VALERIA NORMA RUBIN
Tamoxifen Analogs Bearing Acidic-Side Chain Substituents: Synthesis and Biological Evaluation.
(Under Direction of PETER C. RUENITZ)

The estrogen receptor (ER) ligand 4-[1-(p-hydroxyphenyl)-2-phenylethyl]phenoxyacetic acid (HPPA) was previously found to have differential bone loss suppressive effects in the ovariectomized (OVX) rat approaching those of selective ER modulators (SERMs) like tamoxifen. In an effort to improve efficacy and bioavailability, analogs of this compound were prepared which incorporated features designed to reduce polarity/ionizability. Thus, the acetic acid side chain of HPPA was replaced by n-butanoic acid and 1H-tetrazol-4-ylmethyl moieties, to give **11** and **12**, respectively. Also, the phenolic hydroxyl of HPPA was replaced, giving deoxy analog **13**. In addition, new methods for the synthesis of triarylethylene variants **11**, namely 4-{[1-(p-hydroxyphenyl)-2-phenyl-1-butenyl]phenoxy}-n-butanoic acid (**10**) and its deshydroxy counterpart (9) were developed. The former of these was previously shown to have *in vitro* antiestrogenic effects characteristic of known SERMs.

In the OVX, **9** and **10** were as effective as 17â-estradiol (E2) in suppressing serum markers of bone turnover, namely osteocalcin and deoxypyridinoline, but had only 30% the uterotrophic efficacy of E2. Furthermore, **9** and **10** each lowered serum cholesterol levels by about 30% with respect to vehicle treated controls, and were able to suppress body weight gain to a degree approaching that of E2.

This study has therefore identified two triarylethylene oxybutyric acids, **9** and **10**, that display differential estrogenicity similar to that seen with establishes SERMs.

INDEX WORDS: Tamoxifen, SERMs, Osteoporosis, Raloxifene, Estrogen Replacement Therapy

TAMOXIFEN ANALOGS BEARING ACIDIC-SIDE CHAIN SUBSTITUENTS: SYNTHESIS AND BIOLOGICAL EVALUATION

by

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To Mom and Dad

For never-ending love and support.

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CHAPTER 1

INTRODUCTION

The estrogen receptor (ER) is a member in the superfamily of steroid/thyroid nuclear hormone receptors, which are ligand-activated nuclear transcription factors.[1] In the absence of ligand, the ER is in a transcriptionally inactive form within the nucleus of the cell.[2] Interaction with estrogen, its endogenous ligand, results in receptor dimerization and activation (Figure 1).[3] The ligand-receptor complex interacts with target gene promoters either directly by binding to estrogen response elements in DNA or indirectly through association with other DNA bound proteins.[4] These interactions modulate the transcription levels of target genes resulting in their activation or suppression.[5]

Human ER is composed of 595 amino acids and its sequence is divided into six domains: A, B, C, D, E, and F (Figure 2).[6] Domain C is the most highly conserved region among different members of the family of nuclear receptors, and corresponds to the centrally located DNA-binding domain.[7] This domain mediates the interaction between the activated ER and estrogen response elements in DNA.[8] Domain C also contains a weak dimerization activity.[7] Two distinct transcriptional activation functions are located within the N-terminal domains A/B and within the C-terminal domain E; these are the hormone-independent transcription activation factor 1 (TAF-1) and the hormone-dependent transcription activation factor 2 (TAF-2),

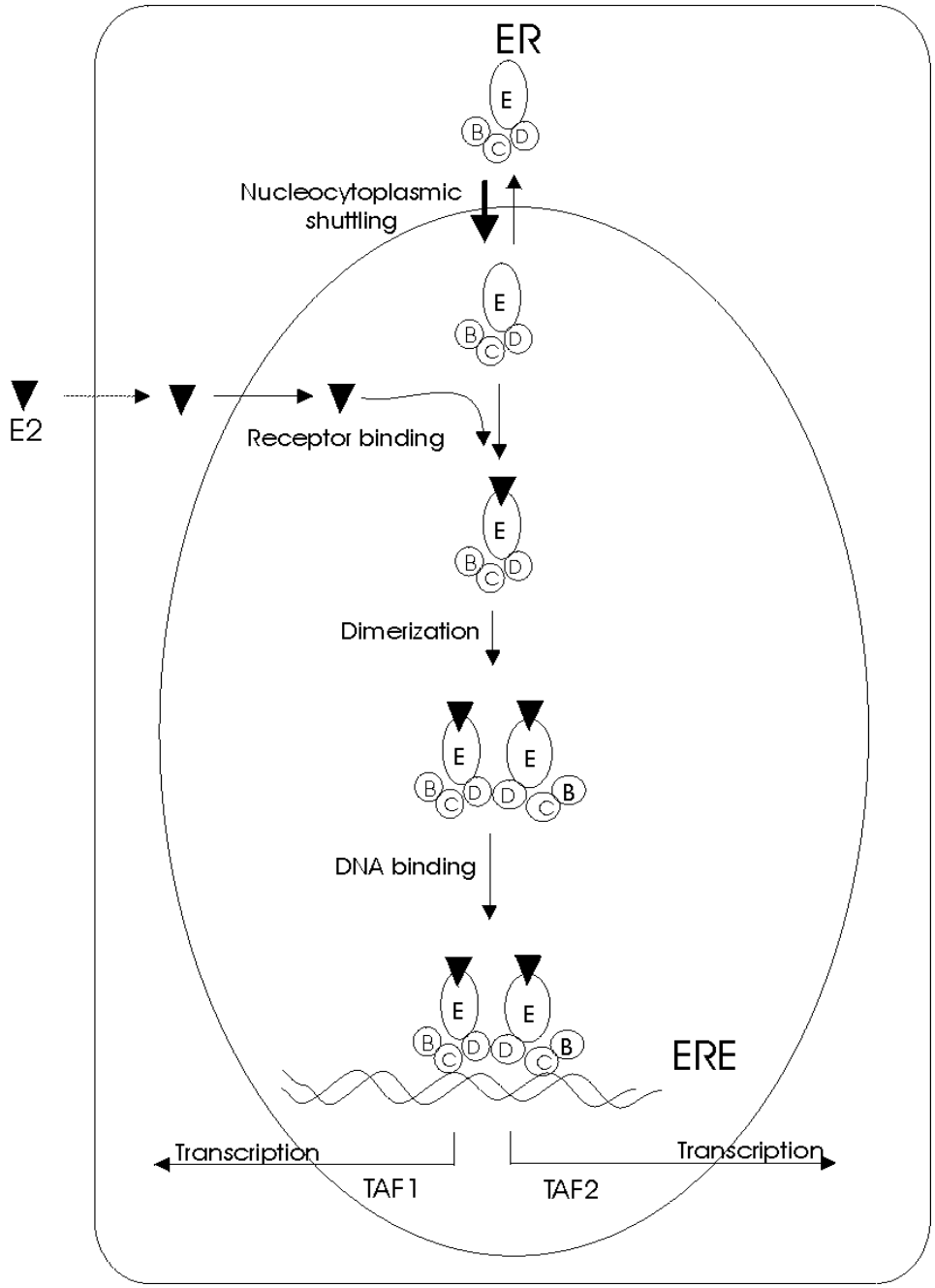


Figure 1. Molecular mechanism of action of E2 [3]

The estrogen receptor (ER) is represented by its four functional domains B,C,D and E. TAF-1 and TAF-2 represent the transcriptional activation factors, and ERE represents the estrogen response elements of DNA.

respectively.[9] TAF-1 and TAF-2 mediate transcriptional activation of the target genes upon binding of the ER-ligand complex to estrogen response elements. Domain E is also involved in dimerization and ligand/steroid binding, and is referred to as the ligand-binding domain.[7] A third, hormone-independent activation factor (TAF-2a) has recently been identified and is located between amino acids 282-351, in regions D and E of the human ER.[4]

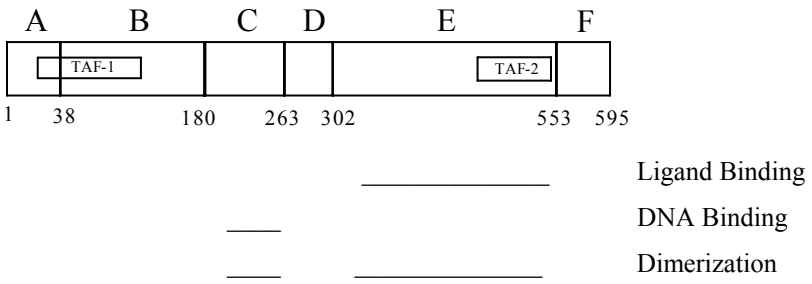


Figure 2. Diagram of ER primary structure and functional domains [6]

Estrogens are female steroid hormones that exert their effect through an interaction with the ER. Their main function is to stimulate the female secondary sex characteristics. In addition to their direct effect on the reproductive organs, estrogens have been found to exert an effect on several other tissues, including those of the skeletal and cardiovascular systems.

The decreased ovarian production of estrogen, mainly 17β-estradiol (E2; Figure 3) is characteristic of menopause. This decrease in endogenous estrogen levels leads to

discomforts and numerous health complications for both menopausal and postmenopausal women. In order to alleviate some of the discomforts and to prevent health complications, postmenopausal women are often administered estradiol, or more commonly, conjugated equine estrogens.[10] This treatment is referred to as estrogen replacement therapy.

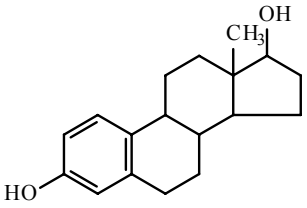


Figure 3. Chemical structure of 17β-estradiol

The major indication for estrogen replacement therapy is for the prevention of osteoporosis, a disease characterized by the progressive loss of bone density.[11-13] Bone remodeling, or bone turnover, is a continuous process that is necessary for the maintenance of healthy adult bone. Two cell types mediate this process: osteoclasts, which are the cells responsible for bone resorption, and osteoblasts, the cells responsible for bone formation or bone deposition.[14] Osteoporosis occurs when this highly balanced process is disrupted, such as in menopause.[14, 15] Estrogen receptors have been found in the nuclei of osteoblasts, and estrogens are known to stimulate their activity.[16, 17]

Therefore, postmenopausal loss of endogenous estrogens is a major cause of osteoporosis and estrogen replacement therapy presents an effective treatment for bone loss.

In addition to protecting against bone loss, estrogen replacement therapy displays beneficial effects on the cardiovascular system and reduces the risk of heart disease in postmenopausal women.[18, 19] Levels of low-density lipoprotein (LDL) increase at menopause by 10-15% and this increase can be prevented by estrogen replacement therapy.[10] In addition, high-density lipoprotein (HDL) levels are also increased by 10-15% with estrogen replacement therapy, and this may play an important role in preventing cardiovascular disease as well.[10] Another cardioprotective effect of estrogen replacement therapy may result from estrogen's antioxidant effect, which inhibits the oxidative modification of LDL, and prevents the formation of oxidized LDL, which may be atherogenic.[20]

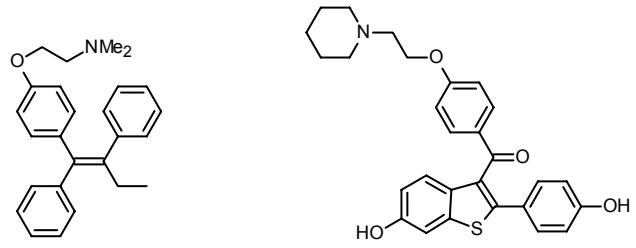
Estrogens are also known to increase vascularity and blood flow to the brain. It is thought that this is the reason why patients on estrogen replacement therapy display an increase age-at-onset and an overall decreased risk for Alzheimer's disease.[21, 22] In addition, several studies have shown that estrogens have a beneficial effect on memory and cognition, and patients with Alzheimer's disease show an improvement in cognition functioning when treated with estrogens.[23, 24]

Even thought the benefits of estrogen replacement therapy are numerous, a number of side effects greatly limit patient compliance to estrogen replacement therapy. Among the side effects are resumption of bleeding, breast tenderness and fluid retention, coupled with an increased risk for breast and uterine cancers and thrombosis.[25-27] The negative effects on the uterus can be decreased by co-administration of a progestin,

known as hormone replacement therapy. However, hormone replacement therapy is contraindicated in women with high risk of cardiovascular disease due to adverse effects on serum lipids.[28]

