J. Steroid Biochem. Molec. Biol. Vol. 38, No. 4, pp. 399–405, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

ESTROGENIC ACTION OF ESTRIOL FATTY ACID ESTERS

JAN E. ZIELINSKI, SHAM L. PAHUJA, JANICE M. LARNER and RICHARD B. HOCHBERG*
Department of Obstetrics and Gynecology and the Comprehensive Cancer Center,
Yale University School of Medicine, P.O. Box 3333, New Haven, CT 06510, U.S.A.

(Received 12 October 1990)

Summary—Recent studies suggest that, estriol, like estradiol, is biosynthetically esterified with fatty acids. We have synthesized the stearate esters of estriol, at C-16α,C-17β and the diester, C-16α,17β and tested these D-ring esters for their estrogenic action both *in vivo* and *in vitro*, comparing them to estradiol, estriol and estradiol-17-stearate. None of the estriol esters bind to the estrogen receptor. They are only weakly estrogenic in a microtiter plate estrogen bioassay: stimulation of alkaline phosphatase in the Ishikawa endometrial cells. However, both estriol monoesters are extremely potent estrogens when injected subcutaneously (in aqueous alcohol) into ovariectomized mice. Compared to the free steroids, they produced a dramatically increased uterine weight with a greatly prolonged duration of stimulation. The 16α,17β-diester also induced a protracted uterotrophic response, but the stimulation of uterine weight was comparatively low. Since the esters of estradiol and estriol do not bind to the estrogen receptor, their estrogenic signal must be generated through the action of esterase enzymes. These naturally occurring esters have the potential of being extremely useful pharmacological agents for long-lived estrogenic stimulation.

INTRODUCTION

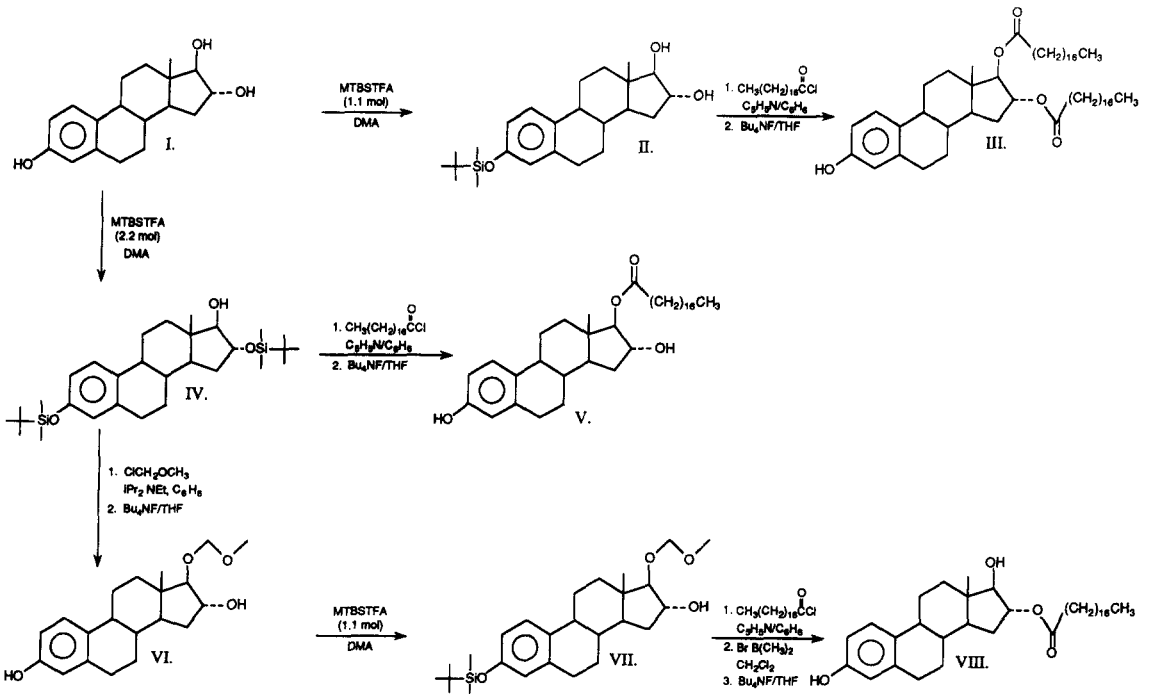
It has become increasingly evident that all of the families of steroid hormones are metabolized into fatty acid esters (for a review, see [1]). While the physiological role of this transformation into an extremely nonpolar "lipoidal" form of the hormone is not clear, its effect on the estrogen, estradiol has been studied in some detail. We have shown that estradiol is biochemically esterified into a mixture of fatty acid esters at C-17β but not at the phenolic C-3 hydroxyl [2, 3]. The biosynthesis of estradiol fatty acid esters has been confirmed in several laboratories [4–8]. We and others have shown that the esterification of estradiol with fatty acids leads to an extremely potent estrogen with an unusually sustained action [9–11]. Esterification protects the steroid nucleus from enzymatic catabolism and prolongs its metabolic survival [12]. The ester is not itself hormonally active, i.e. it does not bind to the estrogen receptor, it requires hydrolysis for activation [13]. Esterases acting on the steroidal ester provide the hormonal stimulus [5, 14, 15], stemming from the long-lived substrate.

