Estradiol-16α-carboxylic Acid Esters as Locally Active Estrogens

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We attempted to design analogues of estradiol to act as locally active estrogens without significant systemic action. We synthesized a series of 16α-carboxylic acid substituted steroids and their esters and tested their action in several assays of estrogenic action, including estrogen receptor (ER) binding, estrogenic potency in Ishikawa cells (human endometrial carcinoma), rat uterine weight (systemic action), and mouse vaginal reductases (local action). All of the estradiol substituted carboxylic acids (formic, acetic and propionic acids) were devoid of estrogenic action. To the contrary, many of the esters had marked estrogenic potency in the receptor and the Ishikawa assays. The esters of the 16α-formic acid series had the highest ER affinity with little difference between the straight-chain alcohol esters (from methyl to *n*-butyl). However, estrogenic action in the Ishikawa assay decreased precipitously with esters longer than the ethyl ester. This decrease correlated well with the increased rate of esterase hydrolysis of longer esters as determined in incubations with rat hepatic microsomes. The most promising candidates, the methyl, ethyl, and fluoroethyl esters of the formate series, were tested for systemic and local action in the in vivo models. All three, especially the fluoroethyl ester, showed divergence between systemic and local estrogenic action. These metabolically labile estrogens will be extremely useful for the therapeutic treatment of the vaginal dyspareunia of menopause in women for whom systemic estrogens are contraindicated.

Introduction

It is well-recognized that pharmacologic estrogen administration (hormone replacement therapy, HRT) can alleviate most, if not all, of the symptomology associated with menopause. These symptoms include but are not limited to bone loss associated with osteoporosis, heart disease associated with changes in blood lipids and lipoproteins, hot flashes, and vaginal dyspareunia.[1] However, there are risks associated with estrogen administration in HRT as well as oral contraceptive use, and they include an association with endometrial cancer, breast cancer, and stroke. Thus, although there are many therapeutic benefits of HRT, there are significant risks.[2−4]

Estrogen therapy affects a number of organs both directly and indirectly, and some of these outcomes are deleterious. Consequently, where possible, symptomology that could be ameliorated by local rather than systemic administration could limit the adverse side effects of estrogen therapy. One such syndrome that can be treated directly, caused by estrogen deprivation or estrogen antagonists, is vaginal dyspareunia, a common disorder which affects a large proportion of women, approximately 40% within 10 years of the onset of menopause.[5] Dyspareunia is associated with a severe physical and psychological impact, for it is not only painful but it can dramatically influence a women's self-image, leading to clinical depression.[6−8] While topical application of estrogens to the vaginal mucosa has been used to treat the vaginal dyspareunia of menopause, these estrogens are adsorbed into the blood and result in significant blood levels of estrogens.[9−12] Thus, this therapy may not be used where systemic estrogens are contraindicated. Another possible use for local estrogens includes the topical administration to aging skin. The skin contains ER and it is an estrogen target organ that can respond to estrogen replacement.[13]

Since topically applied estrogen is adsorbed into the blood, its purpose is defeated. A potent estrogen whose range is limited to the tissue to which it is applied would be ideal for the treatment of these disorders. Similar therapeutic agents with locally limited actions have been termed "soft drugs",[14] compounds that have a limited region of activity due to rapid metabolic inactivation. Ester groups have been used to convey "soft drug" properties to biologically active molecules because hydrolytic enzymes, including esterases, are ubiquitously distributed.[15] In these drugs the esters are the active agents while the hydrolysis products, the corresponding carboxylic acids, are inactive. For example, locally active glucocorticoids have been developed as antiinflammatory agents for the skin. These are carboxy analogues of steroids that are esterified. The parent carboxylic acids do not bind to the glucocorticoid receptor and are biologically inert, while their corresponding esters bind to the glucocorticoid receptor with high affinity.[15−17] The esters are rapidly hydrolyzed to the hormonally inactive parent steroidal carboxylic acid by ubiquitous esterases. Consequently, their effect is localized to the area of the skin to which they are applied because their rapid inactivation prevents systemic action.[18]

Similarly, in a study designed to produce affinity chromatographic supports for the purification of the estrogen receptor (ER) it was found that carboxylic acid

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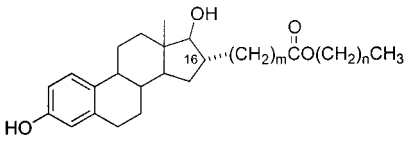


Figure 1. Abbreviation key for the E_2-16α-carboxy esters. E16-(m + 1),(n + 1): where 16 is the position in the steroid nucleus from which the ester chain originates and the quantity (m + 1) is the number of carbon atoms in the acid and (n + 1), the alcohol portion of the chain containing the ester. e.g. E16-2,2; in the figure m and n = 1.

analogues of estradiol (E_2) at C-7α- and C-17α are very poor ligands if they bind at all, but the methyl esters of these same analogues have much improved affinity for the ER.[19] It appears from those results that a charged carboxylic acid group in proximity to the steroid ring interferes with binding to the ER and that masking the charge by esterification reverses this interference. Consequently, we thought it likely that a locally active estrogen could be designed using a concept similar to that used for the glucocorticoids. We synthesized a family of 16α-carboxylic acid analogues of E_2 of varying alkyl chain lengths that were esterified with a number of alcohols of different size and substituents (Figure 1) and performed a structure−activity study in order to determine the feasibility of producing a locally active estrogen. The 16α-position of E_2 was chosen for substitution because it is chemically accessible and because some substitutions there are known to be tolerated by the ER.[20] To produce a potent but locally confined estrogen, specific alcohols of a range of steric bulk, chain length, and leaving group ability were chosen to construct esters of the carboxylic acid containing steroids in order to balance ER affinity and estrogen action with rate of enzymatic hydrolysis. The carboxylic acids and their esters were tested for their affinity for the ER and their ability to activate an estrogen-inducible gene in tissue culture. The results were correlated with the relative rate at which the esters are hydrolyzed by hepatic esterase(s). Finally, candidate esters were tested in in vivo bioassays for their systemic and local action.

Chemistry

The synthesis of the 16α-formyl ester analogues of estradiol **9**−**18** is shown in Scheme 1 and employs methodology used previously by the Katzenellenbogen group to prepare 16α-hydroxymethyl-substituted estradiol derivatives.[20] Deprotonation of 3-benzylestrone with NaH in THF followed by acylation with ethyl formate gave the enol ether **1**, which was protected with EtI as the ethyl enol ether **2**. Stereoselective reduction of the 17-ketone with $LiAlH_4$ gave the 17β-hydroxyl compound **3** that was protected with Ac_2O in pyridine to give the acetate **4**. Acid hydrolysis of **4** with 10% aqueous HCl produced the 16α-aldehyde **5** as the only isomer. Oxidation with CrO_3–H_2SO_4[21] gave the acid **6**, which was deprotected with KOH−MeOH followed by hydrogenolysis with 5% Pd−C/H_2 to produce the acid **8**, E16-1,0.

The methyl, ethyl, propyl, and butyl esters [E16-1,1 (**9**), E16-1,2 (**10**), E16-1,3 (**11**), and E16-1,4 (**13**)] were prepared by reacting **8** with the appropriate alcohol in the presence of $SOCl_2$; the isopropyl, neopentyl, monofluoro-, difluoro- and trifluoroethyl esters [E16-1,3i (**12**), E16-1,5neo (**14**), E16-1,2F_1 (**16**), E16-1,2F_2 (**17**), and E16-1,2F_3 (**18**)] were prepared by reacting **8** with the appropriate alcohol in the presence of pTsOH. The vinyl ester, E16-1,2vin (**15**), was prepared from **8** through a vinyl-exchange reaction with vinyl propionate and $PdCl_2$–LiCl as catalyst.[22] In the ^{1}H NMR spectra of all these esters, the signal for H-17α appears at about δ 3.90 ppm with a coupling constant ($J_{17\alpha-16}$) of 7.4−9 Hz, indicating that these esters have the same stereochemistry at C-16. The magnitude of this coupling constant is consistent with that seen in related C-16α,17β-substituted steroids.[23] Proof of the stereochemistry at C-16 was obtained by reduction of E16-1,2 with $LiAlH_4$, a reagent known not to affect epimerizable asymmetric centers.[24,25] The reduction gives the hydroxymethyl steroid whose ^{1}H NMR spectrum is identical with that of the known 16α-hydroxymethyl estradiol **19**, prepared by the literature procedure.[20]

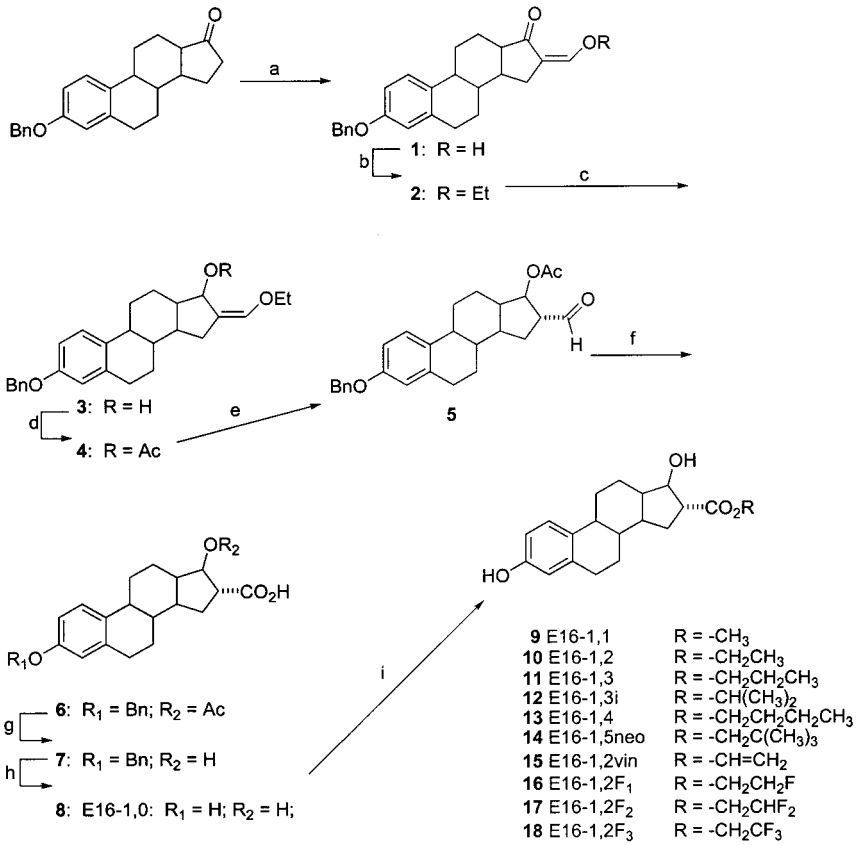
The synthesis of the 16α-carboxymethyl analogues of estradiol (**22**, **23**, **26**) is shown in Scheme 2. Deprotonation of 3-benzylestrone with LDA in THF at 0 °C followed by alkylation with ethyl bromoacetate at <−20 °C gave the keto ester **20** as only the α-isomer in 35% yield with 43% recovered starting material. Reduction of the ketone with lithium tri-*tert*-butoxyaluminum hydride at −78 °C in THF gave the 17β-alcohol **21**. Deprotection of phenolic hydroxyl group with 5% Pd−C/H_2 in EtOH gave the ethyl ester **22** (E16-2,2). Saponification of **22** with 5% KOH−MeOH gave the acid **23** (E16-2,0). The protected ethyl ester **21** was saponified, and the resulting acid **24** was converted to the methyl ester, **25**. Deprotection of **25** with 5% Pd−C/H_2 gave the methyl ester **26** (E16-2,1). Proof of the stereochemistry at C-16 was obtained by Dibal reduction of ethyl ester **21**, followed by Tebbe reaction of the resulting aldehyde **27** to give 16-allyl-3-benzylestradiol **28**. The ^{1}H NMR spectrum is identical to the 16α-allyl-substituted steroid obtained by the literature method.[26]

The synthesis of the 16α-carboxyethyl analogues of estradiol (**34**, **35**) is shown in Scheme 3. The known 16α-allyl-3-benzyloxyestra-1,3,5(10)-trien-17β-ol[26] was first protected as the acetate with Ac_2O in pyridine. Hydroboration of **29** and oxidation with trimethylamine oxide in diglyme produced the alcohol **30**, which was oxidized with CrO_3–H_2SO_4 to give the acid **31**. Saponification of the 17β-acetate with KOH−MeOH followed by esterification of the carboxylic acid with MeOH in the presence of $SOCl_2$ gave **33**. Deprotection of the phenolic hydroxyl group with 5% Pd−C/H_2 provided **34** (E16-3,1). The acid **35** (E16-3,0) was obtained by saponification of **34**.

