PREPARATION OF 14,15-SECOESTRA-1,3,5(10)-TRIEN-15-YNES, INHIBITORS OF ESTRADIOL DEHYDROGENASE

Richard J. Auchus,[a] James O. Palmer,[b] H.L. Carrell,[c] and Douglas F. Covey[a]

Departments of [a]Pharmacology and [b]Pathology, Washington University School of Medicine, St. Louis, MO 63110, USA; and [c]Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, PA 19111, USA

Corresponding author: Douglas F. Covey
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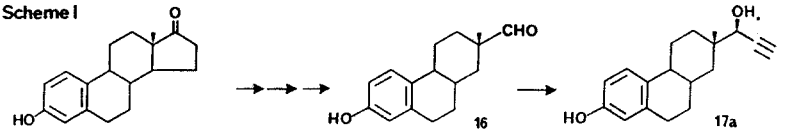
ABSTRACT

The conversion of estrone to 14,15-secoestratrien-15-ynes, inactivators of estradiol dehydrogenase from human term placenta, is described. The optically pure precursor 7-acetoxy-octahydro-2-phenanthrenecarboxylic acid methyl ester is prepared from estrone in five steps and 40% yield. The unsubstituted propargylic secoestratriene diol, a mechanism-based inactivator of estradiol dehydrogenase, and the corresponding acetylenic ketone, an affinity label inactivator of the same enzyme, arise from the phenanthrene ester in three and four steps. The propargylic secoestratriene diol also competes with [^{3}H]estradiol for binding to calf uterus estrogen receptor and possesses weak uterotrophic activity.

INTRODUCTION

As an extension of our efforts to develop mechanism-based inactivators ["suicide substrates" (1,2)] of hydroxysteroid dehydrogenases (3,4) and to evaluate acetylenic steroids as enzyme inhibitors (5-8), 15,16-acetylenic estradiol analogs were prepared as potential active site-directed inactivators of estradiol dehydrogenase (9). The synthesis of such acetylenic secosteroids requires replacing the D-ring with an (ethynyl)hydroxymethyl group. This approach concludes with the reaction of an octahydrophenanthrene carboxaldehyde and an ethynyl organometallic reagent (Scheme I). The synthesis of the intermediate 2-octahydrophenanthrene carbonyl compounds, in turn, requires the selective oxidation of estrone to remove carbons 15 and 16 efficiently. Since literature approaches to estrone D-ring fragmentation were found unsuitable for the target molecules, an economical, high yield approach to the reductive

Scheme I



excision of carbons 15 and 16 from estrone was developed. As described previously, alcohol **17a** is a mechanism-based inactivator of estradiol dehydrogenase from human term placenta (10). Additional in vitro and in vivo data are reported here for alcohol **17a**, as well as for intermediates alcohol **15** and aldehyde **16** (Scheme IV).

EXPERIMENTAL

General Methods

Estrone, NAD^+, and NADH were purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel, mesh 32-60 (flash chromatography) and dry column grade, were purchased from Universal Scientific, Inc. (Atlanta, GA). Thin-layer chromatography (TLC) utilized GF/B 250-μm silica gel plates (Analtech, Inc., Newark, DE). High performance liquid chromatography (HPLC) was performed on a Waters Associates (Milford, MA) instrument using an RCM-100 equipped with 10-μm silica gel or octadecylsilyl-based cartridges. Disposable octadecylsilyl columns were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Mass spectra were recorded on a model 4000 mass spectrometer (Finnigan MAT, San Jose, CA) in electron ionization mode (ionizing energy, 70 eV); fast atom bombardment mass spectra (FAB-MS) were recorded on a ZAB-SE double-focusing mass spectrometer (VG Instruments, Danbury, MA) in 50% glycerol matrix (ion source, 8000 V). NMR spectra, in $CDCl_3$ unless noted, were recorded on an XL-300 spectrometer (Varian Associates, Inc., Palo Alto, CA) at either 300 MHz (^1H) or 75 MHz (^{13}C) or, when indicated, a Varian T-60 (60 MHz, ^1H). Chemical shifts are reported in ppm relative to tetramethylsilane. Infrared (IR) spectra were recorded as films on NaCl disks with a model 1710 FT-IR (Perkin-Elmer Corp., Norwalk, CT). Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Melting points (uncorrected) were determined on a model 40 hot stage (Thomas Scientific, Philadelphia, PA).

3-Hydroxyestra-1,3,5(10),14-tetraen-17-one acetate, (14R,15S)-14-ozonide (2)

Enone **1** (11) (157 mg, 0.5 mmol) in methylene chloride (50 mL) was chilled to -78° C and treated with O_3 from a Welsbach T-408 ozonator (Welsbach Ozone Systems Corp., Philadelphia, PA) until a blue color persisted. The excess ozone was reduced with a few drops of dimethyl sulfide, and the reaction mixture was concentrated in vacuo to a yellow oil. IR 1754 (s,acetate C=O), 1724 (s,C=O), 1212 cm^{-1} (br.C-O); ^1H-NMR (60 MHz) δ 1.24 (s,3,CH_3), 2.28 (s,3,CH_3CO), 6.0-6.1 (m,1,OO(O)CH); FAB-MS, m/e 359 (MH^+).

3-Hydroxy-14,17-dioxo-14,15-secoestra-1,3,5(10)-trien-15-al acetate (3)

Enone 1 (295 mg, 0.95 mmol) in 50 mL CH_2Cl_2 was treated with ozone as above. Crude 2 was dissolved in glacial acetic acid (10 mL) and stirred with zinc dust (762 mg, 11.7 mmol) at room temperature for 30 min. The reaction mixture was diluted with 100 mL diethyl ether and filtered. The filtrate was washed with water (3 x 50 mL) and 5% $NaHCO_3$ (50 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo to a white foam, 290 mg (89% crude yield). The product was homogeneous by TLC (9:1 $CHCl_3$/EtOAc, R_f = 0.20) but appeared to be a mixture (ca. 60:40) of epimers at C-8. ^{1}H-NMR (60 MHz) major isomer, δ 1.42 (s,3,CH_3), 2.30 (s,3,CH_3CO), 3.4-3.8 (m,1,H C-8), 5.44 (d,1,J=6 Hz,CHOH), 7.16 (d,1,J=6 Hz,CH=CHOH); minor isomer, δ 1.24 (s,3,CH_3), 2.30 (s,3,CH_3CO), 3.4-3.8 (m,1,H C-8), 5.64 (d,1,J=5 Hz,CHOH), 7.66 (d,1,J=5 Hz,CH=CHOH).