Recently it has been shown that estriol, 1,3,5(10)-estratrien-3,16α,17β-triol **I**, is converted into a nonpolar metabolite by a human breast cancer cell line in culture [16]. Although it has not been characterized, its polarity suggests a fatty acid ester. We have also found that rat lung converts estriol into a hydrophobic metabolite with characteristics of a fatty acid ester of estriol [17]. This finding has led us to synthesize (Scheme 1) various D-ring esters of estriol, 16α,17β-, and 16α,17β- in order to be able to characterize the nonpolar biosynthetic product and to determine the estrogenic potency of these esters. We have synthesized the stearate esters of estriol as representative stable products for direct comparison with estradiol-17-stearate, whose properties have been extensively investigated [9, 10]. We have determined the extrogenic properties of the various estriol esters **III**, **V** and **VIII**, including: their binding to the estrogen receptor; their ability to induce a hormonal response in an estrogen sensitive cell line (alkaline phosphatase in Ishikawa endometrial cancer cells [18]); and their estrogenic action, and the duration of the response, in stimulating uterine growth in the ovariectomized mouse [19].

EXPERIMENTAL SECTION

Melting points were determined on a Koffler Hot Stage and are uncorrected. NMR spectra

*Author to whom all correspondence should be addressed.



Scheme 1. The synthesis of D-ring esters of estriol.

were recorded on a Bruker WM-500 spectrometer at 500 MHz in $CdCl_3$. High resolution mass spectral data were obtained on a Kratos Ms-80 RFA spectrometer. HPLC was performed on a Waters modular system consisting of a 600E controller and pump, U6K injector and 484 variable wavelength absorbance detector. HPLC analysis was performed at 1 ml/min with the u.v. detector at 280 nm. HPLC system H_1 is: 250 × 46 mm LiChrosphere 100 Diol (Merck) column, CH_2Cl_2/isooctane (3:2); system H_2 is: 250 × 46 mm Ultrasphere Si (Altex) column, iso-octane/isopropanol (49:1).

Estrogen receptor binding assay

Competition for the binding of the estrogen receptor in ovariectomized rabbit uterine cytosol was performed as previously described with minor modification [20]. The various steroids and 2 nM [2,4,6,7-^{3}H]estradiol (115 Ci/mmol) were dissolved in 5 μl dimethylformamide and incubated overnight at 0°C with 100 μl of uterine cytosol in a buffer consisting of 10 mM Tris, 1.5 mM Na_2EDTA, 10 mM Na_2MoO_4 and 1 mM monothioglycerol, pH 7.4. Nonspecific binding was determined by a control containing 100-fold excess of diethylstilbestrol. Free radioactivity was absorbed by dextran-coated charcoal and the bound radioactivity was counted in a liquid scintillation counter.

Ishikawa estrogen bioassay

The induction of alkaline phosphatase in the Ishikawa cells grown in microtiter plates was performed exactly as described [21]. Cells are plated in 96-well microtiter plates at a density of 25,000 cells per well in 150 μl of an estrogen free media (1/1 mixture of phenol red-free Ham's F-12 and DMEM containing 5% charcoal stripped calf serum) and grown in the presence of the indicated concentrations of test steroid. After 3 days, the media is removed, the cells washed, frozen and thawed, and 50 μl of 5 mM *p*-nitrophenyl phosphate in 1 M diethanolamine, pH 9.8, is added. The mixture is incubated at room temperature and alkaline phosphatase activity monitored periodically at 410 nm in a microtiter plate reader.

Mouse uterotrophic assay

Adult female mice CD-1, about 7 weeks old (Charles River) were ovariectomized under Metofane anesthesia. 7 days later they were injected subcutaneously with either 200 μl of vehicle (saline/ethanol 4:1) or estradiol and estradiol-17-stearate at 132 nmol/kg body weight or estriol, **V**, **VIII**, and **III** at 264 nmol/kg body weight, all dissolved in 200 μl of the vehicle. At various times, up to 20 days after the

injection, the animals were killed and their uteri were removed, dissected free from adhering tissue, blotted and weighed.

3-t-butyldimethylsilylestriol II. To a solution of 100 mg (0.347 mmol) of estriol in 0.7 ml of DMA was added 75 μl (0.381 mmol) of MTBSTFA and the mixture was stirred for 4 h at room temperature. After evaporation to dryness under high vacuum, the residue was purified on a silica gel column with $CHCl_3$–ethylacetate–ethanol (5:1:0.3) as the solvent system. After the solvent was evaporated, the residue was recrystallized from methanol to give 110 mg (80% yield) of **II**. Intermediate **II** was homogeneous by TLC: m.p. 196–198°C; ^{1}H-NMR, δ0.192 (s, 6H, $(CH_3)_2Si$), 0.815 (s, 3H, C_{13}-CH_3),) 0.983 (s, 9H, t-BuSi), 3.599 (dd, 1H, J = 5.07 Hz, $C_{17\alpha}$-H), 4.185 (dd, 1H, J = 3.7 and J = 5.6 Hz, $C_{16\beta}$-H), 6.559 (d, 1H, J = 2.6 Hz, C_4-H), 6.619 (dd, 1H, J = 2.6 and J = 8.4 Hz, C_2-H), 7.115 (d, 1H, J = 8.4 Hz, C_1-H); HRMS, found 402.2583, calculated from $C_{24}O_3SiH_{38}$ 402.2580.