Results and Discussion

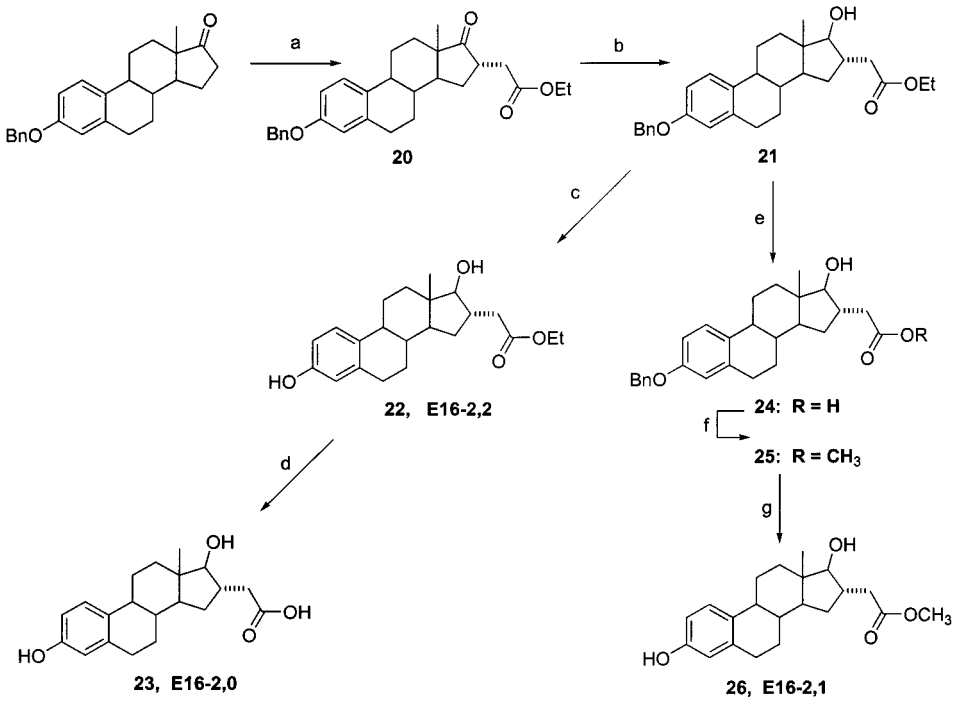
The E_2-16α-alkyl carboxylic acid analogues and their esters were tested in a variety of estrogenic assays and an esterase assay to determine their estrogenic potency and the relative rate of their enzymatic hydrolysis. The receptor studies used the classical assay with rat uterine cytosol (ERα), which is well-recognized as an excellent physiological model. The results show a distinct trend (Table 1). None of the parent carboxylic acid analogues, E16-1,0, E16-2,0, and E16-3,0, showed significant binding to the ER. Of those steroidal esters examined, increasing the distance of the ester function from the steroid ring by elongating the alkyl tether dramatically

Scheme 1[a]



[a] (a) NaH, THF; ethyl formate (estrone-3-benzyl ether → **1**); (b) K_2CO_3, EtI, acetone (**1** → **2**); (c) $LiAlH_4$, Et_2O (**2** → **3**); (d) Ac_2O, pyridine (**3** → **4**); (e) 10% aqueous HCl, THF (**4** → **5**); (f) Jones oxidation (**5** → **6**); (g) KOH−MeOH, 50 °C (**6** → **7**); (h) 5% Pd−C/H_2, EtOH (**7** → **8**); (i) ROH, $SOCl_2$ or ROH, pTsOH or vinyl propionate, $PdCl_2$−LiCl, MeOH (**8** → **9**−**18**).

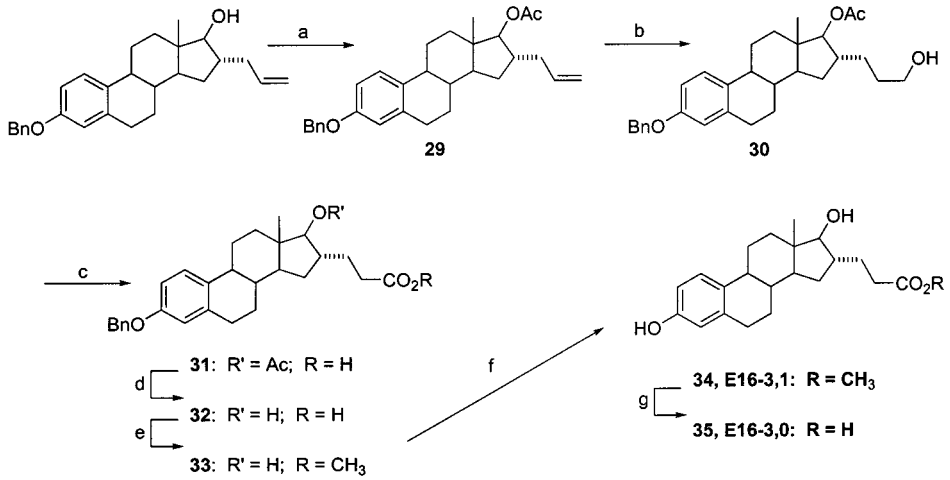
Scheme 2[a]



[a] (a) LDA, THF; ethyl bromoacetate (estrone-3-benzyl ether → **20**); (b) $Li(OtBu)_3AlH$, THF (**20** → **21**); (c) 5% Pd−C/H_2, EtOH (**21** → **22**); (d) 5% KOH−MeOH (**22** → **23**); (e) 5% KOH−MeOH (**21** → **24**); (f) MeOH, $SOCl_2$ (**24** → **25**); (g) 5% Pd−C/H_2 (**25** → **26**).

decreases the binding. Thus, in the series of methyl esters, E16-1,1 has a RBA = 35%; E16-2,1, 5%; and E16-3,1, 1%, where RBA is the relative binding affinity in the rat uterine estrogen receptor. Likewise, in the ethyl ester series, E16-1,2 has a RBA = 40% and E16-2,2, 5%. This precipitous decline with increasing number of

Scheme 3[a]



[a] (a) AcO_2, pyridine (16α-allyl-3-benzylestradiol → 29); (b) BH_3−THF; Et_3NO, diglyme, 150 °C (**29** → **30**); (c) Jones oxidation (**30** → **31**); (d) KOH−MeOH, 55 °C (**31** → **32**); (e) MeOH, $SOCl_2$ (**32** → **33**); (f) 5% Pd−C/H_2, EtOH (**33** → **34**); (g) KOH−MeOH, 60 °C (**34** → **35**).

Table 1. Estrogenic Properties of E_2-16α-alkylesters

compd[a]	ER (RBA[b])	Ishikawa cell AlkP (RSA[c])	esterase (RHA[d])
E_2	100	100	−
E_1	30 ± 11	7 ± 2	−
E16-1,0	0	0	−
E16-1,1	35 ± 4	10 ± 3	45 ± 10
E16-1,2	40 ± 10	11 ± 5	100
E16-1,3	34 ± 9	2 ± 0	230 ± 50
E16-1,3i	7 ± 4	0.1 ± 0.06	140 ± 10
E16-1,4	28 ± 17	1 ± 0	350 ± 50
E16-1,5neo	14 ± 3	1 ± 0.6	50 ± 5
E16-1,2F_1	35 ± 3	13 ± 5	420 ± 40
E16-1,2F_2	10 ± 3	4 ± 1	2350 ± 275
E16-1,2F_3	7 ± 1	3 ±1	8200 ± 1800
E16-1,2vin	6 ± 2	0.4 ± 0.3	20300 ± 5500
E16-2,0	0	0	−
E16-2,1	5 ± 2	0.5 ± 0.1	340 ± 30
E16-2,2	5 ± 1	0.3 ± 0.1	700 ± 120
E16-3,0	0.1 ± 0.1	0	−
E16-3,1	1 ± 1	0	910 ± 100

[a] Abbreviations are shown in Figure 1, with examples as follows: E16-1,0 is the formic acid analogue of E_2. E16-1,1 is the methyl ester of the formate analogue, etc. 3i is the isopropyl ester, 5neo is the neopentyl ester, and 2vin is the vinyl ester. F is fluorine substitution in the 2′-position of an ethyl ester, i.e., F_3 is the 2′,2′,2′-trifluoroethyl ester. [b] RBA is the relative binding affinity in the rat uterine estrogen receptor (ER) assay, where E_2 = 100. [c] RSA is the relative stimulatory activity in the induction of alkaline phosphatase (AlkP) activity in the Ishikawa estrogen bioassay, where E_2 = 100. [d] RHA is the relative hydrolytic activity in the esterase assay with rat hepatic microsomes in comparison to E16-1,2 = 100. The dash (−) indicates not done. All values are ±SD.

carbon atoms did not occur with the alcohol portion of the ester. Here the length of the alcohol portion of the ester had no effect on ER binding: E16-1,1, E16-1,2, and E16-1,3 all had RBAs of approximately 35−40%, and E16-1,4 at 28% was not significantly different from the others. Consequently, the pairs E16-1,2 and E16-2,1, and E16-1,3 and E16-3,1 are all esters with the same number of carbon and oxygen atoms, but their binding to the ER is very different. Noteworthy, all of these E16-1 esters were at least as potent as the natural estrogen, estrone (E_1) (RBA = 30%). However, the E16-1 esters made from bulky alcohols had a markedly decreased affinity for the receptor: the RBA of the neopentyl ester E16-1,5neo was 7%, and that of the isopropyl ester E16-1,3I was 7%. The fluoroethyl ester, E16-1,2F_1, was a good ligand, with an RBA of 35%, comparable to that of E16-1,2. Additional fluorine atoms decreased the affinity for the ER: E16-1,2F_2, RBA = 10%; E16-1,2F_3, RBA = 7%. The vinyl ester, E16-1,2vin, also had a RBA of 6%.