Estra-1,3,5(10),16-tetraene-3,17-diol diacetate (4)

This compound was prepared from estrone as described (12), using p-toluenesulfonic acid (5-10%) in place of sulfuric acid as catalyst and chromatographing the products on dry column grade silica gel with 1:1 methylene chloride/hexanes as eluant. Yields averaged 70-75%.

Methyl 3-hydroxy-16-oxo-16,17-secoestra-1,3,5(10)-trien-17-oate, acetate (11)

A solution of enol acetate 4 (12) (1.42 g, 4 mmol) in methylene chloride (40 mL) and glacial acetic acid (3.2 mL) was chilled to -78°C. Ozone was bubbled into the solution through a gas dispersion tube at a rate of 1.2 L/min until the reaction mixture became bright blue (less than 5 min). Dimethyl sulfide (0.6 mL, 8 mmol) was added with stirring, and the reaction mixture was warmed to 0°C. Glacial acetic acid (36.8 mL), then distilled water (12 mL) were added, and the reaction mixture was stirred at room temperature for 5 h when no anhydride remained (disappearance of 1811 cm^{-1} absorbance). The reaction mixture was poured into 100 mL each water and methylene chloride, shaken, and separated. The organic layer was washed with 100 mL water and 50 mL saturated NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo to a clear, colorless oil. The residue was taken up in 10 mL diethyl ether, chilled to 0°C, and treated with an excess of diazomethane (30 mL of a ca. 0.35 M solution in diethyl ether). The reaction mixture was coated on 3 g silica gel and purified by flash chromatography on 30 g silica gel, eluting with a stepwise gradient of ethyl acetate in hexanes. Compound 11 eluted with 20% ethyl acetate in hexanes. Concentration of the eluate in vacuo afforded 1.31 g white plates (92% yield); mp 98-101°C; IR 2722 (w,ald. C-H), 1757 (s,acetate C=O), 1723 (s,ald. C=O,ester C=O), 1209 cm^{-1} (s,C-O); ^{1}H-NMR δ 1.17 (s,3,CH_3), 2.27 (s,3,CH_3CO), 3.69 (s,3,OCH_3), 9.77 (dd,1,J=1.2 Hz,CHO); ^{13}C-NMR δ 167.7 (CH_3CO), 178.0 (CO_2CH_3), 201.6 (CHO); MS, m/e 358 (M^+); Anal. Calcd for $C_{21}H_{26}O_5$: C, 70.37; H, 7.31. Found: C, 70.51; H, 7.34.

Methyl 3,16-dihydroxy-16,17-secoestra-1,3,5(10),15-tetraen-17-oate, diacetate (12)

A solution of aldehyde 11 (1.31 g, 3.65 mmol) in isopropenyl acetate (20 mL) with p-toluenesulfonic acid (50 mg) was distilled slowly through a short Vigreaux column equipped with a short-path condenser and a drying tube for 22 h. Periodically, about 10 mL distillate was collected by raising the reaction temperature, and fresh isopropenyl acetate plus additional p-toluenesulfonic acid were added (a total of 55 mL

isopropenyl acetate and 100 mg p-toluenesulfonic acid). The reaction mixture was cooled to room temperature, poured into 50 mL each diethyl ether and dilute aqueous $NaHCO_3$, shaken, and separated. The organic layer was washed with 50 mL water and 25 mL saturated NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo to a red oil. The crude product was coated on 3.5 g silica gel and purified by flash chromatography on 25 g silica gel with a stepwise gradient of ethyl acetate in hexanes (enol acetates **12** eluted with 15% ethyl acetate in hexanes). The solvent was removed in vacuo to give 1.23 g of a white foam (84% yield); IR 1757 (s,acetate C=O), 1734 (s,ester C=O), 1669 cm^{-1} (w,C=C); ^{1}H-NMR (E isomer) δ 1.18 (s,3,CH_3), 2.11 (s,3,C=CHOCOCH_3), 2.29 (s,3,CH_3CO), 3.69 (s,3,OCH_3), 5.19 (dd,1,J=11,12 Hz,CH=CHOAc), 7.03 (d,1,J=12 Hz,CHOAc); (Z isomer) δ 1.20 (s,3,CH_3), 2.17 (s,3,CHOCOCH_3), 2.29 (s,3,CH_3CO), 3.62 (OCH_3), 4.70 (dd,1,J=7,11 Hz,CH=CHOAc), 7.09 (d,1,J=7 Hz,CHOAc); MS, m/e 400 (M^+); Anal. Calcd for $C_{23}H_{28}O_6$: C, 68.98; H, 7.05. Found: C, 69.21; H, 7.06.

Methyl 3-hydroxy-15-oxo-15,16-seco-D-norestra-1,3,5(10)-trien-16-oate, acetate (13)

A solution of enol acetates **12** (1.23 g, 3.0 mmol) in methylene chloride (30 mL) and glacial acetic acid (2.4 mL) was chilled to -75°C. Ozone was bubbled into the reaction mixture through a gas dispersion tube (1.2 L/min) until a faint blue color persisted (ca. 5 min). Dimethyl sulfide (0.45 mL, 6 mmol) was added with stirring, and the reaction mixture was warmed to room temperature over 15 min. Distilled water (190 μL) was added, and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was washed twice with 20 mL water and once with 15 mL saturated NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo to a pale yellow oil. Application of a high vacuum (< 1 mm Hg) prompted the formation of white crystals. The crystals were heated in a small amount of diethyl ether and then chilled on ice. The solvent was removed with suction, and the crystals were washed with cold hexanes, affording 895 mg small white leaflets (87% yield), mp 111-115°C. Recrystallization from methylene chloride/hexane afforded white prisms, mp 121-124°C; IR 2736 (w,ald. C-H), 1757 (s,acetate C=O), 1723 (s,ald. C=O,ester C=O), 1211 cm^{-1} (s,C-O); ^{1}H-NMR δ 1.28 (s,3,CH_3), 2.29 (s,3,CH_3CO), 3.74 (s,3,OCH_3), 9.80 (d,1,J=3 Hz,CHO); ^{13}C-NMR δ 169.7 (CH_3CO), 177.2 (CO_2CH_3), 203.9 (CHO); MS, m/e 344 (M^+); Anal. Calcd for $C_{20}H_{24}O_5$: C, 69.75; H, 7.02. Found: C, 69.67; H, 7.13.