Estriol-16α,17β-distearate III. 75 mg (0.25 mmol) of stearyl chloride in 0.1 ml of benzene was added to a solution of 50 mg (0.124 mmol) of **II** in 0.1 ml of benzene and 100 μl (1.25 mmol) of pyridine. After 2 h the reaction mixture was poured into H_2O and extracted with petroleum ether/ether (1:1). The organic layer was washed with aqueous 1% HCl, H_2O and then dried. The residue after evaporation of the solvent was purified on a silica gel column with petroleum ether/ether (20:1). The protecting *t*-butyldimethylsilyl group at C-3 was removed with 0.4 ml 1 M tetra-*n*-butyl-ammonium fluoride (Bu_4NF) in THF. After 1 h, the THF was evaporated and the residue was dissolved in ether and washed with H_2O. The crude product **III** was purified on a silica gel column with $CHCl_3/CH_2Cl_2$ (1:1). Recrystallization from methanol gave 66 mg (65% yield). Compound **III** was homogeneous by TLC and analytical HPLC in system H_1, R_t = 7–8 min; m.p. 40°C; ^{1}H-NMR, δ0.852 (s, 1H, C_{13}-CH_3), 0.888 (t, 6H, J = 6.9 Hz, 2x-OC-$(CH_2)_{16}$-$\mathbf{CH_3}$), 1.262–1.386 (broad, 60H, 2x-OC-CH_2-$(\mathbf{CH_2})_{15}$-CH_3), 2.296 (t, 2H, J = 7.5 Hz, OC-$\mathbf{CH_2}$-$(CH_2)_{15}$-CH_3), 2.336 (t, 2H, J = 7.5 Hz, -OC-$\mathbf{CH_2}$-$(CH_2)_{15}$-CH_3), 4.593 (s, 1H, C_3-OH), 5.016 (d, 1H, $J_{16\beta,17\alpha}$ = 5.8 Hz, $C_{17\alpha}$-H). 5.195 (dd, 1H, $J_{16\beta,17\alpha}$ = 5.8 and $J_{15\beta,16\beta}$ = 7.9 Hz, $C_{16\beta}$-H), 6.566 (d, 1H, J = 2.7 Hz, C_4-H), 6.630 (dd, 1H, J = 2.7 and J = 8.4 Hz, C_2-H), 7.137 (d, 1H, J = 8.4 Hz, C_1H); HRMS, found 820.6944, calculated from $C_{54}O_5H_{92}$ 820.6921.

3,16α-di-(t-butyldimethylsilyl)-estriol IV. A solution of 300 mg (1.04 mmol) of estriol in 2 ml of DMA and 450 μl (2.28 mmol) of MTBSTFA was stirred at room temperature overnight. After evaporation to dryness, the crude product was purified by filtration through a silica gel column with petroleum ether/ether (5:1). Recrystallization from methanol gave 456 mg (85% yield) of **IV**. Intermediate **IV** was pure by TLC analysis: m.p. 65°C; ^{1}H-NMR, δ0.192 (s, 12H, 2x$(CH_3)_2Si$), 0.797 (s, 3H, C_{13}-CH_3), 0.983 (s, 18H, 2xt-BuSi), 3.572 (broad, 1H, $C_{17\alpha}$-H), 4.096 (ddd, 1H, J = 1.7 Hz, J = 5.6 and J = 9.2 Hz, $C_{16\beta}$-H), 6.556 (d, 1H, J = 2.6 Hz, C_4-H), 6.616 (dd, 1H, J = 2.6 and J = 8.4 Hz, C_2-H), 7.115 (d, 1H, J = 8.4 Hz, C_1-H); HRMS, found 516.3440, calculated from $C_{30}O_3Si_2H_{52}$ 516.3441.

Estriol-17β-stearate V. Ester **V** was synthesized in the same manner as **III** starting with 50 mg (0.97 mmol) of **IV** in 0.1 ml of benzene, 50 μl (0.6 mmol) of pyridine and 32 mg (0.106 mmol) stearyl chloride in 0.75 ml benzene. Deprotection of the hydroxyl groups at C-3 and C-16α was performed with 0.3 ml 1M Bu_4NF in THF. Crude ester **V** was purified on a silica gel column with benzene/ethylacetate (25:1). Recrystallization from methanol gave 38 mg (72% yield) of **V**. This compound was homogeneous by TLC and by analytical HPLC in system H_2, R_t = 17–18 min: m.p. 84–85°C; ^{1}H-NMR, δ0.874 (s, 3H, C_{13}-CH_3), 0.889 (t, 3H, J = 6.8 Hz, -OC-$(CH_2)_{16}$-$\mathbf{CH_3}$), 1.266–1.349 (broad, 30H, -OC-CH_2-$(\mathbf{CH_2})_{15}$-CH_3), 2.401 (t, 2H, J = 7.5 Hz, -OC-$\mathbf{CH_2}$-$(CH_2)_{15}$-CH_3), 3.798 (s, 1H, C_3-OH), 4.145 (ddd, 1H, $J_{15\beta,16\beta}$ = 8.3 Hz, $J_{16\beta,17\alpha}$ = 4.3 and $J_{15\alpha,16\beta}$ = 1.6 Hz, $C_{16\beta}$-H), 4.273 (d, 1H, $J_{16\beta,17\alpha}$ = 4.3 Hz, $C_{17\alpha}$-H), 6.572 (d, 1H, J = 2.7 Hz, C_4-H), 6.633 (dd, 1H, J = 2.7 and J = 8.4 Hz, C_2-H), 7.145 (d, 1H, J = 8.4 Hz, C_1-H); HRMS, found 554.4343, calculated from $C_{36}O_4H_{58}$ 554.4320.

