STRUCTURE-ACTIVITY RELATIONSHIPS OF ESTROGENS. EFFECTS OF 14-DEHYDROGENATION AND AXIAL METHYL GROUPS AT C-7, C-9 and C-11

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Received 6-14-83

ABSTRACT

Thirty compounds were evaluated in the rat for uterotropic effects, inhibition of gonadotropin release, and competitive displacement of (^{3}H) estradiol-17β from uterine cytosolic preparations. 7α-Methylestradiol-17β was 150% as active as estradiol-17β as an uterotropic agent. Estradiol-17β was the most active inhibitor of gonadotropin release. 11β-Methylestradiol-17β had 124% of the activity of estradiol-17β in displacing (^{3}H) estradiol-17β from the "estrogen receptor." The 9α-methyl group considerably decreased the potency of estrogens in any of the three assays. The 14-dehydro modification was advantageous only in the estradiol-17β 3-methyl ether series.

Uterotropic activities and inhibition of gonadotropin release did not parallel. The best compound for inhibiting gonadotropin release, as compared to uterotropic activity, was estrone.

The "estrogen receptor" assay data correlated fairly well with uterotropic assay data, but only for compounds having free 3-hydroxyl groups; even so, some exceptions were noted.

INTRODUCTION

The physiological activity of any compound is thought to depend upon its availability to target organs and its affinity for specific receptors contained therein. Therefore, modifications of the structure of a steroid may alter its ability to bind to receptors or enzymes, thereby changing its biologic activity or pharmacokinetics, but these are not necessarily congruent.

The positions for the addition of methyl groups to the structure of estrogenic steroids were selected on the basis of commercial availability (7α-methyl) or ease of synthesis (9α- and 11β-methyl). The 11β-methyl group has been reported to enhance estrogenicity (1). Excluded from present consideration were substitutions

demonstrated to decrease estrogenicity, such as the 6β-methyl group which results in compounds that are only 1/500 as uterotropic as estrone for mice (2), and the relatively inacessible 8β and 12α positions. The 14-dehydro modification was also selected because it enhances the anabolic and andorgenic activities of 19-nortestosterone, particularly when a 7α-methyl group is also present (3).

In this report, we evaluated 30 compounds for their uterotropic effect, inhibition of gonadotropin release, and competitive displacement of (^{3}H) estradiol-17β from the uterine cytosolic estrogen receptor. We used one species of animal (rat) to avoid ambiguities due to species difference when comparing and interpreting data.

MATERIAL AND METHODS

Steroids. -- Estradiol-17β, estrone, and their 3-methyl ethers were purchased from Searle Chemicals Inc., Chicago, IL. 7α-Methylestrone, 7α-methylestrone 3-methyl ether, and 7α-methylestradiol-17β were gifts from Upjohn Co., Kalamazoo, MI. 9α-Methylestrone 3-methyl ether and 9α-methylestradiol-17β 3-methyl ether were prepared according to the methods of Coombs et al (4). 11β-Methylestrone 3-methyl ether and 11β-methylestradiol-17β 3-methyl ether were prepared according to the methods of Baran et al (5). The preparation of 14-dehydroestrone, 7α-methyl-14-dehydroestrone, 14-dehydroestradiol-17β, and 7α-methyl-14-dehydroestradiol-17β 3-methyl ethers have been described previously (3). The 14-dehydro derivatives of 9α- and 11β-methylestrone 3-methyl ethers were synthesized by the methods described for the preparation of 14-dehydroestrone 3-methyl ether (3). 9α- and 11β-methylestrone were obtained in 35-50% yields from the demethylation of their 3-methyl ethers by fusing with 10 times their weight of pyridine hydrochloride for 1 h. The 14-dehydro derivatives of estrone, 7α-methylestrone, and 9α-methylestrone were prepared in 4 steps as follows:

1.) Conversion of the estrone to its 16α-bromo derivative by 2 mole-equivalents of cupric bromide in refluxing chloroform-ethyl acetate (1:1, v/v) for 3 h. This method was described by King and Ostrum for the preparation of hydroxyacetophenones regiospecifically brominated at the α-methylene position (6).