Methyl (2aS,4aS,10aS)-1,2,3,4,4a,9,10,10a-octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxylate, acetate (14)

Aldehyde **13** (1.74 g, 5 mmol) and tris(triphenylphosphine)rhodium [I] chloride (4.77 g, 5 mmol) were heated under nitrogen at 135°C in benzonitrile (25 mL) for 5.5 h as the deep burgundy red solution gradually grew lighter in color. Upon cooling, a yellow solid deposited from the solution. Most of the benzonitrile was removed by careful distillation at reduced pressure, and the warm suspension was stirred while adding 50 mL absolute ethanol. After chilling, the precipitated greenish-yellow solid was filtered off and washed with absolute ethanol, giving 3.53 g of complex. The filtrates were concentrated in vacuo to a red oil which was coated on 7 g silica gel and purified by flash chromatography on a column of 60 g silica gel with a stepwise gradient of ethyl acetate in hexanes. Leftover benzonitrile was thoroughly eluted with

5% ethyl acetate in hexanes before eluting ester **14** with 7.5-10% ethyl acetate in hexanes. Removal of solvents in vacuo afforded 1.40 g (89% yield) of a clear, slightly brownish oil. The oil solidifies on standing at -20°C and may be recrystallized from hexanes to give white prisms, mp 58-61°C. It is advisable to chill the crude reaction mixture and to filter off the yellow complex before distillation to maximize removal of benzonitrile. IR 1757 (s,acetate C=O), 1726 (s,ester C=O), 1213 cm^{-1} (s,C-O); ^{1}H-NMR δ 1.25 (s,3,CH_3), 2.27 (s,3,CH_3CO), 3.69 (s,3,OCH_3); ^{13}C-NMR δ 169.7 (CH_3CO), 178.9 (CO_2CH_3) MS, m/e 316 (M^+); Anal. Calcd for $C_{19}H_{24}O_4$: C, 72.13; H, 7.65. Found: C, 72.15; H, 7.73.

(2S,4aS,10aS)-1,2,3,4,4a,9,10,10a-Octahydro-7-hydroxy-2-methyl-2-phenanthrenemethanol (15)

A solution of ester **14** (1.71 g, 5.5 mmol) in toluene (50 mL) was chilled to -78°C under nitrogen and treated with an excess of diisobutyl aluminium hydride (62 mL of a 1.0 M solution in toluene) in a dropwise stream. After 15 min, the stirred reaction mixture was cautiously treated with 100 mL saturated NH_4Cl solution and 150 mL 10% HCl to decompose the aluminum alkyls (producing vigorous gas evolution) and to dissolve the gelatinous aluminum hydroxides. The aqueous layer was back-extracted twice with 50 mL ethyl acetate, and the pooled organic layers were washed with 75 mL water and filtered. Removal of solvent in vacuo precipitated a white solid that was recrystallized from diethyl ether/hexanes (plus a small amount of ethyl acetate to complete dissolution), affording two crops of white prisms, 1.15 and 0.05 g (total, 1.2 g, 90% yield); mp 139-142°C; IR 3330 cm^{-1} (br,OH); ^{1}H-NMR δ 0.96 (s,3,CH_3), 3.36 (d,2,J=5 Hz,CH_2OH), 4.68 (br,1,ArOH); ^{13}C-NMR (CD_3OD) δ 74.8 (CH_2OH); MS, m/e 246 (M^+); Anal. Calcd for $C_{16}H_{22}O_2$: C, 78.01; H, 9.00. Found: C, 77.79; H, 8.88.

(2S,4aS,10aS)-1,2,3,4,4a,9,10,10a-Octahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxaldehyde (16)

A solution of oxalyl chloride (0.64 mL, 7.3 mmol) in methylene chloride (15 mL) was chilled to -60°C under nitrogen. Dimethyl sulfoxide (1.05 mL, 14.7 mmol) in methylene chloride (3 mL) was added dropwise to the stirred solution, producing vigorous gas evolution during the first half of the addition. The reagent was stirred at -60°C for 5 min, then a solution of alcohol **15** (1.2 g, 4.9 mmol), dissolved in 1.5 mL dimethyl sulfoxide and diluted with 3.5 mL methylene chloride, was added in a steady stream. A white precipitate formed as the reaction mixture was warmed to -10°C over 30 min. The reaction mixture was then chilled to -50°C and treated dropwise with triethylamine (3.4 mL, 24.5 mmol). The suspension was poured into 120 mL ethyl acetate, which was washed twice with water and once with saturated NaCl solution (50 mL each time), dried over Na_2SO_4, and filtered. The crude product was coated on 4 g silica gel and purified by flash chromatography on 25 g silica gel with a stepwise gradient of ethyl acetate in hexanes (aldehyde **16** eluted with 20% ethyl acetate in hexanes). Removal of solvents in vacuo and recrystallization from diethyl ether/hexanes or methylene chloride/hexanes (plus enough ethyl acetate to complete dissolution) gave two crops of white prisms, 712 mg combined. Further chromatography of the mother liquors produced another 104 mg for a total of 816 mg (69% yield); mp 159-163°C;

IR 3405 (br,OH), 2711 (w,ald. C-H), 1721 cm^{-1} (s,ald. C=O); ^{1}H-NMR δ 1.15 (s,3,CH_3), 5.3 (br,1,OH), 9.47 (s,1,CHO); ^{13}C-NMR δ 206.5 (CHO); MS, m/e 244 (M^+); Anal. Calcd for $C_{16}H_{20}O_2$: C, 78.65; H, 8.25. Found: C, 78.40; H, 8.23.