16β-methoxymethylestriol ether VI. 100 mg (0.193 mmol) of **IV** in 0.5 ml of benzene was reacted under Argon with 44 μl (0.58 mmol) of chloromethylmethyl ether in the presence of 101 μl (0.58 mmol) of *N,N*-diisopropylethylamine. After 6 h, the reaction mixture was concentrated under vacuum and the oily residue was dissolved in ether and washed with H_2O. The intermediate, 3,16α-di-(*t*-butyldimethylsilyl)-17β-methoxymethylestriol, was purified by filtration on a silica gel column with

petroleum ether/ether (20:1) and then treated for 1 h with 1.1 ml (6 mmol) of 1 M Bu_4NF in THF in order to remove the two *t*-butyldimethylsilyl protecting groups. The THF was evaporated under vacuum and the residue was extracted from H_2O with ethylacetate. The organic layer was dried and evaporated. The crude product was purified on a silica gel column with $CHCl_3$–ethylacetate–ethanol (5:1:0.1) as the solvent system. Recrystallization from ethylacetate afforded 45 mg (70% yield) of **VI** (homogeneous by TLC): m.p. 172–174°C; ^{1}H-NMR: δ0.8366 (s, 3H, C_{13}-CH_3), 3.230 (d, 1H, $J_{16\beta,17\alpha} = 5.4$ Hz, C-17α), 3.320 (d, 1H, J = 1.7 Hz, C-$_{16\alpha}$-OH), 3.468 (s, 3H-, -O-CH_3), 4.150 (ddd, 1H, $J_{15\beta,16\beta} = 7.3$ Hz, $J_{16\beta,17\alpha} = 5.4$ and $J_{15\alpha,16\beta} = 2.1$ Hz, $C_{16\beta}$-H), 4.763 (dd, 2H, J = 6.7 Hz, -O-CH_2-O-), 6.569 (d, 1H, J = 2.7 Hz, C_4-H), 6.6314 (dd, 1H, J = 8.4 and J = 2.7 Hz, C_2-H), 7.144 (d, 1H, J = 8.4 Hz, C_1-H); HRMS, found 332.1985, calculated from $C_{20}O_4H_{28}$ 332.1980.

3-t-butyldimethylsilyl-17β-methoxymethylestriol VII. *t*-butyldimethylsilylation of the hydroxy group at C-3 of **VI** was performed in the same manner as in the synthesis of **II**. Starting with 100 mg (0.301 mmol) of **VI**, the crude product **VII** was purified on a silica gel column using cyclohexane/ethylacetate (10:1). After evaporation, 114 mg (85% yield) of an oily residue was obtained which, although pure by TLC, resisted crystallization: ^{1}H-NMR: δ0.191 (s, 6H, $(CH_3)_2$Si-), 0.840 (s, 3H, C_{13}-CH_3), 0.982 (s, 9H, t-BuSi-), 3.227 (d, 1H, $J_{16\beta,17\alpha} = 5.4$ Hz, $C_{17\alpha}$-H), 3.467 (s, 3H, -OCH_3), 4.145 (ddd, 1H, $J_{15\beta,16\beta} = 8.3$ Hz, $J_{16\beta,17\alpha} = 5.4$ and $J_{15\alpha,16\beta} = 2.1$ Hz, $C_{16\beta}$-H), 4.761 (dd, 2H, J = 6.7 Hz, -O-CH_2-O-), 6.558 (d, 1H, J = 2.6 Hz, C_4-H), 6.613 (dd, 1H, J = 8.4 and J = 2.6 Hz, C_2-H), 7.111 (d, 1H, J = 8.4 Hz, C_1-H); HRMS, found 446.2866, calculated from $C_{26}O_4SiH_{42}$ 446.2841.