2.) Conversion of the 16α-bromeoestrone to its 3-acetate by acetic anhydride catalyzed by p-toluenesulfonic acid at steam bath temperature for 15 min.

3.) Dehydrobromination of the 16α-bromoestrone 3-acetate to the 14-dehydroestrone 3-acetate, using the same procedures reported for the preparation of 14-dehydroestrone 3-methyl ether (3).

4.) Hydrolysis of the 14-dehydroestrone 3-acetate to the 14-dehydroestrone by HCl in ethanol (100 mL aqueous 75% ethanol containing 1 mL conc HCl for each g steroid 3-acetate) at room temperature for 24 h.

The yield of a 14-dehydroestrone (or its 3-methyl ether) was approximately 10% overall based upon the estrone (or its 3-methyl ether) used as the starting product. Because of extensive decomposition during the dehydrobromination step, 11β-methyl-14-dehydroestrone was not prepared.

All 17β-hydroxysteroids (except estradiol-17β and 7α-methylestradiol-17β which came from commercial sources) were obtained from the reduction of their corresponding 17-ketosteroids with sodium borohydride in aqueous 90% ethanol at room temperature for 30 min.

Table 1 lists the 30 compounds used in this study and their melting points. IR spectra of the compounds were consistent with their structures. X-ray crystallographic data have been reported for estrone 3-methyl ether, 14-dehydroestrone 3-methyl ether, 14-dehydroestradiol-17β, and its 3-methyl ether (7).

Table 1

No.	Name (trivial name given first, then IUPAC name follows)	Mp (°C)[a]
1.	Estradiol-17β	179-183
	1,3,5(10)-estratriene-3,17β-diol	173-179 (8)
2.	7α-Methylestradiol-17β	166-168
	7α-methyl-1,3,5(10)-estratriene-3,17β-diol	... ...
3.	9α-Methylestradiol-17β	113-114
	9α-methyl-1,3,5(10)-estratriene-3,17β-diol	... ...
4.	11β-Methylestradiol-17β	226-229
	11β-methyl-1,3,5(10)-estratriene-3,17β-diol	223-225 (5)
5.	14-Dehydroestradiol-17β	109-112
	1,3,5(10),14-estratetraene-3,17β-diol	... ...
6.	7α-Methyl-14-dehydroestradiol-17β	98-102
	7α-methyl-1,3,5(10),14-estratetraene-3,17β-diol	.. ...
7.	9α-Methyl-14-dehydroestradiol-17β	140-145
	9α-methyl-1,3,5(10),14-estratetraene-3,17β-diol	... ...
8.	Estadiol-17β 3-methyl ether	100-101
	3-methoxy-1,3,5(10)-estratriene-17β-ol	97-98 (8)
9.	7α-Methylestradiol-17β 3-methyl ether	130-131
	3-methoxy-7α-methyl-1,3,5(10)-estratrien-17β-ol	... ...
10.	9α-Methylestradiol-17β 3-methyl ether	65-72
	3-methoxy-9α-methyl-1,3,5(10)-estratrien-17β-ol	70-75 (4)
11.	11β-Methylestradiol-17β 3-methyl ether	109-110
	3-methoxy-11β-methyl-1,3,5(10)-estratrien-17β-ol	101-103 (5)
12.	14-Dehydroestradiol-17β 3-methyl ether	122-123
	3-methoxy-1,3,5(10),14-estratetraen-17β-ol	116-120 (3)
13.	7α-Methyl-14-dehydroestradiol-17β 3-methyl ether	162-165
	3-methoxy-7α-methyl-1,3,5(10),14-estratetraen-17β-ol	162-165 (3)

