8(9)–DEHYDROESTRADIOL DERIVATIVES

BACKGROUND OF THE INVENTION

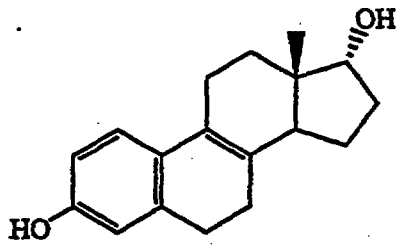
The use of naturally occurring estrogenic compositions of substantial purity and low toxicity such as PREMARIN (conjugated equine estrogens) has become a preferred medical treatment for alleviating the symptoms of menopausal syndrome, osteoporosis/osteopenia in estrogen deficient women and in other hormone related disorders. The estrogenic components of the naturally occurring estrogenic compositions have been generally identified as sulfate esters of estrone, equilin, equilenin, 17-β-estradiol, dihydroequilenin and 17-β-dihydroequilenin (U.S. Patent 2,834,712). The estrogenic compositions are usually buffered or stabilized with alkali metal salts of organic or inorganic acids at a substantially neutral pH of about 6.5 to 7.5. Urea has also been used as a stabilizer (U.S. 3,608,077). The incorporation of antioxidants to stabilize synthetic conjugated estrogens and the failure of pH control with tris(hydroxymethyl)aminomethane (TRIS) to prevent hydrolysis is discussed in U.S. 4,154,820.

8,9-Dehydroestrone is a known compound useful as an intermediate in the synthetic production of estrone by isomerization to 9,11 unsaturation (U.S. Patent 3,394,153) and as an intermediate in the production of 3-cyclopentyloxy-17-ethynyl derivatives of the hormone (U.S. Patent 3,649,621). In addition, 8,9-dehydroestrone is known to possess estrogenic activity and to lower blood lipid levels (U.S. Patent 3,391,169). The alkali metal salts of 8,9-dehydroestrone, 8,9-dehydroestrone-3-sulfate ester and its alkali metal salts, and their use in estrogen replacement therapy, atherosclerosis, and senile osteoporosis are disclosed in U.S. Patents 5,210,081 and 5,288,717.

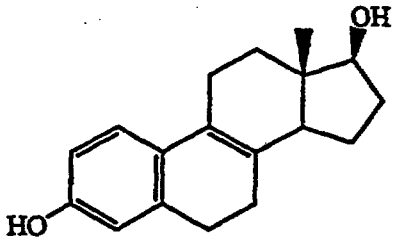
DESCRIPTION OF THE INVENTION

In accordance with this invention, there are provided 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, and 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester. These are collectively referred to as the compounds of this invention. The structures of

17α,Δ8,9-dehydroestradiol and 17β,Δ8,9-dehydroestradiol are shown below as compounds I and II, respectively.



I



II

The compounds of this invention can also be named 17α,Δ8,9-dehydroestrone and 17β,Δ8,9-dehydroestrone; and 17α-estra-1,3,5(10),8-tetraene-3,17-diol and 17β-estra-1,3,5(10),8-tetraene-3,17-diol, depending on which nomenclature system is used.

Pharmaceutically acceptable salts of 17α,Δ8,9-dehydroestradiol 3-sulfate ester or 17β,Δ8,9-dehydroestradiol 3-sulfate ester include, but are not limited to, the alkali metal salts, alkaline earth metal salts, ammonium salts, alkylammonium salts containing 1-6 carbon atoms or dialkylammonium salts containing 1-6 carbon atoms in each alkyl group, and trialkylammonium salts containing 1-6 carbon atoms in each alkyl group.

17α,Δ8,9-dehydroestradiol-3-sodium sulfate and 17β,Δ8,9-dehydroestradiol-3-sodium sulfate are metabolites of Δ8,9-dehydroestrone, that are formed following the administration of Δ8,9-dehydroestrone. This invention therefore also provides 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester in greater than one percent purity, and 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester in greater than one percent purity.

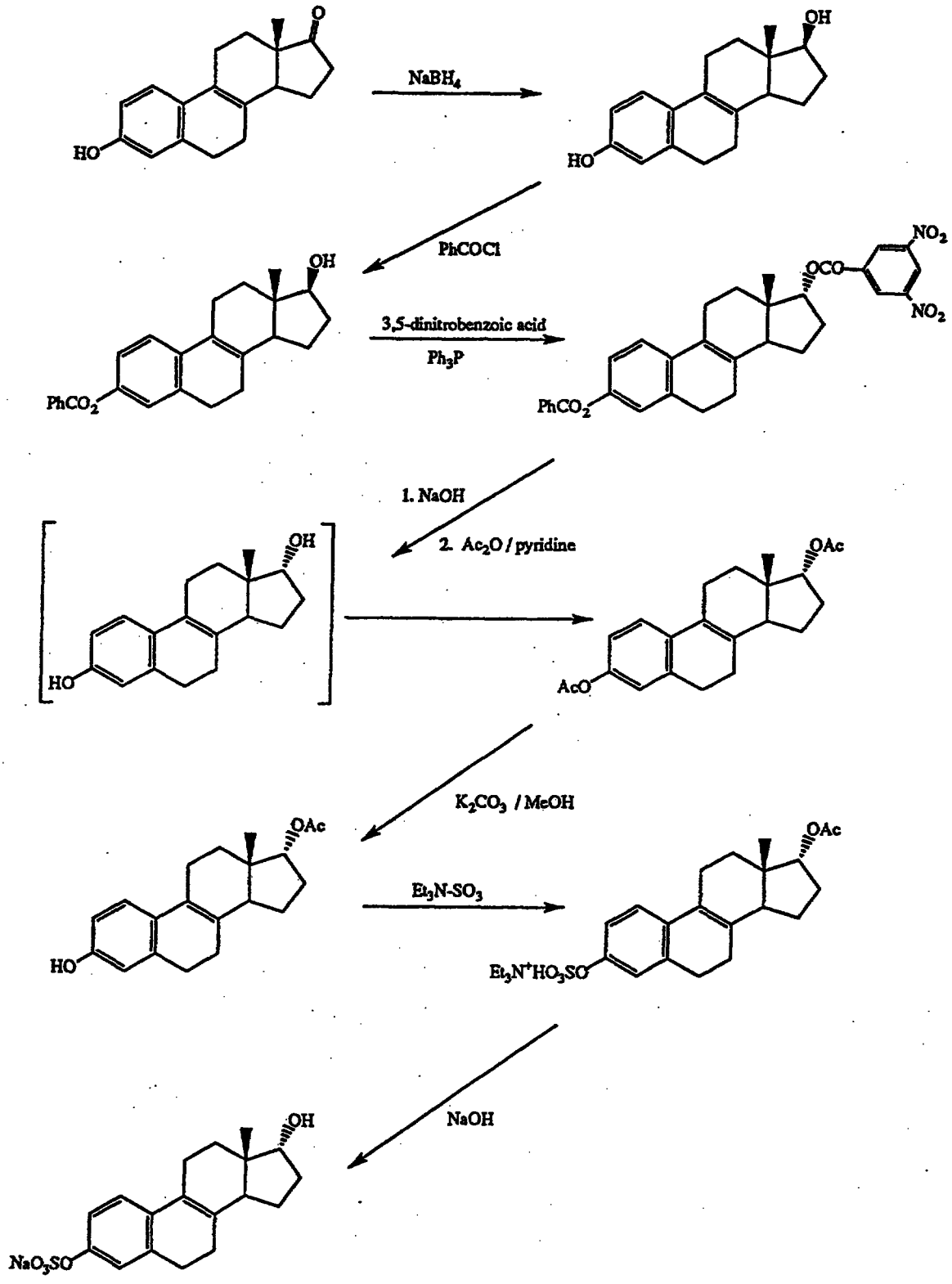
As used in accordance with this invention, treating covers treatment of an existing condition, ameliorating the condition, or providing palliation of the condition and inhibiting includes inhibiting or preventing the progress or development of the condition.

The compounds of this invention can be prepared either by the in vivo metabolism of Δ8,9-dehydroestrone, as shown in Example 5, or can be prepared synthetically from Δ8,9-dehydroestrone as outlined in Schemes I and II.