Research aimed at reducing the side effects while maintaining the numerous potential benefits of estrogen replacement therapy and hormone replacement therapy has lead to the development of nonsteroidal ER ligands collectively known as selective estrogen receptor modulators (SERMs).[29, 30] These are compounds that exert their effect via the ER and display tissue-selective modulation. With SERMs, it is possible to maintain the beneficial effects of estrogen in the liver, brain, bone and cardiovascular system, while reducing the undesired effects on the reproductive organs such as the uterus and the breast.[31]

Tamoxifen (Figure 4) is the first and best known SERM. It is a triarylethylene derivative that was first prescribed to postmenopausal women for the treatment of hormonally dependent breast cancer due to its antiestrogenic effects in the breast.[32, 32] Several studies have shown that tamoxifen possessed estrogen-like activity in the bone and the liver, and thus affords protection against bone loss [34, 35] and cardiovascular disease.[36, 37] However, tamoxifen has also been linked to an increase risk of endometrial cancer due to estrogenic effects on the uterus.[38, 39] The risk of endometrial stimulation has been addressed by what are referred to as 2nd generation SERMs like raloxifene (Figure 4).[40] Raloxifene was also found to protect against bone loss and have beneficial effects on plasma lipoproteins, but it does not stimulate the uterus.[40]



tamoxifen raloxifen

Figure 4. Chemical structures of tamoxifen and raloxifene

The exact mechanism for the tissue selectivity displayed by SERMs has not yet been fully elucidated, however, several theories have been proposed. One explanation comes from the tripartite model for steroid hormone receptors, which involves three distinct mechanisms: ligand-based selectivity, receptor-based selectivity, and effector site-based selectivity (Figure 5).[41] With respect to the ER the last mechanism was thought to be the primary one for tissue selectivity; however, the recent discovery of ERβ, an isoform of the ER, has also validated the possibility of receptor-based selectivity as an important factor in the differential activity displayed by SERMs.[42]

Receptor-based selectivity can occur when more than one isoform of a given receptor exists in different tissues. In humans, ERβ and the first estrogen binding

Type of selectivity	Components			Level of selectivity			Mechanism
	Ligand	Receptor	Effector	Tissue	Cell	Gene	
Ligand-based	Different	Same	Same	Yes	Yes	No	Ligand(s) undergoes different metabolism in different tissues/cells (selective bioactivation selective bioinactivation)
Receptor-based	Same	Different	Same	Yes	Yes	No	Composition of receptors (concentration, subtypes, isoforms, variants) is different in different tissues/cells
Effector-based	Same	Same	Different	Yes	Yes	Yes	The same ligand(s) and same receptor(s) experience different interactions at different effector sites regulating gene transcription

Figure 5. Tripartite model for steroid hormone receptors [41]

receptor discovered (now called ERα) are highly homologous, mainly in the DNA-binding domain, where they share 97% amino acid identity, and in the ligand-binding domain, where the amino acid identity is 60% (Figure 6).[43] Consistent with this, both receptors appear to have similar ligand- and DNA-binding properties. However, the two receptors differ greatly in the sequence homologies of regions A/B (17%) and region D (30%) which contain part of the transcriptional activation functions.[42] In addition, the two ERs appear to have unique tissue distributions and slightly different expression patterns, as shown by studies based on mRNA analysis in rodents and *in situ* hybridization, suggesting that they might be involved in different biological processes.[44, 45]

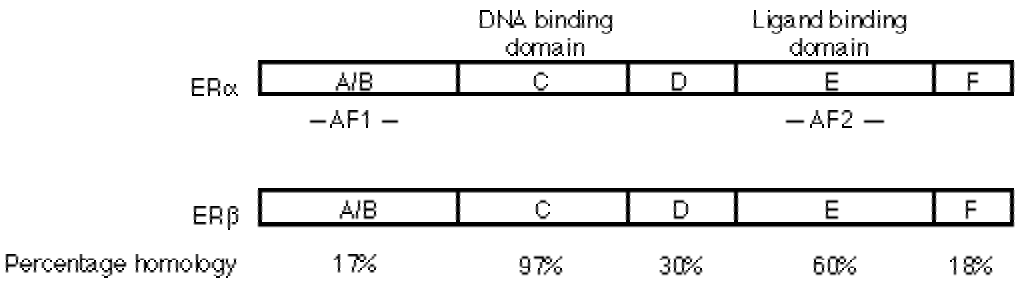


Figure 6: Comparison of amino acid homology between ERα and ERβ [43]

Effector site-based selectivity involves the differential regulation of the two transcriptional activation factors, TAF-1 and TAF-2, in a given tissue. Regardless of the cellular context, E2 activates both TAFs and therefore always acts as an agonist. Compounds with mixed agonist/antagonist activity, like tamoxifen and raloxifene, have been shown to induce conformational changes in the ER upon binding, which are different from those induced by E2.[46, 47] For example, upon binding tamoxifen, the ER dimerizes and binds to DNA, but transcriptional activity mediated by TAF-2 is blocked.[47, 48] TAF-1 gives rise to agonist activity, however the disruption of TAF-2 activity results in antagonism.[47] Therefore, in tissues where TAF-1 predominates, tamoxifen acts as an agonist, and where TAF-2 predominates, tamoxifen acts as an antagonist.[5, 47, 49]

Another explanation for the tissue selectivity of SERMs is based on the presence of intracellular proteins known as coregulators.[31] These are proteins that are known to either enhance (coactivators) or repress (corepressors) the transcription of ligand-bound

ER (Figure 7). Several studies *in vitro* have shown that cellular coactivators and corepressors may potentially contribute to the overall ability of SERMs to regulate ER target gene expression.[50, 51] It appears that all cells contain both forms of coregulators, but the relative expression level of these has not been determined.[50] It is possible that in tissues with a high expression of corepressor protein, a given ligand may act as an antagonist, and/or that the same ligand will act as an agonist where there is a relatively large amount of coactivator protein present.[50] In addition, promotor context may contribute to the agonist/antagonist balance of SERMs by blocking the interaction between ER and coregulators.[50]

Recently, the discovery of alternative estrogen response elements to which the ER-ligand complex can bind has allowed for yet another explanation for the tissue-selective activity of SERMs.[52] The identification of a raloxifene response element, which binds the ER-raloxifene complex, but not the ER-E2 complex, has prompted a search that revealed several genes containing raloxifene reaponse element-like sequences.[52] These genes are known to encode proteins that have important roles in the skeletal, cardiovascular and central nervous systems.[52]

The ovariectomized (OVX) rat has become a widely used model for determining the estrogenic/antiestrogenic activity of ER ligands. Specifically, it is used as an animal model of osteopenia associated with estrogen deficiency [15], but it has also been validated as a model for evaluating estrogen effects on plasma

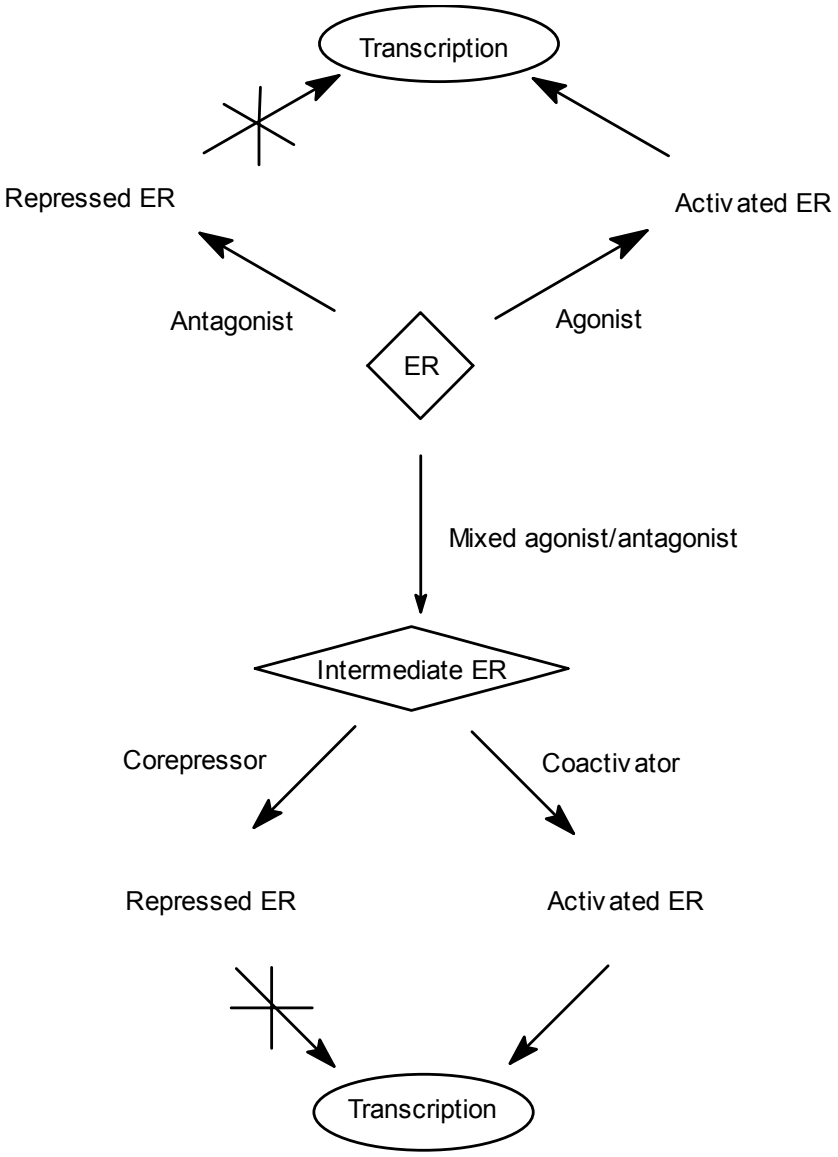


Figure 7. Coregulators in tissue selectivity of SERMs

cholesterol levels.[53] In addition, the OVX rat serves as a model for measuring uterotrophic effects of ER ligands.

OVX rats treated with E2 show a decreased rate of bone turnover and normal bone mass compared to vehicle treated controls.[54, 55] The same is seen with tamoxifen [55-57] or raloxifene [58, 59] treated animals. Similar results are also seen clinically with postmenopausal women on estrogen replacement therapy or hormone replacement therapy [60, 61], or taking either tamoxifen [34, 35, 62, 63] or raloxifene [64] for extended periods of time.

Estrogens and SERMs exert their bone protecting effects by reducing bone turnover rates. The degree to which bone formation and bone resorption are taking place can be monitored by measuring levels of biochemical "bone markers", namely osteocalcin (OC) and deoxypyridinoline (Dpd).[65] OC is a polypeptide produced by osteoblasts during the process of bone formation.[66, 67] During this process, a significant amount of OC escapes into the blood stream. Dpd is a pyridinium crosslink that is released into the circulation during the breakdown of bone collagen, which accompanies resorption.[68] In the OVX rat, the degree to which ER ligands are able to suppress serum OC and Dpd parallels their ability to suppress bone loss and maintain other bone histomorphometric parameters.[15, 69, 70]

Since it's introduction over 30 years ago, numerous analogs of tamoxifen have been synthesized in an attempt to find an "ideal" SERM, a compound which displays estrogenic activity in extra-reproductive tissue, while displaying antiestrogenic or no activity in the reproductive organs. One group of tamoxifen analogs appears to be particularly promising in this regard: those containing acidic side chain substituents.

Oxyacetic acids 4HTA and TA (Figure 8), two *in vivo* metabolites of tamoxifen, were first identified in the feces and urine of female rats treated with radiolabelled tamoxifen, although neither compound was found in the uterine tissue of the treated animals.[71, 72] Subsequent studies with 4HTA revealed that it was only slightly uterotrophic despite being a full estrogen mimetic in MCF-7 cells[73, 74], an estrogen dependant human breast cancer cell line used to assess estrogenic potency and efficacy of ER ligands. These observations were first explained by the selective distribution of 4HTA to tissues not including the uterus. However, studies with a cinnamic acid derivative of tamoxifen, GW 7604, and its putative precursor, GW 5638 (Figure 8), revealed a unique activity profile for these compounds which is thought to be related to their carboxylic acid moiety.[75, 76] Another carboxylic acid-containing analog, HPPA (Figure 8), also a full estrogen agonist in MCF-7 cells, was found to be a selective estrogen in the OVX rat, protecting against bone loss to a degree approaching that of tamoxifen, while displaying no uterotrophic activity.[15] However, bioavailability problems greatly limited this compound usefulness as a clinical agent. Compounds with structural features designed to reduce polarity/ionizability may overcome this problem while maintaining a desirable activity profile.

