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13. ABSTRACT (Maximum 200 Words)

My studies have compared the carcinogenic effects of an E_2-17β-fatty acid ester preparation, E_2 and 4-OH-E_2 in the breast, pituitary and uterus of ACI rats. Results showed that chronic administration of an E_2-17β-fatty acid ester preparation to these rats preferentially induces the development of mammary tumors while chronic administration of E_2 results in the preferential formation of pituitary tumors. The chronic administration of 4-OH-E_2 to intact female ACI rats did not induce the formation of mammary or pituitary tumors, although there was hyperplasia present in the mammary glands. These results are the first report demonstrating that chronic administration of an estrogen fatty acid ester selectively induces the development of mammary tumors in this animal model. In order to study the carcinogenic activity of other estrogen fatty acid esters, such as those of 4-OH-E_2, they must first be synthesized. Therefore, a facile method for the chemical synthesis of large amounts of 4-hydroxyestradiol-17β-stearate, a representative fatty acid ester of the strongly-procarcinogenic estrogen metabolite 4-hydroxyestradiol, has been developed with estrone as the starting material. The ready availability of large amounts of chemically-synthesized 4-hydroxyestradiol-17β-fatty acid esters make it possible for future studies to systematically characterize their hormonal and carcinogenic potency and efficacy.

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INTRODUCTION

Exogenous administration of estrogens has been shown to induce tumor formation in animals (Nandi *et al.*, 1995; Yager and Liehr, 1996; Clifton and Meyer, 1956; Kirkman, 1959; Cutts and Noble, 1964; Newbold *et al.*, 1990; and Li and Li, 1996). In humans, there is strong evidence showing that chronic estrogen administration more readily correlates with an increase in uterine cancer risk than breast cancer risk (Ziel and Finkle, 1975; Sitteri, et al., 1976; Mack et al., 1976; McDonald, et al., 1977; and Grady and Ernster, 1996). Although the difference is not fully understood, it is possible that some of the biologically active estrogen derivatives, such the estrogen fatty acid esters, may play a more significant role than estradiol-17β_2 in the induction of breast cancer.

Recently it was suggested that the mammary adipocytes may serve as a storage site for the lipoidal estrogen fatty acid esters, providing sustained release of bioactive estrogens to mammary glandular cells (Zhu and Conney, 1998; Mills *et al.*, 2001). Data showed that administration of E_2-17β-stearate, a representative estrogen fatty acid ester, to female ovariectomized rats had a differential, strong mitogenic effect in the fat-rich mammary tissues and this effect was not observed in the uterus (Mills *et al.*, 2001). This finding has prompted the hypothesis that the estrogen fatty acid esters may be an important group of endogenous estrogens that can preferentially induce tumor formation in the fat-rich mammary tissues. Therefore, this year was dedicated to testing this hypothesis.

The results of my studies will help to shed light on the physiologic and pathophysiologic roles of endogenously-formed estrogen-fatty acid esters *in vivo*. In addition, the animal studies presented, demonstrated that various endogenous estrogen-fatty acid esters are stronger and more selective than the unesterified parent hormones in stimulating cell growth and in inducing tumor formation in the fat-rich mammary tissues. This data will form the basis for future epidemiological studies to determine the unique importance of endogenous estrogen-fatty acid esters in human breast cancers. In addition, the chemical synthesis of 4-hydroxyestradiol-17β-stearate, a representative fatty acid ester of 4-hydroxyestradiol, is presented. The availability of large quantities of this compound make it possible for future studies to characterize their unique biological activities in animal models as well as in humans.

It is possible that the levels of endogenous estrogen fatty acid esters and the activity of estrogen esterase present in the breast might be of more relevance as a risk factor for human breast cancer than the circulating levels of unesterified estrogens as were usually measured in all earlier epidemiological studies. The results of my studies may also lead to the development of new strategies to mammary cancer prevention through inhibition of the esterase-catalyzed release of bioactive estrogen *in situ*.

BODY

Study 1

Objective: One of the objectives for this year was to chemically synthesize 4-hydroxyestradiol-17β-stearate (Task 1 from the original Statement of Work). Only after this compound is made can Task 2 from the original Statement of Work be accomplished.

Aim: The aim of this study was to develop a facile method for the chemical synthesis of large quantities of 4-hydroxyestradiol-17β-stearate, a representative fatty acid ester of 4-hydroxyestradiol, using estrone as the starting material. Another aim of this study was to chemically synthesize the 3,4-diacetate ester prodrug of 4-hydroxyestradiol-17β-stearate. This prodrug would prevent the catechol ring from premature oxidation.

Training: To accomplish the chemical synthesis of 4-hydroxyestradiol-17β-stearate, I learned many different chemical techniques. These techniques included chemical extractions, recrystallization, chromatographic separations, and how to use and interpret NMR spectrometric data.

Research Accomplishments: A facile method for the chemical synthesis of large amounts of 4-hydroxyestradiol-17β-stearate, a representative fatty acid ester of the strongly-procarcinogenic estrogen metabolite 4-hydroxyestradiol, has been developed with estrone as the starting material. In addition, 4-hydroxyestradiol-3,4,-diacetate 17β-stearate, a stable prodrug of 4-hydroxyestradiol-17β-stearate, which would prevent the catechol structure from premature oxidation, has been designed and synthesized. It is of note that the facile procedure described in the present study for the chemical synthesis of 4-hydroxyestradiol-17β-stearate (a representative fatty acid ester) should be equally applicable to the synthesis of various other fatty acid ester derivatives of 4-hydroxyestradiol. The ready availability of large amounts of chemically-synthesized 4-hydroxyestradiol-17β-fatty acid esters would make it possible for future studies to systematically characterize their hormonal and carcinogenic potency and efficacy in various laboratory animal models as well as to determine the blood and tissue levels of this unique class of procarcinogenic estrogen metabolites in humans and also to probe their roles in the development of human hormonal cancers. Please see **Appendix 1** for a very detailed description of the research accomplishments associated with this study.

Deviations from the original Statement of Work: 4-hydroxyestradiol-17β-stearate was extremely difficult to chemically synthesize in large quantities. It took almost an entire year to synthesize sufficient quantities to implant into rats for carcinogensis studies. Therefore, only carcinogensis studies will be reported with this compound.

Study 2

Objective/Hypothesis: This year, I tested the novel hypothesis that the estrogen fatty acid esters are an especially important group of endogenous estrogens for inducing tumor formation in the rat-rich mammary tissues as compared to the uterus and pituitary. I focused on the following specific aim (Task 2 from the Statement of Work):

Aim: To evaluate the carcinogenic activity of estrogen-fatty acid esters in the breast, uterus and pituitary of female ACI rats in comparison to the carcinogenic activity of E_2 and 4-OH-E_2.

Training: To accomplish the goals set forth in this study, I learned many different scientific techniques. I have learned how to perform live animal experiments including techniques such as oral gavage, subcutaneous implantation of pellets to deliver drugs, how to palpitate for mammary tumors, and how to dissect out organs of interest including the mammary glands, uterus, pituitary and liver. I have also learned how to "fix" and process all of these tissues for histopathological analysis. I processed all of the tissues as well as embedded them in paraffin and cutting them with a microtome. I have also learned immunohistochemical staining techniques with antibodies as well as common H and E stains. I have also learned to read the histopathological as well as immunohistochemical slides.

Research Accomplishments: In this study, the activity of an estrogen-fatty acid ester preparation was compared with E_2 for the induction of tumors in the breast, uterus, and pituitary of intact female ACI rats was compared. The data demonstrated that chronic treatment with E_2 preferentially induces the growth of pituitary tumors while treatment with E_2 fatty acid esters resulted in a higher incidence of mammary tumors compared to the E_2 treated animals. Although animals treated with an estrogen fatty acid ester also developed pituitary tumors, they were statistically smaller than those of animals treated with E_2. These results suggest that the endogenously formed estrogen fatty acid esters are pathophysiologically more important than E_2 for the selective induction of mammary tumor formation. Please see **Appendix 2** for a very detailed description of the research accomplishments associated with this study.

Deviations from original Statement of Work: In the original Statement of Work, I had stated that female Sprague-Dawley rats would be used for carcinogensis studies. However, female ACI rats were chosen as a more appropriate animal model because recent studies have shown that chronic treatment of these rats with E_2 via subcutaneous pellets or silastic implants rapidly induced mammary tumor formation, while spontaneous mammary tumors in the absence of exogenous E_2 are rare (Harvell *et al.*, 2000). It is also known that chronic administration of estrogens induces PRL-producing pituitary tumors in these animals (Shull *et al.*, 1997; Harvell *et al.*, 2000; Harvell *et al.*, 2002) and it has been suggested that the resulting hyperprolactinemia is an important hormonal factor contributing to mammary tumor formation (Henderson *et al.*, 1996; Harvell *et al.*, 2002).

KEY RESEARCH ACCOMPLISHMENTS

- Synthesis of 4-hydroxyestradiol-17β-stearate complete
- Synthesis of 4-hydroxyestradiol-3,4,-diacetate 17β-stearate, a stable prodrug of 4-hydroxyestradiol-17β-stearate complete
- Carcinogenic studies with an estrogen fatty acid ester preparation completed; manuscript in progress
- Carcinogenic studies with 4-hydroxylestradiol-17β-stearate underway

REPORTABLE OUTCOMES

- Presentation titled "Synthesis of 4-hydroxyestradiol-17β-stearate, an estrogen fatty acid ester, and its stable prodrug 4-hydroxyestradiol-3,4-diacetate 17β-stearate" presented at the 54TH Southeastern Regional Meeting of the American Chemical Society
- Abstract titled, "Synthesis of 4-Hydroxyestradiol-17β-Stearate, an Estrogen Fatty Acid Ester, and its Stable Prodrug 4-Hydroxyestradiol-3,4-Diacetate 17β-Stearate" published in the abstracts for the 54TH Southeastern Regional Meeting of the American Chemical Society
- Abstract titled, "Naturally-Occurring Estradiol-17β-Fatty Acid Ester, but not Estradiol-17β, Preferentially Induces the Development of Mammary Tumors in Female ACI Rats" published in the abstracts for the 94th Annual Meeting of the American Association for Cancer Research
- Manuscript in progress titled "Synthesis of 4-hydroxyestradiol-17β-stearate, an estrogen fatty acid Ester, and its Stable Prodrug 4-Hydroxyestradiol-3,4-Diacetate 17β-Stearate"
- Manuscript in progress titled "Naturally-Occurring Estradiol-17β-Fatty Acid Esters, but not Estradiol-17β, Preferentially Induce the Development of Mammary Tumors in Female ACI Rats."
- Laura Hook Mills – Dissertation written and published titled "Studies on the Effects of Estrogen Fatty Acid Esters on Mammary Cell Growth and Carcinogenesis in Female Rats. This dissertation included experiments that were supported by this award
- Laura Hook Mills – Ph.D. degree granted; defense on June 2, 2003
- Laura Hook Mills – Granted a postdoctoral position with Dr. Michael Felder of the University of South Carolina Department of Biological Sciences

CONCLUSIONS

My studies have shown that the estrogen fatty acid esters are a group of mammary selective hormones. They are stronger and more selective than the unesterified parent hormones in stimulating cell growth and in inducing tumor formation in the fat-rich mammary tissues, compared to the uterus and pituitary. Future epidemiological studies should be conducted to determine the unique importance of endogenous estrogen-fatty acid esters in human breast cancers.

In addition, animal studies are currently underway to study the carcinogenic activity of 4-hydroxyestradiol-17β-stearate in female ACI rats. The rats were implanted with pellets containing 18μmol of 4-hydroxyestradiol-17β-stearate and tumor formation should occur very soon. The same procedure was followed for this experiment as IS described in **Appendix 2.**

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Appendix 1

Synthesis of 4-Hydroxyestradiol-17β-Stearate, an Estrogen Fatty Acid Ester, and its Stable Prodrug 4-Hydroxyestradiol-3,4-Diacetate 17β-Stearate

(adapted from dissertation of Laura H. Mills; manuscript in preparation)

1. INTRODUCTION

Estrogen-fatty acid esters represent a unique class of naturally-occurring, nonpolar estrogen derivatives that are metabolically synthesized through esterification of the non-phenolic hydroxyl group of the endogenous estrogens with fatty acids (Hochberg, 1998). Because of their high lipophilicity and slow metabolic clearance, estrogen-fatty acid esters have very long half-lives in animals and humans and tend to be stored in the fat (Larner *et al.*, 1985; Vazquez-Alcantara, *et al.*, 1989; MacLusky *et al.*, 1989; Hochberg *et al.*, 1991; Schatz and Hochberg, 1981). Although the estrogen-fatty acid esters have no binding affinity for the estrogen receptor, they are potent estrogens *in vivo* and their hormonal activity results from the esterase-mediated slow release of the parent hormones (Larner *et al.*, 1992). Extensive earlier studies by Richard B. Hochberg and colleagues (reviewed in Larner *et al.*, 1985; MacLusky *et al.*, 1989; and Hochberg *et al.*, 1991) have demonstrated that the naturally-occurring estradiol-17β-fatty acid esters (when administered as a single *s.c.* or *i.p.* injection) were extremely potent and long-acting hormones in the uterus of the female rats or mice.