17(R,S)-14,15-Secoestra-1,3,5(10)-trien-15-yne-3,17-diols (17)

Acetylene (passed through a -70°C trap and H_2SO_4) was bubbled at room temperature into tetrahydrofuran (THF) (30 mL, freshly distilled from lithium aluminum hydride) for 5 min before and during the dropwise addition of methylmagnesium bromide in diethyl ether (2 mL, 4 mmol), all at room temperature. Acetylene was bubbled into the clear, purple solution for another 30 min. Nitrogen was passed over the ethynylmagnesium bromide solution, and aldehyde **16** (195 mg, 0.8 mmol) in dry THF (2 mL) was added dropwise, followed by two rinses with THF (1 mL each). Vigorous stirring was necessary to disperse the gelatinous white precipitate that formed during the addition of aldehyde **16**. After stirring for 15 min, the reaction mixture was poured into 80 mL each ethyl acetate and saturated NH_4Cl solution, acidified with 5 mL 10% HCl, shaken, and separated. The organic layer was washed with 70 mL saturated NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was coated on 1 g silica gel and purified by flash chromatography on 10 g silica gel with a stepwise gradient of ethyl acetate in hexanes (diols **17** eluted with 20-25% ethyl acetate in hexanes). The concentrated eluates were crystallized with diethyl ether and hexanes, affording 87 mg (40% yield) white powder, mp 191-197°C. A second crop (4.5 mg, mp 195-197°C) was collected, and the mother liquors (ca. 100 mg of a white foam) were reserved for the following oxidation (vide infra). IR 3400 (br,OH), 3261 (s,OH), 2115 cm^{-1} (w,C≡C); ^{1}H-NMR δ 1.06 (s,3,CH_3), 2.50 (d,1,J=2 Hz,C≡CH), 4.03 (dd,1,J=2,6 Hz,CHOH {6 Hz coupling to OH often not observed}), 4.56 (s,1,ArOH); ^{13}C-NMR (CD_3OD) δ 72.9 (CHOH), 74.8 (C≡CH), 84.5 (C≡CH); MS, m/e 270 (M^+); Anal. Calcd for $C_{18}H_{22}O_2$: C, 79.96; H,8.20. Found: C, 79.89; H, 8.18. The alcohols were also prepared using lithium acetylide in THF at -78°C as described elsewhere (13).

3-Hydroxy-14,15-secoestra-1,3,5(10)-trien-15-yn-17-one (18)

A solution of diols **17** (27 mg, 0.1 mmol) in acetone (10 mL) was stirred at 0°C and treated dropwise with Jones reagent (14) (65 μL), producing a thick, brownish green precipitate. The reaction mixture was warmed to room temperature, stirred for 15 min, and quenched with two drops of methanol. The acetone was removed in vacuo at room temperature, and the residue was taken up in 2 mL each ethyl acetate and water, vortexed, and separated. The aqueous layer was extracted with 1 mL ethyl acetate, and the pooled organic layers were washed with saturated NaCl solution (1 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was applied to a Pasteur pipette column of silica gel in a minimal amount of methylene chloride and eluted with a stepwise gradient of ethyl acetate in hexanes under gentle air pressure (ketone **18** eluted with 10 mL 15% ethyl acetate in hexanes). Recrystallization from methylene chloride/hexanes gave 19.5 mg (73% yield) amorphous solid and plates, mp 169-173°C. IR 3434, 3216 (s,OH), 2088 (m,C≡C), 1657 cm^{-1} (s,C=O); ^{1}H-NMR δ 1.27 (s,3,CH_3), 3.29 (s,1,C≡CH), 5.07 (br,1,OH); ^{13}C-NMR δ 80.4 (C≡C), 193.8 (C=O); MS, m/e 268 (M^+); Anal. Calcd for $C_{18}H_{20}O_2$: C, 80.56; H, 7.51. Found: C, 80.69; H, 7.62.

Enzymic Reduction of 18 to 17a

Ketone 18 (3 mg, 11 μmol) was dissolved in absolute ethanol (0.5 mL), treated with NADH (23 mg, 30 μmol) in 4 mL 0.1 M sodium phosphate buffer (pH 7.4, 20% glycerol), and mixed with purified (10) estradiol dehydrogenase (500 μg). After incubating at 25°C for 1.5 h, the reaction mixture was applied to a 3 mL octadecylsilyl column, washed with 6 mL water, and eluted with 5 mL methanol. The methanolic eluate was concentrated in vacuo to a wet residue, which was dissolved in acetone and filtered. The crude material was purified by HPLC (silica cartridge eluted with 10% acetone/90% hexanes, t_r = 5.5 min, ketone 18; 12 min, alcohol 17a at 4 mL/min). The purified alcohol was recrystallized from diethyl ether/hexanes to give 1.8 mg thick white needles (60% yield); mp 199-202°C.

Crystallography of Enzymatically Generated 17a

Crystals of 14,15-secoestra-1,3,5(10)-trien-15-yne-3,17β-diol ($C_{18}H_{22}O_2$) are orthorhombic with a = 11.889(2), b = 16.556(5), and c = 7.620(2) Å. The space group is $P2_12_12_1$ with four molecules of formula weight 270.37 such that ρ_{calc} = 1.197 g·cm^{-3}. Three-dimensional x-ray diffraction intensity data were measured using a Syntex $P2_1$ automated diffractometer (Nicolet, Madison, WI) equipped with a graphite monochromator. Cu(K_α) radiation was used for θ-2θ variable rate scans to a maximum of 138 2θ ($\sin(\theta)/\lambda$ = 0.602 Å^{-1}). A total of 1637 unique data were measured of which 1556 had intensity values of $I_{meas} > 2.0\ \sigma(I)$. The data were Lorentz and polarization corrected, but no correction was made for absorption [μ(Cu K_α) = 5.22 cm^{-1}].

The structure was solved by direct methods using MULTAN80 (15), and the trial structure was first refined by successive structure factor calculations followed by Fourier synthesis. The structure was then refined by full matrix least squares (16,17) first with isotropic temperature factors, then with anisotropic temperature factors. All 22 hydrogen atoms were located in a difference electron density map. The full matrix least squares refinement was then carried out including hydrogen atoms in the refinement (C and O, anisotropic; H, isotropic). For the 1556 data included in the refinement, the final conventional R value was 0.035, and the weighted R (R_w) was 0.042. The quantity minimized was $\Sigma w(F_{obs} - F_{calc})^2$. For the refinement, the observed reflections were assigned a weight $w = 1/\sigma^2$, whereas the unobserved data were assigned w = 0. Values $\sigma(F)$ were determined from the relation $\sigma(F) = (F/2)\{[\sigma^2(I)/I^2] + \delta^2\}^{1/2}$, where δ = 0.022 and represents the instrumental uncertainty as determined from the intensities of four periodically scanned check reflections. The atomic scattering factors used for the calculations were taken from the literature (18).