14.	9α-Methyl-14-dehydroestradiol-17β 3-methyl ether	119-124
	3-methoxy-9α-methyl-1,3,5(10),14-estratetraen-17β-ol	... ...
15.	11β-Methyl-14-dehydroestradiol-17β 3 methyl ether	78-80
	3-methoxy-11β-methyl-1,3,5(10)-estratetraen-17β-ol	.. ..
16.	Estrone	255-257
	3-hydroxy-1,3,5(10)-estratrien-17-one	255-256 (9)
17.	7α-Methylestrone	234-238
	3-hydroxy-7α-methyl-1,3,5(10)-estratrien-17-one	... ...
18.	9α-Methylestrone	226-228
	3-hydroxy-9α-methyl-1,3,5(10)-estratrien-17-one	... ...
19.	11β-Methylestrone	302-306
	3-hydroxy-11β-methyl-1,3,5(10)-estratrien-17 one	302-305 (5)
20.	14-Dehydroestrone	183-189
	3-hydroxy-1,3,5(10),14-estratetraen-17-one	188-191 (10)
21.	7α-Methyl-14-dehydroestrone	190-194
	3-hydorxy-7α-methyl-1,3,5(10),14-estratetraen-17-one	... ...
22.	9α-Methyl-14-dehydroestrone	202-206
	3-hydroxy-9α-methyl-1,3,5(10),14-estratetraen-17-one	... ...
23.	Estrone 3-methyl ether	168-174
	3-methoxy-1,3,5(10)-estratrien-17-one	164-165 (9)
24.	7α-Methylestrone 3-methyl ether	166-168
	3-methoxy-7α-methyl-1,3,5(10)-estratrien-17-one	... ...
25.	9α-Methylestrone 3-methyl ether	191-193
	3-methoxy-9α-methyl-1,3,5(10)-3stratrien-17-one	188-190 (4)
26.	11β-Methylestrone 3-methyl ether	156-159
	3-methoxy-11βmethyl-1,3,5(10)-estratrien-17-one	149-150 (5)
27.	14-Dehydroestrone 3-methyl ether	103-105
	3-methoxy-1,3,5(10),14-estratetraen-17-one	103-104 (11)
28.	7α-Methyl-14-dehydroestrone 3-methyl ether	142-144
	3-methoxy-7α-methyl-1,3,5(10),14-estratetraen-17-one	142-144 (3)
29.	9α-Methyl-14-dehydroestrone 3-methyl ether	112-115
	3-methoxy-9α-methyl-1,3,5(10),14-estratetraen-17-one	... ...
30.	11β-Methyl-14-dehydroestrone 3-methyl ether	110-112
	3-methoxy-11β-methyl-1,3,5(10),14-estratetraen-17-one	... ...

a The melting point for a test compound was determined by a Kofler micro hot stage, is uncorrected, and is given on-line with the trivial name for that compound. The reported mp for a compound is given on-line with the IUPAC systematic name for that compound, and the reference number for the reported mp is enclosed by parentheses.

Bioassays. --- For the uterotropic assay, we use weanling female Fischer rats of our inbred line. The total dose of steroid to be administered is dissolved in 1 mL cottonseed oil. In the late afternoon of the first day, and in the early morning and late afternoon of the following 2 days, each animal is given 0.2 mL subcutaneously. Controls are given the cottonseed oil vehicle. On the morning of the fourth day, animals are sacrificed with ether anesthesia and the uteri dissected free, blotted, and weighed.

The bioassay to assess inhibition of gonadotropin secretion uses Fischer rats weighing from 70 to 80 g. The males are castrated before parabiosis. After a skin incision is made from the neck to the hip, the pair (castrated male on the right and intact female on the left) are joined by sewing through the adjacent scapulae. After incisions through the lateral abdominal muscles and peritoneum, they are joined with a continuous suture. Then, the skin is finally closed throughout. Treatment is started on the day of parabiosis. The steroid, in 0.2 mL cottonseed oil, is administered subcutaneously to the castrated male daily for 10 days. The pairs are sacrificed with ether anesthesia 24 h after the last injection. The ovarian weight in the female is used as the index of gonadotropin secretion by the castrated male.