Table 2. Binding of Selected E_2-16α-Alkylesters to the LBD of Human ERα and ERβ

compd	ERα[a]	ERβ[a]	ERα/ERβ
E_2	100	100	1
E16-1,1	19 ± 9	0.3 ± 0.2	62 ± 10
E16-1,2	19 ± 5	0.3 ± 0.1	77 ± 12
E16-1,2F_1	16 ± 2	0.1 ± 0.1	89 ± 3
E16-1,F_2	13 ± 9	0.2 ± 0.1	67 ± 4

[a] RBA of the indicated ester compared to E_2. Values are ±SD. This assay measures the inhibition of the binding of [^{3}H]E_2 in lysates of *E. coli* in which the LDB of human ERα and ERβ were separately expressed. Abbreviations are in Table 1, LBD is the ligand binding domain.

A few of the E16-1 esters that had high affinity for ERα were also tested (by Drs. Paul Shughrue and Heather Harris) for binding to ERβ. In these experiments binding to the ligand binding domain (LBD) (see Experimental Section) of both the human ERα and ERβ were measured in parallel. The binding of the carboxy analogues to the expressed human ERα (Table 2) is somewhat lower than the binding to ERα of rat uterine cytosol (Table 1) but shows the same trend: E16-1,1 = E16-1,2. ≅ E16-1,2F_1 > E16-1,2F_2 (the RBAs here are not statistically different). More importantly, none of these esters bind well to ERβ, on the order of 1−2% of ERα. The ERα to ERβ ratio range from 60 for E16-1,1 to 90 for E16-1,2F_1. This difference in the affinity of the E_2-16α-alkyl esters for the 2 ERs is not unexpected, because it had previously been reported that another 16α-substituted ER ligand, 16α-iodoestradiol, which binds with high affinity to the classical ER, ERα,[27] binds only poorly to ERβ.[28] Apparently, many substituents at C-16α that do not affect binding to ERα impede binding by ERβ.

All of the E_2-16α-alkyl carboxylic acids and their esters were tested for their estrogenic potency by measuring their effect on the induction of alkaline phosphatase (AlkP) in Ishikawa cells (a human endometrial adenocarcinoma cell line). We have previously

shown that this assay accurately assesses the potency of a wide variety of estrogenic compounds.[29] As can be seen in Table 1, the potencies determined in this experiment are different than those measured in the ER assay. Three of the esters, E16-1,1, E16-1,2, and E16-1,2F_1, had fairly high estrogenic potencies (compared to E_2) with RSAs of 10, 11, and 13%, respectively. They were at least as effective as E_1 (7%) and probably more so, although the differences were not significant. Contrariwise, several of the esters that had relatively high RBAs in the ER binding assay, including E16-1,3 and E16-1,4, had a much lower potency in the Ishikawa cell assay. Most of the other esters also had considerably lower estrogenic action than would have been predicted on the basis of their receptor affinity.

The reason for the discrepancy between ER binding and estrogenic potency became clear when the E_2-16α-alkyl esters were tested as substrates for the esterase(s) in rat hepatic microsomes. It can be seen in Table 1 that there are dramatic differences in the rate of the esterase reaction with the various esters. In general, the longer the alkyl chain, regardless of whether it is in the carboxylic acid or alcohol portion of the ester function, the more rapid the hydrolytic cleavage. This is as expected, for it has been shown that increasing the lipophilicity of the alcohol portion of an ester leads to an increased rate of enzymatic cleavage,[30] and it is clear that the further removed that the ester function is from the bulky steroid nucleus, the more accessible it is to the enzyme. Steric hindrance is also a factor in the hydrolysis of the branched chain alcohols, E16-1,3i and E16-1,5neo, in which the relative rate of reaction is markedly decreased, with RHA = 140 and 50, respectively, compared to E16-1,3, RHA = 230, where RHA is the relative hydrolytic activity in the esterase assay with rat hepatic microsomes. The fluorine-substituted esters, E16-1,2F_1, E16-1,2F_2, and E16-1,2F_3, showed a large increase in enzymatic hydrolysis that was directly related to the number of fluorine atoms at the 2′-position. The rapid rate of hydrolysis of esters of fluorinated alcohols has been ascribed to the increased acidity of the leaving group alcohol.[31] Likewise, vinyl esters have been shown to be excellent substrates for esterases,[32] consistent with the very high rate of cleavage of E16-1,2vin.

In evaluating these compounds, binding to the ER is, of course, the most important factor in the determination of estrogenic potential. However, in biological systems, additional factors such as catabolism must be weighed. In general, in these experiments, the estrogenic potency of each compound as determined by the stimulation of AlkP in the Ishikawa cells (RSA) is consistent with its binding to the ER (RBA), provided that its susceptibility to esterase cleavage (RHA) is considered. Because the carboxylic acid analogues of E_2 are inactive (Table 1), the rate of hydrolysis of the esters is an important factor. This can be seen in the estrogenic potency of the straight chain $E_2$16-1 esters, $E_2$16-1,1 through $E_2$16-1,4. They all have approximately the same RBA, but their RSA decreases with increasing chain length, which reflects their increasing enzymatic cleavage rates with increasing chain length. The E16-2 and E16-3 esters have low RBAs and high RHAs, and consequently, the potency of all of these compounds is low.

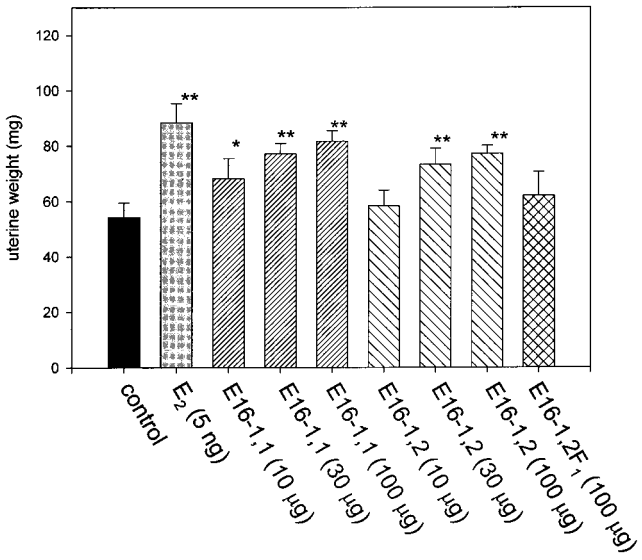


Figure 2. In vivo systemic estrogenic assay (uterotrophic assay). Immature female rats (22 days old) were injected with E16-1,1, E16-1,2, and E16-1,2F_1 as well as E_2 in sesame oil once daily for 3 days. On the fourth day the animals were killed, and the uteri were removed and weighed. The total dose is shown. Controls were injected with sesame oil. Error bars are ±SD. $n = 5$. *$P < 0.05$, **$P < 0.001$ when compared to the control.

The convergence of binding (RBA) and hydrolysis (RHA) as the determinant of potency (RSA) does not hold as well for the fluorinated esters. As can be seen in Table 1, E16-1,2F_1 and E16-1,2 have about the same RBA for ERα, but since the monofluorinated ester is cleaved at about 4 times the rate of the nonfluorinated ester, it might be assumed that E16-1,2 should be considerably more potent. However, both esters have the same RSA. This also appears to be true for the difluoro and trifluoro ethyl esters. While both of these fluorinated esters have approximately the same RBA as E16-1,3i, their RHA is at least 17−60 times greater. If these two factors, enzymatic hydrolysis and ER binding, are considered, then the isopropyl ester should be the more potent. However, the isopropyl ester is almost inactive, while conversely the difluoro and trifluoro ethyl esters, although weak estrogens, are significantly more active. An explanation for these apparently conflicting findings may be that competitive binding studies, which are indirect measurements, do not always accurately reflect the true binding affinity. More likely though, the RBA does not necessarily reflect the ligand-induced conformational changes of the receptor which directly affects the transcriptional stimulation of estrogen responsive genes.[33] Thus, receptor stimulation of genes is more complex than is apparent from ligand binding. In any case, the fluorine-substituted esters are more potent estrogens than would be predicted on the basis of their RBA and RHA.

Three estrogen esters, the methyl (E16-1,1), ethyl (E16-1,2), and fluoroethyl (E16-1,2F_1) esters of E16-1,0, that were most potent in the Ishikawa assay were tested for their systemic estrogenic activity in the classical in vivo assay, uterotrophic stimulation of the immature rat.[34] In this assay all of the test compounds were administered in sesame oil. Again, E_2 is included for comparison. As can be seen in Figure 2, 5 ng of E_2 produced a statistically significant stimulation in the

weight of the uterus. As hypothesized, the systemic potency of the esters was very low. E16-1,1, the methyl ester, produced a small uterotrophic effect ($P < 0.05$) at 10 μg (total dose per animal) in the experiment shown. In two separate experiments, a small effect at this dose was also detected, but it was not statistically significant. This ester produced a reproducible and statistically significant stimulation at 30−50 μg ($P < 0.01$), estimated to be equivalent to a potency ranging from 1/10 000 of E_2 at 30 μg to 1/25 000 at the 100 μg dose. E16-1,2 produced no measurable stimulation at 10 μg and a small increase in uterine weight at 30 μg. The uterotrophic effect of E16-1,2 was estimated as ranging from 1/12 000 at the 30 μg dose to 1/25 000 the potency of E_2 at 100 μg. The fluoroethyl ester, E16-1,2F_1, was less potent; it did not produce a uterotrophic stimulation at 100 μg, and in other experiments (not shown) 300 μg of E16-1,2F_1 produced a small uterotrophic effect, equivalent to less than 1/60 000 of E_2. Consequently, these three esters, which bind with high affinity to the ER and have a high stimulatory activity in the Ishikawa cells, show only a very weak systemic estrogenic action. E16-1,2F_1 has less systemic activity than the other two formyl esters, as might be expected since of these three steroid esters it has the most rapid rate of enzymatic hydrolysis to the inactive steroid carboxylic acid E16-1,0 (Table 1).

These three esters, E16-1,1, E16-1,2, and E16-1,2F_1, were tested to determine whether they were estrogenic in an in vivo assay of local activity, stimulation of vaginal reductase(s) in the ovariectomized mouse. As can be seen in Figure 3a, all of the esters were estrogenic in this assay, producing a statistically significant ($P < 0.001$) stimulation at the level of 250 ng for the methyl and ethyl esters and 100 ng for the monofluoroethyl ester. The potency of the monofluoroethyl ester in this assay was greater than that of the other two esters. However, the vaginal stimulation induced by the esters was considerably lower than that of E_2. In this experiment, as in the original assay,[35] the steroids were instilled in the vagina in aqueous propylene glycol. In this aqueous solvent they are immediately available to the esterase(s) in the vaginal secretions and cells. Consequently, they are rapidly hydrolyzed and have only a short stimulatory period. Since E_2 is not susceptible to esterase attack, it is metabolized relatively slowly in this paradigm and it has a much longer stimulatory action. This contrasts to the systemic, uterotrophic, assay in which the esters (as well as E_2) were injected in an oil, which serves to shield them from metabolism and, thus, prolong estrogenic stimulation. Consequently, we repeated the vaginal assay substituting sesame oil instead of the aqueous medium as the vehicle. In this experiment (Figure 3b) 50 ng of the E16-1,2F_1 (but not E16-1,1 or E16-1,2) produced a significant estrogenic effect equivalent to a dose of 20 pg of E_2. In this and other experiments, there was a consistent stimulation at 10 ng of E16-1,2F_1; however, it was not statistically significant. Thus, in the mouse vaginal assay the monofluoroethyl ester, E16-1,2F_1, had a relative potency of approximately 1/2500 of E_2. As would be expected for an estrogen behaving as a "soft drug", the potency (relative to E_2) of E16-1,2F_1 in the vaginal assay is considerably higher than