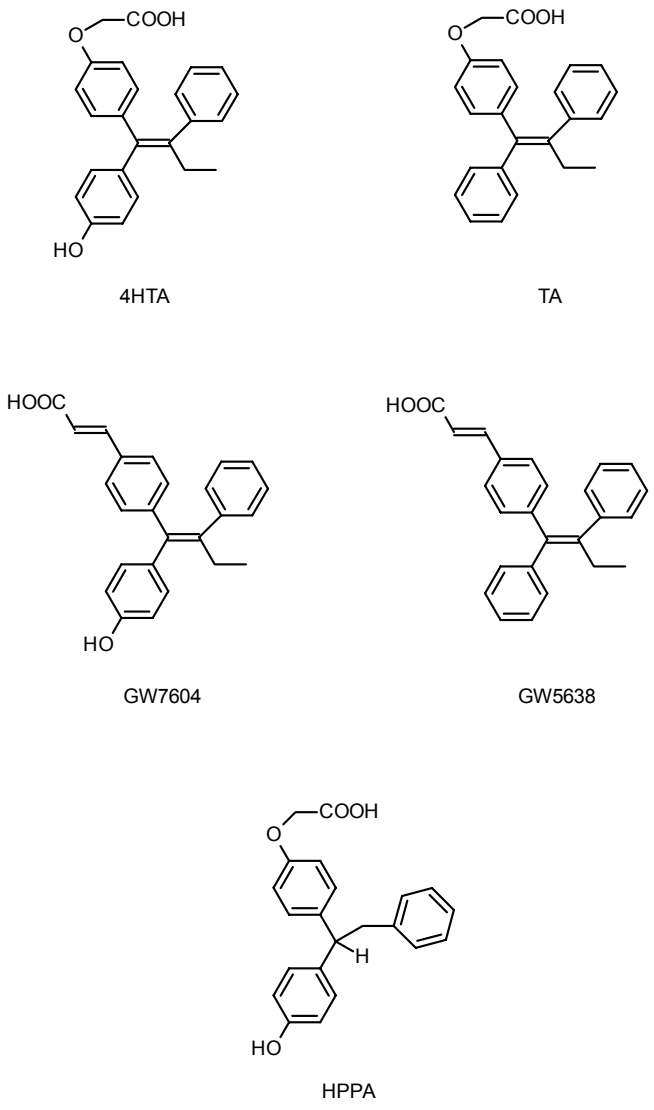


Figure 8. Chemical structures of acidic analogs of tamoxifen

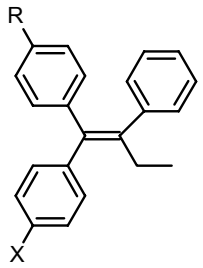
CHAPTER 2

STATEMENT OF PURPOSE

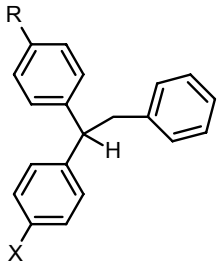
The purpose of this project is to identify analogs of HPPA with improved bioavailability and an activity profile similar to established SERMs. In an attempt to improve bioavailability of HPPA, side chain polarity/ionizability will be reduced by replacing the oxyacetic acid side chain of HPPA with a less acidic oxybutyric acid side chain (**9**-**11**) or a tetrazole ring (**12**) (Figure 9). Differences in ionizability (acidity) of these compounds compared to HPPA will be estimated from the pKa values of their respective phenoxy substituted variants. In addition, a deshydroxy analog of HPPA, **13**, will be prepared (Figure 9).

The synthesis of all the analogs will be carried out using standard literature procedures. Special attention will be paid to the synthesis of **10**. The previously used benzyl ether protecting group will be replaced by the more easily removable pivalate, in an attempt to improve overall yields and aid in final product purification and isolation.

The estrogenic/antiestrogenic *in vivo* activity of these acidic triarylethylenes/ethanes will be evaluated using the OVX rat as an animal model for estrogen deficiency. In particular, their effect on body weight, uterine stimulation, bone protection, and cholesterol and triglyceride levels will be determined.



	R	X
9	$O(CH_2)_3COOH$	H
10	$O(CH_2)_3COOH$	OH [a]



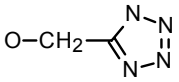
	R	X
HPPA	OCH_2COOH	OH
11	$O(CH_2)_3COOH$	OH
12		OH
13	OCH_2COOH	H

Figure 9: Structures of novel triarylethylene derivatives

[a] 1:1 mixture of *Z*-isomer (shown) and its *E* counterpart.

CHAPTER 3

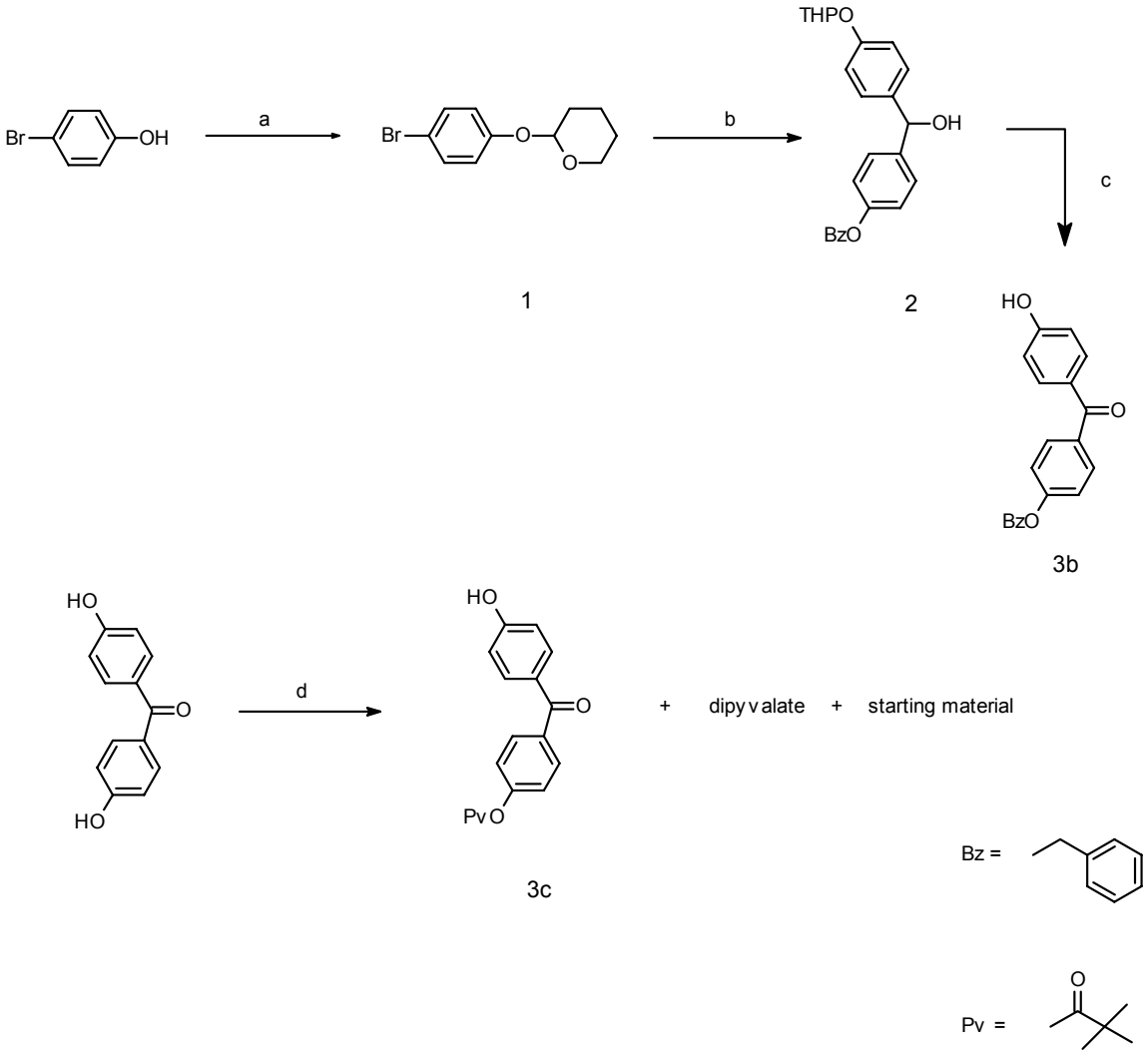
RESULTS

Synthesis. Schemes 1-5 outline the approach used to prepare compounds **9-13**. The triarylethylene backbone of these compounds was obtained through the application of the McMurry olefination reaction using low valent titanium [77] to cross-couple substituted benzophenones (**3a-c**) with propiophenone or benzaldehyde (scheme 2). The desired intermediates (**4a-d**) were easily separated from self-coupling byproducts via column chromatography when needed.

Introduction of the side chain was carried out by *O*-alkylation of monophenols **4a-d** with ethyl-4-bromobutyrate, ethyl bromoacetate, or bromoacetonitrile (scheme 3). Reaction of **7** with sodium azide and ammonium chloride [78] followed by catalytic debenzylation/hydrogenation gave **12** in good yield (scheme 4). Catalytic hydrogenation of intermediates **5** and **6** afforded **11** and **13,** respectively (scheme 5).

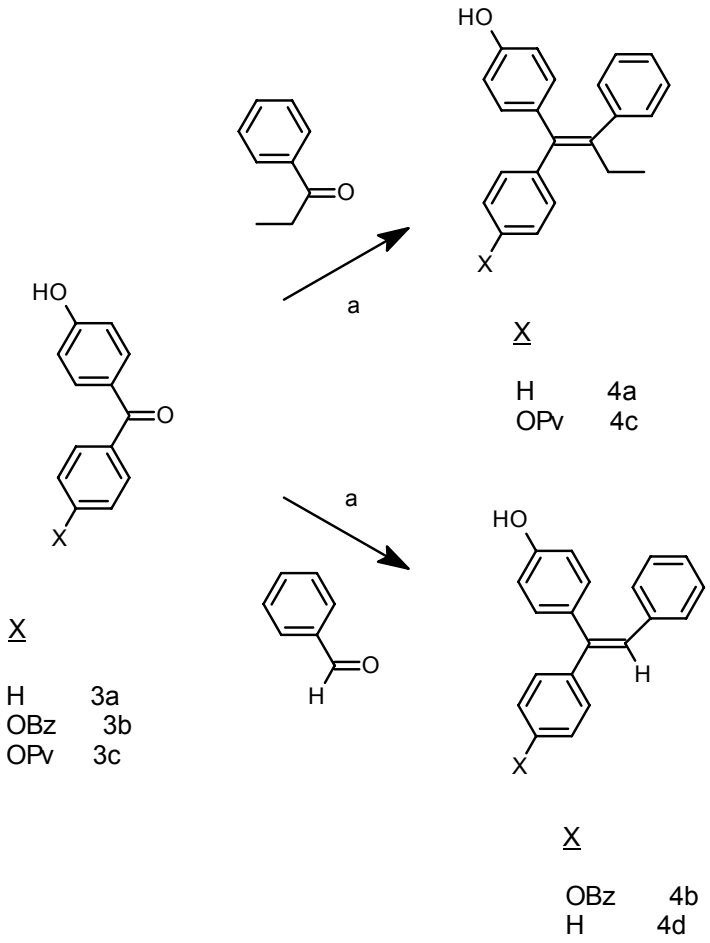
Previously, the synthesis of **10** was carried out starting with **3b** and propiophenone, followed by an alkylation and a debenzylation step (scheme 6).[79] The product obtained in this form was difficult to purify and the overall yield (4%) was very poor. Use of a pivaloyl (Pv) instead of a benzyl (Bz) phenolic protecting group resulted in an improvement of overall yields to 28% after crystalization. Removal of excess 4-bromobutyric acid and pivalic acid from the final product was accomplished by differential liquid-liquid extraction of **10**, taking advantage of **10**'s greater relative

lipophilicity. The starting benzophenone (**3c**) was prepared by a modification of the previously reported alkylation procedure (scheme 1).[80]