Scheme I outlines the preparation of 17α,Δ8,9-dehydroestradiol and salts of its 3-sulfate ester beginning with the reduction of the 17-ketone of Δ8,9-dehydroestrone with a suitable reducing agent, such as sodium borohydride to produce 17β-Δ8,9-

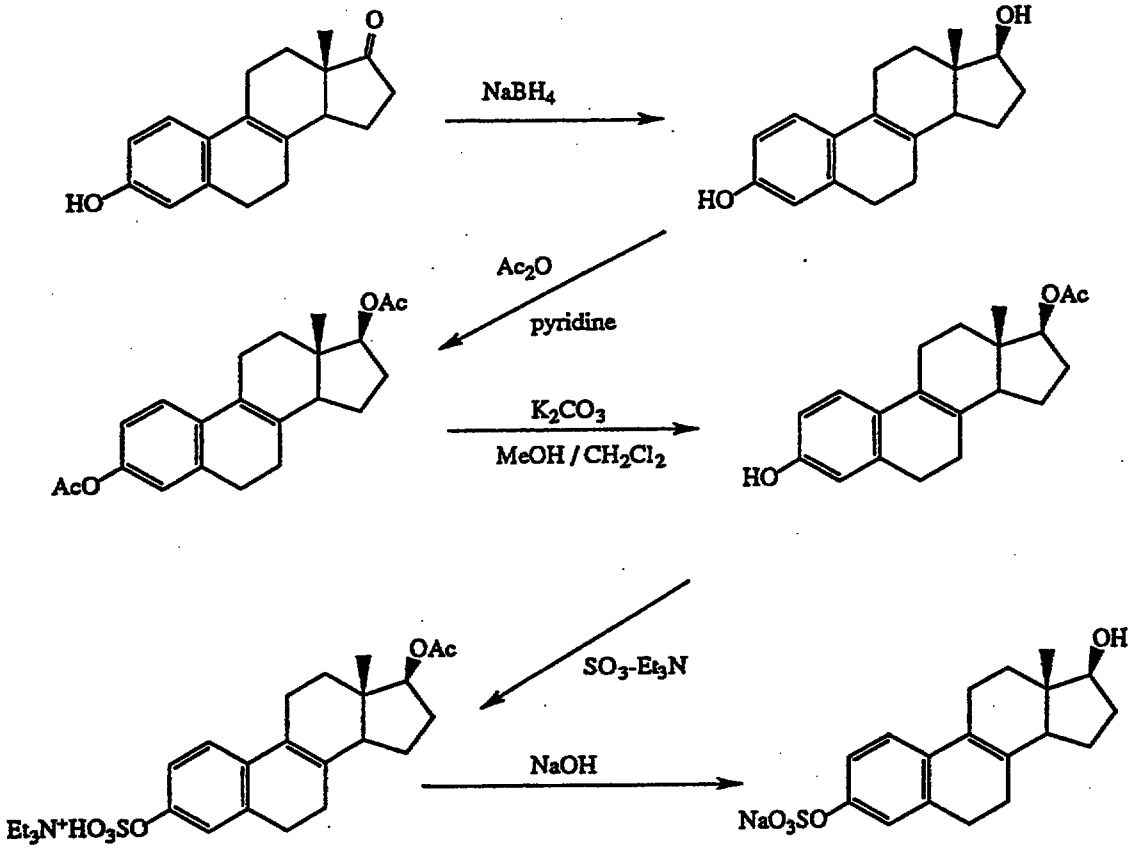
dehydroestradiol. Other hydride reducing agents can be readily used such as sodium cyanoborohydride or lithium aluminum hydride. The 3-hydroxyl group can be selectively acylated with a suitable acylating agent, such benzoyl chloride or acetyl chloride. Inversion of stereochemistry at the 17-position can be accomplished using a Mitsunobu reaction. Hydrolysis of the acyl moieties with a suitable aqueous base, such as sodium hydroxide gives 17α,Δ8,9-dehydroestradiol. To form the 3-sulfate ester, the diol is acylated with a suitable acylating reagent such as acetic anhydride, and the 3-acyl group is selectively cleaved using mild basic conditions, such as potassium carbonate in methanol. Formation of the 3-sulfate ester can be accomplished with a sulfur trioxide -ammonia, -alkylamine, -dialkylamine, or -trialkyamine reagent, such as sulfur trioxide-triethylamine or sulfur trioxide-pyridine. The resultant ammonium, monoalkylammonium, dialkylammonium or trialkylammonium salt of the 3-sulfate ester can be converted into another salt by exchange of cations, optionally via the acid. For instance, conversion to the 3-sulfate metal salt can be accomplished with a metal hydroxide solution. The preparation of 17α,Δ8,9-dehydroestradiol and the sodium and triethylammonium salts of its 3-sulfate ester is provided in Example 1.

Scheme I


NaBH4
PhCOCl
3,5-dinitrobenzoic acid
Ph3P
1. NaOH
2. Ac2O / pyridine
K2CO3 / MeOH
Et3N-SO3
NaOH
HO
OH
OAc
AcO
PhCO2
NO2
OCO
Et3N+HO3SO
NaO3SO

Similarly, 17β,Δ8,9-dehydroestradiol and salts of its 3-sulfate ester can be prepared as shown in Scheme II, starting from Δ8,9-dehydroestrone, except that the inversion of stereochemistry at the 17-position is not needed. Using the process outlined in Scheme II, 17β,Δ8,9-dehydroestradiol and the sodium and triethylammonium salts of its 3-sulfate ester are produced. Other salts of the 3-sulfate ester can be formed by varying the bases used. The preparation of 17β,Δ8,9-dehydroestradiol and the sodium and triethylammonium salts of its 3-sulfate ester is provided in Example 2.

Scheme II



The compounds of Examples 1 and 3 were found to degrade over time. The preparation of TRIS stabilized complexes of the compounds of Examples 1 and 3 are shown in Examples 2 and 4, respectively.

The compounds of this invention are estrogenic, as shown in an in vivo standard pharmacological test procedure which measured uterine growth in immature

female mice and ovariectomized rats as an evaluation of estrogenicity. 17α,Δ8,9-Dehydroestradiol-3-sodium sulfate and 17β,Δ8,9-dehydroestradiol-3-sodium sulfate were evaluated as representative compounds of this invention. Estrone and Δ8,9-dehydroestrone were also evaluated for the purpose of comparison. The compounds to be evaluated were prepared as suspensions in corn oil. Corn oil alone was used as a control.

The following procedure describes the evaluation of estrogenicity in immature mice. Intact immature Charles River CD female mice (23 days of age) were used. The compounds to be evaluated were administered subcutaneously or orally at total doses administered over 3 days of 1 to 1000 μg subcutaneously and 3 to 1000 μg orally. Estrone was administered subcutaneously at total doses of 0.03 to 1 μg over 3 days. The mice were sacrificed approximately 24 hours after the last dose was administered, and the uteri was removed and weighed. The results obtained are summarized in the following table.

ESTROGENIC ACTIVITY - MOUSE UTERINE GROWTH

Treatment[a]	Route	Minimal Effective Dose[b]	Doubling Dose[c]
Estrone	s.c.	0.06-0.1	0.1-0.2
Δ8,9	s.c.	6.2	14.8
17α	s.c.	1.75	7.61
17β	s.c.	0.24	5.0
Estrone	p.o.	5.9	25.9
Δ8,9	p.o.	2.6	26.7
17α	p.o.	2.84	6.67*
17β	p.o.	3.0	18.8

[a] Δ8,9 = Δ8,9-dehydroestrone; 17α = 17α,Δ8,9-dehydroestradiol; 17β = 17β,Δ8,9-dehydroestradiol.

[b] Minimal effective dose given over 3 days required to produce a significant increase in uterine weight over that of vehicle.

[c] Total dose given over 3 days required to produce a 100% increase in uterine weight over that of vehicle.

* A value of dose of 16.6 μg would be obtained if the results from an isolated strong uterine response to the 10 μg dose are deleted.

The following procedure describes the evaluation of estrogenicity in ovariectomized rats. Immature Charles River CD rats were ovariectomized at 30 days of age and allowed 12 days for uterine regression. Administration of compounds to be evaluated was initiated 13 days post ovariectomy and was continued for 7 days. The compounds to be evaluated were administered at daily subcutaneous or oral doses of 3 to 1000 μg/rat/day. The rats were sacrificed approximately 24 hours after the last dose was administered. The uteri were removed and weighed. The results obtained are summarized in the following table.