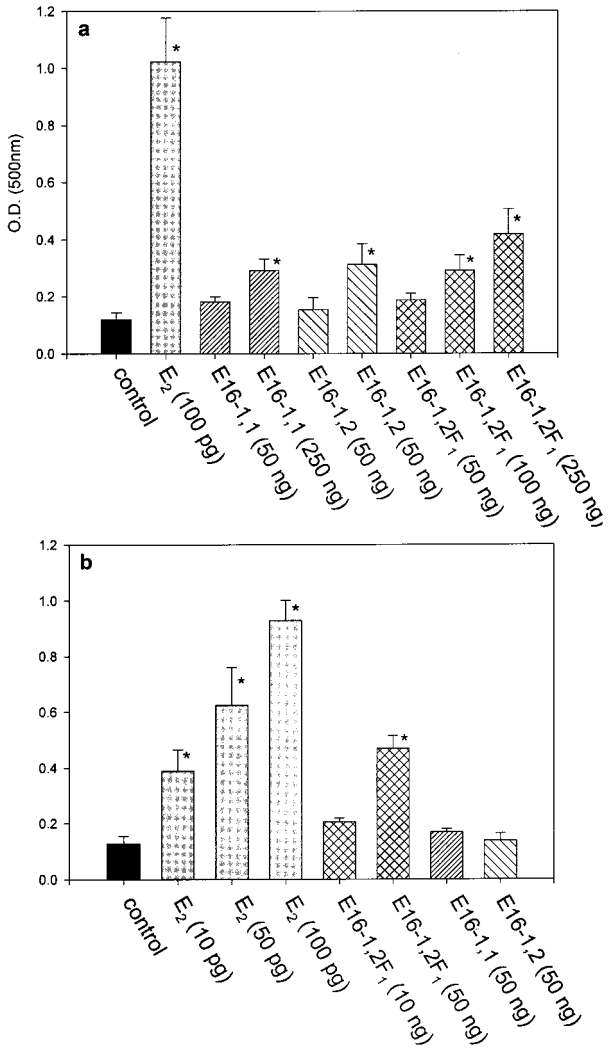


Figure 3. In vivo local estrogenic assay (vaginal assay). Castrated female mice were intravaginally administered E16-1,1, E16-1,2, E16-1,2F_1, and E_2 in a single dose. Controls were injected with vehicle. The next morning, 2,3,5-triphenyltetrazolium chloride was instilled in the vagina and 30 min later the animals were killed and the vaginal reductase activity was determined spectrophotometrically Panel a: the steroids were dissolved in 10 μL of 25% propylene glycol in saline. Panel b: the steroids were dissolved in 10 μL of sesame oil. $n = 5$. Error bars are ±SD. *$P < 0.001$ compared to the control.

that in the systemic assay. Indeed, we are probably underestimating the relative potency of the esters in the vaginal assay, because the tissues of the mouse have a higher level of nonspecific esterase than the rat,[36] the species in which the systemic assay was determined. High levels of esterases would lead to a rapid hydrolysis and deactivation of the esters. Regardless, this experiment demonstrates that these labile esters possess significant estrogenic action when administered directly to an estrogen target tissue.

Vaginal creams containing estrogens are well-known forms of pharmacological treatment of vaginal dyspareunia. In addition to E_2, other weaker estrogens such as estriol and E_1 have been used in vaginal preparations with the intention of producing a local action. However, all of these estrogens, when applied vaginally, are adsorbed into the bloodstream and produce systemic effects.[9−12] Those studies concluded that vaginally administered estrogens are therapeutically efficacious but

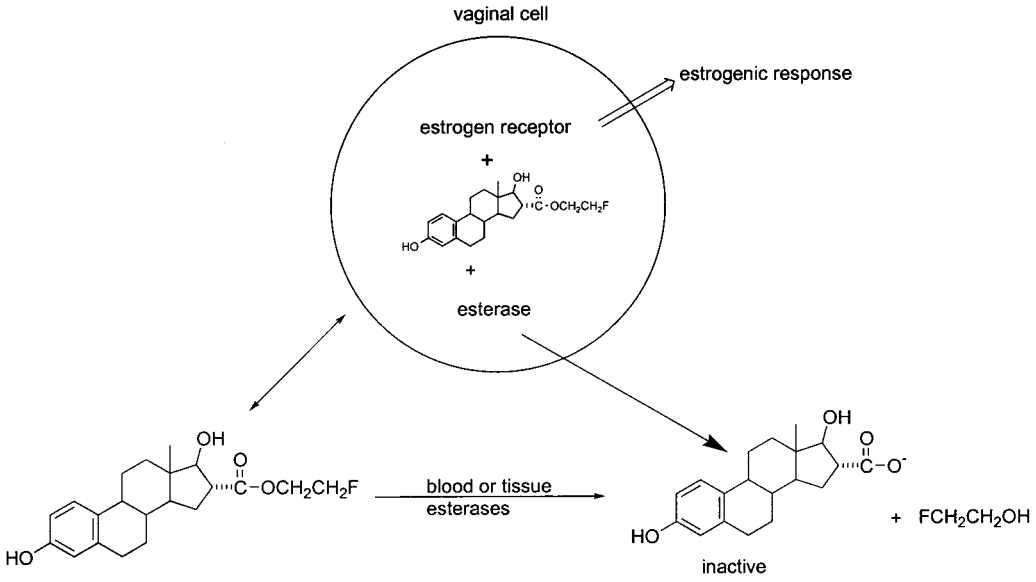


Figure 4. Estrogenic action and biological inactivation of E16-1,2F_1 (**16**). Locally administered compound **16** diffuses into the vaginal cell, where it binds to the estrogen receptor and produces an estrogenic stimulus. Esterases in the vagina or within other tissues and blood rapidly hydrolyze **16** to 2′-fluoroethanol and the biologically inert alkyl carboxylate E16-1,0, (**8**). Consequently, estrogenic stimulation is confined to the area of administration.

that their action is not regionally confined and, therefore, that they should not be used in patients in whom systemic estrogens are contraindicated. In the present study we have synthesized a series of steroids substituted at 16α with carboxylic acid esters as "soft estrogens". These compounds are designed to be rapidly inactivated by hydrolytic esterases in order to confine their activity to the site of application, in this case, the vagina (Figure 4). We found that three of these esters, E16-1,1, E16-1,2, and E16-1,2F_1, had the desired characteristics of high ER binding activity and estrogenic potency (Table 1) and, as required for a "soft estrogen", their enzymatic hydrolysis product, the parent carboxylic acid E16-1,0, was devoid of estrogenic properties. Each of the esters exhibited exceedingly low systemic activity (Figure 2), and yet they were active in the vaginal, local assay (Figure 3). One of these compounds, E16-1,2F_1, was hydrolyzed very rapidly, and thus, it had the lowest systemic action. Conversely, of the three esters, it had the highest vaginal activity, indicating that it could be a potential therapeutic agent for the treatment of women with vaginal dyspareunia. Further, these studies support the concept of using steroidal carboxylic acid esters as local estrogens and indicate that additional exploration of similar esters at other ring positions with greater inherent estrogenic potential could produce estrogens with higher local activity and greater selectivity.

Experimental Section

General. ^{1}H NMR spectra were recorded with a Bruker AM500 and chemical shifts are reported relative to residual $CHCl_3$ (7.27 ppm) or DMSO (2.5 ppm). Purification by flash chromatography was performed according to the procedure of Still[37] using 230−400 mesh silica gel (EM Science, Darmstadt Germany). High-resolution mass spectra were obtained by electrospray ionization on a Micromass Q-Tof spectrometer by Dr. Walter J. McMurray at the Yale University Comprehensive Cancer Center using either PEG as an internal standard, with NH_4OAc, or NaI as an internal standard. Elemental analyses were performed by Schwarzkopf Micro Analytical Laboratory, Woodside, NY. The computer program Prism was purchased from GraphPad Software Inc. (San Diego, CA). The cell culture reagents were obtained from Gibco-BRL (Grand Island, NH). Unless otherwise indicated, solvents (analytical or HPLC grade) and reagents were used as supplied, and all reactions were carried out under nitrogen.

Chromatographic Systems. Thin-layer chromatography (TLC) was performed using Merck silica gel plates (F_{254}) (EM Science) and visualized using phosphomolybdic acid or UV illumination. TLC systems: T-1, hexanes/EtOAc (3:1); T-2, hexanes/EtOAc (2:1); T-3, hexanes/acetone (5:1); T-4, EtOH/EtOAc (1:9); T-5, $CHCl_3$/MeOH (5:1);T-6, hexanes/EtOAc (1:1); T-7, (CH_2Cl_2) Analytical high-performance liquid chromatography (HPLC) was performed on a Waters 600E system (Waters Co. Milford MA) equipped with a 484 variable wavelength detector using the following systems: H-1, Protein I-60 column (7.8 mm × 30 cm, Waters Co.) with HOAc/^{i}PrOH/CH_2Cl_2 (0.1:6:93.9) at 3 mL/min; H-2, Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments, Walnut Creek CA) with HOAc/CH_3CN/H_2O (0.13:35:64.87) at 0.8 mL/min; H-3, Protein I-60 column (7.8 mm × 30 cm, Waters Co.) with CH_2Cl_2 at 3 mL/min; H-4, Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) with CH_3CN/H_2O (45:55) at 1 mL/min; H-5, Ultrasphere ODS (5 μm 10 mm × 25 cm, Altex Scientific Operations Co, Berkeley, CA) with CH_3CN/H_2O (60:40) at 1.3 mL/min; H-6, Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) with CH_3CN/H_2O (60/40) at 1 mL/min; H-7, Ultrasphere ODS (5 μm 10 mm × 25 cm, Altex Scientific Operations Co.) with CH_3CN/H_2O (40/60) at 3 mL/min H-8, Ultrasphere ODS (5 μm 10 mm × 25 cm, Altex Scientific Operations Co.) with H_2O/CH_3CN (50/50) at 3 mL/min; H-9, Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) with THF/CH_3CN/H_2O (5.5:45:49.5) at 0.8 mL/min; H-10, Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) with HOAc/CH_3CN/H_2O (0.13:35:64.87) at 1 mL/min; H-11 Ultrasphere ODS (5 μm 10 mm × 25 cm, Altex Scientific Operations Co.) with THF/CH_3CN/H_2O (5.5:45:49.5) at 1.5 mL/min; H-12 Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) with THF/CH_3CN/H_2O, (6: 40:54) at 0.8 mL/min; H-13, LiChrosorb RP-18, (5 μm, 4.6 mm × 25 cm, EM Science) with HOAc/CH_3CN/H_2O (0.11:45:54.89) at 1 mL/min; H-14, Protein I-60 column (7.8 mm × 30 cm, Waters Co.) with HOAc/^{i}PrOH/CH_2Cl_2 (0.2:5:94.8) at 3 mL/min) H-15,

Microsorb-MV C18 (5 μm, 4.6 mm × 25 cm, Varian Analytical Instruments) with HOAc/CH_3CN/H_2O (0.12:40:59.88) at 1 mL/min.

