 6.53 (d, 1 H, J = 3 Hz, C_4-H), 7.28 (s, 5 H, phenyl H); m/e measured 431.2825, calculated 431.2824.

3,17β-Dihydroxy-6-phenylestra-1,3,5(10),6-tetraene (14). 6-Oxoestradiol[5] (**4**) (500 mg, 1.74 mmol) in anhydrous THF (45 mL) was treated with phenylmagnesium bromide (5.5 mmol) in anhydrous ether (40 mL) as described above. The cooled (ice) mixture was covered with Et_2O (100 mL) and decomposed with 10% aqueous HCl (60 mL), the organic layer separated, and the aqueous layer extracted with Et_2O (2 × 50 mL). The Et_2O solution was washed free of THF and dried (Na_2SO_4), the Et_2O distilled, and the viscous residue extracted with a boiling mixture of Et_2O–petroleum ether (bp 40–60 °C) (1:4, 150 mL). On cooling crystals of the starting ketone **4** (130 mg) were deposited. The mother liquors were partially evaporated, and petroleum ether (bp 40–60 °C) was added to produce a permanent turbidity and left to cool yielding white crystals (85 mg) which on recrystallization from Et_2O–petroleum ether gave the required 6-phenyl-Δ^6-estradiol (**14**): mp 210–212 °C; λ_{max} (EtOH) 224 nm (ϵ 21840), 268 (6310), ~276 sh (6065), 305–308 (1915); $\bar{\nu}$ (KCl) 3390, 3290 (OH), 1603, 1572 (aryl C=C), 700 cm^{-1}(C_6H_5); NMR δ [$CDCl_3$ + $(CD_3)_2SO$] 0.79 (s, ~3 H, C_{18}-Me), 3.72 (m, 1 H, C_{17a}-H), 5.95 (d, 1 H, J = 1.5 Hz, C_7-H), 6.54 (d, 1 H, J = 2.5 Hz, C_4-H), 6.71 (2 d, 1 H, J = 2.5 and 8.5 Hz, C_2-H), 7.15 (d, 1 H, J = 8.5 Hz, C_1-H), 7.33 (s, 5 H, phenyl H); m/e measured 346.1926, calculated 346.1933.

1-Methyl-6-phenyl-Δ^6-estradiol (**15**) was obtained by an analogous process.

3,17β-Diacetoxyestra-1,3,5(10)-trien-6-one Thiosemicarbazone (16). Thiosemicarbazide (100 mg, 1.1 mmol) in EtOH (25 mL) and glacial acetic acid (10 drops) was added to 3,17β-diacetoxyestra-1,3,5(10)-trien-6-one (150 mg, 0.4 mmol) in benzene (15 mL) and heated under reflux for 3 h, the solvent evaporated, and the residue dissolved in the minimum volume of hot EtOH. Unreacted thiosemicarbazide (50 mg) was filtered from the cooled solution and water added to the filtrate to produce a slight turbidity. The required thiosemicarbazone (75 mg) separated on storing in the refrigerator: λ_{max} (EtOH) 225 nm (ϵ 12575) 318 (28530); $\bar{\nu}$ 3392, 3230, 3150 (NH), 1771, 1725 (ester C=O), 1493, 1100 cm^{-1} (thioureido bonds); NMR δ (CD_3SO) 0.76 (s, 3 H, C_{18}-Me), 2.01 (s, 3 H, C_{17}-OCOMe), 2.26 (s, 3 H, C_3-OCOMe), 4.64 (m, 1 H, C_{17a}-H), 7.04 (2 d, 1 H, J = 3 and 8 Hz, C_2-H), 7.34 (d, 1 H, J = 8 Hz, C_1-H), 7.99 (d, 1 H, J = 3 Hz, C_4-H); m/e measured 443.1911, calculated 443.1895.

3,17β-Diacetoxy-6-(2-imino-4-oxothiazolidinyl-1-imino)-estra-1,3,5(10)-triene (18). The thiosemicarbazone **16** (200 mg, 0.45 mmol) and ethyl bromoacetate (200 mg, 1.19 mmol) in EtOH were heated under reflux for 3 h, the ethanol was evaporated, and the residue was extracted with hot chloroform. The yellow solid

which separated on cooling was recrystallized from EtOH–$CHCl_3$ containing a few drops of n-C_6H_{14} to give the required product (175 mg): λ_{max} (EtOH) 216 nm (ϵ 30903), 305 (21097), 326 (18419); $\bar{\nu}$ 3197 (NH), 1770, 1735 (ester C=O), 1632 (C=O of thiazolidinone), 1615 cm^{-1} (C=N); NMR δ [$(CD_3)_2CO$] 0.83 (s, 3 H, C_{18}-Me), 3.82 (s, 2 H, CH_2 of thiazolidinone ring), 4.66 (m, 1 H, $C_{17\alpha}$-H), 6.85 (2 d, 1 H, J = 3 and 9 Hz, C_2-H) 7.18 (d, 1 H, J = 9 Hz, C_1-H), 7.71 (d, 1 H, J = 3 Hz, C_4-H); m/e measured 483.1844, calculated 483.1828.