ESTROGENIC ACTIVITY - MOUSE UTERINE GROWTH

Treatment[a]	Route	Minimal Effective Dose[b]	Doubling Dose[c]
Estrone	s.c.	0.05-0.1	0.12-0.7
Δ8,9	s.c.	0.3	1.2
17α	s.c.	10.4	171.4
17β	s.c.	0.8	2.9
Estrone	p.o.	10.6	22
Δ8,9	p.o.	3.3	21.5
17α	p.o.	67.1	116.0
17β	p.o.	12.0	14.5

[a] Δ8,9 = Δ8,9-dehydroestrone; 17α = 17α,Δ8,9-dehydroestradiol; 17β = 17β,Δ8,9-dehydroestradiol.

[b] Minimal effective dose given over 7 days required to produce a significant increase in uterine weight over that of vehicle.

[c] Total dose given over 7 days required to produce a 100% increase in uterine weight over that of vehicle.

The compounds of this invention are antioxidants. The antioxidant properties of 17α,Δ8,9-dehydroestradiol and 17β,Δ8,9-dehydroestradiol were established in a standard pharmacological test procedure that measured the its ability to inhibit the formation of oxidatively modified low density lipoprotein (LDL) induced by exposure to either Cu++ ions or cultured endothelial cells (Parthasarathy S, Proc Natl Acad Sci USA 86:1046-1050 (1989)) by the TBARS (thiobarbituric acid reactive substances) method for analysis of free aldehydes (Yagi K., Biochem Med 15:212-216 (1976)).

The results obtained in this standard pharmacological test procedure demonstrate that 17α,Δ8,9-dehydroestradiol and 17β,Δ8,9-dehydroestradiol are potent inhibitors of LDL oxidation, inhibiting the process by up to 96%. IC_{50}s of 0.19 μM was obtained in the Cu++ mediated oxidation for both 17α,Δ8,9-dehydroestradiol and 17β,Δ8,9-dehydroestradiol. IC_{50}s of 0.26μM and 0.17μM were obtained in the porcine aortic endothelial cell mediated oxidations, respectively. By comparison, an IC_{50} of 0.56 μM was obtained for estrone in the porcine aortic endothelial cell mediated oxidation test procedure.

To further demonstrate that the antioxidant properties of 17α,Δ8,9-dehydroestradiol and 17β,Δ8,9-dehydroestradiol, two additional standard pharmacological test procedures were conducted using cells in culture. In the first test procedure, radiolabeled-LDL (^{125}I-LDL) (McFarlane AS, In: Munro HN, Allison JB, eds. Mammalian Protein metabolism, Vol. 1. New York: Academic Press 297-341 (1964)) was modified by exposure to Cu++ in the presence and absence of 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol. Next, J774 macrophages, which express scavenger lipoprotein receptors which bind oxidatively modified-LDL, were exposed to the treated ^{125}I-LDL. The results of this experiment demonstrate that binding of the LDL that was oxidized in the presence of 2.5μM concentration of 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol, was reduced by 48% and 54%, respectively, and at a concentration of 0.25 uM was reduced by 34% and 28%, respectively. By comparison, the same concentrations of estrone reduced the binding of LDL that was oxidized by 39% and 0%, respectively. Since binding and metabolism of oxidized LDL by macrophages is though to contribute strongly to the development of foam cells and therefore, atherosclerotic plaque, this effect of reducing LDL oxidation and subsequent binding to scavenger receptors is thought to be of significant benefit.

In the second test procedure, porcine aortic endothelial cells (PAEC) were exposed to LDL that had been modified as above, by exposure to Cu++ in the presence and absence of 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol,. Oxidized LDL has been demonstrated to be cytotoxic to endothelial cells, and this process has also been strongly implicated in the atherogenic process. Subsequent to a 24 hr incubation of the cells with the treated LDL, an MTT assay was performed to assess cytotoxicity (Hansen MB, J Immu Methods 119:203-210 (1989)). This test procedure assesses the percent of cells that are viable (live) in a given assay. In the assay, following exposure to 25 μg/ml LDL oxidized in the absence of compound, only 2% of

the cells remained viable. In contrast, the percent live cells following exposure to LDL Cu++ treated in the presence of 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol (2.5μM) was 100% or greater. Other compounds tested in this same assay had minimal effects on protection of PACE (17β-estradiol = 11% living; Equilin = 4% living; Estrone = 37% living. The results of this test procedure demonstrate that LDL modified in the presence of 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol was not cytotoxic, and therefore, the data is in agreement with the inhibition of oxidative modification by 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol as demonstrated by the TBARS method above.

Based on the results of these standard pharmacological test procedures, 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, and 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester are useful in replacement therapy in estrogen deficiency. The compounds of this invention are therefore useful in providing estrogen replacement therapy following ovariectomy or menopause, and in relieving symptoms related to estrogen deficiency, including vasomotor symptoms, such as hot flushes, and other menopausal related conditions, such as vaginal atrophy, vaginitis, and atrophic changes of the lower urinary tract which may cause increased urinary frequency, incontinence, and dysuria. The compounds of this invention are useful in preventing bone loss and in the inhibition or treatment of osteoporosis. The compounds of this invention are cardioprotective and they are useful in the treatment of atherosclerosis. These cardiovascular protective properties are of great importance when treating postmenopausal patients with estrogens to prevent osteoporosis and in the male when estrogen therapy is indicated. The compounds of this invention are also antioxidants are therefore useful in treating or inhibiting free radical induced disease states. Specific situations in which antioxidant therapy is indicated to be warranted are with cancers, central nervous system disorders, Alzheimer's disease, bone disease, aging, inflammatory disorders, peripheral vascular disease, rheumatoid arthritis, autoimmune diseases, respiratory distress, emphysema, prevention of reperfusion injury, viral hepatitis, chronic active hepatitis, tuberculosis, psoriasis, systemic lupus erythematosus, adult respiratory distress syndrome, central nervous system trauma and stroke. Additionally, the compounds of this invention are useful in the suppression of lactation, and in the prophylaxis and treatment of mumps orchitis.

The compounds of this invention can be formulated neat. More preferably one may prepare a pharmaceutical composition comprising a compound of this invention in association or combination with a pharmaceutically acceptable carrier. The proportion of the pharmaceutical carrier may be determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmacological practice. The pharmaceutical carrier may be solid or liquid.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, lethicins, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by, for example, intramuscular, intraperitoneal or subcutaneous

injection. Sterile solutions can also be administered intravenously. The compounds of this invention can also be administered orally either in liquid or solid composition form.

The compounds of this invention may be administered rectally or vaginally in the form of a conventional suppository. For administration by intranasal or intrabronchial inhalation or insufflation, the compounds of this invention may be formulated into an aqueous or partially aqueous solution, which can then be utilized in the form of an aerosol. The compounds of this invention may also be administered transdermally through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semipermiable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

In addition, the compounds of this invention may be employed as a solution, cream, or lotion by formulation with pharmaceutically acceptable vehicles containing 0.1 - 5 percent, preferably 2%, of active compound which may be administered to a fungally affected area.

The dosage requirements vary with the particular compositions employed, the route of administration, the severity of the symptoms presented and the particular subject being treated. Based on the results obtained in the standard pharmacological test procedures, projected daily dosages of active compound would be 0.02 µg/kg - 500 µg/kg. Treatment will generally be initiated with small dosages less than the optimum dose of the compound. Thereafter the dosage is increased until the optimum effect under the circumstances is reached; precise dosages for oral, parenteral, nasal, or intrabronchial administration will be determined by the administering physician based on experience with the individual subject treated. Preferably, the pharmaceutical composition is in unit dosage form, e.g. as tablets or capsules. In such form, the composition is sub-divided in unit dose containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example, packeted powders, vials, ampoules, prefilled syringes or sachets containing liquids. The unit

dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form.

The following provides the preparation of representative compounds of this invention.