Enzymic Reduction of Aldehyde 16

Aldehyde 16 (5-50 μM) was incubated at 25°C in 1 mL 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.2, 20% glycerol) containing 2 μg estradiol dehydrogenase, 200 μM NADH, and 0.5% ethanol. Loss of absorbance at 340 nm was monitored in a Beckman DU-8 spectrophotometer (10). Using the linear (initial) portion of the assay, K_m and V_{max} values were obtained from least squares fit of the v^{-1} vs. $[S]^{-1}$ plot (means of three experiments, r = 0.999).

Inhibition of Estradiol Oxidation by Alcohol 15 and Aldehyde 16

Estradiol (2.5-60 μM) and 40 μM aldehyde **16** or alcohol **15** were incubated at 25°C in 1 mL 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.2, 0.8% human albumin) containing 2 μg estradiol dehydrogenase, 200 μM NAD^+, and 0.7% ethanol. Absorbance at 340 nm was monitored for 2.25 min. K_m^{app} for estradiol was calculated from double-reciprocal plots (means of two experiments, $r \geq 0.98$), and K_I values were calculated using the equation (19): $K_m^{app} = (K_m/K_I)[I] + K_m$.

Uterotrophic Assays (20)

CD-1 female mice (12-15 g) were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA); olive oil was obtained from Sigma Chemical Co.; and estradiol was obtained from Steraloids, Inc. (Wilton, NH). Mice were injected subcutaneously once daily for 3 days with the compound(s) dissolved in 5 mL/kg olive oil. On the fourth day, the uteri were dissected; their fluid was expressed; and the tissue was blotted and weighed.

Estrogen Receptor Binding Assays

The following reagents were obtained from the indicated sources: [^{3}H]Estradiol (92 Ci/mmol) from Amersham Corp. (Arlington Heights, IL); dextran (MW 71,200), activated charcoal, EDTA, Tris, estradiol, and bovine serum albumin (BSA) from Sigma Chemical Co.; dithiothreitol from Boehringer-Mannheim Diagnostics, Inc. (Houston, TX); and dimethylformamide from Fisher Scientific Co. (Pittsburgh, PA). The following buffers and reagents were prepared: Tris buffer (TED) (10 mM Tris·HCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.4); and dextran-coated charcoal (DCC) (0.5% charcoal, 0.05% dextran, 1 mg/mL BSA in TED). Calf uteri (< 30 g) were obtained at a local abbatoir and held on ice for transport to the laboratory. Cytosols were prepared as described by Puca et al (21). The cytosol was stored in 10-mL aliquots at -70°C. Protein concentrations were estimated by the method of Bradford (22) using BSA as standard. All cytosols were diluted to 1 mg protein/mL with TED before use.

Binding of ligands to the estrogen receptor was evaluated by competition with [^{3}H]-estradiol, and free hormone was removed with DCC (23). Thirteen points, each in triplicate, were obtained for each curve. Unlabeled ligand (10^{-15} - 10^{-7} M final concentration) in 10 μL dimethylformamide was added to a microcentrifuge tube containing 0.2 mL cytosol (78 $\pm$ 5 fmol sites/mg protein) and 0.1 mL of 0.5 nM [^{3}H]estradiol. The tubes were incubated at 4°C for 18 h, then unbound hormone was removed by addition of 0.5 mL DCC. The charcoal was pelleted in a microcentrifuge, and a 0.5-mL aliquot was removed and counted in an model 1217 scintillation counter (LKB Instruments, Inc., Gaithersburg, MD). Counting efficiency was determined using an external standard. The binding data were analyzed by computerized least-squares curve fitting (24). Nonspecific binding was 1.2 $\pm$ 0.1%.

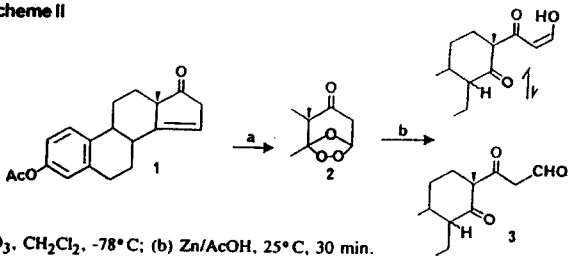
RESULTS AND DISCUSSION

Chemistry

Procedures that cleave the D-ring of estrone derivatives using CCl_4/KOH/t-BuOH (25), KOH fusion (26,27), $Pb(OAc)_4$ (27,28), and O_3 (27,29) have been developed to prepare marrianolic and doisynolic acids or analogs. Further selective, efficient degradation of these steroids, however, could not be realized. Several attempts to cleave α,β- and β,γ-unsaturated estrone derivatives with osmium tetroxide/periodate (30) or Jones reagent (14), or by Tanabe-Eschenmoser fragmentation (31-33) likewise gave low yields with significant side reactions.

Ozonolysis of the β,γ-enone **1** (11) proved unexpectedly simple. Although steroids with electron-rich phenolic A-rings (3-hydroxy or 3-methoxy) are known to undergo further oxidation and decomposition, presumably due to oxidation at the benzylic C-9 (29), ozonolysis of **1** (-78°C, CH_2Cl_2) cleanly produces a stable ozonide **2** which is reduced to the secosteroid **3** with zinc/acetic acid but not with milder reducing agents such as dimethyl sulfide (Scheme II). Analogous chemistry of this sort has been observed in the androstane series (34,35). The simple expedient of deactivating the A-ring with an electron-withdrawing acetate group at C-3 prevents over-oxidation and obviates using premeasured ozone solutions (29) or limiting ozone consumption to 1 equiv (27,36). Attempts to remove the terminal two carbon atoms from the β-keto aldehyde **3** using Jones reagent (14), trimethylene dithiotosylate/KOH (37), or $PhSeCl/H_2O_2$ (38), however, met with limited success. Furthermore, the product was believed to be epimeric at C-8 (see ^{1}H-NMR in Experimental), so routes involving 14-keto secosteroids were abandoned.

