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B-Ring Unsaturated Estrogens: Biological Evaluation of 17α-Dihydroequilein and Novel B-Nor-6-thiaequilenins as Tissue Selective Estrogens

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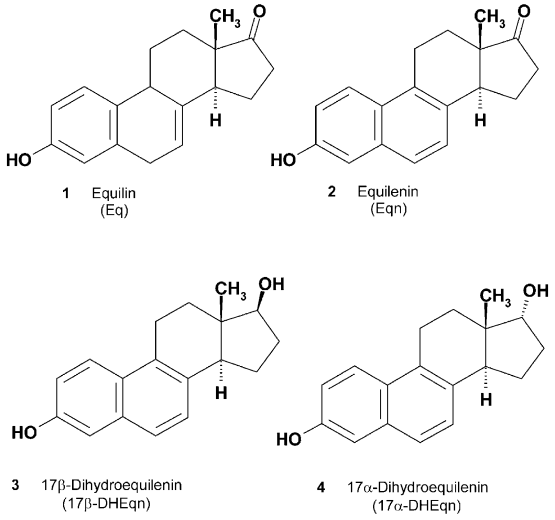
Abstract—The pharmacology and SAR of representative equine estogens is described. 17α-Dihydroequilenin was found to prevent bone loss after 5 weeks of oral administration to ovariectomized rats. The stereochemical significance of the D-ring and the C/D ring juncture was investigated with a series of benzothiophene-based equilenin analogues.

The clinical use of conjugated equine estrogens (CEEs) provides beneficial health outcomes for treating hot flashes and preventing osteoporosis in post-menopausal women. However, concerns regarding the risks[1] for women taking equine estrogens has prompted the search for more selective therapeutic agents particularly with respect to their uterine safety profile. Because CEEs are a complex mixture of estrogen-like steroids, we have evaluated individual representative compounds **1**–**4** of this mixture and herein report the tissue selectivity profile on bone, lipids, and uterus for one such component, 17α-dihydroequilenin (17α-DHEqn, **4**). We have also prepared a series of isosteric benzothiophene-based equilenin analogues in order to investigate their therapeutic potential as tissue selective estrogens.

CEEs contain sulfate esters of two structural classes of estrogen that include ring B saturated steroids such as 17β-estradiol as well as ring B unsaturated estrogens such as equilin (**1**, Eq), equilenin (**2**, Eqn), 17β-dihydroequilenin (**3**, 17β-DHEqn), and 17α-dihydroequilenin (**4**, 17α-DHEqn) among others. In 1991, Bhavnani and co-workers examined a number of ring B unsaturated steroids in their unconjugated form in order to determine their relative binding affinities for the estrogen receptor and their in vivo effects on uterine hypertrophy in ovary-intact rats.[2] All of the individual equine components caused an increase in uterine wet weight with the exception of **4** which lacked a significant effect at the highest dose tested. More recently, the sulfate ester conjugate of 17α-DHEqn has been shown to lower serum cholesterol,[3] increase hippocampal dendritic spine density in rats,[4] and improve arterial vasomotor function in macaques.[5] Neuroprotective effects for this compound have also been observed.[6] The cumulative tissue profile demonstrated in these studies, in which estrogen-like pharmacology is observed on the cardiovascular and central nervous system whereas



1 Equilin (Eq)

2 Equilenin (Eqn)

3 17β-Dihydroequilenin (17β-DHEqn)

4 17α-Dihydroequilenin (17α-DHEqn)

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estrogen-neutral effects are observed on the uterus, prompted us to explore the pharmacology of 17α-DHEqn on uterus, bone, and lipids in order to characterize its overall tissue selectivity profile.

Initially, representative equine estrogens (**1**–**4**) were evaluated in rodents in order to determine their effects on the uterus. While the pharmacology of CEEs on uterine tissue has been well-characterized in ovary-intact rodents,[2] we elected to use ovariectomized (OVX) rats as we have found this model to be a sensitive method for evaluating the agonist characteristics of estrogens and selective estrogen receptor modulators (SERMs) on bone, lipids, and uterus.[7] Previous studies

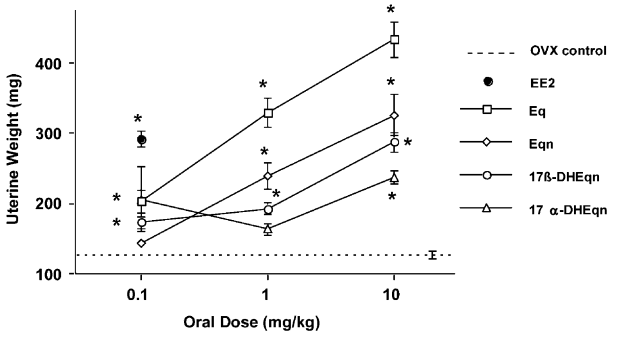


Figure 1. Uterine weight gain in OVX rats after oral exposure to various concentrations of equine estrogens. Points are mean (±SEM) uterine weight (n = 5–6 animals/group). * = $p < 0.05$ versus OVX by one way analysis of variance and Fisher's posthoc analysis.