Because the estrogens under study do not have parallel dose-response curves, for comparative purposes we have adopted a quantal response where the total amount of estrogen which would be required to double the uterine weight of the controls is extrapolated from the dose-response curves obtained by means of a computer program. For the same reason, the results from the gonadotropin release inhibition assay in parabiotic rats are also reduced to quantal assay in which the total amount of steroid required to halve the ovarian weight of the controls is calculated.

Because the uterotropic assay is more sensitive and requires less time than the gonadotropin release inhibition assay, it is difficult to compare the potency of a compound in the uterotropic assay with its potency in the gonadotropin release inhibition assay. Nevertheless, we divide the total dose of a compound needed to halve the weight of the ovaries in the parabiotic assay by its total uterotropic quantal dose to calculate a ratio ("OV/UT ratio") to determine which activity--uterotropic or inhibition of gonadotropin release--is affected the most by a structural modification.

Estradiol-17β Receptor Assay.---The assay buffer contained 1.5 mM Tris (Fisher Scientific), 1 mM EDTA (Fisher Scientific), and 20 mM sodium molybdate (Baker) in deionized glass-distilled water, pH 7.4. The use of 20 mM sodium molybdate to stabilize the "estrogen receptor" was recommended by Anderson et al (12); we have found this to be much better than 0.5 mM dithiothreitol (13) for stabilizing the estrogen receptor preparations.

Five mature female AXC rats were ovariectomized and rested for 5 days before they were sacrificed with ether anesthesia. Uteri were removed, weighed, minced with scissors while on ice, placed in cold assay buffer (1 mL for every 10 mg tissue), and homogenized in a Brinkmann Polytron homogenizer (setting of 7, two 5-sec bursts). The homogenate was centrifuged at 82,200 x g (1 h, 0°C) in a Spinco ultracentrifuge, model L with type 40 rotor. The supernatant (cytosol) was removed with a transfer pipette and kept in an ice bath. The protein content of the cytosol, as determined by the method of Lowry et al (14), averaged 6 mg/mL. The cytosol, as obtained, was sufficient in amount for running triplicate assays on 7 compounds.

(^{3}H) Estradiol-17β was purified by TLC. Ten microliters of an ethanol solution of (6,7-^{3}H) estradiol-17β (5.2 μg/ml; SA: 53 Ci/mmol; New England Nuclear) was spotted on a half-side of

a precoated (silica gel 60, 0.25 mm) 20 x 20 cm glass plate (Brinkmann); the other half-side was spotted with 100 μg unlabeled estradiol-17β on-line with the (^{3}H) estradiol-17β spot. The developing solution was chloroform-ethyl acetate 85:15 v/v. After development, the unlabeled estradiol-17β area was detected by spraying with distilled water and marked. The corresponding area containing (^{3}H) estradiol-17β was cut out and extracted three times with 3 mL ethanol at 60°C. The extract was centrifuged at 3,500 x g (5 min, room temperature), and the supernatant was evaporated to dryness under nitrogen. The residue was dissolved in 37.5 μL ethanol; then 20 mL assay buffer was added and vortexed until clear. One hundred microliters (which contains 93 pg (0.34 pmol) of (^{3}H) estradiol-17β) of this solution was added to each tube in the assay.

The reference compound, estradiol-17β, was studied at five concentrations from 10^{-10} to 10^{-6}M; other competitors (test compounds) were run at four concentrations from 10^{-9} to 10^{-6} M. Each concentration and its blank (no cytosol) were run in triplicate. Table 2 gives the composition of the solutions in the assay tubes. Incubations were at 4°C for 2 h. After incubation, 50 μL of dextran-coated charcoal (5 g Norite-A (Baker) previously washed with methanol and air-dried), 0.5 dextran (immunoassay grade; Schwarz/Mann), and 1 g human gamma globulin (fraction II; Armour Pharmaceutical) in 100 mL deonized glass-distilled water) were added to each tube, vortexed, incubated for 15 min at 4°C, and centrifuged at 3,500 x g (10 min, 0°C) in an IEC refrigerated centrifuge (model RP1). Two hundred microliters of the supernatant were assayed for bound (^{3}H) estradiol-17β by counting in 5 mL Pico-Fluor 15 (Packard Instrument Co.) in a Packard Tri-Carb liquid scintillation spectrometer (model C2425) for 10 min. The efficiency of counting was 45%, and no quenching corrections were made.