Since the mammary glandular cells are surrounded by large amounts of adipocytes, the fat tissue may serve as a storage site for the fatty acid esters of estradiol-17β as well as for the fatty acid esters of the bioactive hydroxylated estrogen metabolites such as 4-hydroxyestradiol. Recently, it was suggested that the estrogen-fatty acid esters may constitute an important class of estrogenic hormones for the fat-rich mammary tissue when compared with some other target tissues (such as the uterus) (Zhu and Conney, 1998; Mills *et al.*, 2001). In partial support of this intriguing idea, recent studies have shown that the naturally-occurring estradiol-17β-stearate has a differential, strong mitogenic effect in the fat-rich mammary tissues over the uterus, and this effect was not observed with estradiol-17β (Mills *et al.*, 2001). Certainly, it would also be of great interest to advance our knowledge on the biological effects of other naturally-occurring estrogen-fatty acid ester derivatives, particularly those of 4-hydroxyestradiol, a unique bioactive estrogen metabolite.

4-Hydroxyestradiol is hormonally-active at the classical estrogen receptors (Zhu and Conney, 1998), potentially genotoxic/mutagenic in mammalian cells (Liehr, 2000; Cavalieri and Rogan, 2002; and Yager and Liehr, 1996), and strongly-procarcinogenic in several animal models tested thus far (Liehr *et al.*, 1986; Li and Li, 1987; Newbold and Liehr, 2000). Moreover, this unique catechol estrogen metabolite may also activate its own signal transduction pathway that is different from the estrogen receptor-mediated signaling pathways (Das *et al.*, 1997; Das *et al.*, 2000). However, this estrogen metabolite generally has a much shorter half-life and much lower bioavailability than estradiol-17β because of its rapid conjugative metabolism. The esterification of this compound would, like the esterification of estradiol-17β, be expected to greatly prolong its hormonal and carcinogenic activity, especially in the fat-rich mammary tissue.

In this part of the study, a facile method for the chemical synthesis of large quantities of 4-hydroxyestradiol-17β-stearate, a representative fatty acid ester of 4-hydroxyestradiol, is described using estrone as the starting material. In addition, the chemical synthesis of the 3,4-diacetate ester prodrug of 4-hydroxyestradiol-17β-stearateis also described. This prodrug would prevent the catechol ring from premature oxidation. The availability of large quantities of the chemically synthesized 4-hydroxyestradiol-17β-

fatty acid esters makes it possible for future studies to systematically characterize their biological activities in animal models as well as in humans.

2. EXPERIMENTAL

2.1. General

Unless otherwise indicated, all the chemicals and solvents used in this study were of analytical grade and were used without any further purification. Estrone [1,3,5(10)-Estratrien-3-ol-17-one] was purchased from the Sigma Chemical Co. (St. Louis, MO). Flash chromatography was performed with 230-400 mesh silica gel and thin layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (EM Science, Gibbstown, NJ) using ethyl acetate/n-heptane as the mobile phase. Melting points (uncorrected) were determined in capillary tubes using an Electrothermal® melting point apparatus. The ^{1}H-NMR and ^{13}C-NMR were recorded on a Varian Mercury 300 spectrophotometer (300-MHz) using tetramethysilane as an internal standard and $CDCl_3$ (deuteriochloroform) as the solvent. Only selected key NMR shifts were listed for each compound. High-resolution mass spectrometry (HRMS) was performed in the Department of Chemistry of the University of South Carolina.

Note that for each steroid compound when described the first time, its trivial name is listed first and is followed by the IUPAC name in a bracket. For convenience, the trivial names are used whenever appropriate.

2.2. 4-Nitroestrone [1,3,5(10)-estratrien-3-nitro-4-ol-17-one; compound 1]

For the synthesis of 4-nitroestrone [1,3,5(10)-estratrien-4-nitro-3-ol-17-one] teo methods were described; one described by Stuenrauch and Knuppen (Stuenrauch and Knuppen, 1976) and an earlier procedure described by Tomson and Horwitz (Tomson and Horwitz, 1959).

Following the procedure of Stuenrauch and Knuppen, estrone (1g, 3.7 mmol) was dissoloved in boiling glacial acetic acid (30 ml). After all of the estrone had dissolved the mixture was allowed to cool down to 50° C. At this time, a nitrating mixture, made of nitric acid (250 µl), water (10 ml) and a few crystals of sodium nitrite, was warmed to 50°C also. The nitrating mixture was then added dropwise to the estrone/acetic acid mixture. The solution was then allowed to cool to room temperature over 24 hours and the yellow precipitate was filtered off. TLC (ethyl acetate/n-heptane, 1:1) showed multiple spots. The product was added to a mixture of boiling acetic acid and water and then immediately filtered (note: the product did not dissolve in this mixture). TLC of the resulting solid (ethyl acetate/n-heptane, 1:1) showed one spot. M.p. 267-270°C (lit. 270-278°C (Stubenrauch and Knuppen, 1976)). R_f (ethyl acetate/n-heptane, 1:2) 0.4. There were obtained 4-nitroestrone (372 mg, 1.2 mmol, 32% yield).

4-Nitroestrone was also synthesized according to the published procedure of Tomson and Horwitz (Tomson and Horwitz, 1959). Estrone (10 g, 37 mmol) was dissolved in glacial acetic acid (522 ml) that was heated to 70-75°C. Next, concentrated nitric acid (2.3 ml) in glacial acetic acid (57.9 ml) was added all at once with stirring to the estrone mixture. The resulting mixture was allowed to cool to room temperature.

After sitting for 24 hours, the yellow precipitate was filtered. It was found to be pure by TLC (ethyl acetate/n-heptane, 1:1). There was obtained 4-nitroestrone (3.1 g, 9.7 mmol). M.p. 273-275°C (lit. 273-276°C (Tomson and Horwitz, 1959)). R_f (ethyl acetate/n-heptane, 1:1). 0.462. In addition, an extra crop of 4-nitroestrone (1.4 g, 4.5mmol) can be collected exactly as described in the paper for a total yield of 38.4 %.

The latter procedure was preferred because it was less cumbersome and gave a slightly better overall yield of 4-nitroestrone. Therefore, the procedure of Tomson and Horwitz (Tomson and Horwitz, 1959) was employed for all further synthetic protocols.

2.3. 4-Aminoestrone [1,3,5(10)-estratrien-4-amino-3-ol-17-one; compound 2]

4-Nitroestrone was reduced to 4-aminoestrone [1,3,5(10)-estratrien-4-amino-3-ol-17-one] according to the procedure described by Stubenrauch and Knuppen (Stubenrauch and Knuppen, 1976).

To begin, 4-nitroestrone (1g, mmol), acetone (300 ml), water (60 ml) and NaOH (1N, 60 ml) were heated to reflux. Sodium hydrosulfte (2 g, 11.5 mmol) was added every 5 minutes. After the addition of 8 g of sodium hydrosulfite, the solution was allowed to stir for an addition 10 minutes. It should be noted that after each addition of sodium hydrosulfite, the solution turned a lighter shade of yellow until it was almost clear. After 25 minutes of refluxing, the acetone was removed in vacuo and acetic acid (10%, 20 ml) was added. The solution was allowed to sit in an ice bath for 2 hours after which time a white precipitate was deposited. The solid was collected and washed with a large amount of water (approximately 70 ml) to remove all the sodium hydrosulfite. The solid was then air dried at room temperature. There was obtained (843.7 mg, 3.0 mmol, 93.2% yield). M.p. 256-260°C (lit. 250-253°C (Stubenrauch and Knuppen, 1976)). R_f (ethyl acetate/n-heptane, 1:1) 0.314.

2.4. 4-Hydroxyestrone [1,3,5(10)-estratrien-3,4-diol-17-one; compound 3]

The C-4 *ortho*-aminophenol group was further converted to an *ortho*-quinone under an inverse oxidation technique as described earlier (Stubenrauch and Knuppen, 1976), and the subsequent reduction of the *ortho*-quionone yielded 4-hydroxyestrone.

To begin, 4-aminoestrone (500 mg, 1.8 mmol) was dissolved completely in glacial acetic acid (150 ml). This mixture was then added over a 3 minute time period to a vigorously stirring solution of sodium metaperiodate (5.0 g, 23.4 mmol) in HCl (0.1N, 350 ml). The solution was allowed to stir for 30 seconds and was then poured onto chloroform (100 ml) and extracted twice with this solvent. The organic layer was then washed two times with water (100 ml). Glacial acetic acid (100 ml) and 1.5 g potassium iodide was then added to the organic layer (note: potassium iodide was first dissolved in a small amount of water) and the mixture was shaken for 2 minutes. Upon the addition of a 5% sodium bisulfite solution (50 ml) the mixture changed from a dark red to a light yellow. The mixture was extracted two more times with chloroform (100 ml) and the organic layer was washed one time with water (100 ml). The aqueous layers were combined and reextracted with chloroform (100 ml). The combined organic layers were dried over anhydrous sodium sulfate. After the addition of glacial acetic acid (2.5 ml), the solvent was removed in vacuo. A yellowish solid formed and was determined to be

4-hydxyestrone. There was obtained 4-hydroxyestrone (313.7 mg, 1.1 mmol, 62.5 % yield). M.p. 258-262 °C. R_f (ethyl acetate/n-heptane, 2:1) 0.567.

2.5. 4-Hydroxyestrone-3,4-dibenzyl ether [1,3,5(10)-estratrien-3,4-diol-17-one dibenzyl ether; compound 4]

To a solution of *compound 3* ([refer to **Scheme I**], 429.5 mg, 1.5 mmol), in ACS-grade acetone (25 ml), was added anhydrous K_2CO_3 (1.382 g, 10 mmol) and benzyl bromide (357 µl, 3 mmol). Upon the addition of these two reagents, the reaction mixture turned a very dark red/brown color. The mixture was refluxed for 2 hrs before addition of more benzyl bromide (357 µl, 3 mmol). A drying tube was added to the condenser and the reaction mixture was allowed to reflux overnight. The reaction mixture was then cooled to room temperature and the solvents were removed *in vacuo*. The crude product was partitioned between water (100 ml), ethyl acetate (100 ml), and hexanes (50 ml). The aqueous layer was removed and the organic layer was washed with 1 N aqueous NaOH (25 ml), water (50 ml), and finally with a saturated aqueous NaCl solution (50 ml). The organic layer was dried with anhydrous Na_2SO_4 followed by removal of the solvents *in vacuo*. After addition of hexanes (5 ml) to the yellow, oily product, a solid formed which was then collected and washed with a small amount of methanol to yield *compound **4*** (514.3 mg, 1.1 mmol, 74% yield). The spectral and physical properties of *compound **4*** are summarized in **Tables 1** and **2**.

2.6. 4-Hydroxyestradiol-3,4-dibenzyl ether [1,3,5(10)-estratrien-3,4,17β-triol 3,4-dibenzyl ether; compound 5]

*Compound **4*** (233.3 mg; 0.5 mmol) was heated gently in methanol (25 ml) to achieve a solution. The solution was then cooled to room temperature and $NaBH_4$ (75.66 mg, 2 mmol) was added. After stirring for 3 hrs at room temperature, water (40 ml) and 1 N HCl (10 ml) were added dropwise to the stirring mixture. The resulting precipitate was collected, washed with a small amount of water, and then air-dried overnight to yield *compound **5*** (218.4 mg, 0.5 mmol, 93% yield). The spectral and physical properties of *compound **5*** are summarized in **Tables 1** and **2**.

2.7. 4-Hydroxyestradiol-3,4-dibenzyl ether 17β-stearate [1,3,5(10)-estratrien-3,4,17β- triol 3,4-dibenzyl ether 17β-stearate; compound 6]

*Compound **5*** (116.9 mg; 0.25 mmol) was dissolved in toluene (1 ml) and pyridine (40.3 µl, 0.5 mmol) was added. Stearoyl chloride (405.2 µl; 1.2 mmol) was then added and the reaction mixture was stirred at room temperature overnight. Ethyl acetate (15 ml) and water (15 ml) were then added to the reaction mixture. The solution was transferred to a separatory funnel and the reaction flask was washed with hexanes (15 ml), which was also added to the funnel. The mixture was extracted and washed with 0.1 N HCl (15 ml), saturated aqueous $NaHCO_3$ (15 ml), and saturated aqueous NaCl (15 ml). The organic layer was dried over anhydrous Na_2SO_4 and the solvents were removed *in vacuo*. The product was purified by column chromatography (ethyl acetate/n-heptane, 1:2) to give *compound **6*** (154.6 mg, 0.2 mmol, 84% yield). The spectral and physical properties of *compound **6*** are summarized in **Tables 1** and **2**.