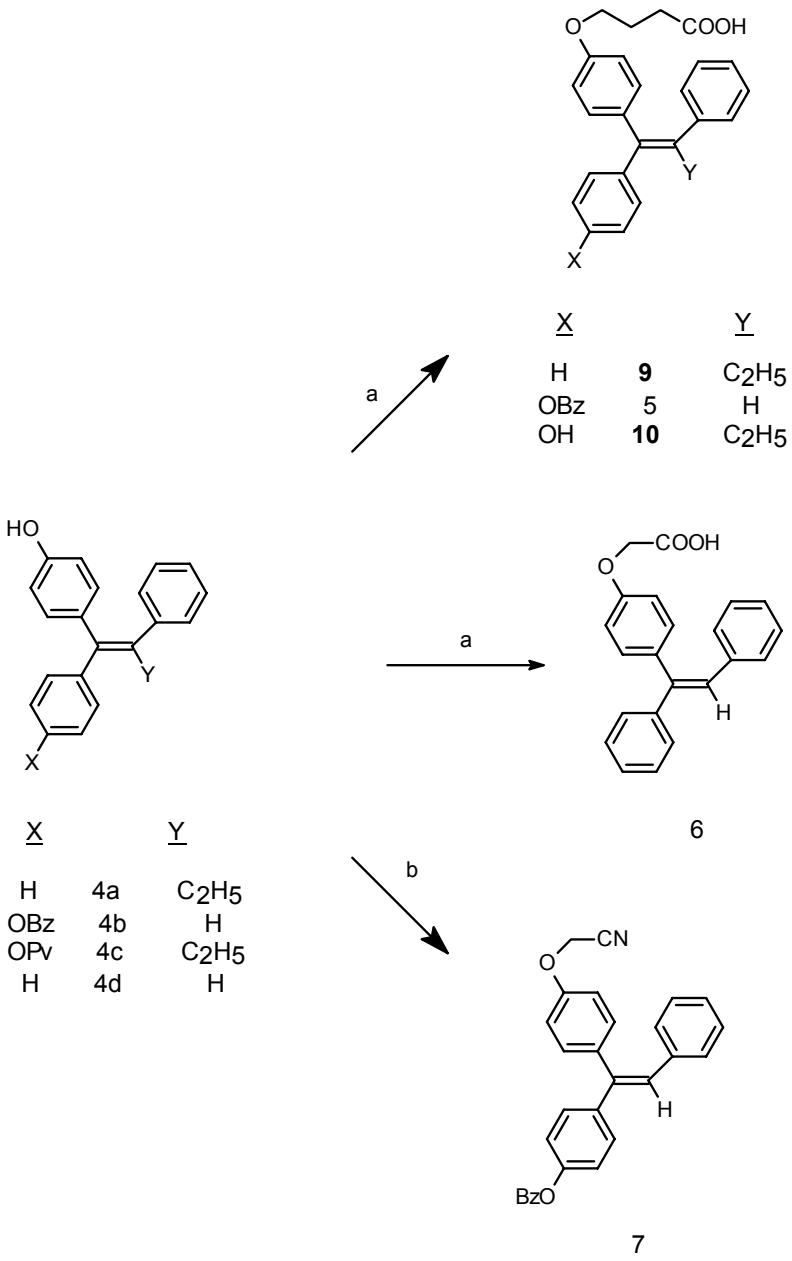
Scheme 1. Synthesis of substituted benzophenones

Reagents: (a) dihydropyran; (b) Mg, *p*-benzyloxybenzaldehyde, THF; (c) $(C_5H_4N)_2Cr_2O_7$, CH_2Cl_2; (d) $(CH_3)_3COCl$, THF



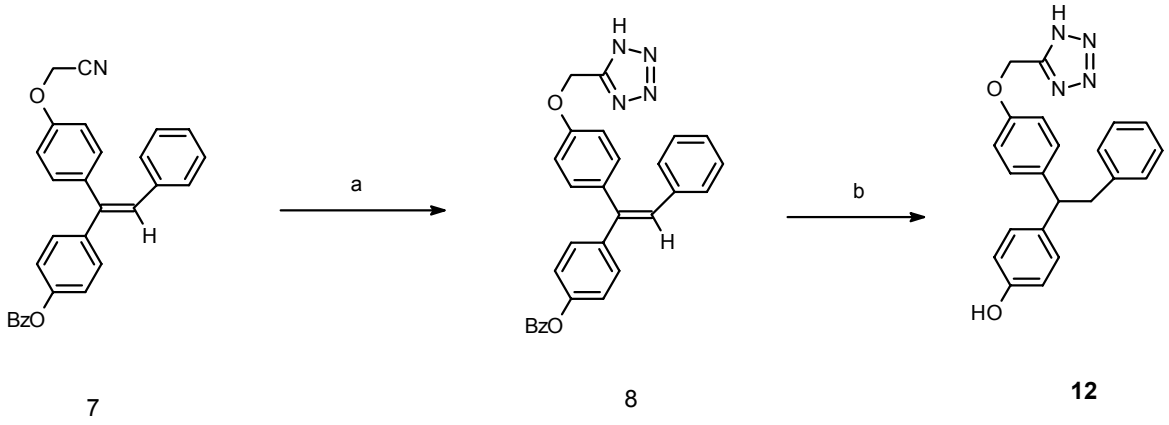
Scheme 2. Synthesis of triarylethylene backbone

Reagents: (a) Ti, THF



Scheme3. *O*-Alkylation of **4a-d**

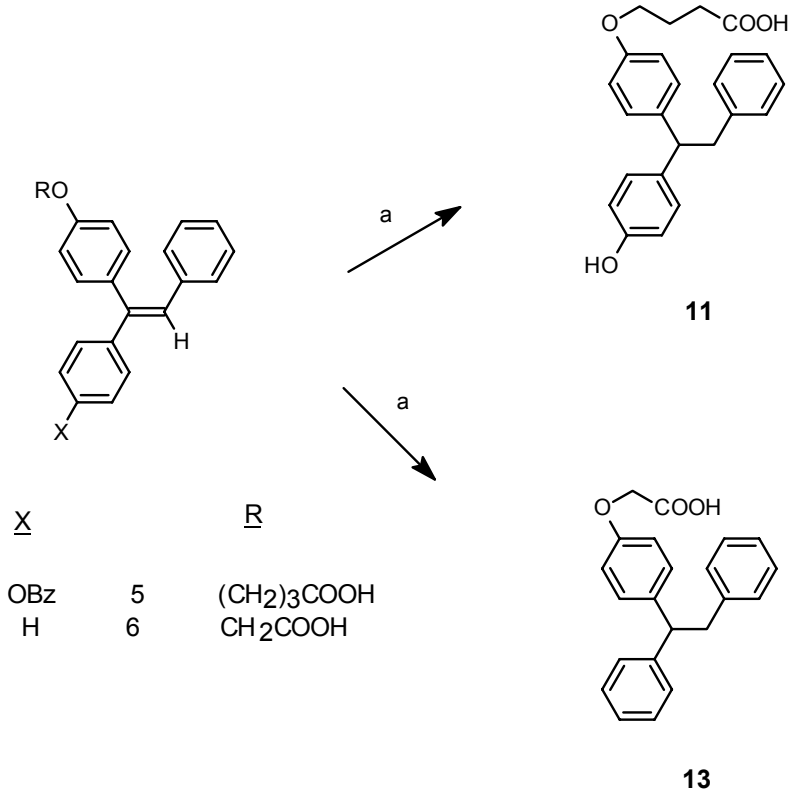
Reagents: (a) $Br(CH_2)_3COOC_2H_5$ or $BrCH_2COOC_2H_5$, acetone, then NaOH, aqueous dioxane; (b) $BrCH_2CN$, K_2CO_3, acetone



7 8 **12**

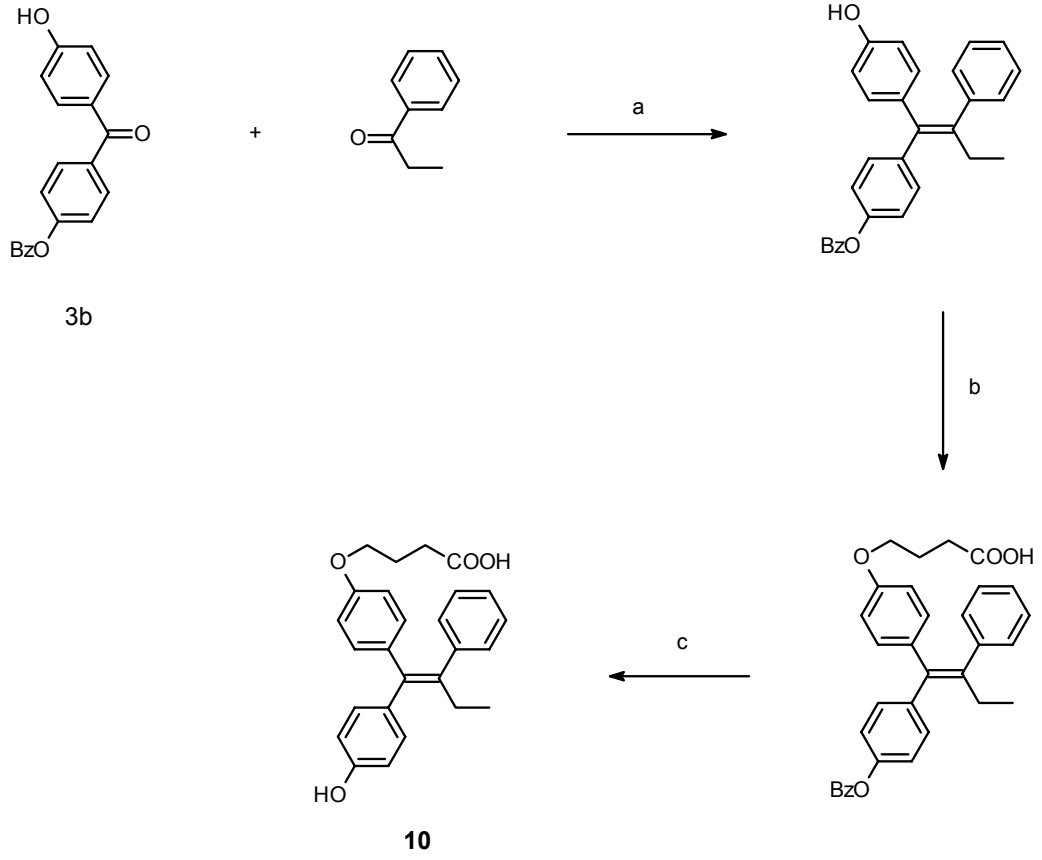
Scheme 4. Synthesis of **12**

Reagents: (a) NaN_3, DMF; (b) H_2, 10% Pd/C, THF/MeOH



Scheme 5. Catalytic hydrogenation of **5** and **6**

Reagents: (a) H_2, 10% Pd/C, THF/MeOH



Scheme 6. Previous synthesis of **10**

Reagents: (a) Ti, THF; (b) $Br(CH_2)_3COOC_2H_5$, K_2CO_3, acetone, then NaOH, aqueous dioxane; (c) HCl, aqueous EtOH

The configuration of final compounds (E/Z isomerism, when relevant) was determined by the relative chemical shifts of the side chain *O*-methylene protons in NMR spectra. For example, the spectrum of **9** contained a single *O*-methylene triplet centered at 3.87 (figure 10). This was assigned to the *Z* configuration of **9** (as shown in figure 9) based on correlation with *O*-methylene shifts of corresponding geometric isomers of tamoxifen and other acidic-side chain analogs.[79] Similarly, the spectrum of **10** contained two *O*-methylene triplets of about equal intensities, centered at 3.87 and 4.03 (figure 11), corresponding to the *Z* and *E* isomers of **10** respectively.

Differences in ionizability (acidity) of compounds **9-13**, compared to HPPA, were determined from the pKa values of their respective phenoxy-substituted variants.[81, 82] The pKa values of 4-phenoxy-*n*-butyric acid and 5-phenoxy-1,2,3,4-tetrazole were 4.44 and 3.49, respectively, and that of phenoxyacetic acid was 3.12. On this basis, **12** was estimated to be 2.3 times less acidic than HPPA; and **9-11** were each approximately 20 times less acidic than HPPA. Furthermore, the side-chain acidity of these oxybutyric acids was suggested to be comparable to that of GW-7804 whose pKa, extrapolated from that of *trans*-cinnamic acid [81], was around 4.40.

ER Affinity. Of all the compounds prepared for this study, **10** exhibited the highest ER affinity, considerably greater than that of its nonphenolic counterpart (**9**) and about one-tenth that of E2 (table 1). Replacement of the oxyacetic side chain of HPPA with an oxybutyric acid moiety (**11**) resulted in about a 70% increase in affinity. HPPA's "tetrazole" analog, **12**, had about 25-30% the ER affinity of HPPA.