Estriol-16α-stearate VIII. Esterification of the 16α-OH in intermediate **VII** was performed in the same manner as for ester **III** using 60 mg (0.134 mmol) of **VII** in 0.125 ml benzene, 60 μl (0.75 mmol) pyridine and 45 mg (0.15 mmol) of stearyl chloride dissolved in 0.75 ml of benzene. The product was purified by filtration on a silica gel column with petroleum ether/ether (20:1). After evaporation the residue was dissolved in 0.13 ml of CH_2Cl_2 and cooled to −78°C. To cleave the methoxymethyl group, 0.27 ml (0.266 mmol) of 1 M dimethylboron bromide in 1,2-dichloroethane was added dropwise under Argon. The reaction mixture was stirred for 1 h at −78°C. Afterwards it was transferred into a stirred solution of 1 ml of saturated $NaHCO_3$ in 2 ml of THF. After a few minutes the mixture was extracted 3 times with ether. The combined organic layer was washed with 3% $NaHSO_3 \cdot H_2O$, dried and evaporated. To remove the *t*-butyldimethylsilyl protecting group at C-3, the residue was treated with 0.4 ml (0.4 mmol) of 1 M Bu_4NF in THF for 1 h. The THF was evaporated and the resulting residue was extracted from H_2O with ether. The ether was evaporated and the crude ester **VIII** was purified on a silica gel column with benzene/ethylacetate (25:1). Recrystallization from methanol gave 45 mg (65% yield) of **VIII**. Ester **VIII** was homogeneous by TLC and by analytical HPLC in system H_2, R_t = 13–14 min: m.p. 68–69°C; ^{1}H-NMR: δ0.872 (s, 3H, C_{13}-CH_3), 0.889 (t, 3H, J = 6.8 Hz, -OC-$(CH_2)_{16}$-$\mathbf{CH_3}$), 1.264–1.367 (broad, 30H,-OC-CH_2-$(\mathbf{CH_2})_{15}$-CH_3), 2.351 (*t*, 2H, J = 7.5 Hz, -OC-$\mathbf{CH_2}$-$(CH_2)_{15}$-CH_3), 3.477 (d, 1H, J = 1.8 Hz, $C_{17\beta}$-OH), 3.602 (dd, 1H, $J_{16\beta,17\alpha} = 4.9$ Hz, $J_{17\alpha,17\beta\text{-}OH} = 1.8$ Hz, $C_{17\alpha}$-H), 4.602 (s, 1H, C_3-OH), 4.832 (ddd, 1H, $J_{15\beta,16\beta} = 9.2$ Hz, $J_{16\beta,17\alpha} = 4.9$ and $J_{15\alpha,16\beta} = 1.6$ Hz, $C_{16\beta}$-H), 6.572 (d, 1H, J = 2.7 Hz, C_4-H), 6.637 (dd, 1H, J = 8.4 and J = 2.7 Hz, C_2-H), 7.157 (d, 1H, J = 8.44 Hz, C_1-H); HRMS, found 554.4320, calculated from $C_{36}O_4H_{58}$ 554.4320.

RESULTS AND DISCUSSION

We have synthesized the ring D stearate esters of estriol, the 2 monoesters **V** and **VIII**, as well as the diester **VIII**. The synthesis of the estriol esters **III**, **V** and **VIII** (Scheme 1) was based on the selective protection of the hydroxyl groups in estriol **I**, then esterification of the appropriate free hydroxyl groups, followed by cleavage of the protecting groups to form the desired esters with high yield. This procedure, which starts with estriol, allows stereochemical retention of the D-ring hydroxyl groups. The hydroxyl groups at C-3 and C-16α were protected by reaction with *N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoracetamide (MTBSTFA) in *N*,*N*-dimethylacetamide (DMA) [22]. The hindered C-17β hydroxyl group does not form a *t*-butyldimethylsilyl ether as has been reported for androst-5-ene-3β,17β-diol [23]. Since MTBSTFA reacts much faster with the phenolic C-3 hydroxyl than with the C-16α hydroxyl group, selective formation of 3-*t*-butyldimethylsilylestriol **II** was accomplished using 1.1/1

molar ratio of the reagent to estriol. Esterification of **II** with stearyl chloride in the presence of pyridine and deprotection under mild conditions with Bu_4NF in THF [24] led to ester **III**. The reaction of MTBSTFA with estriol in a molar ratio of 2.2/1, overnight produced 3,16α-di-*t*-butyldimethylsilylestriol **IV**. Following esterification of the free C-17β hydroxyl group in **IV**, the silyl ethers were cleaved with Bu_4NF/THF resulting in ester **V**. Conversion of the C-17β hydroxyl group in **IV** to the methoxymethyl ether [25] and cleavage of the *t*-butyldimethylsilyl ethers at C-3 and C-16α gave 17β-methoxymethylestriol **VI**. Selective silylation with MTBSTFA at the C-3 hydroxyl group produced 3-*t*-butyldimethylsilyl-17β-methoxymethylestriol **VII**. Following esterification of the hydroxyl group at C-16α with stearyl chloride, the methoxymethyl group was cleaved with dimethylboron bromide [26], and finally the *t*-butyldimethylsilyl group was removed from C-3 with Bu_4NF/THF producing ester **VIII**.

We tested these estriol esters for their estrogenic activity in several systems, comparing them to estradiol, estriol and estradiol-17-stearate. On the basis of the properties of estradiol-17-stearate [13], it would seem unlikely that the estriol esters would bind to the estrogen receptor and induce estrogenic effects directly while still esterified. However, in contrast to the C-17 esters of estradiol, the estriol esters **V** and **VIII** have a free hydroxyl group in the D-ring, a necessary component for binding to the estrogen receptor. Consequently, it was possible that these 2 monoesters, but not the diester **III**, might differ from the estradiol esters and bind to the estrogen receptor. However, as can be seen in Fig. 1, none of the estriol esters **III**, **V** and **VIII** compete for the binding of [^{3}H]estradiol to the estrogen receptor. Both estradiol and estriol inhibited the specific binding of [^{3}H]estradiol to the estrogen receptor. As would be expected [27], estriol is slightly less potent than estradiol.