Example 1

17α-Estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate, sodium salt

A. 17β-Estra-1,3,5(10),8-tetraene-3,17-diol

To a stirred suspension of 3-hydroxyestra-1,3,5(10)-8-tetraene-17-one (24.13 g, methanol (185 mL) and methylene chloride (138 mL) was added sodium borohydride (6.92 g) portionwise over 25 minutes while maintaining the temperature at 28-30°. To the slightly cloudy solution was added distilled water (555 mL) in aliquots. The temperature rose to 30-31°. The resulting slurry was stirred at 25-30° for 0.5 hour and then cooled to 0.5°. The product was collected on a filter and washed with water (40 x 40 mL). Drying the initially white, wet cake (29.66 g) in a vacuum desiccator over phosphorus pentoxide for 4 days provided 23.54 g (96.83%) of yellow product having appropriate spectral (ir and pmr) data.

B. 17β–Estra-1,3,5(10),8-tetraene-3,17-diol 3-benzoate

A 2 L multi-neck flask was equipped with a mechanical stirrer, a condenser with a nitrogen inlet, a pressure equalizing graduated addition funnel and a thermometer. The flask was charged with 17β-estra-1,3,5(10),8-tetraene-3,17-diol (60.00 g, 0.2219 mol.), tetrahydrofuran (500 mL) and triethylamine (46.4 mL, 0.3329 mol., 1.5 molar excess) and the mixture was stirred under nitrogen until a solution was obtained. The addition funnel was charged with benzoyl chloride (30.98 mL, 0.2669 mol., 1.203 molar excess) and tetrahydrofuran up to the 100 mL mark. The benzoyl chloride solution was added dropwise over 25 minutes, under nitrogen, while maintaining the reaction temperature between 15 and 20° using a water/ice bath. The cooling was removed and the stirred suspension (Et_3N.HCl ppt) was allowed to warm to room tempetature (23°). Three and a half hours after completion of the addition of the benzoyl chloride solution, the reaction mixture was chilled in ice and a mixture of saturated brine (204 mL), water (68 mL) and concentrated hydrochloric acid (34 mL) was added rapidly. The temperature rose from 23° to 26°. After stirring 5 minutes, solid precipitated in the lower layer. The aqueous phase was separated from the organic

phase and extracted with tetrahydrofuran (1 x 200 mL and 1 x 100 mL). The combined organic phases were washed with saturated brine (2 x 100 mL) and thrice with a mixture of saturated brine (70 mL) and 5% sodium bicarbonate (30 mL). A further wash with saturated brine (100 mL) did not completely remove residual benzoyl chloride and so the solution was dried by magnetic stirring with anhydrous magnesium sulfate for 15 minutes. The drying agent was removed by filtration and the filter pad washed with tetrahydrofuran. The filtrate and washings were evaporated at 30° to a dry solid. The solid was stirred magnetically with heptane (400mL) for 3 1/2 hours. The resulting white solid was collected on a filter and washed with chilled heptane (0-5°, 2 x 75 mL, 1 x 100 mL). The wet cake (108.09 g) was dried in a vacuum oven at room temperature for 3 days to provide a quantitative yield (83.35 g, 100.3%) of crude 17β-estra-1,3,5(10),8-tetraene-3,17-diol 3-benzoate. This material was suitable for use in the next step.

The crude benzoate (5.0 g) was stirred and heated in 95% ethanol (65 mL) to give a solution which was filtered through a fluted, fast filter paper. The filter was rinsed with hot 95% ethanol and the solution was distilled to 50 mL volume. The solution was allowed to cool and the resulting slurry of crystals was refrigerated. After chilling to -10° the product was collected on a filter and washed with cold (-10°) 95% ethanol. Drying in a vacuum oven at 65° overnight gave purified product (3.82 g, 76.40%). Spectral (ir, pmr & ms) data were appropriate and elemental analyses were acceptable.

C. <u>17α-Estra-1,3,5(10),8-tetraene-3,17-diol 3-benzoate 17-(3,5-dinitrobenzoate)</u>

To a 1 L multi-neck flask equipped with a mechanical stirrer, a reflux condenser with a nitrogen inlet and a thermometer was added, under nitrogen, 17β-estra-1,3,5(10),8-tetraene-3, 17-diol 3-benzoate (73.42 g, 0.1961 mol.), triphenylphosphine (66.85 g, 0.2549 mol.), 3,5-dinitrobenzoic acid (54.06 g, 0.2549 mol.) and toluene (530 mL). To the stirred suspension was added a solution of diethyl azodicarboxylate (40 mL, 0.2549 mol.) and toluene (10 mL) from a 50 mL pressure equalizing dropping funnel, over a period of 7 minutes. A 10 mL rinse of toluene was used to complete the addition (total toluene 550 mL). The stirred suspension was slowly heated to 70° and held at that temperature for 2.5 hours. (After 1/2 hour of heating at 70° crystallization began.)

The slurry of crystals was allowed to cool to 43° and then cooled to -10°. The crystals were collected on a filter and washed with cold (-10°) toluene (2 x 80 mL) cold

(-10°) heptane-toluene (7:3) and cold (-10°) heptane (2 x 80 L). Drying the wet cake (118.79 g) in a vacuum oven overnight at room temperature gave 107.56 g of crude product contaminated with 1,2-dicarbethoxyhydrazine.

To a 1 L Erlenmeyer was added the crude product (107.56 g) and methanol (310 mL). The mixture was stirred magnetically for 5 minutes and the product was collected on a filter. After washing with methanol (2 x 60 mL) the wet cake (83.23 g) was dried at 65° in a vacuum oven overnight. The yellow crystalline product (75.65 g, 67.87%) had appropriate spectral (ir, pmr & ms) data and acceptable elemental analyses.

D. 17α-Estra-1,3,5(10),8-tetraene-3,17-diol 3,17-diacetate

To 17α-estra-1,3,5(10),8-tetraene-3, 17-diol 3-benzoate 17-(3,5-dinitrobenzoate) (71.00 g, 0.1249 mol), in a 3 L multi-neck flask, was added tetrahydrofuran (630 mL) and 320 mL of 2N sodium hydroxide. The mixture was stirred at room temperature (23-27°), under nitrogen, for 24 hours. 2N Hydrochloric acid (196 mL) and ethyl acetate (880 mL) were added rapidly. The resulting layers were separated. The lower aqueous phase was washed with ethyl acetate (3 x 200 mL). The combined organic extracts were washed with saturated brine (2 x 300 mL, pH of last wash 7). The solution was dried by stirring magnetically with anhydrous magnesium sulfate for 15 minutes.

Evaporation gave a red solid (54.08 g) which was subjected to an oil pump vacuum for a few minutes. Pyridine (250 mL) and acetic anhydride (250 mL) were added and the stoppered reaction flask was swirled to achieve solution. The solution was left overnight at room temperature and poured onto ice. The volumn was made up to 2.5 L with water and the resulting suspension was stirred until a manageable red solid was obtained. The solid was collected on a filter and then blended with 300 mL of water. The product was collected and the filter cake was washed with water (total ~ 2 L). Drying over phosphorus pentoxide in a vacuum desiccator over a weekend gave a pink solid (44.23 g). It was stirred magnetically in methanol (100 mL) for a few minutes and collected on a filter. The filter cake was washed thrice with methanol (total 50 mL) and dried in a vacuum oven at room temperature overnight. The pink material weighed 38.60 g (87.21%).

The product (38.60 g) was dissolved in hot methanol (120 mL) and the hot solution was heated briefly with charcoal. The charcoal was removed by filtration through Celite and the filter pad was washed with hot methanol. The product started to

crystallize in the filtrate. The filtrate and washings were reheated to dissolve the product and the solution was distilled to approximately 120 mL volume. The solution was allowed to cool to near room temperature and the resulting slurry of crystals was chilled to 0-5°. The crystals were collected on a filter and washed with cold (0-5°) methanol (2 x 35 mL). Drying in a vacuum oven at room temperature gave the title compound (31.86 g, 82.39%).

E. 17α-Estra-1-3-5(10),8-tetraene-3,17-diol 17-acetate

To 17α-estra-1,3,5(10),8-tetraene-3,17-diol 3,17-diacetate (30.00 g) in dichloromethane (67 mL) and methanol (182 mL) at 0°, under nitrogen, was added dropwise saturated methanolic potassium carbonate (136 mL) while maintaining the temperature at 0° or below. The reaction mixture was stirred at 0° (inside temperature) using a thermostated cooling bath for 1 1/4 hours and then examined by TLC on silica gel with dichloromethane-ethyl acetate (19:1). No starting material was observed. The reaction mixture was distilled under oil pump vacuum while maintaining the reaction flask in the cooling bath. This produced an inside temperature significantly below 0° (e.g. -7 to -10°). To the resulting thick paste as added rapidly 430 mL of 5% (v/v) aqueous acetic acid and the mixture stirred until a filterable solid was obtained. The solid was collected on a filter and then blended with 70 mL of 5% acetic acid and an appropriate volume of water in a blender. The crude product was collected on a filter and washed well with water. Drying in a vacuum desiccator over phosphorus pentoxide for 3 days provided 26.19 g (99.04%) of crude product.