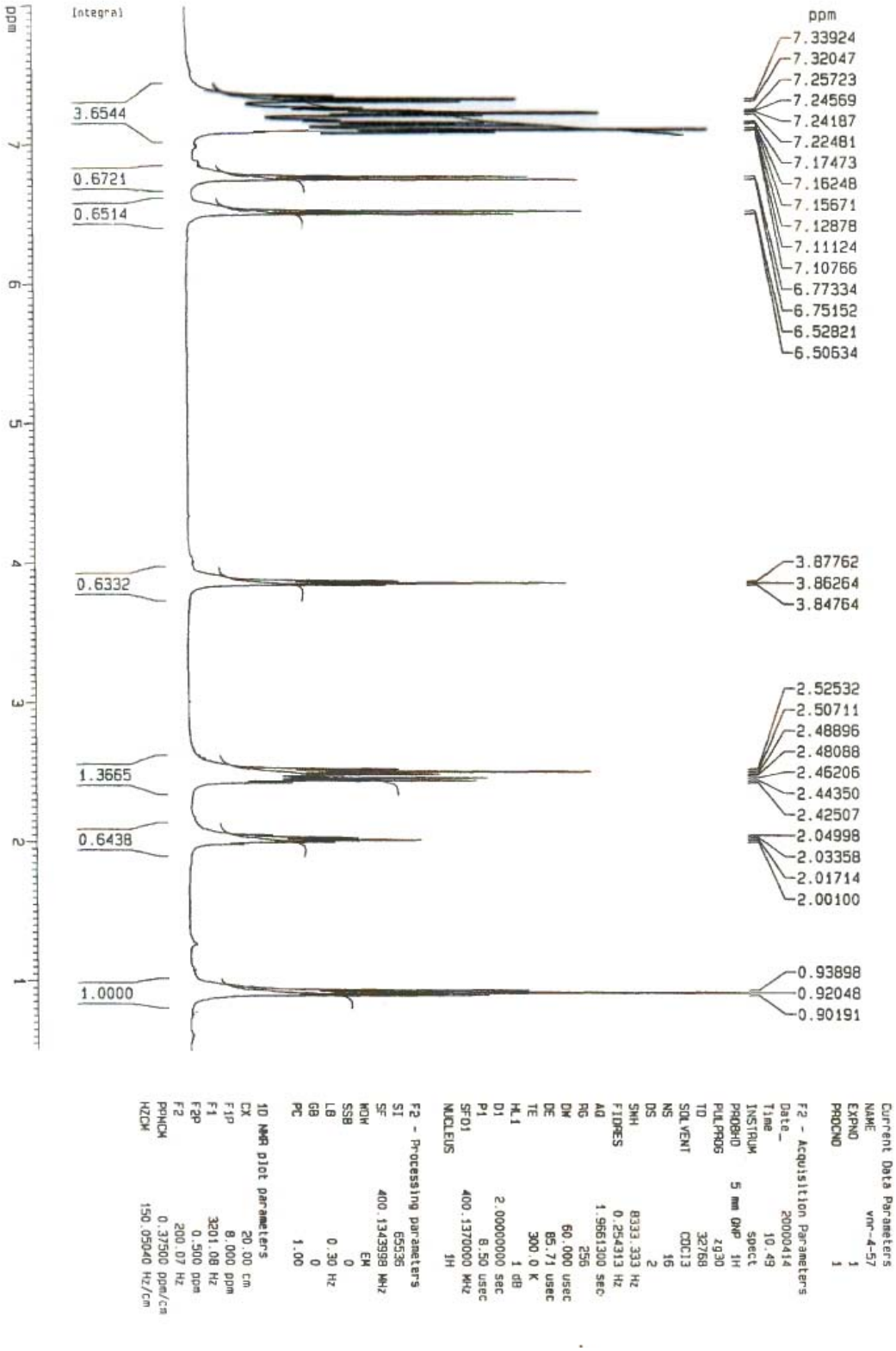


Figure 10. Proton NMR spectrum of *Z*-**9**

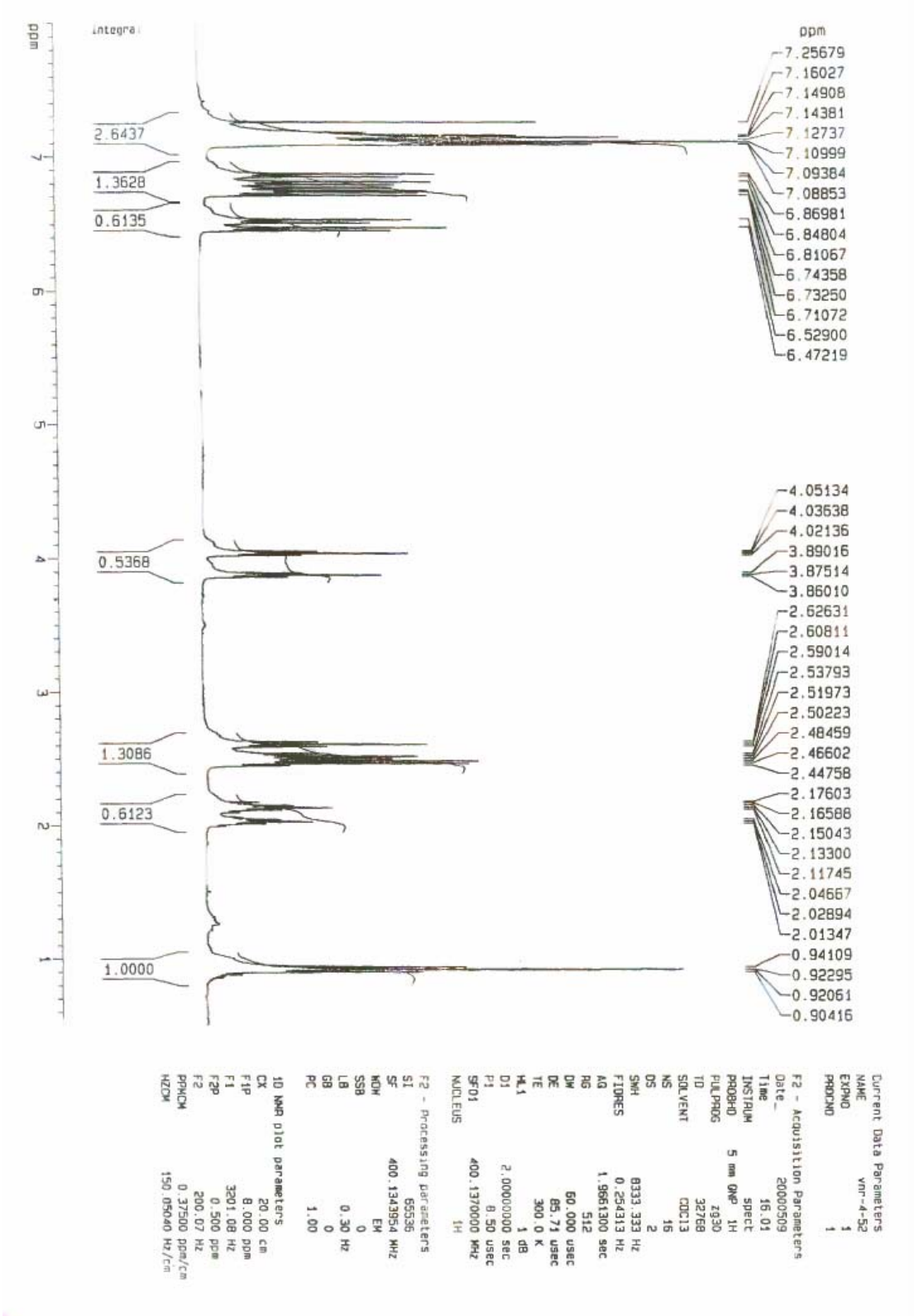


Figure 11. Proton NMR spectrum of *Z*- and *E*-**10**

Estrogenic Effects in the OVX Rat. Oxybutyric acids **9** and **10** exhibited similar bone protecting effects, which were equivalent to that of E2 (table 2). Both compounds significantly lowered serum markers of excessive bone metabolism, OC and Dpd. However, the uterotrophic effect of either **9** or **10** was only about 25-30% that of E2 or EE2 (measured with respect to vehicle treated controls; table 3). In addition, **9** and **10** were able to suppress the gain in body weight seen in vehicle treated OVX rats, although not as effectively as EE2 (table 3). At 10μmol/kg oral doses, **9** and **10** each lowered serum cholesterol levels by about 30% with respect to vehicle treated controls (table 4). And although **10** resulted in a 100% increase in serum triglycerides, the increase seen with **9** was only 41%, similar to that seen with EE2 (table 4).

The observed efficacy of **10** in serum markers of bone and lipid metabolism, and uterus was independent of its route of administration (sc or po).

Compound **12** had only a weak uterotrophic effect (table 2). Unfortunately, it had no effect on serum OC, and is ability to reduce serum Dpd was only of borderline ($p < 0.10$) significance. Neither **11** nor **13**, the oxybutyric acid and nonphenolic analogs of HPPA, exhibited any observable effect on body and uterine weight, or serum markers of bone and lipid metabolism.

Dose Response Effects of 10. Oxybutyric acid **10** exhibited its maximal effects on uterine weight (52% increase), serum OC and Dpd (14% and 29% decrease, respectively), and serum triglycerides (42% increase) at a daily dose of 0.4 μmol/kg (figure 6). Higher doses were required to attain effects on body weight and serum cholesterol.

Table 1. Comparative affinity of acidic triarylethylene derivatives and standard ER ligands for human ERα

compd	tamoxifen	HPPA	**9**	**10**	**11**	**12**	**13**	E2
RBA[a]	6.7	0.91	0.35	10.3	1.57	0.26	[b]	100

[a] The concentration of E2 (5.9 nM) required to displace 50% of specifically bound [^{3}H]E2, divided by the concentration of test compound required to do this, times 100. Each relative binding affinity (RBA) value is the average of three separate determinations in which the calculated individual values differ by < 10%.

[b] At 10mM, **13** displaced 54% of specifically bound [^{3}H]E2.

Table 2. Effect of acidic triarylethylene derivatives on serum markers of bone turnover

Compd[a]	Dosing Route[b]	Serum OC, ng/mL	Serum total Dpd, pmol/mL
9	po	59 (5[c])[d,e]	10.7 (1.2)[d]
10	sc	76 (4)[d]	7.4 (0.8)[d]
	po	62 (8)[d,e]	12.3 (3.9)[d]
11	sc	92 (10)	12.1 (2.3)
12	sc	93 (9)	8.7 (1.5)
13	sc	96 (6)	11.8 (1.7)
E2	sc	65 (12)[d]	6.3 (1.4)[d]
EE2	po	43 (4)[d]	11.0 (2.3)[d]
vehicle	sc	93 (9)	11.3 (1.7)
	po	84 (7)	18.3 (2.9)

a Seven animals per group.

[b] Daily dose of each test compound: 10 μmol/kg of body weight. Those of E2 and EE2 were each 0.35 μmol/kg of body weight.

[c] Standard deviations are in parentheses.

[d] $p < 0.05$ with respect to relevant vehicle-treated control.

[e] $p<0.05$ with respect to relavent estrogen-treated control.

Table 3. Effect of acidic triarylethylene derivatives on body weight gain and uterine weight

Compd[a]	Dosing Route[b]	Uterine wet wgt., mg	Body wgt.gain, g
9	po	195 (30[c])[d,e]	20 (10)[d,e]
10	sc	167 (25)[d,e]	32 (7)[d,e]
	po	184 (17)[d,e]	18 (6)[d,e]
11	sc	75 (13)	70 (10)
12	sc	123 (14)[d]	40 (6)[d,e]
13	sc	72 (8)	64 (10)
E2	sc	355 (73)[d]	14.7 (10)[d]
EE2	po	428 (53)[d]	1 (10)[d]
vehicle	sc	87 (14)	75 (10)
	po	102 (10)	57 (14)

[a] Seven animals per group.

[b] Daily dose of each test compound: 10 μmol/kg of body weight. Those of E2 and EE2 were each 0.35 μmol/kg of body weight.

[c] Standard deviations are in parentheses.

[d] $p < 0.05$ with respect to relevant vehicle-treated control.

[e] $p<0.05$ with respect to relavent estrogen-treated control.