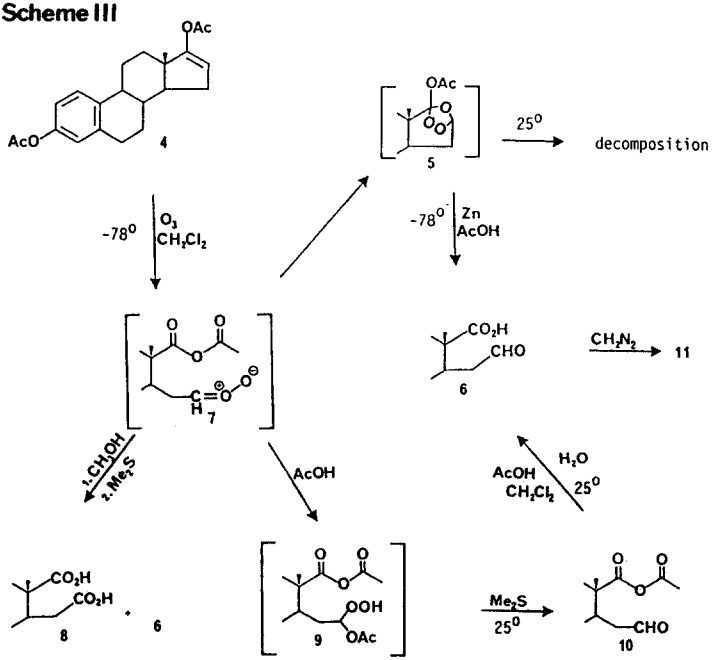
Scheme II



(a) O_3, CH_2Cl_2, -78°C; (b) Zn/AcOH, 25°C, 30 min.

In contrast to the uncomplicated ozonolysis of **1**, initial attempts to effect ozonolysis of enol acetate **4** (12) (Scheme III) in methylene chloride produced multiple uncharacterized products. Nevertheless, if the reaction mixture is maintained at -78°C and rapidly treated with zinc and acetic acid, modest but variable (30-50%) yields of the marrianolic semialdehyde **6** are obtained.

Scheme III

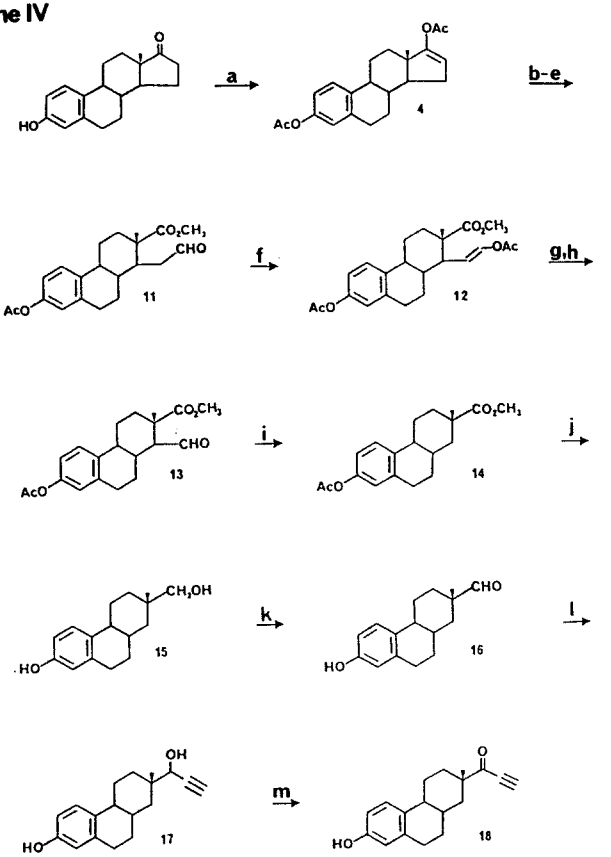


A thorough investigation of various reaction conditions (methylene chloride and/or methanol, plus or minus acetic acid or pyridine) revealed that, in 1:1 methylene chloride/methanol, a substantial portion of the ozonide undergoes further oxidation to diacid **8** (identified by ^{1}H-NMR spectrum of its dimethyl ester). On the other hand, ozonolysis in 8% acetic acid/methylene chloride at -78° C, followed by dimethyl sulfide addition, smoothly generates a single product. The compound was not isolated but was tentatively identified as anhydride **10** by its intense IR absorbance at 1811 cm^{-1}.

The anhydride proved rather stable under the reaction conditions, and the aforementioned variable yields probably derive from failure to subject **10** to adequate hydrolysis conditions. Direct methanolysis of **10** gives a mixture of products, but complete hydrolysis is achieved by stirring crude **10** with silica gel or with 10-13% water in 1:1 acetic acid/methylene chloride for several hours. Esterification of the crude product with diazomethane affords nearly quantitative yields of the marrianolic semialdehyde **11** from **4**.

The lower homolog of aldehyde **11** was similarly prepared by ozonolysis and reduction of enol acetates **12** (Scheme IV). Uncomplicated ozonolysis of E- and Z-enol acetates **12** can be achieved in 8% acetic acid/methylene chloride or in 1:1 methanol/methylene chloride at -78° C, affording crystalline aldehyde **13**. Decarbonylation of **13** with tris(triphenylphosphine)rhodium [I] chloride could not be achieved in either refluxing benzene or toluene, and low yields of **14** with extensive dimerization of reagent occurs in refluxing xylene. When conducted in benzonitrile, a high-boiling, complexing solvent that prevents dimerization of the reagent (39), nearly quantitative decarbonylation is realized with 1 equiv of reagent (Scheme IV).

Scheme IV



(a)$CH_2=C(CH_3)OAc/TsOH$, ↑↓ (75%); (b)O_3, $CH_2Cl_2/AcOH$, -78°C; (c)$(CH_3)_2S$, -78°C→RT; (d)AcOH, H_2O; (e)CH_2N_2 (92%); (f)$CH_2=C(CH_3)OAc/TsOH$, ↑↓ (84%); (g)O_3, $CH_2Cl_2/AcOH$, -78°C; (h)$(CH_3)_2S$, -78°C→RT (87%); (i)$(Ph_3P)_3RhCl$, PhCN, 130-140°C (89%); (j)i-Bu_2AlH, $PhCH_3$, -78°C (90%); (k)$(COCl)_2$/DMSO, CH_2Cl_2/DMSO, -60°→-10°C (69%); (l)HC≡CMgBr, THF, RT (80-85%); (m)Jones reagent, acetone, 0°C→RT (73%).