Table 2. Composition of Solutions in the Assay Tubes

Tube designation	Test solution[a]	Assay buffer	Cytosol	Ligand[b]
A: Competition tube	100 μL	150 μL	100 μL	100 μL
B: Competition blank	100 μL	250 μL	0	100 μL
C: Control tube	0	250 μL	100 μL	100 μL
D: Control blank	0	350 μL	0	100 μL

[a]Competing steroid in assay buffer giving final concentrations from 10^{-10} to 10^{-6} M (estradiol-17β) or 10^{-9} to 10^{-6} M (other compounds).
[b]3.4 pmol (^{3}H) estradiol-17β per mL of assay buffer.

The % (^{3}H) estradiol-17β bound was calculated by the following formula:

$$\frac{A-B}{C-D} \times 100$$

where A: cpm from competition tube; B: cpm from competition blank; C: cpm from control tube; and D: cpm from control blank.

The concentration of a competing substance required to produce 50% binding of (^{3}H) estradiol-17β was determined by plotting the % bound radioactivity versus the logarithm of the concentration.

The relative displacing activity (RDA), which is the ability of a compound to displace 50% of (^{3}H) estradiol-17β from a uterine cytosol preparation as compared to estradiol-17β which is given the value of 100, was calculated by the following formula:

$$\frac{\text{concentration of estradiol-17}\beta\text{ displacing 50\% (}^3\text{H) estradiol-17}\beta}{\text{concentration of competitor displacing 50\% (}^3\text{H) estradiol-17}\beta} \times 100$$

Data.---The results of the three assays are presented in Table 3. The numbers are means between two extremes (the highest and lowest values determined for a compound); ranges are not given, but are not more than $\pm$ 50% of the means. The number of significant figures ranges from three if a number is more than 100 to one if a number is less than 1.

RESULTS

Uterotropic activity.---As expected, the 17β-hydroxy group was more favorable to uterotropic activity than the 17-keto group. On the average, the estradiol-17β series is 11 times more active than the estrone series, and the estradiol-17β 3-methyl ether series is 7 times more active than the estrone 3-methyl ether series.

There were smaller differences in potencies between the 3-methyl ethers and their 3-hyroxy counterparts in the 17-ketosteroid series than those of the 17β-hydroxysteroid series. In the 17-ketosteroid series, a 3-methyl ether is about 1/4 as uterotropic as its 3-hydroxy counterpart, whereas in the 17β-hydroxysteroid series, a 3-methyl ether is about 1/18 as uterotropic as its 3-hydroxy counterpart.

The most active uterotropic agent was 7α-methylestradiol-17β (1 1/2 as active as estradiol-17β); the least active compound was 9α-methyl-14-dehyroestrone (1/2200 as active as estradiol-17β).

The 7α- or 11β-methyl group, but not the 9α-methyl group, favors uterotropic activity. The 7α-methyl group is particularly effective in the 17-ketosteroid series, increasing the potency of estrone or estrone 3-methyl ether by 5-fold. In the estradiol-17β 3-methyl ether

series, the 7α-methyl group and the 14-dehydro modification were synergistic: 7α-methyl-14-dehydroestradiol-17β 3-methyl ether was 9 times as potent as estradiol-17β 3-methyl ether. However, the 14-dehydro modification decreased uterotropic activity in the 17-ketosteroid series.