Table 4. Effect of acidic triarylethylene derivatives on serum levels of cholesterol and triglycerides

Compd[a]	Dosing Route[b]	Cholesterol, mg/dL	Triglycerides, mg/dL
9	po	71 (12[c])[d,e]	92 (14)[d]
10	sc	90 (6)[d]	n/a
	po	76 (10)[d,e]	65 (10)[d]
11	sc	115 (7)	n/a
12	sc	121 (10)	n/a
13	sc	121 (9)	n/a
E2	sc	98 (6)[d]	n/a
EE2	po	24 (7)[d]	78 (18)[d]
vehicle	sc	124 (6)	n/a
	po	101 (7)	46 (9)

a Seven animals per group.

[b] Daily dose of each test compound: 10 μmol/kg of body weight. Those of E2 and EE2 were each 0.03 μmol/kg of body weight.

[c] Standard deviations are in parentheses.

[d] $p < 0.05$ with respect to relevant vehicle-treated control.

[e] $p<0.05$ with respect to relavent estrogen-treated control.

Table 5. Influence of oral dose levels on estrogen mimetic effects of **10**

Compd[a]	Uterine wet wgt., mg	Body wgt. gain, g	Serum OC, ng/mL	Serum total Dpd, pmol/mL	Cholesterol, mg/dL	Triglycerides, mg/dL
10 [0.4]	155 (23[b])[c,d]	42 (6)[c,d]	72 (4)[c,d]	13.0 (2.7)[c]	92 (7)	67 (12)[c]
10 [2]	170 (27)[c,d]	22 (12)[c,d]	66 (6)[c,d]	10.5 (1.5)[c]	89 (15)	73 (5)[c]
10 [10]	184 (17)[c,d]	18 (6)[c,d]	62 (8)[c,d]	12.3 (3.9)[c]	76 (10)[c,d]	65 (10)[c]
EE2	428 (53)[c]	1 (10)[c]	43 (4)[c]	11.0 (2.3)[c]	24 (7)[c]	78 (18)[c]
V	102 (10)	57 (14)	84 (7)	18.3 (2.9)	101 (7)	46 (9)

[a] Daily dose of compound **10** is in brackets in μmol/kg of body weight. That of EE2 was 0.35 μmol/kg of body weight. Seven animals per group.

[b] Standard deviations are in parentheses.

[c] $p < 0.05$ with respect to relevant vehicle-treated control.

[d] $p<0.05$ with respect to relavent estrogen-treated control.

Biotransformation of 9. Incubation of **9** with rat liver 9S fractions from OVX rats resulted in components with HPLC retention times of 32.7 min. and 35.2 min. These were inferred to correspond to the *Z* and *E* isomers of **10**, respectively. The components were observed only when both **9** and the cofactor (NADPH) for oxidative drug metabolism were present in incubation mixtures. The retention times were identical to those of authentic **10**, and the chromatographic peak intensity of each of these extract components was increased by dilution with **10**. However, the respective relative peak intensities of these extract components (70% and 30%) differed from those in **10** (47% and 53%).

The observed conversion rate of **9** to components chromatographically identical to **10** in NADPH-enriched incubations with 9S liver fractions was 2.5% per 20 min.

CHAPTER 4

DISCUSSION

Synthesis. The synthetic procedures used in this study were those typically performed in our lab. Of central importance to the synthesis of all the compounds was the application of the McMurry olefination reaction.[77] The McMurry reaction was used to form the triarylethylene backbone due to its high stereospecificity. Preparations of several tamoxifen analogs by McMurry coupling of two carbonyl groups has shown that the desired *trans* isomer predominated over the *cis* isomer in ratios ranging from 9:1 to 3:1.[77] The stereospecificity of the McMurry reaction is not directly explained by its mechanism (Figure 12).[83]

The formation of the tetrazole ring of **12** was performed by a commonly used procedure using sodium azide and ammonium chloride in dimethylformamide. The mechanism involved a form of cycloaddition reaction between the cyano group of intermediate **7** and the azide aninon (Figure 13).

The synthesis and isolation of **10** was greatly improved by using a pivaloyl (Pv) phenolic protecting group instead of the previously used benzyl (Bz). The pivalate was easily removed from intermediate **4c** during saponification of the side chain ester. This involved stirring the product at room temperature in the presence of sodium hydroxide in aqueous dioxane. Use of the Pv protecting group solved the previously posed problem of deprotection of the 4-hydroxyl group, and shortened the

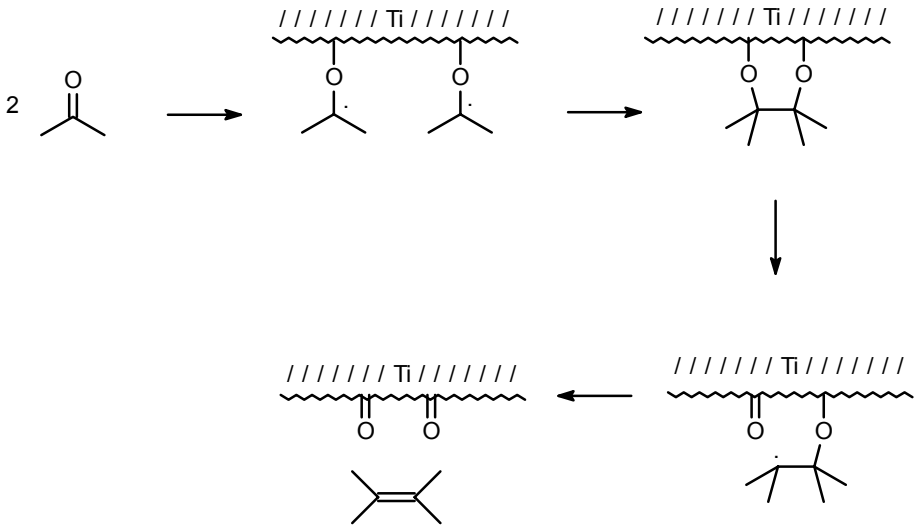


Figure 12. Mechanism of the McMurry olefination reaction [83]

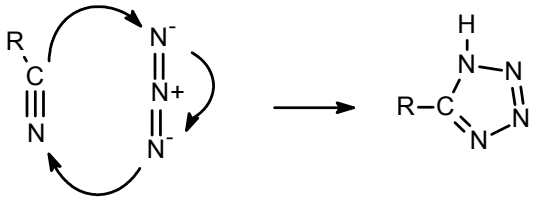


Figure 13. Mechanism of formation of the tetrazole ring of **12**

synthesis of **10** by one step, resulting in an improvement in overall yields. In addition, the fact that excess pivalic acid and bromobutyric acid could be easily removed from the final product via differential liquid-liquid extraction of **10** allowed for the crystalization of **10** as the free acid. Previously, compound **10** had only been isolated as its sodium salt and it was believed that **10** could not be isolated as the free acid due to it's high lipophilicity.

Animal Study. In the OVX rat, compounds **9** and **10** were comparable to established SERMs, like tamoxifen and raloxifene, in terms of differential estrogenic efficacy. The degree of selective bone/uterus estrogenic efficacy of **9** and **10** was similar to that seen with tamoxifen and raloxifene.[55, 70] Thus, like tamoxifen and raloxifene, these compounds suppressed serum markers of bone turnover, namely OC and DPD, to a degree approaching that of E2 and EE2 (table 2), but only had about one-fourth to one-third the uterotrophic efficacy of these steroidal estrogens (table 3). In particular, **9** and **10** caused a reduction in serum OC to about the same degree as tamoxifen and raloxifene, however, none of these compounds were as effective as EE2.[70] On the other hand, tamoxifen and raloxifene, as well as **9** and **10**, were as effective as EE2 in lowering urinary/serum Dpd levels. Although measurement of serum markers of bone turnover as a function of treatment regimen has been proven to be a good assessment of bone density maintenance [15, 69, 70], more time-consuming bone densitometric and microanatomical studies might reveal differences among treatment groups having similar OC and Dpd levels. However, these results, along with the uterotrophic effects observed, indicate that oxybutyric acids **9** and **10** exhibit tissue selective effects in the OVX rat which are comparable to established SERMs.

In addition, compounds **9** and **10** exhibited a reduction in serum cholesterol levels with an efficacy similar to that of tamoxifen and raloxifene, and approaching that of E2 and EE2 (table 4).[53, 84, 85] Compounds **9** and **10** were also found, like tamoxifen and several steroidal estrogens [86-88], to cause an elevation in serum triglyceride levels. The clinical significance of elevated triglyceride levels caused by hormonal/antihormonal therapy is not clear [89, 90], and neither the cellular nor the molecular basis for this effect have been identified, except that those of tamoxifen in the OVX rat were shown to be mediated via the ER.[86]

Another measure of estrogenic activity observed in this study was suppression of body weight gain in the OVX rat. This is a complex process, which also appears to be mediated via the ER.[91] Compounds **9** and **10** were similar to raloxifene, but somewhat less effective than tamoxifen in this regard.[59, 85] The clinical significance of this effect of steroidal estrogens and SERMs remains unclear.

Despite its low ER affinity (table 1), compound **9** was equivalent to **10** regarding the magnitude of its differential estrogenic effects (Figures 14 and 15). This may be attributed in part to *in vivo* enzymatic hydroxylation. In this study, **9** was suggested to undergo regioselective 4-hydroxylation to **10** in the presence of 9S liver fractions from OVX rats. The rate of hydroxylation of **9** observed in this study was similar to that previously seen with tamoxifen in the presence of rat liver enzymes.[92] And in a similar manner to **9** and **10**, 4-hydroxytamoxifen, a major *in vivo* metabolite of tamoxifen, had an RBA at least 50 times greater than tamoxifen for rat uterine ERα.[93, 94] In addition, tamoxifen was also converted in the rat to a host of other Phase 1 metabolites that, based on their ER affinity and tissue distribution and accumulation, might contribute to

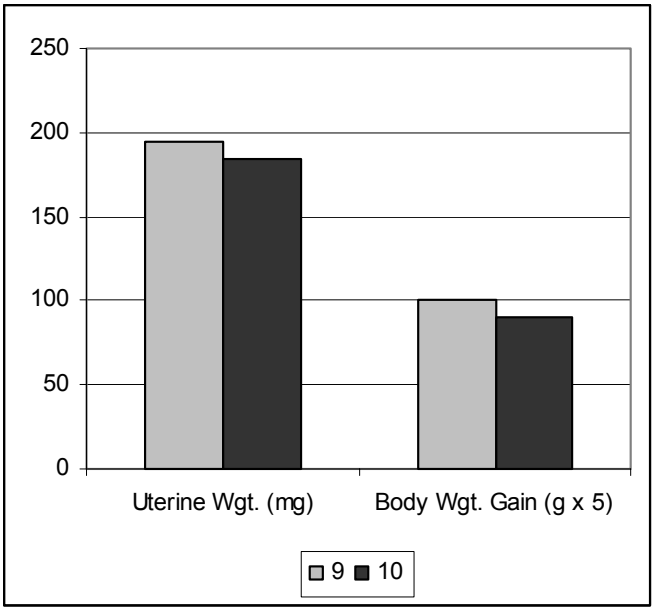


Figure 14. Effects of **9** and **10** on body weight gain and uterine weight

Adapted from data in table 3

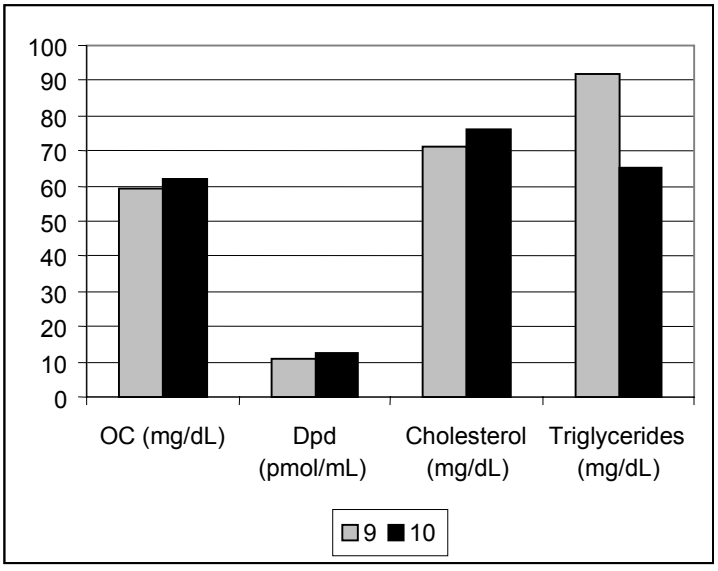


Figure 15. Effects of **9** and **10** on serum levels of markers of estrogenic efficacy

Adapted from data in tables 2 and 4

tamoxifen's observed effects.[93, 94] Therefore, support for the proposition that **9** is a prodrug of **10** would require systemic whole animal biotransformation studies of **9**.