Reduction and deacylation of **14** with lithium aluminum hydride in THF gave low yields, possibly because of the poor solubility of intermediate aluminum alkoxides. Excess diisobutylaluminum hydride in toluene, on the other hand, furnishes excellent yields of diol **15**. Swern oxidation (40) proved superior to pyridinium chlorochromate in generating aldehyde **16**. Reaction of **16** with excess ethynylmagnesium bromide gives alcohols **17**, and cautious oxidation with a minimum of Jones reagent (14) completes the synthesis of ketone **18**.

Although no evidence for epimeric propargyl alcohols was detected by chromatography (TLC and both straight- and reverse-phase HPLC) or spectroscopy (^{1}H- and ^{13}C-NMR), only 40% or less of the purified alcohol can be isolated as a semi-crystalline powder. Chemical shift enhancement of the ^{1}H-NMR spectrum of crude **17**

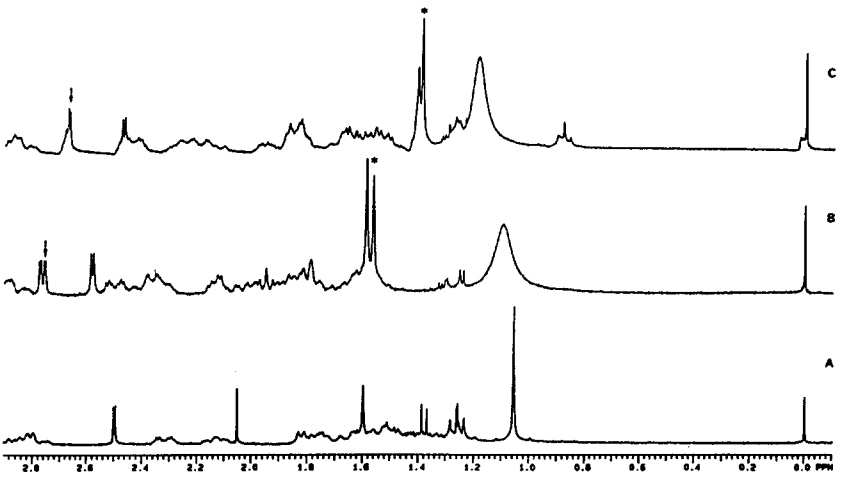


Figure 1. 300-MHz ^{1}H-NMR spectra of (A) unfractionated synthetic alcohols **17**; (B) unfractionated synthetic alcohols **17** plus Eu(FOD)$_3$, showing a 1:1 mixture of isomers; (C) mixture of synthetic alcohols **17** and enzymatically generated alcohol **17a** plus Eu(FOD)$_3$. Note the predominance of the acetylenic proton signals (↓) and methyl protons signal (*) at higher field in (C).

with the lanthanide shift reagent $Eu(FOD)_3$, however, revealed a ≈1:1 mixture of diastereomers arising from reaction with either ethynylmagnesium bromide at room temperature or with lithium acetylide at -78°C (Fig. 1). Even from the 1:1 mixture, the isomer whose resonances shift less downfield preferentially crystallizes and can be obtained at a low crystallization yield. Unfortunately, these crystals were unsuitable for X-ray structure determination.

Reduction of ketone **18** by estradiol dehydrogenase (10) with excess NADH at pH 7.4, on the other hand, smoothly generated a sample of alcohol **17a** from which suitable crystals were grown. The X-ray crystal structure (Fig. 2) establishes the absolute configuration of 17-S. Table 1 contains the final atomic parameters and estimated standard deviations.

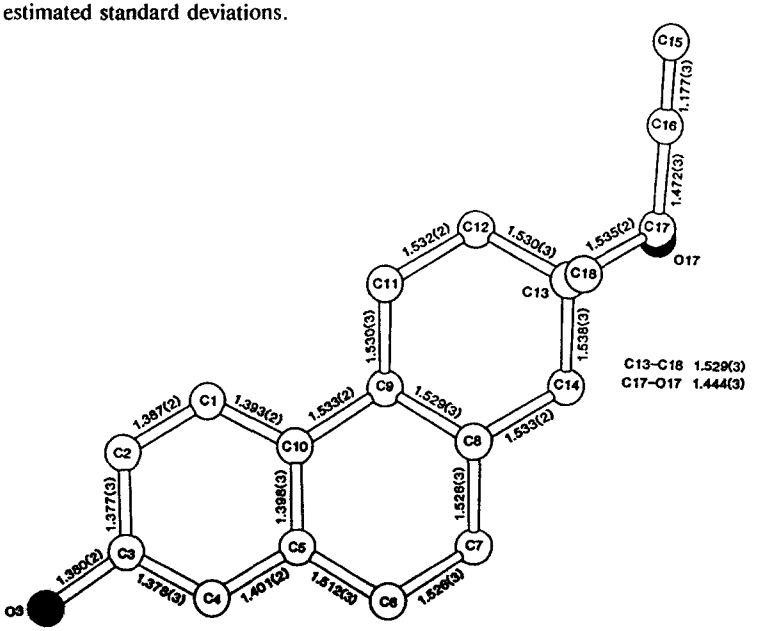


Figure 2. X-ray crystal structure of enzymatically generated alcohol **17a**.

Table 1. Atomic Coordinates (esd) and Average U (esd)