Inhibition of gonadotropin release.---In contrast to the results of the uterotropic assays, the 17-keto group can be more facilitative than the 17β-hydroxyl group in the gonadotropin release inhibition assays. 7α-Methylestrone, 7α-methylestrone 3-methyl ether, and estrone 3-methyl ether were 4, 3, and 1.4 times more active than their 17β-hydroxy counterparts, respectively.

The most potent inhibitor of gonadotropin release was estradiol-17β; the least active compound was 9α-methyl-14-dehydroestrone 3-methyl ether (1/7300 as active as estradiol-17β).

The effects of structural modifications on inhibition of gonadotropin release were more variable than their effects on uterotropic activity. The 11β-methyl group was more favorable than the 7α-methyl group in the 17β-hydroxysteroid series, but in the 17-ketosteroid series it was the other way around: the 7α-methyl group was distinctly more favorable than the 11β-methyl group. The 14-dehydro modification was favorable only in the estradiol-17β 3-methyl ether series: 14-dehydroestradiol-17β 3-methyl ether was the most potent of that series, being 7 times as active as estradiol-17β 3-methyl ether or 1/20 as active as estradiol-17β. In the estradiol-17β series, the 7α-methyl group and the 14-dehydro modification were synergistic: 7α-methyl-14-dehydroestradiol-17β had 54% of the activity of estradiol-17β, while 7α-methylestradiol-17β and 14-dehydroestradiol-17β had

only 10% and 13%, respectively, of the activity of estradiol-17β. The 9α-methyl group was not favorable.

Table 3. In Vitro and In Vivo Data

Compounds	RDA[a]	UT[b] (dose)	OV[c] (dose)	OV/UT[d]
1. Estradiol-17β	100	100 (0.03)	100 (0.4)	13
2. 7α-methyl	104	150 (0.02)	10 (4.0)	200
3. 9α-methyl	35	3.3 (0.9)	1.1 (37)	41
4. 11β-methyl	124	100 (0.03)	67 (0.6)	20
5. Δ^{14}	107	75 (0.04)	13 (3.0)	75
6. 7α-methyl-Δ^{14}	74	75 (0.04)	56 (0.7)	18
7. 9α-methyl-Δ^{14}	41	0.8 (3.8)	0.3 (130)	33
8. Estradiol-17β 3-methyl ether	0.7	100 (1.8)	100 (54)	30
9. 7α-methyl	5.3	360 (0.5)	54 (100)	200
10. 9α-methyl	0.0	16 (11)	7.8 (690)	63
11. 11β-methyl	5.1	600 (0.3)	77 (70)	233
12. Δ^{14}	0.8	360 (0.5)	675 (8.0)	16
13. 7α-methyl-Δ^{14}	3.1	900 (0.2)	98 (55)	275
14. 9α-methyl-Δ^{14}	0.1	43 (4.2)	90 (60)	14
15. 11β-methyl-Δ^{14}	1.2	300 (0.6)	90 (60)	100
16. Estrone	46	100 (0.5)	100 (4.0)	8
17. 7α-methyl	68	556 (0.09)	400 (1.0)	11
18. 9α-methyl	5	8 (6.2)	8.0 (50)	8
19. 11β-methyl	47	100 (0.5)	50 (8.0)	16
20. Δ^{14}	9	24 (2.1)	7.5 (53)	25
21. 7α-methyl-Δ^{14}	52	167 (0.3)	57 (7.0)	23
22. 9α-methyl-Δ^{14}	6	0.8 (65)	0.9 (460)	7
23. Estrone 3-methyl ether	0.0	100 (2.5)	100 (39)	16
24. 7α-methyl	0.0	500 (0.5)	118 (33)	66
25. 9α-methyl	0.0	7.8 (32)	4.2 (930)	29
26. 11β-methyl	0.0	147 (1.7)	24 (160)	94
27. Δ^{14}	0.0	37 (6.7)	9.5 (410)	61
28. 7α-methyl-Δ^{14}	0.0	125 (2.0)	12 (340)	170
29. 9α-methyl-Δ^{14}	0.0	5.8 (43)	1.3 (2920)	68
30. 11β-methyl-Δ^{14}	0.0	61 (4.1)	7.8 (500)	122

[a] RDA: relative displacing activity; the ability of a compound to displace (^{3}H) estradiol-17β from rat uterus cytoplasmic estrogen receptor as compared to estradiol-17β which is given the value of 100.