3-Benzyloxy-16-(ethoxymethylidene)estra-1,3,5(10)-trien-17-one (2). A solution of 5.08 g (14.1 mmol) of benzylestrone in 20 mL of anhydrous THF was added to a suspension of 2.03 g (42.3 mmol) of a 50% dispersion of NaH in 20 mL of anhydrous THF at 0 °C. To this was added 5.7 mL (70 mmol) of ethyl formate, and the reaction was stirred at room temperature for 3 h. The reaction was quenched with saturated aqueous NH_4Cl (50 mL) and extracted with EtOAc (3×, 50 mL). Combined organic extracts were washed with 10% sodium metabisulfite (20 mL) and H_2O (20 mL), dried over Na_2SO_4, and concentrated in vacuo giving a yellow foam.

A solution of 5.61 g of this crude material, 13.3 g (96.2 mmol) of K_2CO_3, and 6.62 mL (82.8 mmol) of EtI in acetone (70 mL) was stirred at room temperature for 48 h. The reaction was poured into H_2O (200 mL) and extracted with CH_2Cl_2 (3×, 100 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving an orange oil. Purification by flash chromatography on a 5 × 18 cm column of silica gel eluting with hexanes/EtOAc (3:1) gave 2.56 g (43%, two steps) of **2**. Data for **2**: TLC, T-1, R_f 0.27; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.93 (s, 3H, H-18), 1.35 (t, 3H, J = 7.2 Hz, $-CH_2CH_3$), 4.05−4.11 (m, 2H, $-CH_2CH_3$), 5.04 (s, 2H, benzylic), 6.74 (d, 1H, J = 2.2 Hz, H-4), 6.80 (dd, 1H, J = 8.7, 2.2 Hz, H-2), 7.21 (d, 1H, J = 8.7 Hz, H-1), 7.30 (s, 1H, −C=CHOEt), 7.32−7.44 (m, 5H, Ar−H).

3-Benzyloxy-16-(ethoxymethylidene)estra-1,3,5(10)-trien-17β-ol (3). A solution of 316 mg (0.759 mmol) of ketone **2** in 2 mL of Et_2O was stirred at room temperature as 43 mg (1.14 mmol) of $LiAlH_4$ was added. The reaction was stirred at room temperature for 1.25 h, poured into EtOAc (1 mL) and saturated aqueous Na−K tartrate (30 mL), and extracted with EtOAc (3×, 50 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving a white gel. Purification of the residue by flash chromatography on a 3 × 21 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 194 mg (61%) of **3**: TLC, T-2, R_f 0.34.

3-Benzyloxy-16-(ethoxymethylidene)estra-1,3,5(10)-trien-17β-yl Acetate (4). A solution of **3** (194 mg) and acetic anhydride (1 mL) in pyridine (3 mL) was stirred at room temperature overnight under N_2. The reaction was poured into saturated aqueous $NaHCO_3$ (150 mL) and extracted with CH_2Cl_2 (3×, 100 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 20 cm column of silica gel using hexanes/EtOAc (4:1) as eluent gave 183 mg (86%) of **4** as a white solid: TLC, T-2, R_f 0.67.

3-Benzyloxy-16α-formylestra-1,3,5(10)-trien-17β-yl Acetate (5). A solution of 183 mg (0.397 mmol) of **4** in THF (1.5 mL) with 4 drops of 10% aqueous HCl and 2 drops of H_2O was stirred at room temperature for 6 h. The reaction was poured into saturated aqueous $NaHCO_3$ (100 mL) and extracted with CH_2Cl_2 (3×, 75 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by repeated (3×) flash chromatography on a 2 × 15 cm column of silica gel using hexanes/acetone (6:1) as eluent gave 97 mg (56%) of **5** as a white solid. Data for **5**: TLC, T-3, R_f 0.22; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.91 (s, 3H, H-18), 2.10 (s, 3H, OAc), 4.86 (d, 1H, J = 8.1 Hz, H-17), 5.04 (s, 2H, benzylic), 6.73 (d, 1H, J = 2.7 Hz, H-4), 6.79 (dd, 1H, J = 8.5, 2.7 Hz, H-2), 7.19 (d, 1H, J = 8.5 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H), 9.83 (d, 1H, J = 3.1 Hz, O=CH); HRMS (ES) calcd for $C_{28}H_{32}O_4Na$ (M + Na^+) m/e 455.2198, found m/e 455.2205.

3-Benzyloxy-17β-acetoxyestra-1,3,5(10)-trien-16α-carboxylic Acid (6). A solution of 824 mg (1.90 mmol) of **5** in 120 mL of acetone was cooled to 0 °C, and 710 μL of Jones reagent solution (8 M solution of CrO_3 in aqueous H_2SO_4)[38] was added. The reaction mixture was stirred at 0 °C for 30 min, diluted with MeOH (20 mL) and H_2O (20 mL), concentrated to about 30 mL, and extracted with CH_2Cl_2 (3×, 70 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using EtOH/CH_2Cl_2 (5:95) gave 784 mg (92%) of **6** as a white foam. Data for **6**: TLC, T-4, R_f 0.78; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (s, 3H, H-18), 2.10 (s, 3H, OAc), 5.02 (d, 1H, J = 7.1 Hz, H-17α), 5.04(s, 2H, benzylic), 6.72 (d, 1H, J = 2.8 Hz, H-4), 6.79 (dd, 1H, J = 8.5, 2.8 Hz, H-2), 7.20 (d, 1H, J = 8.5 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H); HRMS (ES) calcd for $C_{28}H_{32}O_5Na$ (M + Na^+) m/e 471.2147, found m/e 471.2146.

3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16α-carboxylic Acid (7). A solution of 784 mg (1.75 mmol) of **6** and 5% aqueous KOH in MeOH (7 mL) was stirred and heated at 50 °C for 3 h. The reaction was cooled to room temperature, poured into H_2O (50 mL), adjusted to pH 1 with 10% aqueous HCl, and extracted with EtOAc (3×, 100 mL). Combined organic extracts were washed with H_2O (100 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using EtOH/EtOAc (10:90) gave 492 mg (69%) of **7** as a white foam. Data for **7**: TLC, T-4, R_f 0.63; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (2, 3H, H-18), 3.94 (d, 1H, J = 8.3 Hz, H-17α), 5.04 (s, 2H, benzylic), 6.73 (d, 1H, J = 2.2 Hz, H-4), 6.80 (dd, 1H, J = 8.7, 2.2 Hz, H-2), 7.21 (d, 1H, J = 8.7 Hz, H-1), 7.32−7.45 (m, 5H, Ar−H).

3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-carboxylic Acid (8, E16-1,0). A solution of 492 mg (1.21 mmol) of **7** in 5 mL of EtOH was added to a suspension of 50 mg of 5% Pd/C in 10 mL of EtOH, and the reaction was stirred at room temperature under 1 atm of H_2 for 19 h. The reaction was filtered through a 1 in. pad of Celite and washed through with EtOH (100 mL). The filtrate was concentrated in vacuo, giving 343 mg (89%) of **8** as a white solid. Purification of 16 mg of this material by HPLC in system H-1 (t_R = 14 min) gave 13 mg of **8** for bioassay. Data for **8**: TLC, T-5, R_f 0.38; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.69 (s, 3H, H-18), 3.69 (d, 1H, J = 7.5 Hz, H-17α), 4.91 (br s, 1H, OH), 6.42 (d, 1H, 2.3 Hz, H-4), 6.50 (dd, 1H, J = 8.3, 2.3 Hz, H-2), 7.03 (d, 1H, J = 8.3 Hz, H-1), 8.98 (s, 1H, OH), 11.96 (br s, 1H, OH); HRMS (ES) calcd for $C_{19}H_{28}NO_4$ (M + NH_4^+) m/e 334.2018, found m/e 334.2007; HPLC system, H-1, 280 nm, t_R = 14 min, and system H-2, 280 nm, t_R = 9 min, >99% pure.

Methyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)-formate (9, E16-1,1). A solution of 28 mg (0.088 mmol) of carboxylic acid **8** and 9.66 μL (0.132 mmol) of $SOCl_2$ in 2 mL of MeOH was stirred and heated at 40 °C for 2.25 h in a 5 mL flask equipped with a reflux condenser. The reaction mixture was poured into saturated aqueous $NaHCO_3$ (50 mL) and extracted with CH_2Cl_2 (3×, 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving a brown oil. Purification of the residue by flash chromatography on a 2 × 16 cm column of silica gel using hexanes/EtOAc (1:1) as eluent gave 23 mg (79%) of **9** as a white solid. Purification of this material by HPLC in system H-3, 280 nm, gave 17 mg of **9** for bioassay. Data for **9**: TLC, T-6, R_f 0.325; ^{1}H NMR (500 MHz, $CDCl_3$, D_2O) δ 0.84 (s, 3H, H-18), 3.75 (s, 3H, OCH_3), 3.89 (d, 1H, J = 7.7 Hz, H-17α), 6.57 (d, 1H, J = 2.8 Hz, H-4), 6.63 (dd, 1H, J = 8.3, 2.8 Hz, H-2), 7.15 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES) calcd for $C_{20}H_{30}NO_4$ (M + NH_4^+) m/e 348.2175, found m/e 348.2191; HPLC system, H-3, 280 nm, t_R = 14 min, and system H-4, 280 nm, t_R = 8 min, >99% pure.

Ethyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)formate (10, E16-1,2). Compound **10** was prepared by esterification of acid **8 (**14 mg, 0.043 mmol) with EtOH as described for the preparation of **9**. Purification of the residue by flash chromatography on a 1 × 15 cm column of silica gel using hexanes/EtOAc (1:1) as eluent gave 14 mg (94%) of **10** as a white solid. Purification of this material by HPLC (H-3, 280 nm, t_R = 11 min) gave 11 mg of **10** for bioassay. Data for **10**: TLC, T-6, R_f 0.46; ^{1}H NMR (500 MHz, $CDCl_3$, D_2O) δ 0.84(s, 3H, H-18), 1.31 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 3.88 (d, 1H, J = 8.0 Hz, H-17α), 4.21 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 6.57 (d, 1H, J = 2.8 Hz, H-4), 6.63 (dd, 1H, J = 8.4, 2.8 Hz, H-2), 7.15

(d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{21}H_{32}NO_4$ (M + NH_4^+) *m/e* 362.2331, found *m/e* 362.2331. Anal. ($C_{21}H_{28}O_4$) C, H.

Propyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)-formate (11, E16-1,3). Compound **11** was prepared by esterification of acid **8** (66 mg, 0.209 mmol) with *n*-propanol as described for the preparation of **9**. Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 46 mg (62%) of **11** as a white solid. Purification of 22 mg of this material by HPLC (H-5) gave 20 mg of **11** for bioassay. Data for **11**: TLC, T-2, R_f = 0.41; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-18), 0.98 (t, 3H, J = 7.6 Hz, CH_3), 3.88 (d, 1H, J = 7.4 Hz, H-17α), 4.11 (m, 2H, OCH_2), 6.57 (d, 1H, J = 2.5 Hz, H-4), 6.63 (dd, 1H, J = 8.3, 2.5 Hz, H-2), 7.16 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES) calcd for $C_{22}H_{34}NO_4$ (M + NH_4^+) *m/e* 376.3488, found *m/e* 376.2493; HPLC system, H-3, 280 nm, t_R = 12 min, and system H-5, 280 nm, t_R = 9.5 min, >99% pure.

Isopropyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)formate (12, E16-1,3i). A solution of 44 mg (0.14 mmol) of acid **8** and 10 mg (0.053 mmol) of pTsOH in 2-propanol (20 mL) was stirred and heated at 85 °C for 18 h. A Dean−Stark trap filled with 4 Å sieves was added and heating was continued for 18 h. the reaction mixture was allowed to cool to room temperature, poured into saturated aqueous $NaHCO_3$ (20 mL), and extracted with CH_2Cl_2 (3×, 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 21 mg (43%) of **12** as a white solid. Purification of this material by HPLC in system H-3, 280 nm, followed by crystallization from acetone/petroleum ether, gave 8 mg of **12** for bioassay. Data for **12**: TLC, T-6, R_f 0.55; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.84 (s, 3H, H-18), 1.28 (d, 3H, J = 6.5 Hz, CH_3), 1.282 (d, 3H, J = 6.5 Hz, CH_3), 3.85 (br d, 1H, J = 9.3 Hz, H-17α), 5.07 (sept, 1H, J = 6.5 Hz, −CH-), 6.57 (d, 1H, J = 2.7 Hz, H-4), 6.63 (dd, 1H, J = 8.3, 2.7 Hz, H-2), 7.15 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES) calcd for $C_{22}H_{34}NO_4$ (M + NH_4^+) *m/e* 376.2488, found *m/e* 376.2485; HPLC system, H-3, 280 nm, t_R = 11 min, and system H-6, 280 nm, t_R = 7 min, >99% pure.

***n*-Butyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)-formate (13, E16-1,4).** Compound **13** was prepared by esterification of acid **8** (59 mg, 0.19 mmol) with butanol as described for the preparation of **9**. Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel gave 50 mg (73%) of **13** as a white solid. Data for **13**: TLC, T-2, R_f 0.30; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-18), 0.96 (t, 3H, J = 7.4 Hz, $-CH_3$), 3.87 (d, 1H, J = 8.0 Hz, H-17α), 4.15 (m, 2H, OCH_2), 6.57 (d, 1H, J = 2.7 Hz, H-4), 6.63 (dd, 1H, J = 8.4, 2.7 Hz, H-2), 7.15 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{23}H_{36}NO_4$ (M + NH_4^+) *m/e* 390.2644, found *m/e* 390.2647. HPLC system H-3, 280 nm, t_R = 11 min, and system H-6, 280 nm, t_R = 9 min, >99% pure.

2,2-Dimethylpropyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)formate (14, E16-1,5neo). Compound **14** was prepared by esterification of acid **8** (85 mg, 0.27 mmol) with 2 mL of neopentyl alcohol in 10 mL of benzene as described for the preparation of **12**. Purification of the residue by flash chromatography on a 2 × 16 cm column of silica gel using hexanes/EtOAc (3:1) gave 92 mg (89%) of **14** as a white solid. Data for **14**: TLC, T-5, R_f 0.77; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (s, 3H, H-18), 0.97 (s, 9H, CH_3), 3.83 & 3.87 (AB quartet, 2H, J = 10.5 Hz, $-CH_2-$), 3.90 (d, 1H, J = 8.1 Hz, H-17α), 6.57 (d, 1H, J = 2.6 Hz, H-4), 6.63 (dd, 1H, J = 8.5, 2.6 Hz, H-2), 7.15 (d, 1H, J = 8.5 Hz, H-1); HRMS (ES) calcd for $C_{24}H_{38}NO_4$ (M + NH_4^+) *m/e* 404.2801, found *m/e* 404.2809. Anal. ($C_{24}H_{34}O_4$) C, H.

Vinyl (3,17β-Dihydroxyestran-1,3,5(10)-trien-16α-yl)-formate (15, E16-1,2 vin). A solution of 24 mg (0.077 mmol) of acid **8** in 2 mL of vinyl propionate was stirred as 20 μL of a 0.1 M solution of $PdCl_2$–LiCl in vinyl propionate was added. This solution was prepared by combining 17 mg (0.1 mmol) of $PdCl_2$ and 4.2 mg (0.1 mmol) of LiCl in 1 mL of MeOH with heating to dissolve, evaporation of the solvent, and resuspension in 1 mL of vinyl propionate. The reaction mixture was stirred at 92 °C for 4 h, poured into CH_2Cl_2 (50 mL), and washed with saturated aqueous $NaHCO_3$ (20 mL) and H_2O (20 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using hexanes/EtOAc (3:1) gave 20 mg (76%) of **15** as a white solid. Purification of 7 mg of this material in HPLC system H-7, followed by crystallization from Et_2O/hexane, gave 5 mg of **15** for bioassay. Data for **15**: TLC, T-6, R_f 0.57; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (s, 3H, H-18), 3.95 (d, 1H, J = 7.7 Hz, H-17α), 4.63 (dd, 1H, J = 6.3, 1.6 Hz, vinyl-H), 4.94 (dd, 1H, J = 14.0, 1.6 Hz, vinyl-H), 6.57 (d, 1H, J = 2.8 Hz, H-4), 6.64 (dd, 1H, J = 8.3, 2.8 Hz, H-2), 7.16 (d, 1H, J = 8.3 Hz, H-1), 7.33 (dd, 1H, J = 14.0, 6.3 Hz, vinyl-H); HRMS (ES) calcd for $C_{21}H_{30}NO_4$ (M + NH_4^+) *m/e* 360.2175, found *m/e* 360.2171; HPLC system H-3, 280 nm, t_R = 12.21 min, and system H-8, 280 nm, t_R = 11.5 min, >99% pure.

2′-Fluoroethyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)formate (16, E16-1,2 F1). Compound **16** was prepared by esterification of acid **8** (16 mg, 0.05 mmol) with 1.5 mL of fluoroethanol in 1.5 mL of toluene as described for the preparation of **12**. Purification of the residue by flash chromatography on a 2 × 16 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 11 mg (59%) of **16** as a white solid. Purification of this material by HPLC in system H-3, followed by crystallization from Et_2O/petroleum ether, gave 8 mg of **16** for bioassay. Data for **16**: TLC, T-6, R_f = 0.375; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-18), 3.91 (d, 1H, J = 8.2 Hz, H-17α), 4.36−4.44 (m 2H, CH_2CH_2F), 4.64 (dt, 2H, J = 47.4, 4.2 Hz, CH_2CH_2F), 6.57 (d, 1H, J = 2.8 Hz, H-4), 6.63 (dd, 1H, J = 8.3, 2.8 Hz, H-2), 7.16 (d, 1H, J = 8.3, H-1); HRMS (ES) calcd for $C_{21}H_{31}FNO_4$ (M + NH_4^+) *m/e* 380.2237, found *m/e* 380.2248; HPLC, system H-3, 280 nm, t_R = 13.0 min, and system H-8, 280 nm, t_R = 8.5 min, >99% pure.

2′,2′-Difluoroethyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)formate (17, E16-1,2 F2). Compound **17** was prepared by esterification of acid **8** (25 mg, 0.08 mmol) with 2,2-difluoroethanol as described for the preparation of **16**. Purification of this residue by flash chromatography on a 2 × 17 cm column of silica gel using hexanes/EtOAc (3:1) followed by hexanes/EtOAc (2:1) as eluent gave 29 mg of **17** as a yellow oil. Purification of this material by HPLC with system H-3 gave 23 mg (74%) of **17** as a clear colorless oil. Further HPLC purification of 6 mg of this material with system H-7, followed by crystallization from Et_2O/hexanes, gave 5 mg of **17** for bioassay. Data for **17**: TLC, T-6, R_f 0.5; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-18), 3.91 (d, 1H, J = 8.3 Hz, H-17α), 4.32−4.39 (m, 2H, CH_2CHF_2), 5.99 (tt, 1H, J = 55.2, 4.0 Hz, CH_2CHF_2), 6.57 (d, 1H, J = 2.8 Hz, H-4), 6.64 (dd, 1H, J = 8.4, 2.8 Hz, H-2), 7.16 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{21}H_{30}F_2NO_4$ (M + NH_4^+) *m/e* 398.2143, found *m/e* 398.2148; HPLC system H-3, 280 nm, t_R = 12.8 min, and system H-8, 280 nm, t_R = 10.5 min, >99% pure.

2′,2′,2′-Trifluoroethyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)formate (18, E16-1,2 F3). Compound **18** was prepared by esterification of acid **8** (25 mg, 0.079 mmol) with 2,2,2-trifluoroethanol as described for the preparation of **16**. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using hexanes/EtOAc (4:1) as eluent gave 19 mg of **18**. HPLC purification of this material with system H-3 gave 17 mg (54%) of **18** as a slightly yellow oil. Further HPLC purification of 3 mg of this material with system H-8, followed by crystallization from Et_2O/hexanes, gave 2 mg of **18** as an amorphous solid. Data for **18**: TLC, T-6, R_f 0.58; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (s, 3H, H-18), 3.92 (d, 1H, J = 8.3 Hz, H-17α), 4.52−4.58 (m, 2H, CH_2CF_3), 6.57 (d, 1H, J = 3.0 Hz, H-4), 6.64 (dd, 1H, J = 8.4, 3.0 Hz, H-2), 7.15 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{21}H_{29}F_3NO_4$ (M + NH_4^+) *m/e* 416.2049, found *m/e* 416.2051; HPLC system H-3, 280 nm, t_R = 11.6 min, and system H-8, 280 nm, t_R = 13.5 min, >99% pure.

16α-Hydroxymethylestra-1,3,5(10)-trien-3,17β-diol (19). A solution of 5 mg (0.015 mmol) of ethyl ester **10** and 5 mg (0.13 mmol) of $LiAlH_4$ in 1 mL of anhydrous THF was stirred at 0 °C for 1 h under N_2. The reaction was quenched with 1 mL of EtOAc, poured into saturated aqueous Na−K tartarate (5 mL), and extracted with EtOAc (3×, 5 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo giving 3.5 mg (75%) of **19**[20] as a white solid: TLC, T-5, R_f 0.51; ^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 0.68 (s, 3H, H-18), 3.15 (d, 1H, J = 8.1 Hz, H-17α), 3.28 (dd, 1H, J = 10.3, 7.1 Hz, CH_2OH), 3.51 (dd, 1H, J = 10.3, 4.1 Hz, CH_2OH), 6.42 (d, 1H, J = 2.6 Hz, H-2), 6.49 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 7.02 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{19}H_{26}O_3Na$ (M + Na^+) *m/e* 325.1780, found *m/e* 325.1782.

Ethyl (3-Benzyloxy-17-oxoestra-1,3,5(10)-trien-16α-yl)-acetate (20). A solution of 2.196 g (6.09 mmol) of estrone benzyl ether in 20 mL of anhydrous THF was added in one portion at 0 °C to a solution of 5.79 mmol of LDA (2.89 mL of a 2 M solution in heptane, THF, ethylbenzene) in THF (10 mL). The resulting mixture was cooled to −45 °C and a solution of 3.05 g (2.0 mL, 18.3 mmol) of ethyl bromoacetate in 8 mL of THF was added dropwise over 5 min. The reaction was stirred at <−20 °C for 7.5 h under N_2, poured into H_2O (500 mL), and extracted with CH_2Cl_2 (2×, 300 mL). Combined organic extracts were washed with 10% aqueous sodium metabisulfite, dried over Na_2SO_4, and concentrated in vacuo giving a yellow oil. Purification of the residue twice by flash chromatography on a 5 × 17 cm column of silica gel using CH_2Cl_2 as eluent gave 944 mg (35%) of **20** and 954 mg (43%) of recovered starting material. Data for **20**: TLC, T-7, R_f = 0.075; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.00 (s, 3H, H-18), 1.29 (t, 3H, J = 7.4 Hz, CH_3), 4.18 (q, 2H, J = 7.4 Hz, OCH_2), 5.05 (s, 2H, benzylic), 6.74 (d, 1H, J = 2.6 Hz, H-4), 6.80 (dd, 1H, J = 8.6, 2.6 Hz, H-2), 7.21 (d, 1H, J = 8.6 Hz, H-1), 7.31−7.45 (m, 5H, Ar−H); HRMS (ES) Calcd for $C_{29}H_{34}O_4Na$ (M + Na^+) *m/e* 469.2355, found *m/e* 469.2354.