Atom	x	y	z	$U(A^2)$
C1	0.0769(1)	0.0678(1)	0.1387(2)	0.0394(8)
C2	-0.0365(1)	0.0619(1)	0.1798(3)	0.0426(9)
C3	-0.0727(1)	0.0892(1)	0.3409(3)	0.0413(9)
C4	0.0022(1)	0.1241(1)	0.4560(2)	0.0433(9)
C5	0.1168(1)	0.1304(1)	0.4149(2)	0.0389(8)
C6	0.1946(2)	0.1700(1)	0.5464(2)	0.049(1)
C7	0.3185(1)	0.1502(1)	0.5164(2)	0.0447(9)
C8	0.3478(1)	0.1588(1)	0.3223(2)	0.0376(8)
C9	0.2815(1)	0.0980(1)	0.2125(2)	0.0350(8)
C10	0.1554(1)	0.1002(1)	0.2546(2)	0.0359(8)
C11	0.3098(1)	0.1088(1)	0.0181(2)	0.0418(9)
C12	0.4363(1)	0.0984(1)	-0.0130(2)	0.0418(9)
C13	0.5081(1)	0.1557(1)	0.0976(2)	0.0382(8)
C14	0.4744(1)	0.1482(1)	0.2918(3)	0.0396(9)
C15	0.7090(2)	0.1348(2)	-0.2454(3)	0.067(1)
C16	0.6758(2)	0.1362(1)	-0.0999(3)	0.052(1)
C17	0.6347(2)	0.1329(1)	0.0822(3)	0.0444(9)
C18	0.4972(2)	0.2430(1)	0.0342(3)	0.055(1)
O3	-0.1817(1)	0.0790(1)	0.3991(2)	0.0538(8)
O17	0.6594(1)	0.05444(9)	0.1552(2)	0.0487(7)
H1	0.101(2)	0.045(1)	0.030(3)	0.041(6)
H2	-0.088(2)	0.039(1)	0.098(3)	0.039(5)
H4	-0.025(2)	0.142(1)	0.572(3)	0.050(6)
H6a	0.169(2)	0.161(2)	0.670(3)	0.072(8)
H6b	0.181(2)	0.232(1)	0.537(3)	0.059(7)
H7a	0.336(2)	0.093(2)	0.557(3)	0.064(7)
H7b	0.364(2)	0.187(1)	0.587(3)	0.056(7)
H8	0.322(2)	0.216(1)	0.279(3)	0.045(6)
H9	0.309(2)	0.039(1)	0.250(3)	0.046(6)
H11a	0.282(2)	0.163(1)	-0.019(3)	0.051(6)
H11b	0.272(2)	0.067(1)	-0.056(3)	0.046(6)
H12a	0.451(2)	0.107(1)	-0.135(3)	0.047(6)
H12b	0.456(2)	0.042(1)	0.021(3)	0.043(6)
H14a	0.518(2)	0.189(1)	0.366(3)	0.047(6)
H14b	0.493(2)	0.094(1)	0.326(3)	0.040(6)
H15	0.741(2)	0.131(2)	-0.356(4)	0.074(9)
H18a	0.525(2)	0.248(2)	-0.095(4)	0.072(8)
H18b	0.430(2)	0.263(2)	0.048(4)	0.084(9)
H18c	0.551(2)	0.281(2)	0.103(4)	0.086(9)
H17	0.679(2)	0.174(1)	0.160(3)	0.053(7)
H30	-0.218(2)	0.063(2)	0.323(4)	0.069(9)
H170	0.659(2)	0.014(2)	0.069(4)	0.102(9)

Mixing unfractionated synthetic and enzymatic material showed, by chemical shift enhanced ^{1}H-NMR, that the isomer from which the resonances at higher field arise is the 17-S isomer--the enzymic substrate (Fig. 1). Similar mixing experiments showed that semicrystalline-synthetic product contains predominantly (typically ≈90%) the 17-S isomer. Accordingly, the semicrystalline-synthetic and enzymatically generated alcohols are indistinguishable as substrates and inhibitors of estradiol dehydrogenase (10), which is known to utilize only the 17β (17-S) epimer of estradiol.

Enzymology

As previously reported (10), propargyl alcohol **17a** is a mechanism-based inactivator of estradiol dehydrogenase from human term placenta (K_m = 79 μM, V_{max} = 8.1 nmol/min/mg enz; K_i = 2.0 μM, limiting $t_{1/2}$ = 82 min [pH 9.2]). The enzyme reduces aldehyde **16** at pH 7.4 in the presence of excess NADH (K_m = 4.4 μM, V_{max} = 1.2 μmol/min/mg enz). Alcohol **15**, on the other hand, is not a substrate, even at pH 9.2 with 200 μM NAD^+, conditions favoring steroid oxidation (10). Other examples of hydroxysteroid dehydrogenases that catalyze reduction of steroidal aldehydes but not oxidation of the corresponding primary alcohols have been reported (41). Likewise, estradiol dehydrogenase will reduce 16-keto-17β-estradiol to estriol, but the reverse reaction has not been demonstrated (42). Although not a substrate, alcohol **15** is a competitive inhibitor with good affinity for the enzyme at pH 9.2 with 200 μM NAD^+ (K_i = 4.8 μM). Under these conditions, aldehyde **16** has no effect on the enzymatic oxidation of estradiol. This result suggests that abortive ternary complexes (E· NAD^+ · **16**) are not formed, as is the case for estrone and estradiol (43).

Estrogen Receptor Binding and Uterotrophic Assays

Since four of the compounds were shown to bind to the active site of estradiol dehydrogenase, their ability to interact with the estrogen receptor was investigated. Competitive binding experiments show that alcohol **17a** competes with [^{3}H]estradiol binding to calf uterus estrogen receptor with a K_a of 2.5 ± 0.6 × 10^{-9} M. In simultaneous assays, the K_a of estradiol was 2.8 ± 0.9 × 10^{-10} M and that of estrone was 6.4 ± 1.7 × 10^{-9} M.

Since competition with [^{3}H]estradiol binding does not distinguish agonists from antagonists, uterotrophic assays were conducted. Alcohol **17a** is a very weak uterotrophic agent, about 10^3 times less potent than estradiol, with no apparent antagonistic activity (Table 2). Alcohol **15** appears to be an even weaker estrogen. The discrepancy between the apparent high affinity of alcohol 17a for the receptor and its weak *in vivo* activity might reflect rapid metabolic inactivation of **17a**. No attempt was made to establish the *in vivo* half-life of alcohol **17a**.

Table 2. Uterotrophic Agonistic and Antagonistic Activity

dose (µg/kg/day)	Uterine Weight (mg)[a] estradiol	alcohol 17a	estradiol + 17a[b]	alcohol 15	estradiol + 15[b]
0	16.2±1.0	--	--	--	--
0.1	20.9±1.2	--	28.9±1.8	--	--
0.3	24.5±2.0	17.7±5.6	36.4±1.5	--	--
1	39.3±2.2	19.8±1.1	47.3±2.0	--	37.3±2.9
3	61.9±2.8	20.8±1.4	61.4±3.6	18.7±4.3	--
10	57.5±2.9	21.6±2.0	78.9±4.6	--	53.7±7.1
300	73.1±3.4	27.6±1.5	86.4±3.2	--	--
3000	--	45.5±2.5	--	31.3±2.0	--

[a]Values indicate mean ± SEM of 10 animals per dose of estradiol and/or alcohol 17a; 15 animals for control; and 5 animals per dose of alcohol 15 ± estradiol.
[b]Alcohols 17a and 15 were administered at 300 µg/kg/day at all doses of estradiol.

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