3,17β-Dihydroxy-6-(2-imino-4-oxothiazolidinyl-1-imino)-estra-1,3,5(10)-triene (19). Thiosemicarbazone **16** (200 mg) and KOH (1 g) in methanol (30 mL) were left at room temperature for 24 h, the solution was diluted with water (350 mL) and made acid with 10% aqueous HCl, and the precipitated 3,17β-dihydroxyestra-1,3,5(10)-trien-6-one thiosemicarbazone (95 mg) was filtered and recrystallized from EtOH–C_6H_{14}: mp 255–256 °C; λ_{max} (EtOH) 213 nm (ϵ 24236), 315 (29857). Condensation of this derived dihydroxythiosemicarbazone (50 mg, 0.14 mmol) with ethyl bromoacetate (30 mg, 0.18 mmol) as described above gave the required product as a yellow amorphous powder (27 mg): λ_{max} (EtOH) 222 nm (ϵ 20054), 309 (19632), 325 (18576).

Biological Methods. Competitive Binding Assay Using Mouse Uterine Cytosol. Mice (Tuck No. 1 strain, 6 weeks old) were ovariectomized under ether anesthesia. One week later they were killed by cervical dislocation and the uteri removed, dissected free from adhering connective tissue, and frozen in liquid nitrogen. The uteri were powdered using a Thermovac tissue pulverizer and homogenized in Tris buffer (0.01 M, pH 7.4, containing 0.5584 g of EDTA and 77.16 mg of dithiothreitol per liter) at 4 °C using an Ultra Turrax homogenizer (2 × 2 s bursts with 1-min cooling between). Four to five uteri per milliliter of buffer was a satisfactory concentration. The homogenate was centrifuged for 1 h at 4–5 °C and 105000g in a Christ Omega II ultracentrifuge and the supernatant (cytosol) decanted.

Test compounds were dissolved in ethanol or, for hydrochlorides of bases, in Tris buffer. The required volumes of the ethanol solutions were added to incubation tubes, the ethanol was evaporated, and 50 μL of buffer was added or the required quantity of the base hydrochloride added in 50 μL of buffer. Aliquots of cytosol (200 μL) were added to the tubes and the mixtures incubated for 15 min at 5–7 °C. [6,7-^{3}H]-17β-Estradiol (0.5×10^{-12} g mol) (sp act 49.3 Ci/mmol, New England Nuclear) in 50 μL of Tris buffer was added and incubation continued for 1 h.

Each incubate (200 μL) was layered onto 10–30% sucrose gradients (4.75 mL in Tris buffer in 5-mL polyallomer tubes) and centrifuged at 275000g for 15 h at 4–5 °C in a Christ Omega II ultracentrifuge using a 6 × 5 mL titanium swing bucket rotor. Fractions (100 μL) were collected from the gradients into counting vials containing 5 mL of liquid scintillation cocktail (6 g of Bu-PBD, 100 g of naphthalene, 135 mL of toluene, 720 mL of dioxane, and 45 mL of methanol) and counted for 10 min in a Packard liquid scintillation spectrometer (Model 3320). Counting efficiency was determined by external standardization.

The inhibition of 17β-estradiol binding was obtained from the expression

$$\% \text{ inhibition} = \frac{\text{E8S cpm}}{\text{Etot cpm}} \times \frac{\text{Ctot cpm}}{\text{C8S cpm}} \times 100$$

where E8S cpm and C8S cpm are the counts per minute in the "8S" peak of experimental and control gradients, respectively, and Etot cpm and Ctot cpm are the total counts per minute in the experimental and control gradients, respectively. No difference in result was obtained if dpm's were calculated.

Uterine Weight Tests. Immature rats (Wistar) or mice (Tuck No. 1) were randomly distributed into suitably sized groups (5–6 rats, 9–11 mice). Arachis oil solutions containing 17β-estradiol, the test compound, or a mixture of 17β-estradiol and the test compound were prepared. Animals were injected daily for 3 days sc, the injection volumes being 0.1 mL for rats and 0.05 mL for mice, with either arachis oil alone, 17β-estradiol alone, or a combination of 17β-estradiol and test compound. For rats receiving estradiol and test compound two separate injections were used, but for mice the drugs were combined in a single solution. On the fourth day the animals were killed by cervical dislocation, the uteri were removed and dissected free from connective tissue, and the intraluminal fluid was removed by pressing on filter paper and weighed.

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