We have developed the Ishikawa microtiter plate bioassay as means of easily determining the potency of estrogenic compounds [21]. These cells actively metabolize estrogens [28], and unlike the estrogen receptor assay in which metabolism is minimal, in the Ishikawa bioassay metabolic intervention is an important determinant of the magnitude of the estrogenic signal. With most estrogens there is a good correlation in potency between *in vivo* studies and the Ishikawa assay [21]. However, this is not true of estradiol-17-stearate which is a very strong estrogen *in vivo* [9, 10] and only weakly estrogenic in this assay [21]. The most likely explanation for this disparity is that the ester requires hydrolysis for its action and the Ishikawa cells have low esterase activity. Since the fatty acid protects the steroid esters from metabolic inactivation [12], we hypothesized that if the estriol esters could act directly with the estrogen receptor, they would be very active in the Ishikawa assay. However, the results of the induction of alkaline phosphatase in the Ishikawa cells show that they do not produce a strong estrogenic response (Fig. 2). In this experiment the order

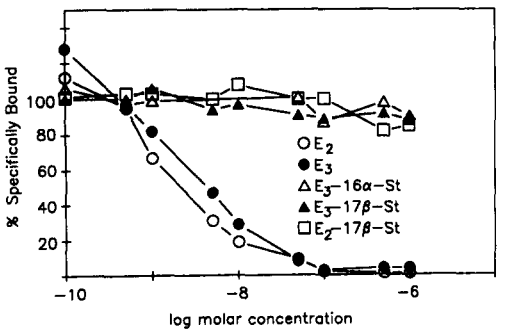


Fig. 1. Competition for binding to the estrogen receptor. The indicated C_{18}-steroids were incubated with rabbit uterine cytosol and [^{3}H]estradiol at 0°C overnight. The diester **III** did not compete for the binding of [^{3}H]estradiol. The curve for **III** has been omitted for clarity. The amount of [^{3}H]estradiol specifically bound in the absence of any competitor was 17%, and nonspecific binding in the presence of 200 nM diethylstilbestrol was less than 2% of the total radioactive ligand. E_2 = estradiol; E_3 = estriol; E_3-16α-St = estriol-16α-stearate **VIII**; E_3-17β-St = estriol-17β-stearate **V**; E_2-17β-St = estradiol-17β-stearate. Each point is the average of duplicate determinations.

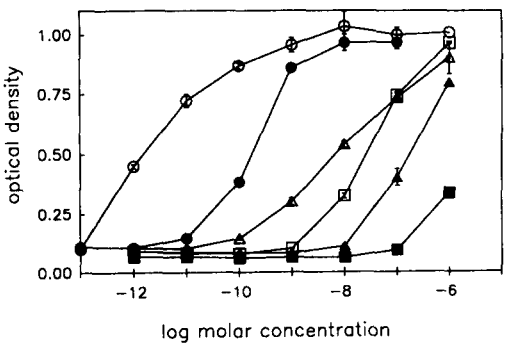


Fig. 2. Induction of alkaline phosphatase in Ishikawa cells. Ishikawa cells were grown in estrogen-free media in 96-well microtiter plates for 3 days with the various steroids. Alkaline phosphatase activity was determined with the chromogenic substrate *p*-nitrophenyl phosphate at 410 nm. ○ = estradiol; ● = estriol; △ = estradiol-17β-stearate; □ = **V**; ▲ = **VIII**; ■ = **III**. Each concentration of steroid was assayed in quadruplicate. Error bars are SEM, those not visible are smaller than the symbol.

of potency is estradiol > estriol > estradiol-17-stearate > V > VIII > III. This experiment agrees well with the study (Fig. 1) which showed that V, VIII, and III do not bind to the estrogen receptor. The lower potency of the estriol esters compared to estradiol-17-stearate, which must be hydrolyzed to stimulate Ishikawa alkaline phosphatase, indicates that in order for the estriol esters to act they too must be acted on by an esterase.

In contrast to the *in vitro* assays, when the esters are administered *in vivo* in a single subcutaneous injection, they are markedly more potent than both of the unesterified C_{18}-steroids (Fig. 3). Predictably estriol, was much less active than estradiol (note that estriol and its esters were administered in twice the dose as estradiol and estradiol-17-stearate). The uterotrophic stimulation was gone by 2 days after the injection of estriol and by about 3 days for estradiol. In comparison to the free steroids, all of the esters produced a sustained uterotrophic effect which lasted about 2 weeks. There was also a much greater stimulation of uterine weight caused by the esters. At maximum stimulation estradiol increased uterine weight over the controls at 24 h, 103%; estriol at 24 h, 45%; V at 6 days, 464%; VIII at 4 days, 362%; estradiol-17-stearate at 6 days, 442%.

The diester III produced a much less pronounced uterotrophic weight gain than either V and VIII. After the injection of III: at 4 days the uterine weight was stimulated 41%, at 8 days 31%, at 14 days 31%, returning to baseline between 2 and 3 weeks. We had expected that the diester III might elicit an even greater prolonged effect since it requires hydrolysis at both D-ring esters to liberate the estrogenic signal, estriol. However, while it did bring about a protracted uterine stimulation, the duration was not substantially greater than the monoesters.