Table 1. Effects on estrogen antagonism for 17α-DHEqn (**4**) and ICI 182780 expressed as the ability of test compound to block the stimulatory effects of EE2 in immature female rats

Compd	Dose (mpk)	% Inhibition[a]
ICI 182780	0.10	40.3±7.2*
	1.00	100±4.2*
	10.0	117±1.1*
4	0.10	3.4±8.2
	1.00	14.7±3.3
	10.0	−22.8±8.1*

* = $p < 0.05$ vs EE2 stimulated control. Statistical evaluations were made by one way analysis of variance (ANOVA).
[a]% Inhibition = [uterine weight$_{EE2}$ − uterine weight$_{test}$/uterine weight$_{EE2}$ − uterine weight$_{control}$] × 100. Negative signs indicate an increase relative to vehicle control.

with the sulfate ester conjugate of **4** in OVX rats have shown that this compound reduces total cholesterol without inducing uterine growth.[3] However, the pharmacology of unconjugated equine estrogens has not been reported in OVX rats to our knowledge. As shown in Figure 1, Eq (**1**) elicits the most pronounced agonist response at 10 mg/kg (mpk), a 263% increase in uterine wet weight relative to OVX control, followed by Eqn (2, 168%), 17β-DHEqn (**3**, 150%), and 17α-DHEqn (**4**, 100%). Thus, **4** is the least uterotrophic of the compounds that we examined based on rank order of uterine weight gain at the high dose (10 mpk). The trend we observe for **1**–**4** is consistent with data previously reported in ovary-intact rats although in these studies 17α-DHEqn did not induce uterine weight gain after daily subcutaneous administration of 2 μg/kg for 3 days.[2]

In order to assess whether **4** antagonizes the effects of estrogen in the uterus it was evaluated in ovary-intact rats that were co-administered with test compound and ethynyl estradiol (EE2, 0.1 mpk) for three days. As shown in Table 1, the known pure antagonist ICI 182,780[8] effectively blocks the stimulatory response of EE2 in a dose dependent manner. 17α-DHEqn (**4**) shows no inhibitory activity and, in fact, causes a slight but statistically significant increase in uterine weight at the 10 mpk dose. Taken together, this data demonstrates that **4** increases uterine weight in OVX rats and does not antagonize the effects of estogen-induced uterine weight gain in immature rats.

We subsequently examined the effects of longer term administration of **4** on bone, uterus, and lipids in the OVX rat.[9] Oral treatment with 17α-DHEqn for five weeks prevents bone loss in the proximal metaphysis of excised femurs in a dose-dependent manner as shown in Table 2. In addition, **4** efficaciously lowers total serum cholesterol with an ED50 of <0.1 mpk. Uterine wet weight is significantly increased by 100% and 142% relative to OVX controls at doses of 1 and 10 mpk, respectively. Thus, the efficacy demonstrated by 17α-DHEqn as an estrogen agonist in preventing bone loss and lowering cholesterol is observed only at doses at which agonist effects are also observed in the uterus as determined by induction of uterine weight gain.

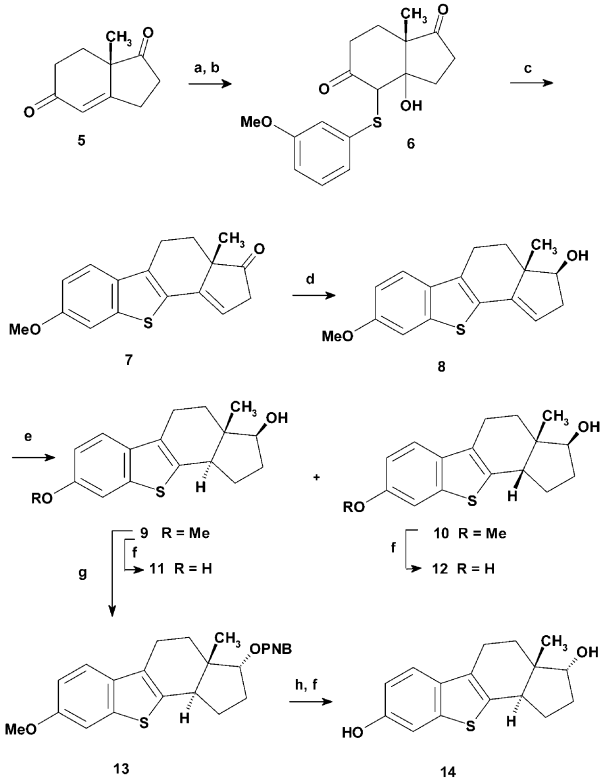
In order to identify compounds with an improved uterine profile relative to **4** we chose to explore structure–activity studies on the annulated cyclopentane ring of