2.8. 4-Hydroxyestradiol-3,4-diacetate 17β-stearate [1,3,5(10)-estratrien-3,4,17β-triol 3,4-diacetate 17β-stearate; compound 7]

Compound 6 (130.0 mg, 0.177 mmol) was dissolved in acetone (40 ml) and acetic anhydride (10 ml) followed by addition of 10% Pd/C (250 mg) as catalyst. The reaction mixture was hydrogenated at an initial pressure of 42 psi until no starting material remained as determined by TLC. Following hydrogenation, the catalyst was removed by filtration and acetone was removed *in vacuo*. Pyridine (14 ml, 0.172 mmol) was added to the remaining acetic anhydride solution and stirred at room temperature overnight. The reaction mixture was poured into ice-water and extracted with ethyl acetate (100 ml). The organic layer was washed with 1 N HCl (25 ml) and saturated aqueous NaCl (25 ml), and then dried over anhydrous Na_2SO_4. The solvents were removed *in vacuo*. Purification was carried out by column chromatography (ethyl acetate/n-heptane, 3:1) followed by recrystallization (MeOH) to give *compound 7* (43.5 mg, 0.068 mmol, 39% yield). The spectral and physical properties of *compound 7* are summarized in **Tables 1** and **2**.

2.9. 4-Hydroxyestradiol-17β-stearate [1,3,5(10)-estratrien-3,4,17β-triol 17β-stearate; compound 8]

From compound 6. A 500 ml pressure bottle was charged with *compound 4* (50 mg, 0.068 mmol), tetrahydrofuran (10 ml), glacial acetic acid (40 ml), and 10% Pd/C (250 mg). The mixture was then hydrogenated for 2 hrs at an initial pressure of 42 psi. The Pd/C was removed by filtration and the solvents were removed *in vacuo* until ~1 ml solvent remained. Ice water was then added to the solution and the resulting solid was filtered and dried to afford *compound 8* (26 mg, 0.047 mmol, 69% yield). The spectral and physical properties of *compound 8* are summarized in **Tables 1** and **2**.

From compound 7. *Compound 7* (192.2 mg, 0.309 mmol) was dissolved in warm MeOH (60 ml). $NaBH_4$ (175 mg, 4.63 mmol) was then added and the mixture was allowed to stir at room temperature until no starting material remained as determined by TLC (~2 hrs). Ice was then added and the solution was cooled in a freezer. The solid was collected by filtration and dried under high vacuum to obtain *compound 8* (74.6 mg, 0.135 mmol, 45% yield). The spectral and physical properties of *compound 8* prepared this way are listed below. M.p. 52-56°C. R_f (ethyl acetate/n-heptane, 1:2) 0.362. ^{1}H-NMR ($CDCl_3$ 300 MHz) δ 6.72 (d, 2H, C-1); δ 6.61 (d, 2H, C-2); δ 4.61 (t, 1H, C-17); δ 2.2 (t, 2H, CO-CH_2-R); δ 1.03-1.32 (m, 27H, C-18 CH_3, $(CH_2)_{12}$-CH_3); δ 0.80 (t, 3H, $(CH_2)_{12}$-CH_3). ^{13}C-NMR ($CDCl_3$, 300 MHz) δ 117.195 (C-1); δ 112.575 (C-2); δ 140.958, 141.586 (C-3, C-4); δ 82.836 (C-17); δ 174.611 (O-CO-R). HRMS calculated for $C_{36}H_{58}O_4$: 554.4335; Found: 554.4340.

3. RESULTS AND DISCUSSION

3.1. Synthesis of 4-hydroxyestrone

Although 4-hydroxyestrone is commercially available (extremely expensive), it would be almost unrealistic to use it as the starting material for preparing large amounts (grams of quantity) of 4-hydroxyestradiol-17β-stearate. Therefore, it was decided to

chemically synthesize 4-hydroxyestrone from estrone as the starting material, which is readily available in large quantities at a very low cost.

The synthesis of 4-hydroxyestrone from estrone was done by following the basic schemes described earlier by Stuenrauch and Knuppen (Stuenrauch and Knuppen, 1976). Estrone was first converted to 4-nitroestrone. During the synthesis, a comparison of the nitration procedure used by Stuenrauch and Knuppen (Stuenrauch and Knuppen, 1976) was compared with an earlier nitration procedure used by Tomson and Horwitz (Tomson and Horwitz, 1959). It was found that the latter was less cumbersome and yet it produced a slightly better overall yield of 4-nitroestrone as a product. Therefore, the procedure of Tomson and Horwitz was adopted for the synthesis of 4-nitroestrone from estrone.

The next step involved the reduction of 4-nitroestrone to 4-aminoestrone, which was carried out by following the procedure as described by Stuenrauch and Knuppen (Stuenrauch and Knuppen, 1976). Following this step, 4-aminoestrone was then converted to 4-hydroxyestrone according the same procedure (Stuenrauch and Knuppen, 1976). Here a few minor modifications are worth noting. During the workup, it was found that in order for KI to be effective we had to first dissolve the KI in a few drops of water before adding it to the acetic acid (100 ml), which was then introduced into the separatory funnel. Otherwise the KI would remain as a solid and would not be as effective. Second, it was determined that the product 4-hydroxyestrone produced by this step was already very pure as determined by TLC and, therefore, further purification by using recrystallization and column chromatography (which were described in the original procedure (Stuenrauch and Knuppen, 1976) was not absolutely necessary for the purpose of this study. In addition, it was also noted that pouring small amounts of MeOH over the product helped in yielding pure 4-hydroxyestrone. It is of note that the modified procedures described here were very easy to follow and highly efficient for the chemical synthesis of relatively large amounts of 4-hydroxyestrone from estrone.

It is also worth noting that 4-hydroxyestrone was attempted to be synthesized from estr-4-ene-3,17-dione by following a procedure recently reported by Majgier-Baranowska *et al.* (Majgier-Baranowska et al., 1998). According to their report, this procedure would be more efficient for the synthesis of 4-hydroxyestrone than the above method by Stubenrauch and Knuppen. However, besides several of the experimental problems encountered, the synthetic procedures failed to work as described in our hands after several months of trials, and we were unable to produce any 4-hydroxyestrone.

3.2. Synthesis of 4-hydroxyestradiol-17β-stearate

In order to selectively esterify the 17β-hydroxyl group, the strategy was to first protect the C-3 and C-4 phenolic hydroxyl groups of 4-hydroxyestrone, and then stereospecifically convert the C-17 ketone to C-17β hydroxyl group. Benzyl ether groups were chosen as protecting groups for the C-3 and C-4 hydroxyls because these groups would be stable for all subsequent synthetic reactions and because they could be selectively removed at the last step through catalytic hydrogenation to yield the original hydroxyl groups. Experimentally, benzyl bromide was reacted with 4-hydroxyestrone to convert its C-3 and C-4 phenolic hydroxyl groups to the corresponding benzyl ethers, with a yield of 74%.

The stereospecific reduction of the C-17 ketone of 4-hydroxyestrone-3,4-dibenzyl ether to the corresponding C-17β hydroxyl group was carried out in methanol with

$NaBH_4$ in excess. After several hrs of reaction time, the solution was acidified, and the resulting precipitate was collected and confirmed to be the desired compound, 4-hydroxyestradiol-3,4-dibenzyl ether (*compound* ***5***), on the basis of its physical and spectral properties as previously described. This reaction gave a yield of 93%.

Next, the C-17β hydroxyl group was esterified by dissolving *compound* ***5*** in toluene and then reacting with an excess of stearoyl chloride in the presence of pyridine. This was a very slow reaction and, therefore, it was allowed to stir at room temperature overnight. TLC was performed the next morning to ascertain that no starting material remained. The mixture was then extracted and the organic extracts were washed sequentially with 0.1 N HCl, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl. Flash column chromatography of the dried product (ethyl acetate/n-heptane, 1:2) was performed to remove unreacted stearoyl chloride. Following these procedures, 4-hydroxyestradiol-3,4-dibenzyl ether 17β-stearate (*compound* ***6***) was prepared from 4-hydroxyestradiol-3,4-dibenzyl ether (*compound* ***5***) with an overall yield of 84%.

Catechols are well known to be extremely unstable as they are subject to rapid oxidation when exposed to air. To search for an effective method to selectively remove the benzyl ether groups at the C-3 and C-4 positions without oxidizing the resultant catechol structure, several different methods were tested. We first tried a method that involved removing the benzyl ether groups via catalytic hydrogenation using acetone as the solvent. However, after filtration of the catalyst and removal of the solvents *in vacuo*, the dried product yielded multiple substantial spots on TLC. The hydrogenation was also attempted in toluene and again the same problem existed. In both cases, we were unable to readily separate these multiple products by standard chromatography methods. It was then attempted to remove the benzyl ether groups with HCO_2NH_4 (ammonium formate) as used in many similar synthetic procedures (reviewed in Hanson, 1997). To do so, *compound* ***6*** (4-hydroxyestradiol-3,4-dibenzyl ether 17β-stearate) was dissolved in methanol followed by the addition of HCO_2NH_4 and 10% Pd/C. After stirring at room temperature overnight, the catalyst was removed by filtration and the solvents removed *in vacuo*. Water and ethyl acetate were then added and the organic layer was extracted, washed with saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. Upon analysis, the dried product again displayed multiple substantial spots on TLC that were difficult to be separated by standard chromatography methods.

During the search for ideal methods, an earlier study, which suggested that catechols could be purified by using flash chromatography on silica gel impregnated with ascorbic acid was noted (Stuenrauch and Knuppen, 1976; Gelbke and Knuppen, 1972). Since ascorbic acid is a powerful antioxidant, it is generally thought that the inclusion of this compound may aid in the stability of the catechol by preventing it from oxidization. In light of this idea, different experimental methods with ascorbic acid were attempted. When ascorbic acid was introduced into the solvent or mobile phase, it eventually became present in all the fractions collected off the chromatographic steps. The ascorbic acid and its oxidized products could not be easily removed from the fractions and, consequently, this method was not as useful as we had expected. It was also attempted to include 1% ascorbic acid in MeOH during the recrystallization of 4-hydroxyestradiol-17β-stearate. It was hoped that the desired compound would precipitate out and thus be separated from ascorbic acid. However, this method also did not work as effectively as

expected and a mixture of compounds was obtained, which showed multiple spots on TLC.

Largely based on earlier experiences that catechol estrogen solutions appeared to be far more stable in acidic solutions than in neutral or basic conditions, the benzyl ether groups were attempted to be removed via catalytic hydrogenation in the presence of glacial acetic acid (which can be easily removed *in vacuo*). It was found that simply including acetic acid in the solution solved the problems and it was possible to obtain the compound as a single spot on TLC without any further purification. Briefly, *compound **6*** was dissolved in a small amount of tetrahydrofuran, and then glacial acetic acid was added in 10 ml aliquots for a total of 40 ml. Pd/C (10%) was added and the mixture was hydrogenated at an initial pressure of 42 psi. After 2 hrs on the hydrogenator, Pd/C was filtered and the solvents were removed *in vacuo* until ~1 ml remained which yielded a very concentrated mixture. After addition of ice water, a solid precipitated out of the solution. This solid was quickly filtered to avoid overexposure to air and was then placed on the high vacuum to remove any remaining solvent. The solid was comfiremed to be the desired compound, 4-hydroxyestradiol-17β-stearate (*compound **8***). The overall yield for the synthesis of *compound **8*** from 4-hydroxyestradiol-3,4-dibenzyl ether 17β-stearate (*compound **6***) was 69%.

3.3. Synthesis of 4-hydroxyestradiol-3,4-diacetate 17β-stearate

Because of the extremely unstable nature of the catechol estrogen derivatives, a stable prodrug of 4-hydroxyestradiol-17β-stearate has also been designed and synthesized. This would allow for better delivery of the desired compound without the fear of premature oxidation. The availability of such a stable prodrug (such as 4-hydroxyestradiol-3,4-diacetate 17β-stearate) would be very valuable for future studies to evaluate the *efficacy* of the hormonal and carcinogenic actions of the naturally-occurring 4-hydroxyestradiol-17β-fatty acid esters in animal models.

Specifically, 4-hydroxyestradiol-3,4-diacetate 17β-stearate was synthesized in this study. Acetate groups were chosen as hydroxyl protecting groups for the following reasons: **(i)** The formation of acetate at the hydroxyl group has been widely used as a protection in many other synthetic schemes (Borgman RJ, 1973). **(ii)** Many commonly-present esterases can efficiently catalyze the removal of the acetate group *in vivo* and thereby release the desired parent compound (Hochberg RB, 1998). **(iii)** Earlier studies have shown that acetate groups could be more readily removed *in vitro* and *in vivo* by esterases than the longer chain esters (Hochberg, RB, 1998; Janocko et al., 1984). It is of the opinion that these reasons give a high likelihood that the prodrug will be readily removed *in vivo* to yield the desired compound.