Ethyl (3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16α-yl)acetate (21). A solution of 51 mg (0.11 mmol) of ketone **20** and 0.34 mmol of $Li(OtBu)_3AlH$ (340 μL of a 1 M solution in THF) in 2 mL of THF was stirred at −78 °C for 5.5 h and then at room temperature for 1 h. The reaction was poured into saturated aqueous NH_4Cl (50 mL) and extracted with CH_2Cl_2 (3×, 50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving a clear colorless oil. Purification of the residue by flash chromatography on a 2 × 16 cm column of silica gel using $CHCl_3$/EtOAc (5:0.15) gave 36 mg (71%) of **21** as a white solid. Data for **21**: TLC, T-6, R_f 0.63; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-18), 1.28 (t, 3H, J = 7.12 Hz, CH_3), 3.35 (d, 1H, J = 7.4 Hz, H-17α), 4.16 (q, 2H, J = 7.2 Hz, $-OCH_2-$), 6.72 (d, 1H, J = 2.5 Hz, H-4), 6.78 (dd, 1H, J = 8.8, 2.5 Hz, H-2), 7.21 (d, 1H, J = 8.8 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H); HRMS (ES) calcd for $C_{29}H_{36}O_4Na$ (M + Na^+) *m/e* 471.2511, found 471.2518.

Ethyl (3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl) acetate (22, E16-2,2). Compound **22** was prepared by hydrogenolysis of **21** (125 mg, 0.279 mmol) as described for the preparation of **8.** Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 78 mg (78%) of **22**. Further purification of 30 mg of this material by HPLC with system H-3 gave 20 mg of **22** for bioassay. Data for **22**: TLC, T-6, R_f 0.57; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (s, 3H, H-18), 1.28 (t, 3H, J = 7.1 Hz, CH_3), 3.38 (d, 1H, J = 7.3 Hz, H-17α), 4.16 (q, 2H, J = 7.1 Hz, OCH_2), 6.56 (d, 1H, J = 2.6 Hz, H-4), 6.63 (dd, 1H, J = 8.3 Hz, H-2), 7.16 (d, 1H, J = 8.3 Hz, H-1); HRMS (ES) calcd for $C_{22}H_{34}NO_4$ (M + NH_4^+) *m/e* 359.2222, found *m/e* 359.2232; HPLC system H-3, 280 nm, t_R = 11.5 min, and system H-9, 280 nm, t_R = 12 min, >99% pure.

3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-ylacetic Acid (23, E16-2,0). Compound **23** was prepared by saponification of ethyl ester **22** (43 mg, 0.12 mmol) as described for **7**, giving 40 mg (100%). Further purification of 6 mg of this material by HPLC with system H-1, followed by acid/base extraction, gave 5 mg of **23** for bioassay. Data for **23**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.70 (s, 3H, H-18), 3.12 (d, 1H, J = 7.8 Hz, H-17α), 6.42 (d, 1H, J = 2.5 Hz, H-4), 6.49 (dd, 1H, J = 8.7, 2.5 Hz, H-2), 7.03 (d, 1H, J = 8.7 Hz, H-1), 8.96 (s, 1H, OH); HRMS (ES) calcd for $C_{20}H_{30}NO_4$ (M + NH_4^+) *m/e* 348.2175, found *m/e* 348.2188; HPLC system H-1, 280 nm, t_R = 10 min, and system H-10, 280 nm, t_R = 12 min, > 99% pure.

3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16α-yl-acetic Acid (24). Compound **24** was prepared by saponification of ester **21** (107 mg, 0.238 mmol) as described for **7**, giving 60 mg (60%) of **24** as a white solid. This material was used without further purification in the next step. TLC, T-5, R_f 0.36.

Methyl (3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16α-yl)acetate (25). Compound **25** was prepared by esterification of crude **24** (60 mg) with MeOH as described for the preparation of **9**. Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel using $CHCl_3$/EtOAc (5:0.15) gave 57 mg (92%) of **25** as a white solid. Data for **25**: TLC, T-5, R_f 0.8; ^{1}H NMR (500 MHz, $CDCl_3$ + D_2O) δ 0.85 (s, 3H, H-18), 3.37 (d, 1H, J = 7.3 Hz, H-17α), 3.71 (s, 3H, OCH_3), 5.04 (s, 2H, benzylic), 6.72 (d, 1H, J = 2.8 Hz, H-4), 6.79 (dd, 1H, J = 8.5, 2.8 Hz, H-2), 7.21 (d, J = 8.5 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H); HRMS (ES) calcd for $C_{28}H_{34}O_4Na$ (M + Na^+) *m/e* 457.2355, found 457.2342.

Methyl 3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl-acetate (26, E16-2,1). Compound **26** was prepared by hydrogenolysis of **25** (55 mg, 0.126 mmol) as described for the preparation of **8**. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica using hexanes/EtOAc (2:1) as eluent gave 33 mg of **26**. HPLC purification in six portions with system H-3 gave 28 mg (64%) of **26** as a white solid. Data for **26**: TLC, T-6, R_f 0.456; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (s, 3H, H-18), 3.38 (d, 1H, J = 7.3 Hz, H-17α), 3.71 (s, 3H, OCH_3), 6.56 (d, 1H, J = 2.5 Hz, H-4), 6.63 (dd, 1H, J = 8.4, 2.5 Hz, H-2), 7.16 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{21}H_{32}NO_4$ (M + NH_4^+) *m/e* 345.2066, found *m/e* 345.2082; HPLC system H-3, 280 nm, t_R = 11 min, and system H-11, 280 nm, t_R = 18 min, >99% pure.

3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16β-yl-acetaldehyde (27). A solution of 10 mg (0.022 mmol) of **21** in anhydrous toluene (200 μL) was stirred at −60 °C as 0.0669 mmol of Dibal (44 μL of a 1.5 M solution in toluene) was added. The reaction was stirred at −60 °C for 2 h, quenched with MeOH (2 mL), poured into H_2O (5 mL), and extracted with EtOAc (3×, 5 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using hexanes/EtOAc (1:1) as eluent gave 5 mg (59%) of **27** as a clear colorless oil. Data for **27**: TLC, T-6, R_f 0.3; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.86 (s, 3H, H-18), 3.33 (d, 1H, J = 7.3 Hz, H-17α), 5.04 (s, 3H, benzylic), 6.72 (d, 1H, J = 2.5 Hz, H-4), 6.79 (dd, 1H, J = 8.8, 2.5 Hz, H-2), 7.21 (d, 1H, J = 8.8 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H), 9.83 (s, 1H, CHO); HRMS (ES) calcd for $C_{27}H_{36}NO_3$ (M + NH_4^+) *m/e* 422.2695, found *m/e* 422.2681.

16α-Allyl-3-benzyloxyestra-1,3,5(10)-trien-17β-ol (28). A solution of 5 mg (0.013 mmol) of **27** in anhydrous toluene (100 μL), pyridine (1 μL), and THF (33 μL) was stirred at −78 °C as 0.0156 mmol of Tebbe reagent (31 μL of a 0.5 M solution in toluene) was added by syringe. The reaction was stirred at −78 °C for 2 h, at 40 °C for 2 h, and then at 0 °C for 1 h. The reaction was quenched with 15% NaOH (25 μL), allowed to stir for 0.5 h, warmed to room temperature, and passed through a 1 in. plug of Celite. The filter was washed with EtOAc and the filtrate was concentrated in vacuo. Purification of the residue on a 1 × 17 cm column of silica gel using hexanes/EtOAc (2:1) followed by hexanes/EtOAc (4:1) gave 1.3 mg (25%) of **28**[26] as a clear colorless oil and 1.8 mg (34%) of recovered **27**. Data for 28: TLC, T-6, R_f 0.76; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.83 (s, 3H, H-18), 2.86 (m, 2H, H-6), 3.33 (d, 1H, J = 7.4 Hz, H-17α), 5.03−5.12 (m, 2H, $=CH_2$), 5.04 (s, 2H, benzylic), 5.85−5.93 (m, 1H, $-CH=CH_2$), 6.72 d, 1H, J = 2.6 Hz, H-4), 6.79 (dd, 1H, J = 8.7, 2.6 Hz, H-2), 7.21 (d, 1H, J = 8.7 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H).

16α-Allyl-3-benzyloxyestra-1,3,5(10)-trien-17β-yl Acetate (29). A solution of 1.874 g (4.65 mmol) of **28**[26] and 5.2 mL (55.1 mL) of acetic anhydride in 10.4 mL of anhydrous pyridine was stirred at room temperature for 16.5 h. The reaction was poured into H_2O (300 mL) and extracted with CH_2Cl_2 (3×, 200 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 3 × 20 cm column of silica gel using CH_2Cl_2 as eluent gave 1.73 g (84%) of **29** as a white solid. Data for **29**: TLC, T-7, R_f 0.48; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.84 (s, 3H, H-18), 2.07 (s, 3H, Ac), 2.84 (m, 2H, H-6), 4.64 (d, 1H, J = 7.3 Hz, H-17α), 4.99−5.07 (m, 2H, =CH_2), 5.04 (s, 3H, benzylic), 5.78 (m, 1H, −CH=), 6.72 (d, 1H, J = 2.0 Hz, H-4), 6.78 (dd, 1H, J = 8.6, 2.0 Hz, H-2), 7.19 (d, 1H, J = 8.6 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H).

3-Benzyloxy-16α-(3′-hydroxypropyl)estra-1,3,5(10)-trien-17β-yl Acetate (30). A solution of 629 mg (1.41 mmol) of **29** in anhydrous diglyme (21 mL) was stirred at 0 °C as 1.49 mmol of borane−THF (1.49 mL of a 1 M solution in THF) was added. The reaction was stirred at 0 °C for 0.5 h, allowed to warm to room temperature, and stirred for 2 h. To this was added 660 mg (5.96 mmol) of trimethylamine oxide, and the reaction was stirred and heated at 150 °C for 2 h, cooled to room temperature, poured into H_2O (150 mL), and extracted with CH_2Cl_2 (3×, 100 mL). The combined organic extracts were washed with 10% sodium metabisulfite (70 mL), H_2O (70 mL), dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by flash chromatography on a 3 × 22 cm column of silica gel using hexanes/EtOAc (1.5:1) as eluent gave 530 mg (81%) of **30** as a white solid. Data for **30**: TLC, T-6, R_f 0.34; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.83 (s, 3H, H-18), 2.09 (s, 3H, OAc), 2.85 (m, 2H, H-6), 3.85 (t, 2H, J = 5.7 Hz, −CH_2O−), 4.63 (d, 1H, J = 7.8 Hz, H-17α), 5.04 (s, 2H, benzylic), 6.72 (d, 1H, J = 2.6 Hz, H-4), 6.78 (dd, 1H, J = 8.2, 2.6 Hz, H-2), 7.19 (d, 1H, J = 2.6 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H); HRMS (ES) calcd for $C_{30}H_{38}O_4Na$ (M + Na^+) *m/e* 485.2668, found *m/e* 485.2679.