Crude product (26.19 g) was magnetically stirred in methanol (270 mL) containing a few drops of glacial acetic acid (pH of sol. = 4) and heated to achieve solution. The solution was filtered through a fluted, fast filter paper and rinsed through with 20 mL of hot methanol. The solution was distilled to a volume of 175 mL and allowed to cool. Cooling in ice and scratching enhanced crystallization. After cooling in the freezer (-20°) for 1 hour, the crystals were collected on a filter and washed with cold (-20°) methanol (3 x 20 mL). Drying at 60° in a vacuum oven provided purified product (22.72 g, 86.75%) which had appropriate spectral (ir, pmr & ms) data and acceptable elemental (C & H) analyses.

A second crop (172 g, 6.57%) was obtained from the mother liquor. This was combined with material remaining from the first crop (22.27 g) and the total (22.99 g) was stirred magnetically and heated in methanol (230 mL) to produce a yellow solution. The solution was filtered through a fluted, fast filter paper and rinsed through with 20

mL of hot methanol. The solution was distilled to a volume of 125 mL, allowed to cool to room temperature and then refrigerated. The resulting crystals were collected on a filter and washed with cold (0°) methanol (3 x 15 ml). Drying at 60° in a vacuum oven for 20 hours provided 18.79 g (81.73% recryst. step, 71.06% overall) of white product having appropriate spectral (ir, pmr & ms) data and acceptable elemental (C & H) analyses.

F. 17α-Estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate 17-acetate, triethylammonium salt

To a stirred solution of 17α-estra-1,3,5(10),8-tetraene-3,17-diol 3,17-acetate (17.50 g, 0.0560 mol.) in tetrahydrofuran (204 mL) at room temperature, under nitrogen, was added triethylamine-sulfur trioxide complex (20.30 g, 0.1120 mol., 2 molar excess). The solid dissolved rapidly and the solution was stirred 4 hours at room temperature under nitrogen. TLC on silica gel with dichloromethane-ethyl acetate (19:1) had showed no starting material after 3.75 hours. Anhydrous ether (612 mL) was added and an oil formed. Shortly afterwards, the oil crystallized. The mixture was stirred for 1/2 hour longer and the crystals were collected on a filter. The crystals were washed with ether (2 x 125 mL). Drying the wet crystals (43.31 g) in a vacuum oven at room temperature overnight gave 28.90 g (104.51%) of product contaminated with excess reagent.

To a stirred solution of the crude product (28.90 g) in methanol (70 mL) was carefully added triethylamine (2-3 mL) to bring the pH to 7-8. Ether (800 mL) was added rapidly in aliquots. Again, an oil formed which quickly crystallized. Stirring was continued for 15 minutes and the slurry of crystals was cooled to -15°. The crystals were collected on a filter and washed with ether (3 x 80 mL). Drying in a vacuum oven at room temperature overnight gave 25.09 g (90.74%) of white material containing some solidified lumps.

To a stirred solution of the above material (25.09 g) in methanol (40 mL) at room temperature was carefully added triethylamine (a few drops) to adjust the pH to 7. The solution was filtered into a 1 L multi-neck flask and the filter was washed with methanol (20 mL). To the mechanically stirred solution was added ether (600 mL.). Once again, the product formed as an oil which quickly crystallized. The mixture was stirred vigorously for 1/2 hour and then cooled to -10°. The white crystalline product was collected on a filter and washed with ether (3 x 60 mL). Drying at room temperature in a vacuum oven provided title product (23.64 g, 94.22% and 85.49%

overall) having appropriate spectral (ir, pmr & ms) data and acceptable elemental (C, H & N) analyses.

G. 17α-Estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate sodium salt

To a solution of 17α-estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate 17-acetate triethylammonium salt (22.50 g, 0.04558 mol.), in methanol (144 mL), under nitrogen, was added 144 mL of 1.6N sodium hydroxide and the mixture stirred magnetically for 4.5 hours. The resulting yellow solution was evaporated at room temperature until 150 mL of distillate was collected. n-Butanol (450 mL) was added and the mixture was stirred until the sticky white crystals dissolved and two phases were obtained. The mixture was transferred to a separatory funnel with a mixture of 20 mL n-butanol, 10 mL of saturated brine and 10 mL of water. The layers separated slowly. The upper organic phase was washed consecutively with saturated brine (120 mL, 2 x 100 mL, 3 x 75 mL and 1 x 50 mL) until the pH of the final wash was 7. The cloudy organic phase was filtered through a small pad of solka Floc (prewashed with n-butanol) on a 7 cm Buchner funnel and rinsed through with n-butanol (2 x 25 mL). The solution was transferred to a 2 L round bottom flask with a 50 mL rinse of n-butanol. The solution was evaporated at 35-40° under oil pump vacuum until a thick slurry of crystals was obtained and 300 mL of distillate had been collected. Ether (500 mL) was added and mixed with the slurry. The white crystals were collected on a 10 cm filter and washed with ether (4 x 80 mL). The filter cake was transferred to a 1 L Erlenmeyer and magnetically stirred with 400 mL of ether for 5 minutes. The crystals were collected once more and washed with ether (5 x 70 mL). The wet cake was dried in a vacuum oven with a nitrogen bleed for 6 days to give 17.17 g (96.49% based on a hydrate) of white solid.

The above crude product (17.17 g) was stirred magnetically in U.S.P. ethanol (400 mL) until almost all the solid had dissolved. The mixture was filtered and the filtrate transferred to a 5 L multi-neck flask with 20 mL of U.S.P. ethanol. The solution was stirred mechanically and ether (1.4 L) was added to cause crystallization. An additional 1.9 L of ether was used to complete the process. The slurry of crystals was cooled to -10° and the crystals were collected on a filter. The filter cake was washed with ether (2 x 100 mL). Drying at room temperature in a vacuum oven for two days provided white crystals of title product (17.67 g, 86.34% based on MW=448.989).

Calc. for $C_{18}H_{21}O_5NaS$. 0.9 C_2H_5OH. 1.3 H_2O. 0.2 NaCl (448.989): C, 52.97; H, 6.51; Na, 6.14; Cl, 1.58; EtOH, 9.23; H_2O, 5.22

Found: C, 52.78; H, 6.27; Na, 6.17; Cl, 1.70; EtOH, 9.12; H_2O, 4.01

Example 2

17α-Estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate, sodium salt, with Tris (hydroxymethyl) aminomethane (TRIS)

To a solution of tris(hydroxymethyl)aminomethane (8.48 g) in distilled water (1,400 mL) was added 17α-estra-1,3,5(10),8-tetraene-3,17α-diol 3-sulfate (ester) sodium salt (14.69 g, MWW-7332-3, P.R. 215-20) and the mixture was stirred magnetically to dissolve the steroid. The solution was filtered and the filter was washed with water (100 L). The solution was transferred to a tray at -40° in a large Virtis Freeze-dryer using a 500 mL water rinse. The solution was frozen and the solid was freeze dried for 5 days. The resulting white, soft, flaky material was pressed, scraped, and mixed well in a bottle. The material was dried for a further 4 days at room temperature in the freeze-drier. The white product weighed 20.93 g (98.54%).

Example 3

A. 17β-Estra-1,3,5(10),8-tetraene-3,17-diol

To a stirred suspension of 3-hydroxyestra-1,3,5(10)-8-tetraene-17-one (24.13 g, methanol (185 mL) and methylene chloride (138 mL) was added sodium borohydride (6.92 g) portionwise over 25 minutes while maintaining the temperature at 28-30°. To the slightly cloudy solution was added distilled water (555 mL) in aliquots. The temperature rose to 30-31°. The resulting slurry was stirred at 25-30° for 0.5 hour and then cooled to 0.5°. The product was collected on a filter and washed with water (40 x 40 mL). Drying the initially white, wet cake (29.66 g) in a vacuum desiccator over phosphorus pentoxide for 4 days provided 23.54 g (96.83%) of yellow product having appropriate spectral (ir and pmr) data.