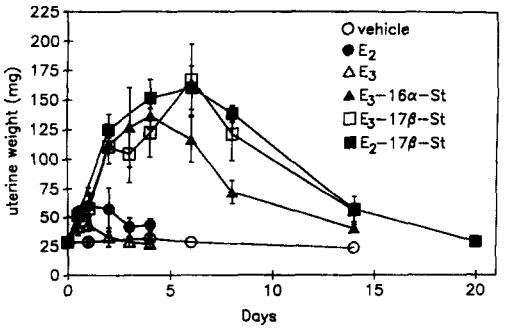


Fig. 3. Mouse uterotrophic assay. Adult ovariectomized mice were injected once with 132 nmol/kg of estradiol, estradiol-17-stearate, or 264 nmol/kg of estriol, III, V, VIII in 200 μl of aqueous ethanol. At the indicated times the animals were killed and the uteri removed and weighed. Abbreviations are defined in the legend to Fig. 1. Error bars are SD. At each point, *n* is not less than 4.

The mode of administration of the steroids in the uterotrophic experiment (Fig. 3) differs from our previous studies with estradiol-17-stearate [9, 10]. In those experiments the estrogens were injected intravenously rather than subcutaneously, as they are in the present study. The present route was chosen because we recently found that the steroidal esters are present in much higher amounts in tissues than in blood (unpublished observations), probably sequestered where they are synthesized. Consequently, we chose subcutaneous injections in an attempt to allow the esters to equilibrate with tissues before passing through the liver. Interestingly, with estradiol there is virtually no difference between the 2 forms of injection. However, comparing the results in Fig. 3 with those previously obtained with estradiol-17-stearate, there is a very large increase in potency when the ester is injected subcutaneously. Obviously these hydrophobic esters given in aqueous alcohol, mimic the increased estrogenic effect that is obtained when nonesterified estrogens are administered in a vehicle of oil rather than an aqueous one [19].

Estriol is a major metabolite of estradiol. In human pregnancy it is the predominant C_{18} estrogen, synthesized by the placenta from fetal 16α-hydroxylated C_{19} steroids [29]. While estriol is known to be estrogenic, it is relatively weak estrogen compared to estradiol [19]. This is borne out by all of our experiments (Figs 1–3). Estriol, was called an "impeded" estrogen [30], because it, like other weak estrogens, can inhibit the estrogenic action of estradiol. The low potency, as well as the inhibitory action of estriol, is caused by the short duration of its occupancy of the nuclear estrogen receptor when it is administered in a bolus injection [31]. However, when estriol is given in a sustained, more physiological manner, then it is a potent estrogens [31, 32]. It is apparent that the C-16α- and C-17β-estriol monoesters, V and VIII, are also producing a sustained stimulus. Most likely, the esters are sequestered in tissues, slowly releasing estriol over a period of many days. Thus, a dramatic estrogenic effect is produced. The biosynthetic formation of estriol esters would lead to a pool of preformed estrogen that can maintain estrogen dependent tissues for a considerable period. Our experiments with the estriol

esters show that these esters are extremely potent estrogens and that they could play an important physiological role. In addition, these esters would make exceptional pharmacological agents for prolonged estrogen stimulation.

Acknowledgements—This work was supported by NIH Grants CA37799 and CA29591. We thank Marcie Raeffer for her assistance in the preparation of this manuscript.