[b] UT: relative uterotropic activity; the ability of a compound to double the weight of rat uterus with the reference compound (which appears first in each of the four divisions) given the value of 100. (Total dose in μg enclosed by parentheses).

[c] OV: relative gonadotropin release inhibition activity; the ability of a compound to halve the weight of parabiotic rat ovary with the reference compound (which appears first in each of the four divisions) given the value of 100. (Total dose in μg enclosed by parentheses.)

[d] OV/UT: the total gonadotropin release inhibition dose divided by the total uterotropic dose.

Comparison of inhibition of gonadotropin release with uterotropic activity.---An examination of the OV/UT dose ratios (Table 3) indicates that the 17-keto group favors inhibition of gonadotropin release, when compared with uterotropic activity, more than the 17β-hydroxyl group does. On the average, the dose to inhibit gonadotropin release is 14 times the uterotropic dose for the estrone series; the corresponding OV/UT dose ratios are 56 for the estradiol-17β series, 79 for the estrone 3-methyl ether series, and 116 for the estradiol-17β 3-methyl ether series.

A structural modification can favor uterotropic activity over inhibition of gonadotropin release, particularly in the estradiol-17β 3-methyl ether series. 7α-Methyl-14-dehydroestradiol-17β 3-methyl ether is the most potent uterotropic agent in that series, being 9 times as active as estradiol-17β 3-methyl ether or 1/7 times as active as estradiol-17β. However, its gonadotropin release inhibition activity is no greater than that of estradiol-17β 3-methyl ether. The result was a compound that required a 275-fold increase of its quantal uterotropic dose to halve the weight of the ovaries in the parabiotic assay.

The compound that showed the best (lowest) OV/UT dose ratio at the lowest possible dose was estrone, which had an OV/UT dose ratio of 8 (the uterotropic dose was 0.5 μg, whereas the dose required for inhibition of gonadotropin release was 4 μg).

Competitive displacement of (^{3}H) estradiol-17β from the uterine cytosolic estrogen receptor (RDA assay).---It apparently requires a free hydroxyl group at C-3 for a compound to effectively displace (^{3}H) estradiol-17β in the RDA assay. Estradiol-17β 3-methyl ether and its derivatives have low RDA values (0.0-5.3, Table 3), while estrone 3-methyl ether and its derivatives consistently have RDA values of 0. However, the 3-methyl ethers are not devoid of estrogenic activity (Table 3). There are two caveats about interpreting RDA data: while an RDA value of 0 does not necessarily mean that the compound will be inactive as an estrogen, a RDA value of more than 100 does not necessarily mean that the compound will be more estrogenic than estradiol-17β. 14-Dehydroestradiol-17β has an RDA of 107, but is only 75% as uterotropic as estradiol-17β. 11β-Methylestradiol-17β, despite its RDA of 124, is no more active than estradiol-17β as an uterotropic agent.

The 14-dehydro modification showed remarkable differences in the RDA assay depending on what was on C-17. 14-Dehydroestradiol-17β has an RDA of 107, while 14-dehydroestrone has an RDA of 9. The low RDA value of the latter compound correlated with its low uterotropic potency (24% of that of estrone, or 1.4% of that of estradiol-17β). The 7α- and 11β-methyl derivatives also showed C-17 dependent effects on the RDA: the 11β-methyl group increased the RDA value of estradiol-17β by 24% but had no effect on that of estrone, while the 7α-methyl group increased the RDA value of estrone by 50% but had no significant effect on that of estradiol-17β. There have been previous reports on the interesting C-17 dependence in steroid-receptor interactions. For example, Schmit et al found

that the 9(11)-dehydro modification increased the affinity of 4-androstene-3,17-dione but decreased that of testosterone for the rat prostate cytosolic androgen receptor (15).