Experimentally, *compound **6*** was first dissolved in acetone and then acetic anhydride and Pd/C were added. Acetic anhydride was added to ensure that any hydroxyl group formed during the hydrogenation procedure would immediately react with acetic anhydride to form acetates at the C-3 and C-4 positions. The mixture was allowed to hydrogenate until no starting material remained as determined by TLC. Following this procedure, Pd/C was filtered off and pyridine was added. The reaction mixture was allowed to stir overnight to ensure that both hydroxyl groups were acetylated. After pouring over ice water, the solution was extracted with ethyl acetate the organic layer was washed with a 1 N HCl solution and then with a saturated NaCl

solution. The solvent was then removed *in vacuo* and the dried product was subjected to flash column chromatography (ethyl acetate/n-heptane, 3:1) for purification. Fractions were collected and TLC was performed to determine which fractions contained the desired compound. Fractions that contained the desired compound were combined and dried to obtain pure 4-hydroxyestradiol-3,4-diacetate 17β-stearate (*compound 7*). Its synthesis from 4-hydrxoyestradiol-3,4-dibenzyl ether 17β-stearate (*compound 6*) gave a yield of 39%.

To probe whether the acetate groups of *compound 7* could be readily removed, a preliminary test was performed to evaluate the release of 4-hydroxyestradiol-17β-stearate from its diacetate prodrug, 4-hydrxoyestradiol-3,4-diacetate 17β-stearate, under a mild chemical reaction condition. Experimentally, *compound 7* was dissolved in MeOH and gently heated to get it into solution. $NaBH_4$ was added in excess and was allowed to react for 2-3 hrs at room temperature, and then acetic acid was added to the solution to reduce potential oxidation of the catechol structure. MeOH was then removed *in vacuo* and ice was added to the remaining solution. A precipitate formed out of the solution and was filtered and placed on high vacuum to remove any remaining solvent present. The product was confirmed to be the desired compound, 4-hydroxyestradiol-17β-stearate, with an overall yield of 45%. Although the facile removal of the diacetate groups with mild chemical catalysts does not necessarily mean a similarly facile removal by esterase(s), this information does suggest that this prodrug likely will also be readily removable biochemically, on the basis of the extensive available information regarding the esterase-mediated hydrolytic removal of acetate groups.

Lastly, in comparison with the design of 4-hydroxyestradiol-3,4-diacetate 17β-stearate as a prodrug, it also worth a brief note that the 3,4-dibenzyl ether derivative (*compound 4*) would be far less suitable as a prodrug. The main reason is that the benzyl ether groups at the C-3 and C-4 positions are attached via the ether bonds, which are completely different from the ester bonds (in the case of the 3,4-diacetate derivative) and cannot be metabolically cleaved by esterases.

4. Conclusion

A facile method for the chemical synthesis of large amounts of 4-hydroxyestradiol-17β-stearate, a representative fatty acid ester of the strongly-procarcinogenic estrogen metabolite 4-hydroxyestradiol, has been developed with estrone as the starting material. In addition, 4-hydroxyestradiol-3,4,-diacetate 17β-stearate, a stable prodrug of 4-hydroxyestradiol-17β-stearate, which would prevent the catechol structure from premature oxidation, has been designed and synthesized. It is of note that the facile procedure described in the present study for the chemical synthesis of 4-hydroxyestradiol-17β-stearate (a representative fatty acid ester) should be equally applicable to the synthesis of various other fatty acid ester derivatives of 4-hydroxyestradiol. The ready availability of large amounts of chemically-synthesized 4-hydroxyestradiol-17β-fatty acid esters would make it possible for future studies to systematically characterize their hormonal and carcinogenic potency and efficacy in various laboratory animal models as well as to determine the blood and tissue levels of this unique class of procarcinogenic estrogen metabolites in humans and also to probe their roles in the development of human hormonal cancers.

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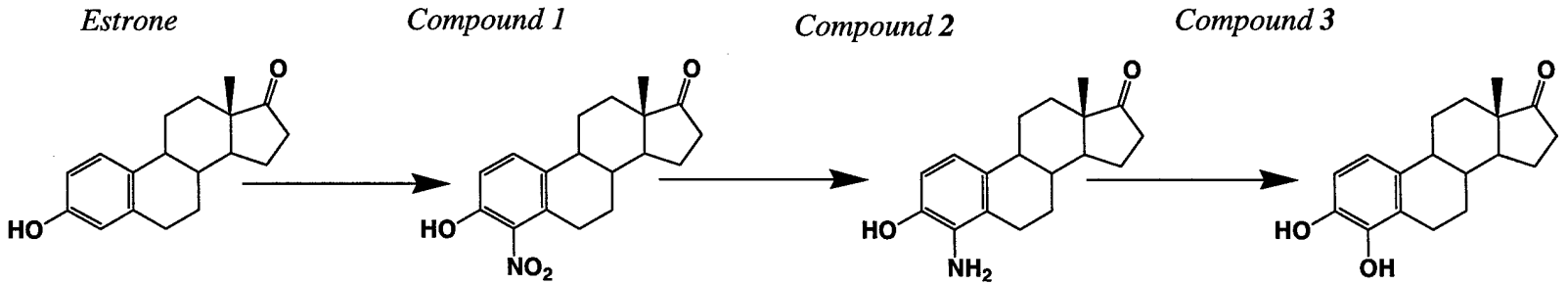
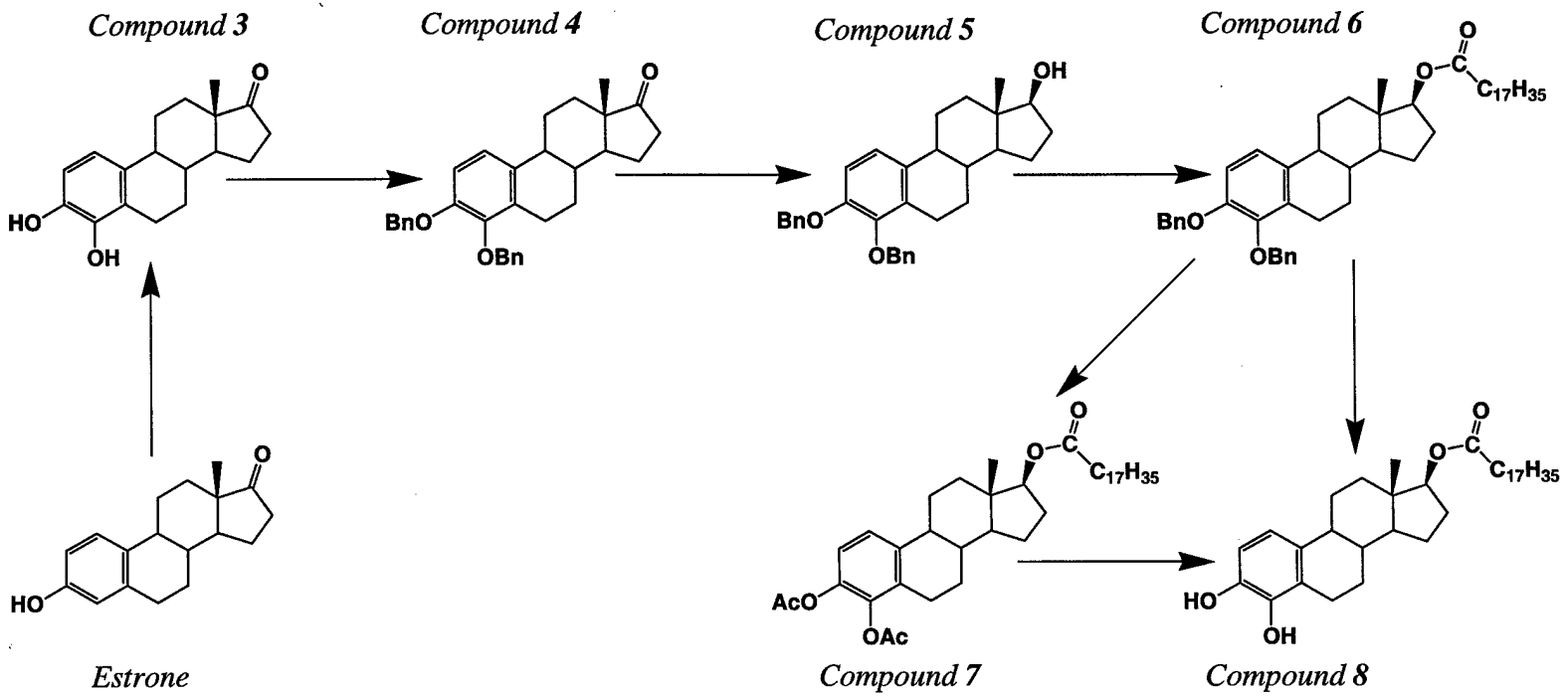
Scheme I. Scheme for the Chemical Synthesis of 4-Hydroxyestradiol with Estrone as the Starting Material. The synthesis of 4-hydoxyestrone from estrone was done largely according to the methods previously described by Stubenrauch and Knuppen (Stubenrauch and Knuppen, 1976). The methods for synthesis of *compounds1-3* details of are described under the *Experimental* section. For convenience, the trivial and IUPAC names of each synthesized compound are listed.



Comp'd No.	Trivial Name	IUPAC Name
	Estrone	1,3,5(10)-Estratrien-3-ol-17-one
1	4-Nitroestrone	1,3,5(10)-Estratrien-4-nitro-3-ol-17-one
2	4-Aminoestrone	1,3,5(10)-Estratrien-4-amino-3-ol-17-one
3	4-Hydroxyestrone	1,3,5(10)-Estratrien-3,4-diol-17-one

Scheme II. Scheme for the Chemical Synthesis of 4-Hydroxyestradiol-17β-Stearate and its Stable Prodrug 4-Hydroxyestradiol-3,4-Diacetate 17β-Stearate, with 4-Hydroxyestrone as the Starting Material. The methods for synthesis of *compounds4-8* were developed in this study, and details of each synthetic step are described under the *Experimental* section. For convenience, the trivial and IUPAC names of each synthesized compound are listed.



Comp'd No.	Trivial Name	IUPAC Name
3	4-Hydroxyestrone	1,3,5(10)-Estratrien-3,4-diol-17-one
4	4-Hydroxyestrone-3,4-dibenzyl ether	1,3,5(10)-Estratrien-3,4-diol-17-one dibenzyl ether
5	4-Hydroxyestradiol-3,4-dibenzyl ether	1,3,5(10)-Estratrien-3,4,17β-triol 3,4-dibenzyl ether
6	4-Hydroxyestradiol-3,4-dibenzyl ether 17β-stearate	1,3,5(10)-Estratrien-3,4,17β-triol 3,4-dibenzylether 17β-stearate
7	4-Hydroxyestradiol-3,4-diacetate 17β-stearate	1,3,5(10)-Estratrien-3,4,17β-triol 3,4-diacetate 17β-stearate
8	4-Hydroxyeatradiol-17β-stearate	1,3,5(10)-Estratrien-3,4,17β-triol 17β-stearate

Table 1. Spectral and Physical Properties of the Synthesized Compounds.

Compound	Melting point (°C)	R_f [1]	HRMS[2]	Characteristic mass fragments (*m/z*)
2	104-106	0.452	466.2504 ($C_{32}H_{43}O_3$: 466.2508)	91 (base peak), 466, 375, 347, 181
3	69-73	0.219	469.2762 ($C_{32}H_{36}O_3$: 469.2743)	468 (base peak)
4	54-57	0.767	735.5322 ($C_{50}H_{70}O_4$: 735.5352)	735 (base peak), 644, 452, 360, 302
5	72-73	0.671	638.4531 ($C_{40}H_{62}O_6$: 638.4546)	554 (base peak), 638, 596, 355, 313, 270, 175
6	52-55	0.362	554.4340 ($C_{36}H_{58}O_4$: 554.4335)	554 (base peak), 271, 175, 149, 57

1. The mobile phase used was ethyl acetate/n-heptane (1:2).
2. High-resolution mass spectrometry. The observed value for each compound was shown on the top, and the calculated value according to the listed formula is shown in parenthesis.

Table 2. ^{13}C-NMR and ^{1}H-NMR Values for Selected Key Carbons and Protons of the Synthesized Compounds.