REFERENCES

1. Hochberg R. B., Pahuja S. L., Larner J. M. and Zielinski J. E.: Estradiol-fatty acid esters: endogenous long lived estrogens. *Proc. N.Y. Acad. Sci.* **595** (1990) 74–92.
2. Schatz F. and Hochberg R. B.: Lipoidal derivative of estradiol: the biosynthesis of a nonpolar estrogen metabolite. *Endocrinology* **109** (1981) 697–703.
3. Mellon-Nussbaum S., Ponticorvo L., Schatz F. and Hochberg R. B.: Estradiol fatty acid esters: the isolation and identification of the lipoidal derivative of estradiol synthesized in the bovine uterus. *J. Biol. Chem.* **257** (1982) 5678–5684.
4. Abul-Hajj Y. J.: Formation of estradiol-17β fatty acyl 17-esters in mammary tumors. *Steroids* **40** (1982). 149–155.
5. Adams J. B., Hall R. T. and Nott S.: Esterification-deesterification of estradiol by human mammary cancer cells in culture. *J. Steroid Biochem.* **24** (1986) 1159–1162.
6. Lee F. T. and Adams J. B.: Solubilisation and reconstitution of acylcoenzyme A: estradiol-17β acyltransferase. *Biochem. Biophys. Res. Commun.* **144** (1987) 569–575.
7. Martyn P., Smith D. L. and Adams J. B.: Properties of fatty acyl-coenzyme A: estradiol-17β acyltransferase in bovine placenta microsomes. *Molec. Cell. Endocr.* **60** (1988) 7–13.
8. Paris A. and Rao D.: Biosynthesis of estradiol-17β fatty acyl esters by microsomes derived from bovine liver and adrenals. *J. Steroid Biochem.* **33** (1989) 465–472.
9. Larner J. M., MacLusky N. J. and Hochberg R. B.: The naturally occurring C-17 fatty acid esters of estradiol are long-acting estrogens. *J. Steroid Biochem.* **22** (1985) 407–413.
10. MacLusky N. J., Larner J. M. and Hochberg R. B.: Actions of an estradiol-17-fatty acid ester in estrogen target tissues of the rat: comparison with other C-17 metabolites and a pharmacological C-17 ester. *Endocrinology* **124** (1989) 318–324.
11. Vazquez-Alcantara M. A., Menjivar M., Garcia G. A., Diaz-Zagoya J. C. and Garza-Flores J.: Long-acting estrogenic responses of estradiol fatty acid esters. *J. Steroid Biochem.* **33** (1989) 1111–1118.
12. Larner J. M. and Hochberg R. B.: The clearance and metabolism of estradiol and estradiol-17-esters in the rat. *Endocrinology* **117** (1985) 1209–1214.
13. Janocko L., Larner J. M. and Hochberg R. B.: The interaction of C-17-esters of estradiol with the estrogen receptor. *Endocrinology* **114** (1984) 1180–1186.
14. Katz J., Finlay T. H., Banerjee S. and Levitz M.: An estrogen-dependent esterase activity in MCF-7 cells. *J. Steroid Biochem.* **26** (1987) 687–692.
15. Lee F-T., Adams J. B., Garton A. J. and Yeaman S. J.: Hormone-sensitive lipase is involved in the hydrolysis of lipoidal derivatives of estrogens and other steroid hormones. *Biochim. Biophys. Acta* **963** (1988) 258–264.
16. Poulin R., Poirier D., Theriault C., Couture J., Belanger A. and Labrie F.: Wide spectrum of steroids serving as substrates for the formation of lipoidal derivatives in ZR-75-1 human breast cancer cells. *J. Steroid Biochem.* **35** (1990) 237–247.
17. Pahuja S. L., Zielinski J., Giordano G., McMurray W. J. and Hochberg R. B.: The biosynthesis of D-ring esters of estriol. Submitted for publication.
18. Holinka C. F., Hata H., Kuramoto H. and Gurpide E.: Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). *Cancer Res.* **46** (1986) 2771–2774.
19. Emmens C. W.: *Methods in Hormone Research* (Edited by R. I. Dorfman). Academic Press, New York (1962) pp. 59–111.
20. Brown T. J., MacLusky N. J., Toran-Allerand C. D., Zielinski J. E. and Hochberg R. B.: Characterization of 11β-methoxy-16α-[^{125}I]iodoestradiol binding: neuronal localization of estrogen binding sites in the developing rat brain. *Endocrinology* **124** (1989) 2074–2088.
21. Littlefield B. A., Gurpide E., Markiewcz L., McKinley B. and Hochberg R. B.: A simple and sensitive microtiter plate estrogen bioassay based on stimulation of alkaline phosphatase in Ishikawa cells: Estrogenic action of Δ^5 adrenal steroids. *Endocrinology* (In press).
22. Mawhinney T. P. and Madson M. A.: N-Methyl-N-(tert-butyldimethylsilyl)trifluoro-acetamide and related N-tert-Butyldimethylsilyl amides as protective silyl donors. *J. Org. Chem.* **47** (1982) 3336–3339.
23. Hosoda H., Fukushima D. K. and Fishman J.: Convenient, high yield conversion of androst-5-ene-3β-17β-diol to dehydroisoandrosterone. *J. Org. Chem.* **38** (1973) 4209–4211.
24. Corey E. J. and Venkateswarlu A.: Protection of hydroxyl groups as tert-butyldimethylsilyl derivatives. *J. Am. Chem. Soc.* **94** (1972) 6190–6191.
25. Stork G. and Takahashi T.: Chiral synthesis of prostaglandins (PGE_1) from D-glyceraldehyde. *J. Am. Chem. Soc.* **99** (1977) 1275–1276.
26. Guindon Y., Morton H. E. and Yoakim C.: Dimethylboron bromide and diphenylboron bromide. Acetal and ketal cleavage. Cleavage of MEM, MOM and MTM ethers. *Tetrahedron Lett.* **24** (1983) 3969–3972.
27. Korenman S.: Comparative binding affinity of estrogens and its relation to estrogenic potency. *Steroids* **13** (1969) 163–177.
28. Hata H., Holinka C. F., Pahuja S. L., Hochberg R. B., Kuramoto H. and Gurpide E.: Estradiol metabolism in Ishikawa endometrial cancer cells. *J. Steroid Biochem.* **26** (1987) 699–704.
29. Siiteri P. K. and MacDonald P. C.: The utilization of circulating dehydroisoandrosterone sulfate for estrogen synthesis during human pregnancy. *Steroids* **2** (1963) 713–730.
30. Huggins C. and Jensen E. V.: The depression of estrone-induced uterine growth by phenolic estrogens with oxygenated functions at positions 6 or 16: the impeded estrogens. *J. Exp. Med.* **102** (1955) 335–346.
31. Clark J. H. and Markaverich B. M. III: Receptors, mechanism of action and biological responses. The agonistic and antagonistic actions of estriol. *J. Steroid Biochem.* **20** (1984) 1005–1013.
32. Clark J. H., Paszko Z. and Peck E. J.: Nuclear binding and retention of the receptor estrogen complex: relation to the agonistic and antagonistic properties of estriol. *Endocrinology* **100** (1977) 91–96.