DISCUSSION

Although the RDA assay data may be used to predict the uterotropic activity of a 3-hydroxy ring A-aromatic steroid when (^{3}H) estradiol-17β is used as the ligand, it is not consistent. As a consequence, the *in vitro* RDA assay cannot serve as a substitute for *in vivo* experiments which, in reality, determine estrogenicity or antagonism to estrogens.

The bioassay data (Table 3) may suggest that vulnerability or resistance to metabolism of structurally modified estrogens may explain the difference in the biological activities that were observed. However, such suggestions would be speculative. We do not know whether the 3-methyl ethers or the 17-ketosteroids are active *per se*, or that they need further metabolism (by 3-demethylase or 17β-hydroxysteroid oxidoreductase, for examples) to more active compounds. We have not investigated the pharmacokinetics of the compounds *in vivo*, and we have not ascertained their affinities for metabolic enzymes *in vitro*.

The nature of the "estrogen receptor" (as exemplified by the RDA assay) remains unresolved. There have been numerous reports on the variability of the RDA assay depending on what was used as the (^{3}H) ligand. We do not know whether the uterine cytoplasmic preparation contains only one estrogen receptor with multiple binding sites capable of binding structurally different ligands (one ligand not necessarily capable of displacing another), or more than one

estrogen receptor, each one specifically binding with its own ligand that is structurally different from the others. If the binding of a compound to an estrogen receptor is sine qua non for eliciting estrogenic activity, then it can be safely said that it is not estradiol-17β that a compound must "compete with." 11-Keto-9β-estrone is equal to estrone in the uterotropic and gonadotropin release inhibition assays (16). Here, we disclose that the RDA value of 11-keto-9β-estrone is only 1.1, which is nowhere close to the RDA value of 46 that was reported for estrone.

We have established that the 11β and 7α positions are logical candidates for additions there of various groups to develop potent uterotropic agents and/or gonadotropin release inhibitors. Moxestrol (17-ethinyl-11β-methoxy-1,3,5(10-estratriene-3,17β-diol) is an example of a compound modified at the 11β position which is a highly potent estrogen for humans (17,18). There are no reports to our knowledge of estrogens modified at the 7α position by groups other than methyl.

The 14-dehydro modification was advantageous only in the estradiol-17β 3-methyl ether series. We have no explanation for the apparent "structural specificity."

Although the introduction of the 9α-methyl group resulted in compounds with extremely low estrogenic potency, this cannot be taken to rule out the 9α position as a candidate for modifications. The 9α-hydroxy-11β-nitro modification has resulted in potent estrogens (especially by the peroral route) as determined by rat bioassays (19). A caveat seems to be in order here: if a modification at a position on the steroid structure is found to be deleterious to biological activity, one should try a different modification

at that position before ruling out that position as a candidate for further development of potent steroids.

We have shown that uterotropic activity and inhibition of gonadotropin release do not parallel. For that reason, it should be quite possible to develop compounds that are better inhibitors of gonadotropin release than they are as uterotropes. Such compounds would have clinical value as contraceptives or in the treatment of hormone-dependent cancer.

ACKNOWLEDGMENTS

The work was supported in part by the annual gift program of the Alton Ochsner Medical Foundation through donors who have designated their gifts to this project. We thank Dr. Robert V. Coombs of Sandoz-Wander, Inc. for a sample of 9α-methylestrone 3-methyl ether and Dr. John C. Babcock of the Upjohn Co. for samples of 7α-methylestrogens and C,H analyses. The technical assistance and advice of members of our group--Byrnes Carriere, Roy Coleman, Albert Flores, Leslie Hamer, and Luxurie Sorenson--are greatly appreciated.

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