Cpd	^{13}C-NMR values of selected key carbons								^{1}H-NMR values of selected key protons
	C-1	C-2	C-3	C-4	C-17	C-17 ester carbonyl	(CH_2) of benzyl ethers	Acetate carbonyls	
2	120.926	112.018	146.180	149.944	221.283	-	71.144 74.280	-	δ 6.95 (d, 1H, C-1); δ 6.78 (d, 1H, C-2); δ 4.96, δ 5.05 (s, 2H, O-CH_2); δ 7.20-7.41 (m, 10H, O-CH_2-ArH)
3	120.926	111.988	146.191	149.820	82.159	-	71.177 74.246	-	δ 6.95 (d, 1H, C-1); δ 6.75 (d, 1H, C-2); δ 3.66 (t, 1H, C-17); δ 4.95, δ 5.06 (s, 2H, O-CH_2); δ 7.22-7.41 (m, 10H, O-CH_2-ArH)
4	120.941	111.958	146.183	149.824	82.656	174.229	71.166 74.246	-	δ 6.93 (d, 1H, C-1); δ 6.76 (d, 1H, C-2); δ 4.65 (t, 1H, C-17); δ 4.94, δ 5.04 (s, 2H, O-CH_2); δ 7.20-7.41 (m, 10H, O-CH_2-ArH); δ 2.25 (t, 2H, CO-CH_2-R); δ 1.06-1.30 (m, 27H, C-18 CH_3, $(CH_2)_{12}$-CH_3); δ 0.80 (t, 3H, $(CH_2)_{12}$-CH_3
5	122.564	118.938	139.082	138.596	81.267	172.963	-	167.207 167.719	δ 7.14 (d, 1H, C-1); δ 6.89 (d, 1H, C-2); δ 4.62 (t, 1H, C-17H); δ 2.19, 2.23 (s, 3H, CO-CH_3); δ 1.12-1.30 (m, 27H, C-18 CH3, $(CH_2)_{12}$-CH_3); δ 0.83 (t, 3H, $(CH_2)_{12}$-CH_3
6	117.132	112.571	141.006	141.668	82.836	174.611	-	-	δ 6.70 (d, 2H, C-1); δ 6.61 (d, 2H, C-2); δ 4.62 (t, 1H, C-17); δ 2.23 (t, 2H, CO-CH_2-R); δ 1.05-1.30 (m, 27H, C-18 CH_3, $(CH_2)_{12}$-CH_3); δ 0.80 (t, 3H, $(CH_2)_{12}$-CH_3)

Appendix 2

Naturally-Occurring Estradiol-17β-Fatty Acid Esters, but not Estradiol-17β, Preferentially Induce the Development of Mammary Tumors in Female ACI Rats

(adapted from dissertation of Laura H. Mills; manuscript in preparation)

1. INTRODUCTION

Endogenous estrogens are female sex hormones that are among the most potent non-peptidal mitogens in various target organs, such as the breast, uterus, and anterior pituitary (Spady *et al.*, 1999). Excessive, prolonged growth stimulation of the target cells by endogenous or administered estrogens has long been considered to be a major cause for the development of estrogen-dependent cancers (Yager and Liehr, 1996). However, a number of earlier studies have shown that the levels of estradiol-17β (E_2) in circulation and urine generally have a much higher degree of correlation with an increased risk of human uterine endometrial cancer (Ziel and Finkle, 1975; Siiteri *et al.*, 1976; Mack *et al.*, 1976; McDonald *et al.*, 1977; Grady and Ernster, 1996) than with the risk of human breast cancer (Jick *et al.*, 1980; Kelsey and Gammon, 1991; Pike *et al.*, 1993; Feigelson and Henderson, 1996). The mechanism underlying this discrepancy is largely not understood. We recently have hypothesized that the hormonal activity of estrogens in the breast may be, in part, mediated by biologically-active derivatives, such as the fatty acid esters of endogenous E_2, 4-hydroxy-E_2, and 16α-hydroxyestrone.

The naturally-occurring estrogen fatty acid esters are a unique group of estrogen derivatives that are formed from enzymatic esterification of E_2 (only at the C-17 position) using various fatty acyl-CoAs as cofactors (Schatz and Hochberg, 1981; Mellon-

Nussbaum *et al.*, 1982; Abul-Hajj, 1982; Martyn *et al.*, 1987; Pahuja and Hochberg, 1989; Xu *et al.*, 2001). Although the estrogen fatty acid esters cannot bind to the estrogen receptors, their strong, long-term hormonal activity results from the slow release of the parent hormone catalyzed by esterases (Larner *et al.*, 1992). Because of their high lipophilicity, estrogen fatty acid esters are found at much higher concentrations in the fat than in the blood (Larner *et al.*, 1992). Recently it was suggested that the mammary adipocytes may serve as a storage site for the lipoidal estrogen fatty acid esters, providing sustained release of bioactive estrogens to mammary glandular cells (Zhu and Conney, 1998; Mills *et al.*, 2001). Data showed that administration of E_2-17β-stearate, a representative estrogen fatty acid ester, to female ovariectomized rats had a differential, strong mitogenic effect in the fat-rich mammary tissues and this effect was not observed in the uterus (Mills *et al.*, 2001). This finding has prompted the hypothesis that the estrogen fatty acid esters may be an important group of endogenous estrogens that can preferentially induce tumor formation in the fat-rich mammary tissues.

Therefore, in the present study, the activity of an estrogen-fatty acid ester preparation with E_2 for the induction of tumors in the breast, uterus, and pituitary of intact female ACI rats was compared. The female ACI rats were chosen because recent studies have shown that chronic treatment of these rats with E_2 via subcutaneous pellets or silastic implants rapidly induced mammary tumor formation, while spontaneous mammary tumors in the absence of exogenous E_2 are rare (Harvell *et al.*, 2000). It is also known that chronic administration of estrogens induces PRL-producing pituitary tumors in these animals (Shull *et al.*, 1997; Harvell *et al.*, 2000; Harvell *et al.*, 2002). It has been suggested that the resulting hyperprolactinemia is an important hormonal factor contributing to mammary tumor formation (Henderson *et al.*, 1996; Harvell *et al.*, 2002).

The data demonstrated that chronic treatment with E_2 preferentially induces the growth of pituitary tumors while treatment with E_2 fatty acid esters resulted in a higher incidence of mammary tumors compared to the E_2 treated animals. Although animals treated with an estrogen fatty acid ester also developed pituitary tumors, they were

statistically smaller than those of animals treated with E_2. These results suggest that the endogenously formed estrogen fatty acid esters are pathophysiologically more important than E_2 for the selective induction of mammary tumor formation.

2. MATERIALS AND METHODS

2.1 Chemicals

Cholesterol, E_2 and an estrogen fatty acid ester preparation, that was suppose to contain pure (>99%) E_2-17β-stearate (E_2-17β-S), were purchased from Steraolids (Newport, RI). A further analyses of the preparation, using high-performance liquid chromatography and mass spectrometry-liquid chromatography, showed that the estrogen fatty acid ester preparation was, in fact, composed of 63% E_2-17β-S and 37% E_2-17β-palmitate (structures shown in **Fig. 1**). 4-OH-E_2 was synthesized according to the procedure of Stubenrauch and Knuppen (Stubenrauch and Knuppen, 1976).

2.2 Animal experiments

All experimental protocols involving live animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina. Intact, female ACI rats (average age 55 days old) were purchased from Harlan Laboratories (Houston, TX). After arrival, they were allowed to acclimatize for a week before the experiment began. The animals were housed under controlled conditions of temperature and photoperiod (12-h light/12-h dark cycle) and the animals were allowed free access to food and water throughout the experiment. At 62 days of age, the animals were randomly divided into 4 groups (control, E_2, and 4-OH-E_2, and an E_2 fatty acid ester preparation), with 25-26 animals per group. They were implanted under the back skin with a 20 mg pellet (containing an estrogen or only cholesterol) while the animals were under halothane anesthesia. Each pellet contained 18 μmol of an estrogen plus cholesterol or contained only cholesterol for the control group. The animals were weighed weekly and examined for the presence of palpable mammary tumors. The decision to terminate an animal during the course of the long-term experiment was made when apparent moribund signs were observed (usually due to the presence of a mammary or pituitary tumors).

2.2.1. Pellet formulations

Four different pellet formations were made. They consisted of cholesterol alone for the control group or a mixture of 18 μmol of E_2, or an estrogen fatty acid ester preparation, or 4-OH-E_2 plus cholesterol. The components were thoroughly mixed together and compressed into a 20 mg cylindrical pellet on a Parr Pellet Press (Parr Instrument Company Moline, IL).

2.2.2. Collection and evaluation of mammary tissues, uteri and pituitaries

The rats were killed by decapitation. Trunk blood was collected in Vacutainer test tubes containing sodium heparin and stored for further analysis. At the time of death, the location and size of mammary tumors were recorded and the size and weight of pituitaries (both normal and tumorous) were determined. Tumor volumes were calculated using the formula V = height x length x width x $\pi/6$ (Di Chiro and Nelson, 1962). Representative sections of mammary tissues and pituitaries, both normal and tumorous, and uteri were removed and stored in 10% buffered neutral formalin overnight followed by dehydration through a sequential transfer through 80-100% ethanol and then 100% xylene. The tissues were then embedded in paraffin blocks, cut into 7-μm sections, and placed on Superfrost microscope slides (Fisher Scientific). The sections were stained with hematoxylin and eosin and evaluated under light microscopy by a pathologist.

2.2.3. RIA of the plasma levels of PRL, FSH and LH

The collected whole blood samples from each animal were kept in a Vacutainer test tube containing sodium heparin (purchased from Fisher Scientific, Suwanee, GA) at 4°C for 2 hours followed by centrifugation at 3000 rpm for 15 min. Aliquots of the plasma samples were transferred to small vials with sealed caps and stored at -80°C until analysis. The plasma levels of PRL, FSH and LH were analyzed at the National Hormone and Pituitary Program (Torrance, CA) by using ^{125}I-labeled RIAs.

3. RESULTS

3.1. Induction of pituitary tumors by chronic treatment with estrogens

In this experiment, an estrogen was released to female intact ACI rats through a pellet implanted under the back skin of the animal at ~62 days of age. The carcinogenic effect of each estrogen in the pituitary was determined by measuring both the pituitary wet weight and volume.

All of the control animals as well as the 4-OH-E_2-treated animals remained healthy during the course of the whole experiment. Upon pathological examination of these animals, at the termination of the study, it was found that no animals had developed a pituitary tumor. The average pituitary weight of control animals was 11.7 ± .8 mg with an estimated average pituitary volume of 4.6 ± 0.9 mm^3 (**Fig. 2**). The average pituitary weight of 4-OH-E_2-treated animals was 17.3 ± 1.3 mg with an average pituitary volume of 20.9 mm^3 (**Fig. 10**). Although the pituitaries of 4-OH-E_2-treated animals were slightly larger in weight and volume, no histological differences between these two groups were observed. Histological analysis of the pituitaries revealed no abnormalities, as both the anterior and posterior portions of the pituitary gland appeared to be normal (**Fig. 3**).

Approximately 5 months after treatment started, some of the animals in the E_2 treatment group began to get very sick and it was necessary to kill them. By approximately 6.5 months after treatment started, all animals in this group were rapidly losing a lot of weight and were becoming morbid; therefore, all of these animals were sacrificed. Upon pathological examination, all animals in this group were found to have very large pituitary tumors. It is important to note that the majority of these animals failed to develop palpable mammary tumors. The treatment of female ACI rats with E_2

resulted in a 100% incidence of pituitary tumors, with an average tumor weight of 254.4 ± 19.0 mg and an average tumor volume of 284.6 ± 24.0 mm^3 **(Fig. 2)**. Major histological changes were seen in the anterior pituitary lobe. These changes included extensive hyperplasia and adenomas with focal hemorrhage, cystic change, and apoplectic necrosis. The histopathological features of the adenomas suggest that they are of a benign type **(Fig. 3)**.

On the other hand, the majority of animals in the E_2 fatty acid group remained healthy until the experiment was terminated approximately 10 months after the estrogen treatment started. Only 2 animals died during the course of the long-term experiment, apparently due to the development of large pituitary tumors. Upon macroscopic examination, the animals in this group differed in their pituitary tumors as compared to the E_2-treated animals. The treatment of female ACI rats with the E_2 fatty acid esters resulted in an average pituitary weight of 76.0 ± 12.8 mg and an average pituitary volume of 81.6 ± 14.8 mm^3 **(Fig. 2)**. Upon histopathological examination, hyperplasia and adenoma were found exclusively in the anterior lobe of the pituitary. Only in a few instances were the hyperplasia and adenoma coupled with cystic changes, focal hemorrhage, or apoplectic necrosis **(Fig. 3)**. Notably, the extent and severity of these changes were markedly less than those seen in the E_2-treated animals.

3.2. Effect of estrogen treatment on the induction of mammary tumors

The carcinogenic effect of chronic estrogen administration to female intact ACI rats was also studied in the mammary gland. During the course of the experiment, no animals in the control or 4-OH-E_2 treatment group developed mammary tumors. However, under histological analysis, several changes were noted in the mammary tissues of 4-OH-E_2-treated animals. In the control animals, normal mammary histology was observed with the small terminal duct lobular units scattered throughout the mammary adipose tissue **(Fig. 5)**. In the 4-OH-E_2-treated animals, typical changes in the mammary glands included hyperplasia of the terminal duct lobular units with more secretory changes such as the presence of intracytoplasmic vacuoles and luminal secretions **(Fig. 5)**. Notably, the hyperplastic units were smaller and less dense compared to the changes observed in either the E_2 or E_2 fatty acid ester groups.