B. 17β-Estra-1,3,5(10),8-tetraene-3,17-diol 3,17-diacetate

A mixture of 17β-estra-1,3,5(10)-8-tetraene-3, 71-diol (23,.09 g), was swirled with a mixture of acetic anhydride (120 mL) and pyridine (120 mL) in a stoppered 500 mL flask. The resulting yellow solution was left at room temperature overnight. The solution was added to crushed ice (~600 mL). A white precipitate formed. The slurry

was stirred magnetically and water (220 mL) was added. Stirring was continued and the temperature was allowed to rise to 18-20°. The product was collected on a filter and washed with water (100 mL). Drying the wet cake (90.65 g) over phosphorus pentoxide in a vacuum desiccator gave 28.19 g (93.13%) opf white, crude product.

To a 2 L multi-neck flask equipped with a mechanical stirrer, a thermometer and a one piece distillation apparatus, was added, under nitrogen, crude 17β-estra-1,3,5(10),8-tetraene-3,17-diol 3,17-diacetate (28.19 g) and methanol (675 mL). The slurry was heated and the product dissolved at 59-60° after the addition of a further 200 mL of methanol. The solution was distilled to approximately 500 mL and 404 mL of distillate was collected. The resulting slurry of crystals was allowed to cool to 35° and then cooled to -10°. The crystals were collected on a filter and washed with cold (-10°) methanol (3 x 50 mL). The wet crystals (30.14 g) were dried in a vacuum oven at 65° for 4 days to provide 25.15 g (89.24%) of white, crystalline 3, 17β-diacetate, having consistent spectral (ir, pmr and ms) data and acceptable elemental (C & H) analyses.

C. 17β-Estra-1,3,5(10),8-tetraene-3,17-diol 17-acetate

To a stirred suspension of 17β-estra-1,3,5(10),8-tetraene-3,17-diol 3,17-diacetate (24.16 g, 0.0682 mol. in methanol (150 mL) and dichloromethane (56 mL) cooled to -5° under nitrogen in a constant temperature bath was added 113 mL of solution of saturated methanolic potassium carbonate over a period of 45 minutes. (The temperature of the reaction ranged between -3 and -5° during the addition). Stirring at -5° under nitrogen was continued for a total period of 22.5 hours. A precipitate (product) formed after 1 3/4 hours. TLC on silica gel with dichloromethane-ethyl acetate (9.5:0.5) was used to judge reaction completion. The reaction flask was equipped with a one piece distillation apparatus. Distillation under diminished pressure (initially with an aspirator and later with an oil pump) while maintaining the reaction flask in the -5° constant temperature batch was used to remove all of the solvent. Five percent acetic acid (350 mL) was added rapidly to the dry solid and the resulting slurry was stirred at -5 to 0° for 2 hours. The resulting pH was 4.5. The crude product was collected on a filter and washed with 4 x 50 mL of water. The white crude material was stirred magnetically with 300 mL of water and collected once more on a filter. Washing with water (9 x 40 mL0 removed traces of acid and the pH of the last wash was 5.5. Drying the wet cake (37.06 g) in a vacuum desiccator over phosphorus pentoxide overnight provided 20.02 g (94.03%) of crude 17β-monoacetate.

To a 500 mL round bottom multi-neck flask equipped with a mechanical stirrer, a thermometer and a one piece distillation apparatus was added, under nitrogen, crude product (20.02 g), methanol (100 mL) and dichloromethane (200 mL). The pH (wetted pH paper) of the solution was 8; 5 drops of glacial acetic acid were added to bring the pH to 5. The solution was distilled at atmospheric pressure until crystallization was copious (volume of distillate 220 mL). The slurry was cooled to 40° and then to -10° with a dry ice-acetone bath. The product was collected on a filter and washed with cold (-10°) methanol (3 x 25 mL). The wet cake (21.65 g) was dried at 70° overnight and then at 85° for 5.5 hours in a vacuum oven. The resulting white crystalline material (18.07 g, 90.25%) had appropriate specral (ir, pmr and ms) data.

D. 17β-Estra-1,3,5(10),8-tetraene-3,17-diol 3-Sulfate 17-acetate triethylammonium salt

To a stirred solution of 17β-estra-1,3,5(10),8-tetraene-3,17-diol 17-acetate (17.18 g, 0.05499 mol. in tetrahydrofuran (200 mL) udner nitrogen was added sulfur trioxide-triethylamine (19.93 g, 0.110 mol.) and the solution was stirred for 4 hours at room temperature. Reaction completion was judged by TLC on silica gel with CH_2C1_2:EtOAc:MeOH:NEt_3 (3:3:1.5:2.5). Ether (600 mL) was added and the resulting slurry was stirred for 30 minutes at room temperature. The crude product was collected on a filter and washed with ether (3 x 27 mL). Drying the wet cake (36.50 g) for 2 days in a vacuum oven at room temperature afforded 30.13 g (100.98%) of crude, white product contaminated with excess reagent.

To a 1 L multi-neck flask equipped with a mechanical stirrer, a reflux condenser with a nitrogen inlet and a thermometer was added, under nitrogen, crude product (30.13 g) and methanol (80 mL). The temperature dropped from 26 to 20° and so a warm water bath was used to bring the temperature to 25-25°. Another 50 mL of methanol was added to achieve solution at that temperature. The pH (wetted pH paper) of the solution was 3. The pH was carefully adjusted to pH 7-8 by the addition of triethylamine (2.5 mL).

To the clear solution was immediately added ether (1 L). The resulting slurry was transferred to a 2 L Erlenmeyer with ether (2 x 75 mL) and stirred magneticaly. Aa additional 50 mL of ether (total 1,200 mL) was added and the resulting slurry was stirred at room temperature for 10 minutes. It was then cooled to -10° with an acetone-dry ice bath. The product was collected on a filter and washed with ether (3 x 30 mL). The wet cake (32.63 g) was dried overnight in a vacuum oven at room temperature to

give 23.25 g (85.64%) of purified material having appropraite spectral (ir, pmr and ms) data and acceptable elemental (C,H and N) analyses.

E. 17β-Estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate Sodium salt

To a solution of 17β-estra-1,3,5(10),8-tetraene-3,17-diol 3-Sulfate17-acetate (diester) triethylammonium salt, (22.27 g, 0.09511 mol. in methanol (145 mL) was adced 145 mL of 1.6 N sodium hydroxide and the mixture was stirred at room temperature (25° until 140 mL of distillate had been collected. The white, sticky solid (which precipitated during the evaporation) was dissolved by stirring with n-butanol (150 mL), under nitrogen, for 30 minutes. The mixture was transferred to a separatory funnel along with 20 mL of n-butanol, 10 mL of saturated brine and 10 mL of water. The separation of phases was slow so a further 60 mL of brine was added. The lower aqueous phase (~126 mL) was separated and the organic phase was washed with saturated brine (1 x 40 mL, followed by 3 x 30 mL, final wash pH 13-14). Solid product started to form. n-Butanol (500 mL), saturated brine (100 mL) and water (20 mL) were added. The product redissolved on mixing and the pH of the wash was 12. The organic phase was washed further with saturated brine (6 x 100 mL) until the final wash had pH 7-8. The slightly cloudy organic phase was filtered through Solka Floc and the filter pad was washed with n-butanol (50 mL). The solution was transferred with 50 mL of n-butanol to a round bottom flask and was evaporated under oil pump vacuum at 35-38° until crystallization occurred (volume of concentrate, 208 mL, distillate volume 630 mL.) To the stirred slurry was added ether (700 mL) and the slurry was stirred for 5 minutes at room temperature. The product was collected on filter and washed with ether (4 x 100 mL). The wet cake (39.99 g) was dried in a vacuum oven at room temperature (25°) to give 16.83 g off-white, crude product.

To the above crude material (16.83 g) was added 95% ethanol (165 mL) and the mixture was stirred for 10 minutes to achieve a cloudy solution. The solution was filtered. To the magnetically stirred, clear filtrate was added ether (1,500 mL.) (Crystallization was apparent after 500 mL had been added.)