Compound **9** was synthesized and isolated as a single isomer, however **10** (shown in figure 9 as the *Z* isomer) was always accompanied by its *E* isomer. Therefore, hydrolysis of **10**'s *Z*-ester precursor (**4c**) always gave *Z*- and *E*-**10** in a ca. 1:1 ratio. Ratios of **10** and its *E* isomer were determined directly by ^{1}H-NMR spectroscopy, and confirmed by HPLC analysis, and differed only slightly for the separate batches of **10** prepared. In addition, "metabolic" **10**, produced enzymatically form **9**, was found to have undergone isomerization to the extent of about 30%. Furthermore, *para*-hydroxyl substituted triarylethylenes are found to undergo isomerization in protic solvents, the mechanism for which involves the formation of a quinone methide due to tautomerization of the α ring (Figure 16). Thus, resolution of **10** from its geometric isomer prior to biological evaluation was not attempted. It should be considered, however, that in many cases, the constituent geometric isomers of this structural type have been shown to exhibit contrasting ER affinity and estrogenic/antiestrogenic potencies and efficacies, as in the case of TAM.[95-97] Thus, **10** might owe its observed ER affinity and estrogenic effects in part to its *E* isomer.

The molecular basis for tissue selective estrogenic effects expressed by tamoxifen, raloxifene and other SERMs arises from the distinct conformation they induce in the ligand binding domain of the ER, compared to E2.[98] The crystal structures of SERM- and E2-liganded hERα ligand-binding domain indicate a more "open" conformation exists in the former cases. This is due to an inability of helix 12 of the

ligand-binding domian to fold down completely and enclose the ligand.[46, 99] The resulting complexes are less able to activate DNA estrogen response elements in uterine tissue, but are nearly as effective as E2-ER complexes in activating functionally distinct estrogen response elements [86] such as those present in bone remodeling cells.

Compound **10** appears to interact differently than tamoxifen and raloxifene with the ER, especially regarding the positioning of its side chain. This is due in part to **10**'s inability to interact with a complimentary amino acid residue (Asp 351) in the hERα ligand-binding domain, with which 4-hydroxytamoxifen and raloxifene appear to bind through interaction with their protonated side chains. Furthermore, the side chain of **10** may interfere sterically with the ligand binding process. Despite these observations, the

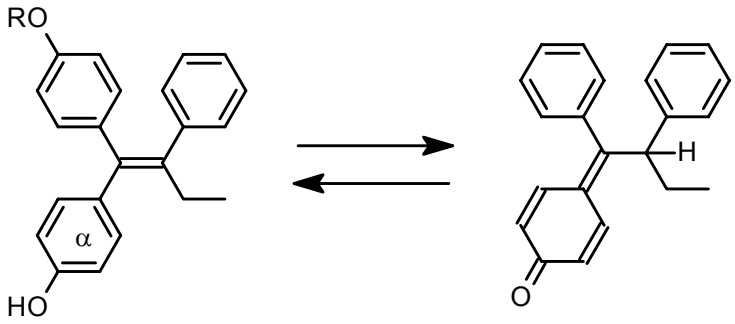


Figure 16. Isomerization of 4-hydroxylated triarylethylenes

current results indicate a similarity of differential bone/uterus estrogenic efficacy of **10** compared to tamoxifen and raloxifene, suggesting that the conformation of the ER ligand-binding domain liganded with **10** does not differ greatly from that arising from interactions with these SERMs.

Of the compounds prepared for this study, triarylethylenes **9** and **10** were the only two that showed any significant estrogenic activity in the OVX rat. "Tetrazole" **12** appeared to have a slight effect regarding body weight gain and uterotrophic activity (table 3), and also lowered serum Dpd levels, however, this effect was only of borderline ($p < 0.10$) significance. It was believed that administration of a higher dose of **12** may result in greater estrogenic efficacy, and possibly the observation of other estrogenic effects such as those seen with **9** and **10**, however, a compound that would require a daily dose higher than 10 μmol/kg (i.e. a less efficacious compound) was not of interest in this study.

The observed lack of systemic effects of **11** seems to be counter-intuitive. Its ER affinity was greater than that of HPPA, shown previously to have differential bone protective activity.[15] And its polarity, and thus predicted bioavailability, was estimated to be comparable to those of GW 7604, **9** and **10**, ER ligands shown previously [75, 76], or in this study to exhibit SERM-like effects. This may be due in part to poor efficacy of **11** due to excessive drug metabolism. It is speculated that **11** may be a better substrate for metabolic conjugation than either HPPA or **10**. However, the degrees to which HPPA, **10**, and **11** undergo metabolic conjugation have not been assessed.

The goal of this study was to modify the polarity of HPPA in order to identify ER ligands with SERM-like activity. The current results indicate that triarylethylene oxybutyric acids **9** and **10** are capable of such differential estrogenicity.

CHAPTER 5

EXPERIMENTAL

General. Solvents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI) and the University of Georgia Central Research Stores. Moisture-sensitive and air-sensitive reactions were carried out in flame or oven dried glassware under dry nitrogen atmosphere. Workup of organic extracts, filtrates and column fractions was carried out by concentration *in vacuo* at 40 °C. Analytical thin-layer chromatography (TLC) using 0.25 mm Analtech silica gel GF_{254} plates was used to monitor reaction progress and analyze column chromatography fractions and purity of products. TLC plates were developed using chloroform/2-propanol/glacial acetic acid (90/10/0.5, v/v/v) [solvent 1] or toluene/chloroform (50/50, v/v) [solvent 2], and viewed under UV light at 254 nm wavelength. Chromatographic mobilities are expressed as R_f values. Melting points were determined using an Electrothermal 9100 apparatus and are uncorrected. 400 MHz proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained using a Bruker AMX 400 spectrometer. NMR samples were prepared using acetone-d_6 as solvent unless otherwise stated. Chemical shifts (δ) are reported in parts per million and were calculated using tetramethylsilane as standard. Positive ion liquid secondary ion mass spectra (LSIMS) were obtained on a Micromass AutoSpec series-M high-resolution magnetic sector mass spectrometer of EBE geometry. Sample solutions

were prepared using glycerol as the matrix. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

Unless indicated otherwise, each unsaturated intermediate and final compound characterized in this study was composed of approximately equal amounts of its constituent geometric isomers. Furthermore, no attempt was made to resolve putative optical isomers of **11-13**.

Starting Materials. 4-hydroxy-4'-benzyloxy-benzophenone (**3b**) was prepared as reported.[20] 4-hydroxy-4'-(trimethylacetoxy)benzophenone (**3c**) was prepared by a modification of a previously reported procedure.[24] A mixture of 4,4'-dihydroxy-benzophenone (5 g, 23.3 mmol), potassium carbonate (3.2 g, 23.3 mmol) and trimethylacetylchloride (2.8 g, 3.0 mL, 23.3 mmol) in dry tetrahydrofuran (THF; 40 mL) was refluxed for 4 h. After cooling, the reaction was quenched with water (25 mL) and extracted with ethyl acetate (3 x 50 mL). The organic layer was dried with magnesium sulfate, filtered and concentrated to give a yellow oil which solidified upon standing at 8 °C. This was chromatographed on silica gel (45 g, CH_2Cl_2/EtOAc, 19/1). The first 190 mL of eluate was discarded. The next 220 mL was collected and concentrated to give a white solid (0.81 g, 12 %). TLC (solvent 1), one spot, R_f 0.64; ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H, Pv), 6.90, 7.17, 7.77, 7.80 (d, 8H, ArH); LSIMS m/z calcd for $C_{18}H_{19}O_4$ 299.1285 $(M+H)^+$, found 299.1264.

General Method for Olefination of 11a-c. 1-phenyl-1-(*p*-hydroxyphenyl)-2-phenylbut-1-ene (4a) was prepared by a procedure previously reported [23] with slight modifications. To a cold (-15 °C), stirred suspension of zinc powder (4.9 g, 75.5 mmol) in dry tetrahydrofuran (35 mL) was added slowly titanium tetrachloride (5.7 g, 3.3 mL,

30.2 mmol). The reaction mixture was refluxed for 2 h and then cooled to 40 °C at which point a solution of 4-hydroxybenzophenone (**3a**) (3.0 g, 15.1 mmol) and propiophenone (2.5 g, 2.5 mL, 18.2 mmol) in THF (15 mL) was added dropwise to the stirred suspension. The mixture was refluxed for another 4 h, then cooled and poured into an aqueous solution of 10% potassium carbonate (300 mL). After standing overnight, the mixture was filtered and the filtrate was concentrated to give 5.06 g (>100%) of yellow oil. This was used in the next step without further purification. TLC (solvent 2), one major spot (>95%), R_f 0.35.

By this same procedure the following compounds were prepared. Crude **1-[(*p*-(benzyloxyphenyl)-1-(4-hydroxyphenyl)-2-phenyl]ethene (4b)** was obtained as a golden oil after workup. This was chromatographed on silica gel (49 g, $CHCl_3$/toluene, 50/50). The product solidified from $CHCl_3$-hexanes as a white solid (34%): TLC (solvent 2), one spot, R_f 0.34; ^{1}H NMR ($CDCl_3$) δ 5.06 (s, 2H, CH_2Ph), 6.76-7.42 (m, 13H, ArH).

1-(4-trimethylacetoxyphenyl)-1-(*p*-hydroxyphenyl)-2-phenylbut-1-ene (4c) (69 %): TLC (solvent 2), one spot, Rf 0.20; ^{1}H NMR δ 0.89 (t, 3H, CH_2CH_3), 1.35 (s, 9H, Pv), 2.46 (q, 2H, CH_2CH_3), 6.49-6.70 (d, 4H, C_6H_4OH), 7.10-7.29 (m, 9H, ArH); LSIMS m/z calcd for $C_{27}H_{28}O_3$ 400.2038 ($M^{+\cdot}$), found 400.2032.

1,2-diphenyl-1-(*p*-hydroxyphenyl)ethene (4d) (30 %): TLC (solvent 2), one spot, R_f 0.29; ^{1}H NMR ($CDCl_3$) δ 1.59 (br s, 1H, OH), 6.77-7.65 (m, 14 H, ArH).

General Method for Alkylation of Phenols 4a-d. The synthesis of ***Z*-4-[*p*-(1,2-diphenyl-1-butenyl)phenoxy]-*n*-butanoic acid (9)** is typical. To a solution of **4a** (2.53 g, 8.45 mmol) in acetone (20 mL) was added potassium carbonate (1.4 g, 10.1 mmol) and ethyl-4-bromobutyrate (4.45 g, 3.25 mL, 22.7 mmol). The reaction mixture was refluxed

while stirring for 6 h, and then cooled to room temperature, filtered and concentrated. The resultant yellow syrup was dissolved in dioxane (15 mL) and 10% aqueous NaOH (15 mL) was added. After 1 h of stirring the reaction was cooled in an ice bath and 10% aqueous HCl was added until the mixture was slightly acidic. The suspension was extracted with ether (3 x 20 mL). After workup, the product was crystallized from hot $CHCl_3$-hexanes at 8 °C and collected as white crystals (1.27 g, 39.0 %). TLC (solvent 1), one spot, R_f 0.50; mp 120.1-125.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3H, $CH_2\mathit{CH_3}$), 2.03 (m, 2H, $\mathit{CH_2}CH_2COOH$), 2.42-2.52 (m, 4H, $\mathit{CH_2}CH_3$, CH_2COOH), 3.86 (t, 2H, $\mathit{CH_2}CH_2CH_2COOH$), 6.51, 6.76 (d, 4H, C_6H_4OH), 7.08-7.35 (m, 10H, ArH); LSIMS m/z calcd for $C_{26}H_{26}O_3$ 386.1881 ($M^{+\cdot}$), found 386.1863. Anal. ($C_{26}H_{26}O_3 \cdot 0.25\ H_2O$) C, H.

By this same procedure the following compounds were prepared. ***Z*-4-{[1-(*p*-benzyloxyphenyl)-2-phenyl-1-ethenyl]phenoxy}-*n*-butanoic acid (5)** crystallized from $CHCl_3$-hexanes at 8 °C (52%): TLC (solvent 1), one spot, R_f 0.75; ^{1}H NMR ($CDCl_3$) δ 2.13 (m, 2H, $\mathit{CH_2}CH_2COOH$), 2.60 (t, 2H, $\mathit{CH_2}COOH$), 4.00 (t, 2H, $\mathit{CH_2}CH_2CH_2COOH$), 5.06 (s, 2H, CH_2Ph), 6.81-7.46 (m, 18H, ArH); nominal mass calcd for $C_{31}H_{28}O_4$ 464, LSIMS m/z found 464 ($M^{+\cdot}$).