The first palpable mammary tumor in the E_2 treatment group was observed 126 days following the initiation of hormone treatment. Because of morbidity, apparently due to the development of pituitary tumors, 15 animals (57.7%) in this group had to be sacrificed during the course of the experiment. 203 days after the initiation of hormone treatment, all remaining animals in this group became morbid and therefore were sacrificed. At the time of death, only 9 animals total (34.6%) had developed a palpable mammary tumor **(Fig. 4)**. However, the pathology revealed that an additional 4 animals (15.4%) displayed characteristics of the beginning stage of tumor formation. Histological changes in the mammary glands of these animals included hyperplasia in the terminal duct lobular units, which were extensive and very pronounced. Notably, the hyperplastic units were generally very large in size, consisting on average of ~ 50 end buds that were densely located in clusters **(Fig. 5)**. The majority of the mammary tumors were classified as ductal carcinoma in situ (DCIS).

The first palpable mammary tumor in animals treated with the E_2 fatty acid esters was observed 140 days following the initiation of hormone treatment. However, only 2

animals in this group died during the course of the experiment due to a cause other than a mammary tumor. In addition, the animals in this group were able to live much longer than the animals in the E_2 group and were very healthy until the time of death (due to the presence of a mammary tumor or the termination of the experiment). The experiment was terminated 300 days after the initiation of hormone treatment and at this time, 65.4% of the total population displayed palpable mammary tumors (**Fig. 4**). The pathology also revealed that one additional animal had the beginning stages of tumor formation and one other animal had an intraductal papilloma (benign tumor). The histological changes in the mammary glands of these animals included extensive and pronounced hyperplastic changes in the terminal duct lobular units. These changes were close in comparison to those observed in the E_2-treated animals (**Fig. 5**). The total percentage of animals in this group with tumors (malignant and benign) was 73.1%. The majority of the malignant tumors were classified as ductal carcinoma in situ (DCIS).

3.3. Effect of estrogen treatment on the uterus

The uterus of control animals did not show any histological changes. The endometrium contained spaced, nondilated endometrium glands. However, in animals treated with 4-OH-E_2 or E_2, there were a significant number of animals that displayed cystically-dilated glands. Only a few animals in the E_2 fatty acid ester group displayed cystically-dilated glands in the uterus.

3.4. Effect of estrogen treatment on animal body weight

It is known that chronic estrogen administration decreases an animal's body weight gain in a dose-dependent manner (Ratka, 1990). The results from this study also showed that chronic administration of E_2 to animals reduced the animals' body weight gain when compared to the control animals. However, ~163 days after the initiation of hormone treatment, the animals in the E_2 treatment group not only stopped gaining weight but actually began to lose weight, whereas all animals in the control, 4-OH-E_2, and estrogen fatty acid esters groups continuously gained weight throughout the course of the experiment. At no time was there a significant reduction of weight seen in the control, 4-OH-E_2 or estrogen fatty acid ester group animals (**Fig. 6**). The reduction of weight in E_2-treated animals most likely was due to the morbidity associated with large pituitary tumors.

3.5. Effect of estrogen treatment on plasma levels of PRL, FSH and LH

The plasma levels of PRL, FSH and LH in intact female rats treated chronically with an estrogen was also determined. In control animals, the levels of PRL, FHS and LH are in agreement with earlier reports (Ratka, 1990; Shull *et al.*, 1997; Harvell *et al.*, 2002). In animals treated with 4-OH-E_2, there was no significant difference observed in the levels of PRL. However, compared with the control animals, the levels of FSH and LH in 4-OH-E_2-treated animals were, on average, suppressed by 34.3% and 55.4%, respectively (**Fig. 7**).

In the E_2 treatment group, the level of PRL was significantly increased over all groups. The levels of FSH were decreased by 56.3% when compared to the control animals while the pituitary secretion of LH was almost completely suppressed (< 0.5 ng/ml of LH was detected in all of the rats in this group) (**Fig. 7**).

In the estrogen fatty acid esters treatment group, very similar patterns of PRL stimulation and FSH and LH suppression were observed, but to a lesser extent when compared to the E_2-treated animals. The PRL levels were increased significantly over control animals and the levels of FSH and LH were suppressed by 47.2% and 88.4% respectively, compared with control animals (**Fig. 7**).

It is important to note that in all four treatment groups, there was a high degree of positive correlation between the pituitary weight and prolactin levels. The levels of FSH and LH did not show appreciable correlation with the pituitary weights (**Fig. 8**).

4. DISCUSSION

A previous study showed that chronic administration of 0.5 or 5 nmol/day of E_2-17β-S (a representative estrogen fatty acid ester) to ovariectomized female rats for 10 or 23 days had a markedly stronger stimulatory effect on mammary glandular cell growth than did E_2 at equimolar doses, while E_2 showed a preferential growth stimulatory effect in the uterus of these animals (Mills *et al.*, 2001). This observation has led to the hypothesis that the estrogen fatty acid esters may be a group of endogenous estrogens that have preferential activity in stimulating cell growth and, possibly, for inducing tumor formation in the fat-rich mammary tissues

In the present study, the carcinogenic effects of an E_2-17β-fatty acid ester preparation, E_2 and 4-OH-E_2 was compared in the breast, pituitary and uterus of intact, female ACI rats. Results showed that chronic administration of an E_2-17β-fatty acid ester preparation to these rats preferentially induces the development of mammary tumors while chronic administration of E_2 results in the preferential formation of pituitary tumors. The chronic administration of 4-OH-E_2 to intact female ACI rats did not induce the formation of mammary or pituitary tumors, although there was hyperplasia present in the mammary glands. These results are the first report demonstrating that chronic administration of an estrogen fatty acid ester selectively induces the development of mammary tumors in this animal model.

Earlier studies have shown that chronic administration of E_2 to intact female ACI rats led to rapid development of mammary tumors and that a 100% tumor incidence was achieved (Shull J, *et al.*, 1997; Li *et al.*, 2002; Harvell *et al.*, 2002). Notably, the animals in these studies received either a 20 mg pellet containing 3 mg E_2, similar to the ones we made, or a silastic tubing insert consisting of 27.5 mg of crystalline E_2. The initiation of hormone treatment in these animals ranged from at 49 days of age to 63 days of age. In this study, an estrogen pellet consisting of 5 mg of E_2, or equivalent moles of an E_2-17β-fatty acid ester preparation or 4-OH-E_2, and cholesterol was implanted in female intact ACI rats that were at an average age of 63 days. However, a 100% mammary cancer incidence in the ACI rats treated with E_2 was not seen; rather the incidence of mammary tumors was only 50%, and only 34.6% of the animals had palpable tumors after 6.5 months of estrogen implantation. The difference is not fully understood.

However, it is important to note that the pituitary weights in the animals treated with E_2, in the present study, were appeared to be significantly larger than previously reported (Shull *et al.*, 1997; Harvell *et al.*, 2000; Harvell *et al.*, 2002). The average pituitary weight of the E_2 treated animals in the present study was 254.4 ± 19.0 mg while earlier reports show that administration of E_2 results in a pituitary weight of only 41 mg

(Harvell *et al.*, 2000), 60 mg (Shull *et al.*, 1997) or 70 mg (Harvell *et al.*, 2002). In addition, the average serum PRL levels in the rats treated with E_2 in the current study was 6059 ng/ml which is much higher than earlier reports showing PRL levels of 2300 ng/ml (Shull *et al.*, 1997) and 3224 ng/ml (Harvell *et al.*, 2002) following treatment with E_2.

The results of this present study showed that in animals given the estrogen fatty acid esters, there was a 69.2% mammary cancer incidence, with 65.4% of the animals displaying palpable tumors. In addition, these animals had a much smaller average pituitary weight compared with E_2-treated animals (76 mg vs. 254 mg). Although the circulating PRL levels in the estrogen fatty acid ester treatment group were also increased significantly over control values (1695 ng/ml vs. 70 ng/ml), this difference was much less pronounced compared with the E_2 treated animals. Therefore, it is concluded that E_2 is much stronger in promoting the formation and growth of pituitary tumors while estrogen fatty acid esters have a preferential, strong carcinogenic effect in the mammary glands.

Rarely have the levels of PRL, FSH, and LH been measured simultaneously in animals treated chronically with an estrogen. However, in this study, it was found that the measurement of these hormones after estrogen administration produced interesting results. Data showed that pituitary LH and FSH secretion have different sensitivities to estrogen's feedback regulation. While the levels of FSH are greatly reduced by estrogen administration, the levels of LH are almost entirely suppressed. In the E_2 treatment group, the animals had the highest levels of PRL yet the majority of these animals failed to get palpable mammary tumors. In addition, the animals that were treated with E_2 showed the greatest effects on the pituitary secretion of PRL, FSH and LH. This lends support to the theory that the estrogen fatty acid esters are a group of mammary-selective hormones.

In summary, it was demonstrated that an E_2-17β-fatty acid ester preparation has a differential, strong carcinogenic effect in the fat-rich mammary tissues, and this effect was not observed with E_2. Administration of E_2 accompanying to animals resulted in preferential formation of pituitary tumors with accompanying hyperprolactinemia. It is of the opinion that the estrogen fatty acid esters may have a much stronger hormonal effect in the mammary gland because mammary glandular cells are surrounded by large amounts of adipocytes, which serve as storage depots for the estrogen esters. Also, the estrogen fatty acid esters that are stored in the mammary fat tissue could be slowly released following their metabolic cleavage catalyzed by esterases. The results of the present study raise the possibility that the endogenously-formed estrogen-17β-fatty acid esters may also play an important role in the formation of human breast cancer. More studies are needed to determine their precise roles in the formation of human breast cancer.

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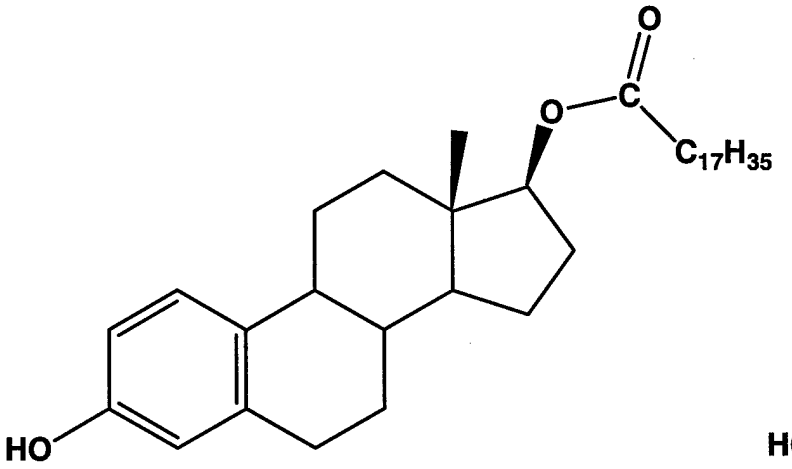
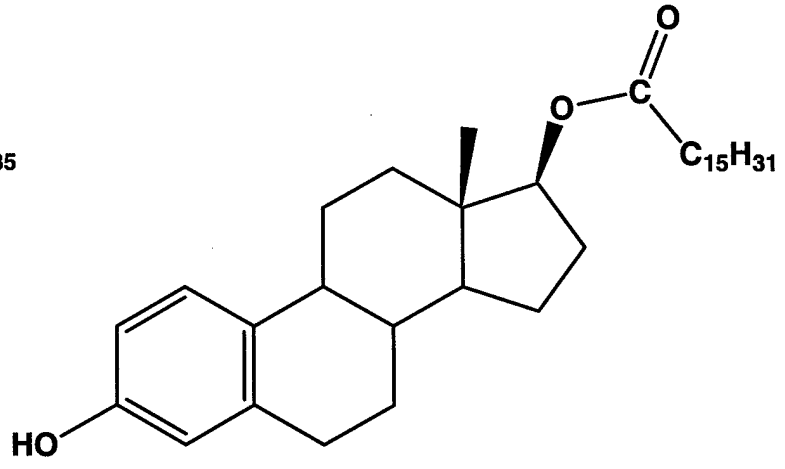
Figure 1. Structures of Estradiol-17β-Stearate (E_2-17β-S) and Estradiol-17β-Palmitate (E_2-17β-P).