The resulting slurry was stirred for 10 minutes at room temperature and then filtered. The filter cake was washed with ether (5 x 100 mL.) The resulting white, powdery solid (17.28 g) still smelled of ethanol and so it was magnetically stirred with ether (300 mL) for 10 minutes and collected once more on a filter. The filter cake was washed with ether (4 x 50 mL) and the wet cake (25.18 g) was dried at room

temperature overnight in a vacuum oven. The white product (15.50 g, 86.51%) had appropriate spectral (ir, pmr and ms) data.

Example 4

17β-Estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate sodium salt with tris (hydroxymethyl)aminomethane (TRIS)

To a stirred solution of 17β-estra-1,3,5(10),8-tetraene-3,17-diol 3-sulfate (ester) sodium salt (13.11 g) in distilled water (1,400 mL) was added TRIS (7.56 g) and the solution was filtered. The filter was washed with 100 mL of distilled water. The filtrate and washing was transferred to a tray at -40° using 500 mL of distilled water. The frozen solution was freeze dried over a period of 4 days. The resulting white shiny material (20.92 g) was pressed, mixed, placed in a preweighed bottle, and dried a further 4 days at room temperature (23°) in the freeze dryer. The material weighed 19.69 g (97.55%).

Example 5

Preparation of 17α,Δ8,9-dehydroestradiol or 17β,Δ8,9-dehydroestradiol by the in vivo metabolism of Δ8,9-dehydroestrone

A. Dose administration and sample collection

Four female dogs, weighing 9 - 13 kg were given 1 mg/kg of $\Delta^{8,9}$-dehydroestrone, po in capsules. The dogs were housed throughout the study in individual stainless steel metabolism cages. The dogs were fasted overnight prior to dosing and food was returned 4 hr afterwards. Water was provided ad libitum throughout the study. Blood (8 ml) was collected at 0, 0.5, 1, 2, 4, 6 and 8 hr post-dose and plasma was obtained. Urine was collected for 24 hr post-dose over dry ice.

B. Determination of metabolite profiles by HPLC

1. Urinary metabolite profiles

Urinary metabolites were determined by analyzing 0-24 hr samples after enzyme hydrolysis. To hydrolyze the conjugates, an aliquot (1 ml) of urine was combined with 1 ml of 0.05 M sodium acetate buffer, pH 5.0, and incubated overnight at 37°C with 2000 units of Glusulase (Helix Pomatia, DuPont). The hydrolyzed samples were

centrifuged at 2500 rpm for 10 min. Supernatants of hydrolyzed urine samples were extracted using C-18/N+ cartridges (Bifunctional cartridges containing C-18 and quaternary amine sorbents from Chemical Separations, PA). The cartridge extractions were performed by vacuum pull (Speed Mate 30 Applied Separations). The C-18/N+ cartridges were first conditioned with 2 x 1 ml acetone, 2 x 1 ml methanol, 2 x 1 ml 0.1N acetic acid and 2 x 1 ml H_2O. The sample (supernatant) was applied and the cartridges were washed with 2 x 1 ml H_2O, 2 x 1 ml 0.1N acetic acid, 2 x 1 ml H_2O and 2 x 1 ml hexane. The metabolites were then eluted in 2 x 1 ml acetone. The extracts were dried under N_2 and reconstituted in 1 ml of mobile phase [60% buffer (0.05 M KH_2PO_4, pH 3.0), and 40% organic (2:1 acetonitrlie; methanol)]. Twenty-five microliters of each extract were injected onto a C-6 5µ HPLC column (Alltech) using an SP8780 autosampler (Spectra-Physics) and eluted at a flow rate of 1 ml/min with an ESA-420 pump. Metabolites were detected with an electrochemical detector (Coulochem II-ESA). The potentials for the detector were set at 0.75 V (guard cell), 0.35 V (electrode 1) and 0.70 V (electrode 2) and the signals from electrode 2 were recorded on a chart recorder (Kipp and Zonen BD 40).

2. Plasma metabolite profiles

Plasma samples were also analyzed by HPLC after hydrolysis. Hydrolysis of the conjugates was achieved by incubating the samples with Glusulase as described for the urine samples above. Following centrifugation, the plasma samples were extracted using C-18/N+ cartridges by the above described procedure and analyzed by HPLC-electrochemical detection.

3. Hydrolysis in the presence of enzyme inhibitor

The type(s) of conjugates present in the plasma and urine samples were determined by conducting the hydrolysis of all samples in the presence or absence of 200 µM of saccharolactone, an inhibitor of β-glucuronidase. The hydrolysis, extraction and HPLC analyses were performed as described above.

C. Identification of the metabolites by GC/MS analysis of hydrolyzed urine and plasma samples

The structures of the metabolites were identified by EI-GC/MS analysis of TMS derivatives of extracts of hydrolyzed urine and plasma samples. The structures of the metabolites were confirmed by analyzing and comparing the mass spectra of authentic reference standards.

1. GC/MS analysis of hydrolyzed urine samples

Five ml of 0-24 hr urine from one dog was mixed with 5 ml of pH 5.0 sodium acetate buffer (0.05M) and 10,000 units of Glusulase. The mixture was incubated overnight at 37°C in a shaking water bath (Precision Scientific). The following morning, the hydrolysates were centrifuged at 2500 rpm for 10 min. C-18/N^+ cartridge extraction was carried out as previously described. Following acetone elution of the metabolites, N_2 was used to dry the extracts. Each extract or reference standard was then reconstituted in 80 µl of toluene; then 10 µl of BSTFA and 5 µl of pyridine were added and reacted at 65°C for 1 hr to form TMS derivatives of the metabolites. The derivatized samples were analyzed using a Finnigan-MAT 8230 high resolution mass spectrometer directly interfaced to a Varian 3400 gas chromatograph. The data were acquired on an SS-300 data system and printed on a Printronix. The source ionization mode was positive electron impact. The column used was a J&W DB-5MS 30 M x 0.32 mm ID. The initial column was 80°C with a -1 minute hold programmed to 260°C at 10°C/min. The injection temperature was 250°C. The injection volume was 2 µl.

2. GC/MS analysis of hydrolyzed plasma samples

Two ml of plasma was mixed with 2 ml of pH 5.0 sodium acetate buffer (0.05 M) and hydrolyzed with 2000 units Glusulase (DuPont). The mixture was incubated overnight at 37°C in a shaking water bath and extracted as described previously. Expermiental samples, control plasma and control plasma containing synthetic standards were analyzed as described above.

D. Metabolite Profile

1. Urine

The HPLC chromatograms of enzyme hydrolyzed 0-24 hour urine resulted in three peaks which were not present in the chromatogram of an extract from control dog urine. The retention time of the third eluting peak was identical to that of Δ8,9-dehydroestrone. Peaks 1 and 2 had the same retention times (17.4 min and 19.3 min, respectively), as 17β,Δ8,9-dehydroestradiol and 17α,Δ8,9-dehydroestradiol. Also, co-chromatography with reference standards clearly indicated the presence of 17β,Δ8,9-dehydroestradiol and 17α,Δ8,9-dehydroestradiol as peaks 1 and 2, respectively. Analysis of samples that were extracted before hydrolysis did not reveal

any metabolites, suggesting that little of the metabolites exist in unconjugated forms. Samples from all dogs gave similar metabolite profiles.

2. Plasma

Analysis of plasma samples after hydrolysis also showed the presence of the same three peaks as in the urine samples at all time points examined. In both urine and plasma sampes, addition of saccharolactone inhibited the hydrolysis of the conjugates, indicating that most of the metabolites were in glucuronide forms.

E. Identification of 17α,Δ8,9-dehydroestradiol

The mass spectrum of peak 2 in the GC/MS chromatograms displayed a molecular ion m/z 414 suggesting that it is a diol. A weak M^+ ion and a base peak at m/z 309 [M-105 (CH_3, TMS OH)] are characteristic of 17α-dihydro ring-B unsaturated estrogens. Other fragments at m/z 399 [M-15(CH_3)], 324 [M-90 (TMS)] and 283 [M-131] are also characteristic for 17-hydroxy estrogens. The mass spectrum of peak 2 was identical to that of authentic reference 17α,Δ8,9-dehydroestradiol. Based on these data the structure of this metabolite was identified as 17α,Δ8,9-dehydroestradiol.