Table 2. Effects of 17α-DHEqn (**4**) on uterine weight, serum cholesterol, and bone in 6-month old OVX rats after 5 weeks of oral dosing

Compd	Dose (mpk)	Uterine weight % increase versus OVX	Serum cholesterol % decrease versus OVX	Bone (femur) % protection[a]
EE2[b]	0.10	213.5±12.6*	83.3±6.6*	90.9±17.8*
4	0.01	17.2±9.2	9.8±6.8	6.1±17.2
	0.10	23.1±7.5	66.3±4.2*	26.5±20.1
	1.00	100.4±6.8*	82.6±4.3*	59.5±14*
	10.0	142.3±14.4*	93.8±1.2*	119.0±7.9*

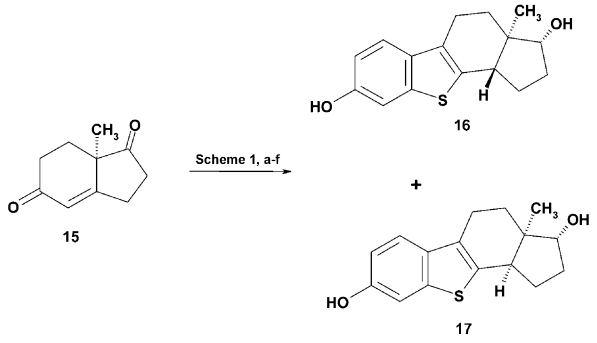
*$p \leq 0.05$, statistical evaluations were made by one way analysis of variance (ANOVA).
[a]Bone density was determined at the proximal metaphysis of excised femur. Percent protection was calculated for individual animals by the following formula: % protection = [BMD$_{test}$ − BMD$_{ovx}$/(BMD$_{sham}$ − BMD$_{ovx}$)] × 100.
[b]EE2, ethynylestradiol.

the equilenins. The biological effects of modifying the C-17 hydroxy group of CEEs and other estrogens has been well documented.[2] We were specifically interested in how alterations at the C/D ring juncture with respect to the C-17 hydroxyl group might influence receptor affinity and, ultimately, uterine pharmacology. However, due to the synthetic complexity involved in the enantioselective preparation of such derivatives, we chose to investigate the benzothiophene, or B-nor-5-thiaequilenin, scaffold. The benzothiopene moiety is an established A/B-ring surrogate for estrogen receptor ligands[10] and was readily accessed, albeit in racemic form, via existing synthetic technology developed by Crenshaw.[11] The asymmetric preparation of the thiaequilenin scaffold was accomplished by modification of Crenshaw's procedure as shown in Schemes 1 and 2 in which tetrahydro-7-methyl-indene-1,5-dione (**5** or **15**) is used to establish the absolute stereochemistry of the quaternary angular methyl group.[12] For the analogues maintaining this angular methyl group in the β configuration (Scheme 1), epoxidation of **5** followed by condensation with *m*-methoxythiophenol yielded **6** as a mixture of diastereomers.[10] Cyclization of the dione was achieved under Friedel–Crafts conditions with $AlCl_3$ to yield **7** along with $<10\%$ of the 5-methoxy regioisomer which was separated by crystallization. X-ray crystallography of **7** confirmed the absolute stereochemistry. Stereoselective hydride reduction of the ketone from the α-face gave **8** which was subsequently reduced by hydrogenation of the olefin using 5% palladium on carbon to give a mixture of diastereomers, **9** and **10**, that were readily separated by silica gel chromatography. Catalyst selection was important in determining the ratio of the diastereomers. Use of 5% Pd/C resulted in nearly equivalent amounts of the *trans* and *cis* C/D ring juncture while use of $Pd/CaCO_3$ resulted in a 4:1 mix favoring the *trans* C/D ring juncture. Demethylation with sodium ethanethiolate in DMF at reflux provided **11**. Inversion of the 17β hydroxy alcohol was accomplished using modified Mitsunobu conditions.[13] Compounds **16** and **17** in which the angular methyl group is in the α configuration were prepared in optically pure fashion as shown in Scheme 2 in analogous fashion to **11** and **12** using the antipode of the starting dione (**15**) and carrying it through an identical reaction sequence.[14]



Scheme 1. Reagents and conditions: (a) H_2O_2, NaOH, (b) *m*-methoxythiophenol, NaOH, 31%; (c) $AlCl_3$, CH_2Cl_2, 68%; (d) $NaBH_4$, THF/MeOH, 99%: (e) H_2, 5% Pd/C, THF/EtOH, 38% **9**, 45% **10**; (f) NaSEt, DMF, 30%; (g) *p*-nitrobenzoic acid, DEAD, PPh_3, 70%; (h) potassium carbonate, 69%.