Estradiol-17β-stearate



Estradiol-17β-palmitate

Figure 2. Effect of Chronic Administration of Estradiol-17β (E_2), 4-OH-Estradiol (4-OH-E_2), or Estradiol-17β-Fatty Acid on the Weight of the Pituitary Gland (top graph) and Pituitary Volume (bottom graph). The intact female ACI rats (62 days old) were randomly divided into one control group, an E_2 treatment group, a 4-OH-E_2 treatment group and an estrogen fatty acid ester group. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol, or cholesterol alone for the control group. At the time of death, the pituitary was removed and weighed to determine a pituitary weight. The pituitary was also measured and the volume was determined by the formula V=height x length x width x $\pi/6$. Each y value represents the mean ± S.E.M. of the animals and x values represent the mean ± S.D. of the animals

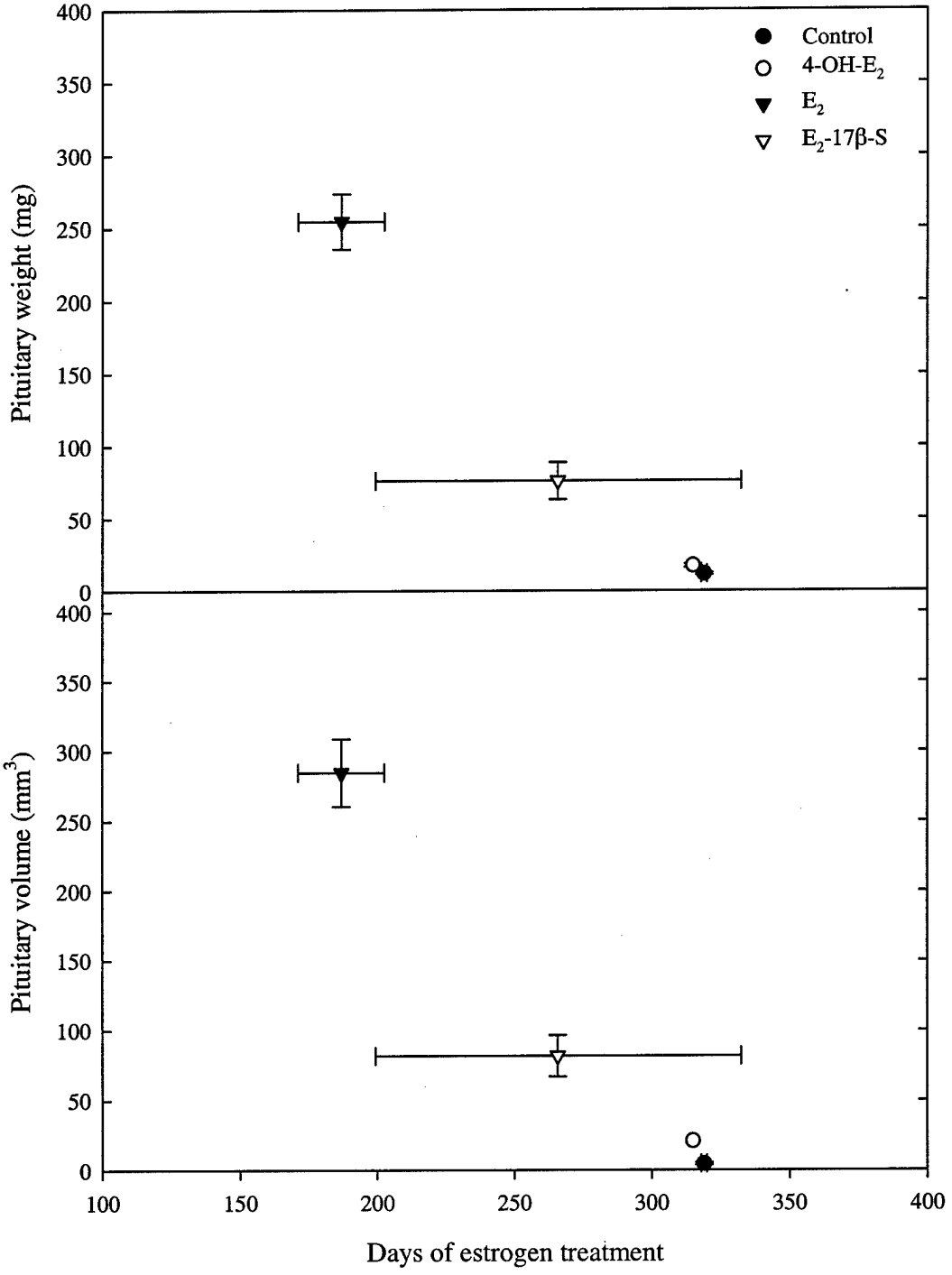

Control
4-OH-E2
E2
E2-17β-S
Pituitary weight (mg)
Pituitary volume (mm3)
Days of estrogen treatment
0
50
100
150
200
250
300
350
400

Fig 3. Hematoxylin and Eosin (H and E) Staining of Pituitaries in Control Intact Female ACI Rats and in Animals Treated Chronically with an Estrogen. The intact female ACI rats (62 days old) were randomly divided into one control group, a 4-OH-E_2 treatment group, and E_2 treatment group, and an estrogen fatty acid esters group. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol, or cholesterol alone for the control group. Frames A and B are representative H and E pituitary stains from control animals. Frames C and D are representative H and E pituitary stains from 4-OH-E_2 treated animals. Frames E and F are representative H and E pituitary tumor stains from E_2 treated animals. Frames G and H are representative H and E pituitary stains from the estrogen fatty acid esters group. Note: the descriptions of the pituitary tumors as well as pituitary edits are described in the Results section.

Figure 4. Effect of Chronic Estrogen Administration on the Induction of Mammary Tumors. The intact female ACI rats (62 days old) were randomly divided into one control group, an E_2 treatment group, a 4-OH-E_2 treatment group and an estrogen fatty acid ester group. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol, or cholesterol alone for the control group. No animals in the control or 4-OH-E_2 treatment group developed mammary tumors, and therefore the data for these two groups is not shown.

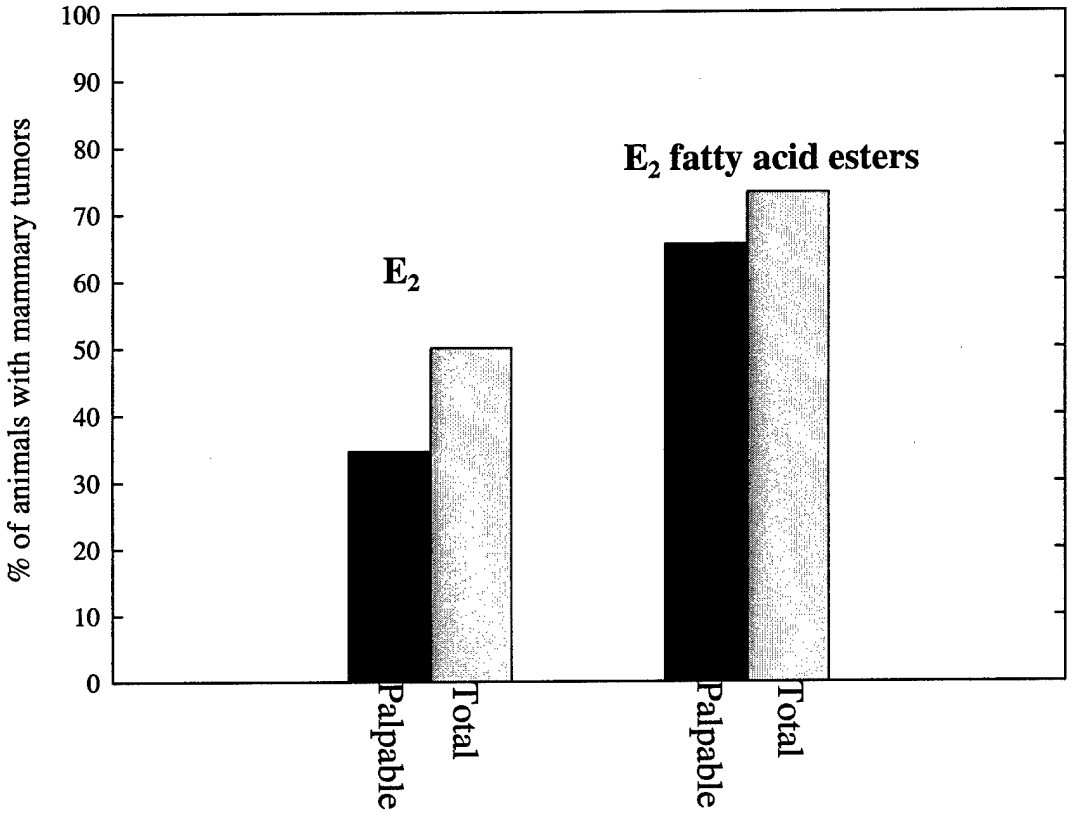

% of animals with mammary tumors
100
90
80
70
60
50
40
30
20
10
0
E2
E2 fatty acid esters
Palpable
Total
Palpable
Total

Fig 5. Hematoxylin and Eosin (H and E) Staining of Mammary Tissue in Control Intact Female ACI Rats and in Animals Treated Chronically with an Estrogen. The intact female ACI rats (62 days old) were randomly divided into one control group, a 4-OH-E_2 treatment group, and E_2 treatment group, and an estrogen fatty acid esters group. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol, or cholesterol alone for the control group. Frames A and B are representative H and E mammary tissue stains from control animals. Frames C and D are representative H and E mammary tissue stains from 4-OH-E_2 treated animals with significant hyperplasia. Frames E and F are representative H and E pituitary tumor stains from E_2 treated animals. Frames E and G are representative H and E mammary tissue stains from animals with hyperplasia that were treated with E_2 or the estrogen fatty acid esters, respectively. Frames F and H are representative mammary tumor stains from animals with DCIS that were treated with E_2 or the estrogen fatty acid esters, respectively.

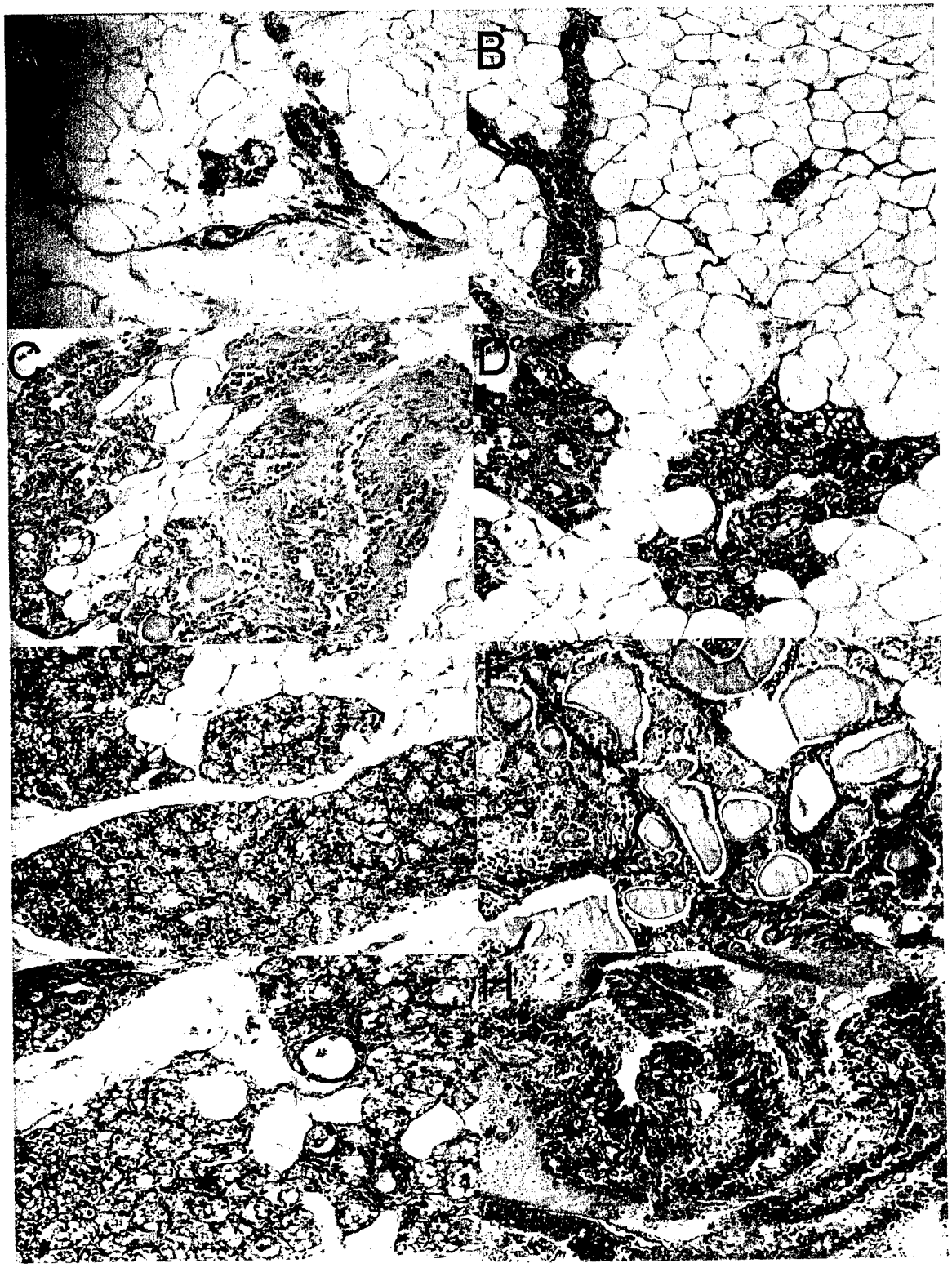

B
C
D
F
H

Figure 6. Effect of Chronic Estrogen Administration on the Body Weight of Intact, Female ACI Rats. The intact female ACI rats (62 days old) were randomly divided into one control group, one E_2 treatment group, one 4-OH-E_2 treatment group and one E_2-17β-fatty acid ester treatment groups. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol or cholesterol alone for the control group. The animal's body weight was measured at regular intervals throughout the experiment.