E. Identification of 17β,Δ8,9-dehydroestradiol

The mass spectrum of peak 1 in the GC/MS chromatograms displayed a molecular ion m/z 414 and fragment ions at m/z 399 [M-15 (CH_3)], 324 [M-90 (TMS)], 309 [M-105 (CH_3, TMS)] and 283 (M-131) that are characteristic of ring-B unsaturated estrogen diols. Unlike 17α,Δ8,9-dehydroestradiol, this compound gave a strong molecular ion with a relative intensity of about 95%, which is characteristic of 17β-dihydro ring-B unsaturated estrogens. The mass spectrum of peak 3 was identical to that of reference 17β,Δ8,9-dehydroestradiol. Based on these data the structure of this metabolite was determined as 17β,Δ8,9-dehydroestradiol.

THIS SHEET HAS BEEN REPLACED

WHAT IS CLAIMED IS:

1. A compound which is 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester.

2. The compound of claim 1 wherein the pharmaceutically acceptable salt of the 3-sulfate ester is an alkali metal salt, alkaline earth metal salt, ammonium salt, alkylammonium salt containing 1-6 carbon atoms, or dialkylammonium salt containing 1-6 carbon atoms in each alkyl group, or trialkylammonium salt containing 1-6 carbon atoms in each alkyl group.

3. The compound of claim 1 which is 17α,Δ8,9-dehydroestradiol 3-sulfate sodium salt.

4. The compound of claim 1 which is 17α,Δ8,9-dehydroestradiol 3-sulfate triethylammonium salt.

5. A compound which is 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester.

6. The compound of claim 5 wherein the pharmaceutically acceptable salt of the 3-sulfate ester is an alkali metal salt, alkaline earth metal salt, ammonium salt, alkylammonium salt containing 1-6 carbon atoms, or dialkylammonium salt containing 1-6 carbon atoms in each alkyl group, or trialkylammonium salt containing 1-6 carbon atoms in each alkyl group.

7. The compound of claim 5 which is 17β,Δ8,9-dehydroestradiol 3-sulfate sodium salt.

8. The compound of claim 5 which is 17β,Δ8,9-dehydroestradiol 3-sulfate triethylammonium salt.

9. A method of inhibiting or treating free radical induced disease states by administering an antioxidant amount of 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, to a mammal in need thereof.

THIS SHEET HAS BEEN REPLACED

10. A method of inhibiting endogenous free radical involvement in disease development of cancers, central nervous system disorders, Alzheimer's disease, bone disease, aging, inflammatory disorders, peripheral vascular disease, rheumatoid arthritis, autoimmune diseases, respiratory distress, emphysema, prevention of reperfusion injury, viral hepatitis, chronic active hepatitis, tuberculosis, psoriasis, systemic lupus erythematosus, adult respiratory distress syndrome, central nervous system trauma and stroke, or injury during reperfusion procedures which comprises administering 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, to a mammal in need thereof.

11. A method of inhibiting or treating free radical induced disease states by administering an antioxidant amount of 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, to a mammal in need thereof.

12. A method of inhibiting endogenous free radical involvement in disease development of cancers, central nervous system disorders, Alzheimer's disease, bone disease, aging, inflammatory disorders, peripheral vascular disease, rheumatoid arthritis, autoimmune diseases, respiratory distress, emphysema, prevention of reperfusion injury, viral hepatitis, chronic active hepatitis, tuberculosis, psoriasis, systemic lupus erythematosus, adult respiratory distress syndrome, central nervous system trauma and stroke, or injury during reperfusion procedures which comprises administering 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, to a mammal in need thereof.

13. A method of providing estrogen replacement therapy or treating estrogen deficiency in a mammal in need thereof, which comprises administering an estrogenic amount of 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

14. A method of providing estrogen replacement therapy or treating estrogen deficiency in a mammal in need thereof, which comprises administering an estrogenic amount of 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

This Sheet Has Been Replaced

THIS SHEET HAS BEEN REPLACED

* C L M S *

15. A method of treating vasomotor symptoms related to estrogen deficiency in a mammal in need thereof, which comprises administering 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

16. The method of claim 15, wherein the vasomotor symptom is hot flushes.

17. A method of treating vasomotor symptoms related to estrogen deficiency in a mammal in need thereof, which comprises administering 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

18. The method of claim 17, wherein the vasomotor symptom is hot flushes.

19. A method of treating or inhibiting osteoporosis in a mammal in need thereof which comprises administering an anti-osteoporosis effective amount of 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

20. A method of treating or inhibiting osteoporosis in a mammal in need thereof which comprises administering an anti-osteoporosis effective amount of 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

21. A method of treating or inhibiting atherosclerosis in a mammal in need thereof which comprises administering an anti-atherosclerosis effective amount of 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

22. A method of treating or inhibiting atherosclerosis in a mammal in need thereof which comprises administering an anti-atherosclerosis effective amount of 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester to said mammal.

23. A pharmaceutical composition which comprises 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester and a pharmaceutical carrier.

This Sheet Has Been Replaced

24. A pharmaceutical composition which comprises 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester and a pharmaceutical carrier.

25. 17α,Δ8,9-Dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, which is at least 1% pure.

26. 17β,Δ8,9-Dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester, which is at least 1% pure.

27. A process for preparing 17α,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester which comprises

(a) reducing the 17-ketone of Δ8,9-dehydroestrone with a hydride reducing agent to give 17β,Δ8,9-dehydroestradiol;

(b) acylation of the 3-hydroxyl group of 17β,Δ8,9-dehydroestradiol with a suitable acylating agent to produce a 3-acylated 17β,Δ8,9-dehydroestradiol;

(c) reacting the 3-acylated 17β,Δ8,9-dehydroestradiol with 3,5-dinitrobenzoic acid and triphenylphoshphine to give 3-acylated, 17-(3,5-dinitrobenzoyl) 17α,Δ8,9-dehydroestradiol; and

(d) treatment of 3-acylated, 17-(3,5-dinitrobenzoyl) 17α,Δ8,9-dehydroestradiol with an aqueous base to give 17α,Δ8,9-dehydroestradiol.

28. The process of claim 27 which further comprises

(a) acylation of 17α,Δ8,9-dehydroestradiol with a suitable acylating reagent to give 3,17-diacyl 17α,Δ8,9-dehydroestradiol;

(b) hydrolysis of the 3-acyl group with a mild base to give 17-acyl 17α,Δ8,9-dehydroestradiol; and

(c) sulfation of the 3-hydroxyl group with a sulfur trioxide -ammonia, -alkylamine, -dialkylamine, or -trialkylamine reagent to give 17α,Δ8,9-dehydroestradiol 3-sulfate ammonium, alkylammonium, dialkylammonium, or trialkylammonium salt.

29. The process of claim 28 which further comprises treatment of the 17α,Δ8,9-dehydroestradiol 3-sulfate ammonium, alkylammonium, dialkylammonium, or trialkylammonium salt with a suitable metal hydroxide base to give 17α,Δ8,9-dehydroestradiol 3-sulfate metal salt.

THIS SHEET HAS BEEN REPLACED

30. A process for preparing 17β,Δ8,9-dehydroestradiol or a pharmaceutically acceptable salt of its 3-sulfate ester which comprises reducing the 17-ketone of Δ8,9-dehydroestrone with a hydride reducing agent to give 17β,Δ8,9-dehydroestradiol.

31. The process of claim 30 which further comprises

(a) acylation of 17α,Δ8,9-dehydroestradiol with a suitable acylating reagent to give 3,17-diacyl 17α,Δ8,9-dehydroestradiol;

(b) hydrolysis of the 3-acyl group with a mild base to give 17-acyl 17β,Δ8,9-dehydroestradiol; and

(c) sulfation of the 3-hydroxyl group with a sulfur trioxide -ammonia, -alkylamine, -dialkylamine, or -trialkylamine reagent to give 17β,Δ8,9-dehydroestradiol 3-sulfate ammonium, alkylammonium, dialkylammonium, or trialkylammonium salt.

32. The process of claim 31 which further comprises treatment of the 17β,Δ8,9-dehydroestradiol 3-sulfate ammonium, alkylammonium, dialkylammonium, or trialkylammonium salt with a suitable metal hydroxide base to give 17β,Δ8,9-dehydroestradiol 3-sulfate metal salt.

This Sheet Has Been Replaced