3-(3-Benzyloxy-17β-acetoxyestra-1,3,5(10)-trien-16α-yl)-propanoic Acid (31). Compound **31** was prepared by CrO_3 oxidation of **30** (530 mg, 1.14 mmol) as described for **6**, giving 474 mg of **31** as a white foam: TLC, T-5, R_f 0.64. This material was used without further purification in the next step. HRMS (ES) calcd for $C_{30}H_{36}O_5Na$ (M + Na^+) *m/e* 499.2460, found *m/e* 499.2449.

3-(3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16α-yl)propanoic Acid (32). Compound **32** was prepared by saponification of crude **31** (474 mg) as described for **7**, giving 436 mg of **32** as a white foam: TLC, T-5, R_f 0.54. This material was used without further purification in the next step.

Methyl 3-(3-Benzyloxy-17β-hydroxyestra-1,3,5(10)-trien-16α-yl)propanoate (33). Compound **33** was prepared by esterification of crude **32** (436 mg) with MeOH as described for the preparation of **9**. Purification of the residue by flash chromatography on a 3 × 21 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 268 mg (52%, three steps) of **33** as a white solid. Data for **33**: TLC, T-5, R_f 0.76; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81 (s, 3H, H-18), 2.84 (m, 2H, H-6), 3.31 (d, 1H, J = 7.3 Hz, H-17α), 3.69 (s, 3H, OCH_3), 5.04 (s, 2H, benzylic), 6.72 (d, 1H, J = 2.8 Hz, H-4), 6.79 (dd, 1H, J = 8.6, 2.8 Hz, H-2), 7.21 (d, 1H, J = 8.6 Hz, H-1), 7.31−7.44 (m, 5H, Ar−H); HRMS (ES) calcd for $C_{29}H_{36}O_4Na$ (M + Na^+) *m/e* 471.2511, found *m/e* 471.2513.

Methyl 3-(3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)-propanoate (34, E16-3,1). Compound **34** was prepared by hydrogenolysis of **33** (37 mg, 0.083 mmol) as described for the preparation of **8**. Purification of the residue by flash chromatography on a 1 × 20 cm column of silica gel using hexanes/EtOAc (2:1) as eluent gave 25 mg (84%) of **34**. Purification of 20 mg of this material by HPLC with system H-3 gave 18 mg of **33** for bioassay. Data for **33**: TLC, T-6, R_f 0.45; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81 (s, 3H, H-18), 3.31 (d, 1H, J = 7.4 Hz, H-17α), 3.69 (s, 3H, OCH_3), 6.57 (d, 1H, J = 2.6 Hz, H-4), 6.63 (dd, 1H, J = 8.4, 2.6 Hz, H-2), 7.16 (d, 1H, J = 8.4 Hz, H-1); HRMS (ES) calcd for $C_{22}H_{34}NO_4$ (M + NH_4^+) *m/e* 359.2222, found *m/e* 359.2231; HPLC system H-3, 280 nm, t_R = 14 min, and system H-12, 280 nm), t_R = 14.5 min, >99% pure.

3-(3,17β-Dihydroxyestra-1,3,5(10)-trien-16α-yl)propanioc Acid (35, E16-3,0). Compound **35** was prepared by saponification of **34** (35 mg, 0.097 mmol) as described for **7**. Purification of the residue by HPLC in 10 portions with system H-13 gave 20 mg (59%) of **35** as a white solid. Data for **35**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.68 (s, 3H, H-18), 3.08 (d, 1H, J = 7.2 Hz, H-17α), 6.42 (d, 1H, J = 2.1 Hz, H-4), 6.49 (dd, 1H, J = 8.2, 2.1 Hz, H-2), 7.03 (d, 1H, J = 8.2 Hz, H-1), 8.96 (s, 1H, OH); HRMS (ES) calcd for $C_{21}H_{32}NO_4$ (M + NH_4^+) *m/e* 362.2332, found *m/e* 362.2344; HPLC system H-14, 280 nm, t_R = 12 min, and system H-13, 280 nm, t_R = 23 min, >99% pure.

Competitive Binding to the Estrogen Receptor ERα and ERβ. Binding affinities relative to E_2 were performed in incubations with the ER (ERα[39]) in rat uterine cytosol. Female Sprague−Dawley rats were castrated and sacrificed 24 h later. The uterus was removed, homogenized in ice-cold TEGDMo buffer (10 mM Tris, 1.5 mM Na_2-EDTA, 10% (v/v) glycerol, 1.0 mM dithiothreitol, 25 mM sodium molybdate, pH 7.4 at 4 °C), and centrifuged at 105000*g* for 45 min at 4 °C. The supernatant (cytosol) was frozen on dry ice and stored at −80 °C until assay. For assay, the cytosol was defrosted, diluted, and incubated with 1 nm [^{3}H]E_2 in the presence and absence of nonradioactive E_2, estrone (E_1), or the E_2-carboxy analogues over a range of concentrations from 10^{-12} to 10^{-6} M. Incubations were carried out on ice overnight, and bound radioactivity was separated from free by adsorption with dextran-coated charcoal and quantified by counting.[27] Relative binding affinity (RBA) was determined by analysis of the displacement curves by the curve-fitting program Prism. The results shown in Table 1 are from at least three separate experiments performed in duplicate. A subset of E_2-16α-alkyl esters was also compared by Drs. Paul Shughrue and Heather Harris for binding to the LBD of human ERα (M_{250}–V_{595})[40] and human ERβ (M_{214}–Q_{530}).[41] The assay was performed in competition with [^{3}H]E_2 in lysates of *Escherichia coli* in which the LBDs are expressed as described, with the exception that the incubation was performed overnight at 0−2 °C.[28] The results, as the RBAs compared to that of E_2 and the ratio of the RBAs of ERα/ERβ, are shown in Table 2.

Estrogenic Potency in Ishikawa Cells. The estrogenic potency of the E_2 analogues was determined in a estrogen bioassay, the induction of AlkP in human endometrial adenocarcinoma cells (Ishikawa) grown in 96-well microtiter plates as we have previously described.[29] In short, the cells are grown in phenol red free medium with estrogen depleted (charcoal stripped) bovine serum in the presence or absence of varying amounts of the steroids, across a dose range of at least 6 orders of magnitude. E_2 and E_1 were included for comparison. After 3 days, the cells are washed, frozen, thawed, and then incubated with 5 mM *p*-nitrophenyl phosphate, a chromogenic substrate for the AlkP enzyme, at pH 9.8. To ensure linear enzymatic analysis, the plates are monitored kinetically for the production of *p*-nitrophenol at 405 nm. The relative stimulatory activity (RSA) represents the ratio of 1/EC_{50} of the steroid analogue to that of E_2 × 100, using the curve fitting program Prism to determine the EC_{50}. Each compound was analyzed in at least three separate experiments performed in duplicate.

In Vivo Estrogen Bioassays: Uterine Weight. Systemic estrogenic potency was determined by a uterotrophic assay in immature rats as described.[34] Female Sprague−Dawley rats, 22 days old, were injected subcutaneously daily for 3 days with an injection volume of 0.1 mL of the 16α-alkyl esters, for a total dose of between 1 and 300 μg, or of E_2, for a total dose of between 0.001 and 0.1 μg, in sesame oil. Control animals received sesame oil. On the fourth day, the animals were killed, and the uteri were removed, dissected, blotted, and weighed. Each compound was assayed in three separate experiments with n = 5. A typical experiment is shown in Figure 2.

In Vivo Estrogen Bioassays: Vaginal Reductases. The estrogenic action of locally applied E_2-16α-alkyl esters on the vagina was determined by measuring the induction of vaginal reductases.[35] Female CD-1 mice were ovariectomized and 1 week later were instilled with the E_2-16α-alkyl esters or E_2 in 10 μL of 25% propylene glycol in saline. In some experiments, as indicated, the method was modified by dissolving and injecting the estrogens in 10 μL of sesame oil. The next morning 0.5 mg of 2,3,5-triphenyltetrazolium chloride in 20 μL of saline was instilled in the vagina. Thirty minutes later the animals were killed, and the vaginas removed, washed thoroughly with saline, and then blotted on filter paper. Each vagina was placed in a 12 × 75 mm test tube and extracted for 1 h with ethanol/tetrachloroethylene (3:1). Afterward, the solvent was removed and the formazan product in the organic extract was quantified at 500 nm. Each compound was assayed on at least three separate occasions with at least five replicates each time. A typical experiment is shown in Figure 3.

Esterase. Esterase activity was measured in rat hepatic microsomes essentially using the conditions described.[42] Liver obtained from Sprague−Dawley rats was washed with phosphate-buffered saline, homogenized in 3 volumes of cold 0.25 M sucrose, and centrifuged at 700*g* for 10 min and then at 10000*g* for 20 min. The resulting supernatant was centrifuged at 105000*g* for 60 min. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4) and washed by centrifugation at 105000*g* for 60 min. The washed pellet was suspended in 0.1 M Tris-HCl (pH 8.0) at a concentration of ∼13 mg of protein/mL and frozen at −80 °C. For assay, the pellets were thawed and diluted with the same buffer. The incubation mixture consisted of the microsomal enzyme preparation, 0.28 mg of protein/mL, 50 μM E_2-16α-alkyl esters, added in 10 μL of ethanol, all in a final volume of 1 mL of pH 8.0 Tris buffer. Since the rates of reaction are widely different for the various esters, the incubation times were varied accordingly to obtain linear kinetics. At several appropriate time points, 100 μL aliquots were withdrawn and the reaction was quenched with 33 μL of CH_3CN, followed by 33 μL of a solution of THF containing 1 μg of the internal standard, 6-ketoestradiol. The quenched aliquot was centrifuged for several minutes on a benchtop centrifuge, and 80 μL of the supernatant was analyzed for the esterase hydrolysis product [the corresponding E_2-16α-carboxylic acid: E16-1,0 (**8**), E16-2,0 (**23**), E16-3,0 (**35**)] by reversed-phase HPLC with system H-10 for $E_2$16-1 and $E_2$16-2 esters and with system H-15 for $E_2$16-3 esters. The E_2-16α-carboxyl products (t_R for E16-1,0 (**8**) = 7 min and for E16-2,0 (**23**) = 9 min, E16-3,0 (**35**) = 6.5 min) and the internal standard, 6-ketoestradiol (t_R 7.5 min in system H-10, and 5 min in system H-15), were quantified at 280 nm on the HPLC UV detector. The UV absorbance was converted to moles of product by comparison to standard curves and corrected for recovery of the internal standard, 6-ketoestradiol. The velocity of the reaction for each ester, in nmol product/min/mL, was then normalized to the ester, E16-1,2 (**10**) and is shown in Table 1. as relative hydrolytic activity (RHA). The enzymatic velocity for the hydrolysis of E16-1,2 (**10**) was 0.9 ± 0.2 (SD) nmol product/min/mL over the various experiments. Since all of the esters could not be tested simultaneously, in each case we compared the rate of hydrolysis of the test compound to that of E16-1,2 (**10**) run concurrently. All compounds were tested in triplicate in three separate experiments.

The various E_2-16α-alkyl esters, including the reactive E16-1,2vin (**15**), were stable under the conditions used in the esterase assay. Each of the substrates was incubated with heat-denatured (1 h at 80 °C) enzyme at 37 °C for periods that exceeded the incubation times of the enzyme assay. Only insignificant amounts of carboxyl products were formed from any of these esters during the incubations with denatured enzyme.

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