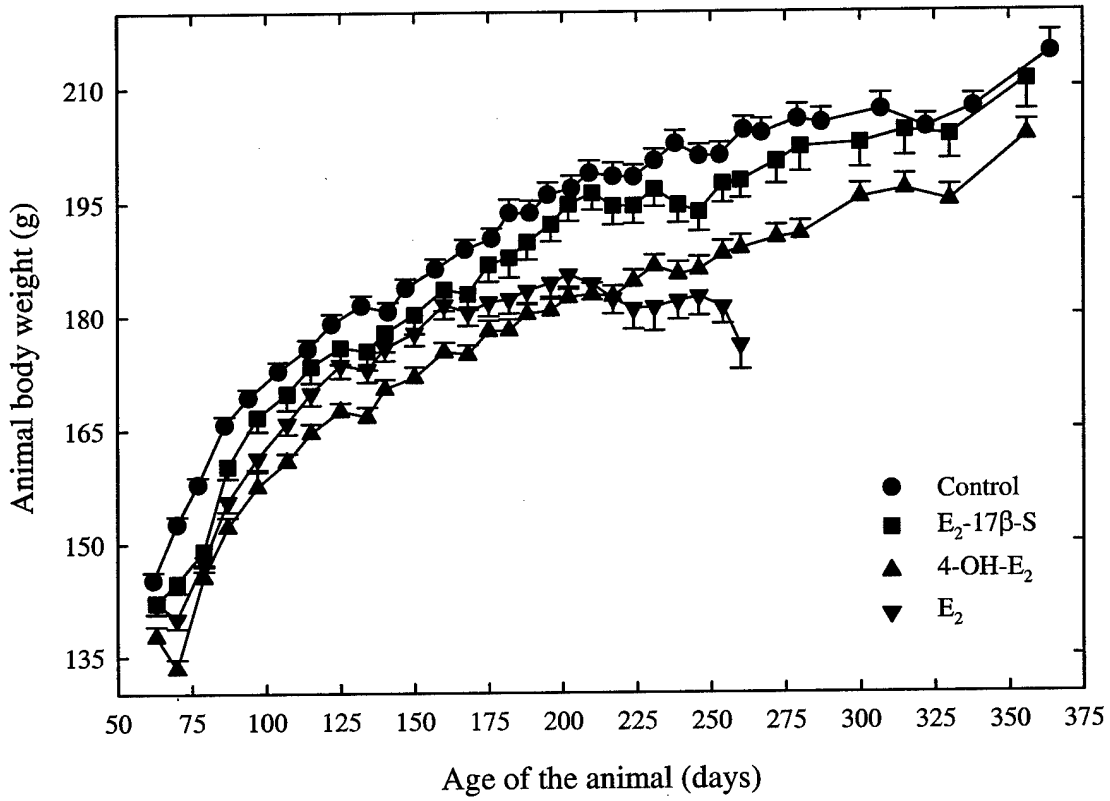

Animal body weight (g)
210
195
180
165
150
135
50
75
100
125
150
175
200
225
250
275
300
325
350
375
Age of the animal (days)
Control
E2-17β-S
4-OH-E2
E2

Fig 7. The Effect of Chronic Administration of an Estrogen on the Pituitary Secretion of PRL, FSH and LH. The intact female ACI rats (62 days old) were randomly divided into one control group, one E_2 treatment group, one 4-OH-E_2 treatment group and one E_2-17β-fatty acid ester treatment groups. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol or cholesterol alone for the control group. When the animals were sacrificed by decapitation, their blood samples were collected in Vacutainer test tubes containing heparin as described in "Materials and Methods." The plasma levels of LH, FSH, and PRL were analyzed at the National Hormone and Pituitary Program by using ^{125}I-labeled RIAs. Each value is the mean ± SE of each group.

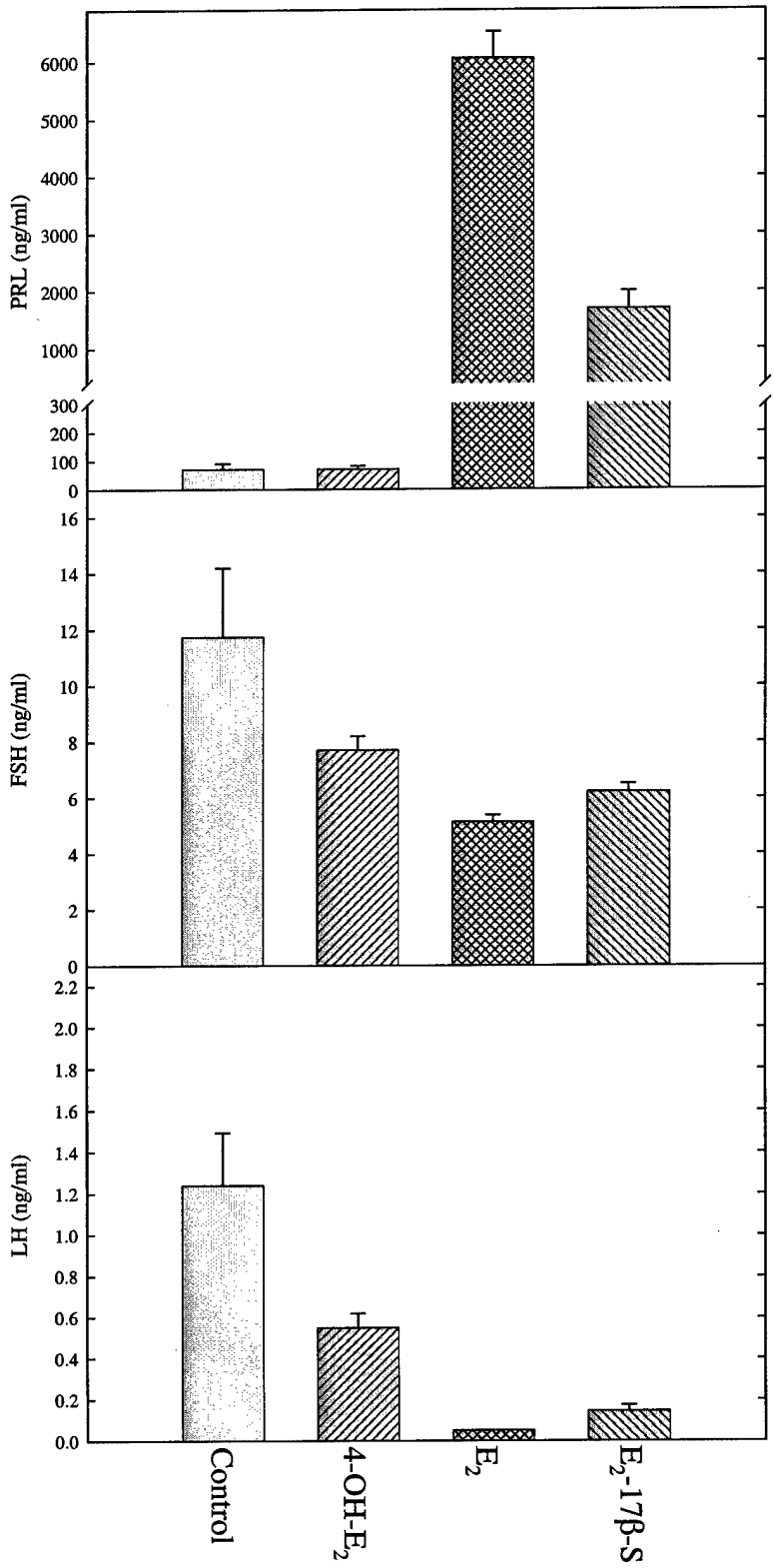
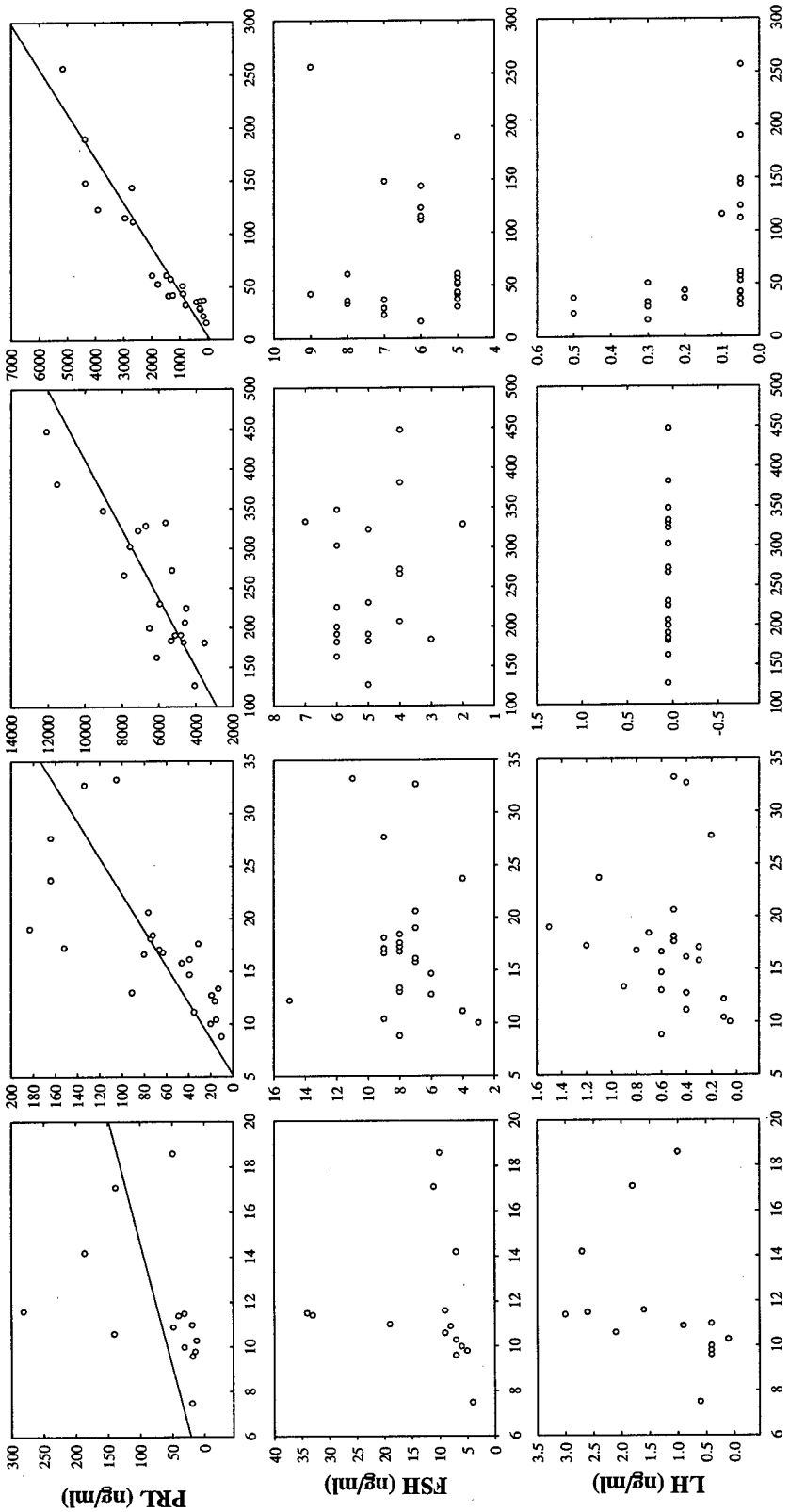

PRL (ng/ml)
6000
5000
4000
3000
2000
1000
300
200
100
0
FSH (ng/ml)
16
14
12
10
8
6
4
2
0
LH (ng/ml)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
4-OH-E2
E2
E2-17β-S

Fig 8. The Effect of Chronic Administration of an Estrogen on the Pituitary Secretion of PRL, FSH and LH. The intact female ACI rats (62 days old) were randomly divided into one control group, one E_2 treatment group, one 4-OH-E_2 treatment group and one E_2-17β-fatty acid ester treatment groups. Each animal was surgically implanted under the back skin with a 20 mg pellet containing a mixture of the hormone and cholesterol or cholesterol alone for the control group. When the animals were sacrificed by decapitation, their blood samples were collected in Vacutainer test tubes containing heparin as described in "Materials and Methods." The plasma levels of LH, FSH, and PRL were analyzed at the National Hormone and Pituitary Program by using ^{125}I-labeled RIAs. Each value is the mean ± SE of each group.


CONTROL
4-OH-E2
E2
E2-17β-S
PRL (ng/ml)
FSH (ng/ml)
LH (ng/ml)
PITUITARY WEIGHT (mg)