As shown in Table 3, the thiaequilenin ligands bind to ERα with K_i's ranging from 4 to 24 nM.[15] Weaker relative affinity to ERβ is observed in each case. The ligand with the highest affinity to ERα is **11** ($K_i = 4$ nM) while its enantiomer **16** has 3-fold weaker affinity ($K_i = 13$ nM). Inversion of the 17β-OH to 17α-OH results in a >5-fold loss in potency (see **11** and **14**), data which is consistent with known trends for estradiol and analogues.[16] While the effects of modifying the C-17 position in the steroid backbone are well documented, structure–activity studies examining the variations in the C/D ring juncture are limited.[16] In the thiaequilenin series, the C/D *trans* 17β-OH analogue **11** binds with higher affinity to ERα than its *cis* counterpart **12**. A similar trend is observed for the analogous compounds in the 17α-OH series (**16** and **17**). The orientation of the angular methyl group has little influence on binding in the 17α-OH series as shown by analogues **14** and **17** that differ only by the orientation of the methyl group and have identical affinity. When comparing ligands that are



Scheme 2.

Table 3. hERα and hERβ binding affinity for 17α-DHEqn and analogues

Compd	ERα	ERβ
	K_i, nM	K_i, nM
17α-DHEqn (**4**)	8.0	24
11	4.0	11
12	10	47
14	23	73
16	13	17
17	24	120

Table 4. Effects of 17α-DHEqn (**4**), **11**, and **14** on uterine weight in OVX rats after 4 days of oral dosing

Compd	Dose (mpk)	Uterine weight/ body weight	Uterine weight % increase versus OVX
OVX	—	0.32±0.03	—
EE2	0.10	1.03±0.03	219.6
4	0.01	0.33±0.13	1.5
	0.10	0.29±0.13	−11.1
	1.00	0.45±0.02*	38.1
	10.0	0.75±0.06*	132.5
11	0.01	0.37±0.03	13.7
	0.10	0.33±0.01	1.4
	1.00	0.32±0.01	0.2
	5.00	0.48±0.01*	48.4
14	0.01	0.34±0.02	5.5
	0.10	0.36±0.02	10.2
	1.00	0.31±0.02	−5.3
	10.0	0.66±0.05*	103.1

*$p < 0.05$ verses OVX by one way analysis of variance and Fisher's posthoc analysis. $n = 5$ animals/group.

enantiomeric pairs, **11** and **16** or **12** and **17**, the binding affinity of the enantiomer with the 17β-OH is more potent than its 17α counterpart. In general, compounds with the 17β-OH functionality have higher affinity to ERα regardless of whether the ring C/D ring juncture is *cis* or *trans*. However, the overall differences in affinity between ligands are relatively small indicating that the receptor can readily accommodate changes in the spatial arrangement of the C/D-ring juncture without dramatic consequences to the binding affinity. Overall, this data supports the hypothesis that there is a flexible binding pocket in the estrogen receptor that allows for D-ring modifications in the ligand.[16]

In order to determine the effects of thiaequilenins on the uterus, **11** and **14** were evaluated in the OVX rat. As shown in Table 4, 17α-DHEqn (**4**) causes an increase in uterine weight relative to OVX controls at both 1 and 10 mpk after 4 days of oral administration. Uterine hypertrophy is also observed with compounds **11** and **14** at the 5 and 10 mpk dose, respectively, but not at 1 mpk. Based on this data the thiaequilenins are less uterotrophic than **4** at equivalent doses. Further studies will be required in order to determine if this observation is due to differences in pharmacokinetics between these compounds and whether the improvement in uterine pharmacology translates to a better overall tissue selectivity profile with respect to bone, lipids, and uterus.

In summary, we have evaluated a series of equine estrogen analogues to determine whether these agents demonstrate tissue selective pharmacology. 17α-DHEqn (**4**) effectively prevents bone loss and lowers serum cholesterol in the OVX rat at doses that also causes a statistically significant increase in uterine weight versus OVX controls. Structure–activity studies with B-nor-6-thiaequilenin analogues of **4** indicate that the orientation of the C-17 hydroxy group plays a more significant role than the C/D ring juncture with respect to determining affinity to the receptor. The uterine profile of the thiaequilenins **11** and **14** is encouraging and suggests that this structural platform may be useful for the design of tissue selective estrogens or SERMs.

Acknowledgements

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