4-{[1-(*p*-hydroxyphenyl)-2-phenyl-1-butenyl]phenoxy}-*n*-butanoic acid (10). A solution of **4c** (0.93 g, 2.33 mmol) in acetone (15 mL) was alkylated with ethyl-4-bromobutyrate (2.27 g, 1.67 mL, 11.63 mmol). Upon saponification with 10% aqueous NaOH (20 mL) in dioxane (20 mL), and workup, the crude extract was shaken with 25 mL of ether and 3 x 20 mL 0.05 M potassium phosphate buffer, pH 7.04. The ether layer was worked up and the residue was crystallized from ether-petroleum ether upon standing

at 8 °C for several days. Three batches of white crystals (0.37 g, 40%) were collected: TLC (solvent 1), one spot, R_f 0.51; mp 141.6-147.6 °C; ^{1}H NMR ($CDCl_3$) δ 0.92 (t, 3H, CH_2CH_3), 2.02, 2.13 (q, 2H, CH_2CH_2COOH), 2.44-2.62 (m, 4H, CH_2CH_3, CH_2COOH), 3.87, 4.03 (t, 2H, $CH_2CH_2CH_2COOH$), 6.45, 6.51, 6.71, 6.73, 6.75, 6.79, 6.85 (d, 7H, 1.75 O-C_6H_4), 7.08-7.25 (m, 6H, C_6H_5, 0.25 O-C_6H_4); LSIMS m/z calcd for $C_{26}H_{26}O_4$ 402.1831 ($M^{+\cdot}$), found 402.1828. Anal. ($C_{26}H_{26}O_4 \cdot 0.5\ H_2O$) C, H.

4-(1,2-diphenyl-1-ethenyl)phenoxyacetic acid (6) solidified from $CHCl_3$-hexanes at 8 °C (56 %): TLC (solvent 1), one spot, Rf 0.55; ^{1}H NMR ($CDCl_3$) δ 4.70 (s, 2H, CH_2COOH), 6.85-7.32 (m, 14H, ArH).

***Z*-4-[1-(*p*-benzyloxyphenyl)-2-phenyl-1-ethenyl]phenoxyacetonitrile (7)** was synthesized by a similar procedure as described above. A solution of **4b** (1.03 g, 2.72 mmol) in acetone (25 mL) was stirred with K_2CO_3 (1.09 g, 2.9 mmol) and bromoacetonitrile (1.45 g, 0.84 mL, 12.1 mmol). The mixture was refluxed for 2 h after which it was filtered. The filtrate was then concentrated to give a yellow oil (1.8 g), which solidified upon standing. This was dissolved in toluene and the solution was filtered through a silica gel column (36 g, toluene) to remove impurities. A total of 250 mL of eluate were collected, combined and concentrated to give white crystals (1.0 g, 88%). TLC (solvent 1), one spot, R_f 0.83; ^{1}H NMR ($CDCl_3$) δ 4.77 (s, 2H, CH_2CN), 5.07 (s, 2H, CH_2Ph), 6.81-7.44 (m, 13H, ArH).

***Z*-1-(*p*-benzyloxyphenyl)-1-[4-(1H-tetrazol-2-ylmethoxy)phenyl]-2-phenyl-ethene (8)**. A solution of **7** (1.0 g, 2.4 mmol) and NH_4Cl (0.38 g, 7.2 mmol) in dimethylformamide (DMF; 25 mL) was stirred and NaN_3 (0.31 g, 4.8 mmol) was carefully added. The reaction was refluxed for 48 h, then filtered and concentrated to give

a brown oil. The oil was dissolved in DMF (20 mL) and 10% aqueous HCl (10 mL) was added. The mixture was extracted with ether (3 x 15 mL). The combined ether extracts were shaken with 10% aqueous NaOH (30 ml). The aqueous extract was acidified to pH 4 by addition of 10% HCl. A white solid separated. This was filtered: (0.9 g, 82 %). TLC (solvent 1), one spot, R_f 0.56; ^{1}H NMR δ 5.14 (s, 2H, CH_2Ph), 5.56 (s, 2H, CH_2-Ar), 6.91-7.27 (m, 13H, ArH).

Catalytic Hydrogenation/Hydrogenolysis of 5, 6 and 8. To a solution of **5** (0.7 g, 1.5 mmol) in ethanol (40 mL) and THF (10 mL) was added 10% palladium on activated carbon (70 mg). The mixture was shaken under ~45 psi of H_2 for 1.5 h. The mixture was filtered after addition of methylene chloride (20 mL). The filtrate was concentrated *in vacuo* to give a yellow oil. This was chromatographed on silica gel (25 g, 15% EtOAc in hexanes) to give **4-{4-[1-(*p*-hydroxyphenyl)-2-phenylethyl]phenoxy}-*n*-butanoic acid (11)** (0.34 g, 59.6%) which crystallized from $CHCl_3$-hexanes. TLC (solvent 1), one spot, R_f 0.72; mp 124.7-128.8 °C; ^{1}H NMR δ 2.47 (t, 2H, *CH_2*COOH), 3.29 (d, 2H), 3.97 (t, 2H, *CH_2*CH_2CH_2COOH), 4.17 (t, 1H), 6.70, 6.79 (d, 4H, C_6H_4OH), 7.00-7.17 (m, 9H, ArH); nominal mass calcd for $C_{24}H_{24}O_4$ 376, LSIMS m/z found 377 $(M+H)^+$. Anal.($C_{24}H_{24}O_4 \cdot H_2O$) C, H.

By this same procedure the following compounds were prepared. **4-(1,2-diphenylethyl)phenoxyacetic acid (13)** (28%): TLC (solvent 1), one spot, R_f 0.57; mp 161.0-167.0 °C; ^{1}H NMR δ 3.36 (d, 2H), 4.31 (t, 1H), 4.64 (s, 2H, *CH_2*COOH), 6.82 (d, 2H, 0.5 O-C_6H_4), 7.07-7.32 (m, 12H, 0.5 O-C_6H_4, ArH). Anal. ($C_{22}H_{20}O_3 \cdot H_2O$) C, H.

1-(*p*-hydroxyphenyl)-1-[4-(1H-tetrazol-4-ylmethoxy)phenyl]-2-phenyl-ethane (12) crystallized as white crystals from acetone-H_2O at 8 °C (72%): TLC (solvent 1), one

spot, R_f 0.40; mp 108.2-109.5 °C; ^{1}H NMR δ 3.33 (d, 2H), 4.24 (t, 1H), 5.49 (s, 2H, CH_2-Ar), 6.73, 6.95 (d, 4H, C_6H_4OH), 7.12-7.17 (m, 7H, C_6H_5, 0.5 O-C_6H_4), 7.26 (d, 2H, 0.5 O-C_6H_4); LSIMS m/z calcd for $C_{22}H_{21}N_4O_2$ 373.1664 $(M+H)^+$, found 373.1643. Anal. ($C_{22}H_{20}N_4O_2 \cdot 2H_2O$) C, H, N.

Estrogen Receptor Affinity. The ability of **9-13**, and standard ER ligands tamoxifen, raloxifene, and tamoxifen-"bisphenol", to displace specifically bound [^{3}H]17β-estradiol from human recombinant ERα was determined as described previously.[21]

Animal Studies. OVX Sprague Dawley rats (10-12 weeks old) were obtained from Harlan, Inc., Indianapolis, IN. Animals were housed and fed as described [8] at the AAALAC accredited animal facility at the University of Georgia College of Pharmacy, in accordance with a protocol approved by the University of Georgia Institutional Animal Care and Use Committee. Test compounds (**10-13**) for subcutaneous (sc) dosing were administered in 5% benzyl alcohol - corn oil. For oral (po) dosing, compounds **9** and **10** were each dissolved in 80% ethanol containing an equimolar amount of tromethamine. At the time of use, such stock solutions were diluted to 1/10 the original compound concentration by addition of 0.11% aqueous methylcellulose. Daily dosing was carried out five days per week for three weeks. Each treatment group had seven animals, and each experiment included groups treated in turn with vehicle or estrogen, in addition to groups receiving test compounds.

At the end of the 21-day study period, the animals were euthanized under carbon dioxide. The body weight of each animal was recorded. Blood was aspirated by syringe from the abdominal aorta and allowed to coagulate in a Vacutainer tube at room

temperature for 2 h. Serum was obtained by centrifugation for 10 min at 3000 rpm, and samples were stored at -80 °C until analyzed for OC or Dpd. Uterine tissue was removed, dissected free of fat and connective tissue, and weight was recorded.

For drug metabolism studies, livers from four of the aqueous vehicle-treated animals were dissected, combined, minced, and homogenized in three volumes of 1.15% ice cold aqueous KCl using a tissue homogenizer. The homogenate was centrifuged at 9000 x g for 25 min at 4 °C. Aliquots (5 mL) of the supernatant (9S fraction) were lyophilized and stored at -80 °C prior to use.

ELISAs for Serum OC and Dpd. Serum samples were thawed by placing containers on ice for 2 h. Properly diluted samples were assayed for OC using an enzyme immunoassay kit (Biomedical Technologies, Inc., Stoughton, MA). The procedure was carried out in a 96 well polystyrene plate in which a monoclonal antibody to the N-terminal region of rat OC was bound to each well surface. After overnight incubation with the diluted serum sample, wells were washed and incubated with a second antibody (goat polyclonal), which interacted with the C-terminal region of the immobilized OC. Subsequent incubation with horseradish peroxidase (HRP) conjugated donkey-anti goat IgG, and then a solution of HRP substrate, 3,3',5,5'-tetramethylbenzidine, was carried out. Absorbance at 405 nm, which accompanied substrate oxidation, was determined using a plate reader. The amount of OC in the sample was calculated by comparing its absorbance with those of standards, which contained known amounts of rat OC. Absorbance intensity was directly proportional to the amount of OC present in the sample.

Alternatively, thawed serum samples were analyzed for total Dpd, using a hydrolysis/competitive enzyme immnunoassay procedure (Metra Biosystems, Inc., Mountain View, CA). Each serum sample was mixed with 6 N HCl plus solubilizing agent. Precipitated protein was separated by centrifugation at 10,000 x g for 10 min. An aliquot of the supernatant was heated at 99 °C for 18 h to hydrolyze that portion of serum Dpd linked to polypeptides. This was neutralized by addition of 10 N NaOH, and an aliquot was transferred to the 96 well assay plate, each well containing monoclonal anti-Dpd antibody. Then a fixed amount of Dpd-alkaline phosphatase conjugate was added. After a 2 h incubation, wells were washed and a solution of *p*-nitrophenyl phosphate was added. After a second 2 h incubation, alkaline stop solution was added and absorbance of formed *p*-nitrophenoxide was determined at 405 nm using a plate reader. The amount of Dpd in the sample was calculated by comparing absorbance of the sample with that of standards, run in parallel, which contained known amounts of Dpd. Absorbance intensity was inversely proportional to the amount of Dpd originally present in the sample.

Colorimetric Assays for Serum Cholesterol and Triglycerides. Materials and methods for these determinations were obtained from Sigma Chemical Co., St. Louis, MO. Total serum cholesterol was determined by initial enzymatic hydrolysis of esterified cholesterol, followed by cholesterol oxidase catalyzed formation of an equimolar amount of hydrogen peroxide that accompanied the conversion of cholesterol to cholest-4-en-3-one. The hydrogen peroxide produced was coupled with 4-aminoantipyrine and *p*-hydroxybenzenesulfonate, in the presence of horseradish peroxidase, to give a quinoneimine adduct which was quantitated at 500 nm. Amounts of cholesterol in serum samples were calculated by comparing absorbances of the sample with those of

standards, run in parallel, which contained known amounts of cholesterol. All samples and standards were run in duplicates.

Serum triglycerides were determines by initial enzymatic formation of glycerol, followed by sequential enzymatic conversion of this to dihydroxyacetone phosphate, with concominant reduction of NAD to NADH. Diaphorase-catalyzed dehydrogenation of NADH was accompanied by simultaneous oxidation of 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride to formazan, which was quantitated at 500 nm. Amounts of triglycerides in serum samples, run in duplicates, were calculated by comparing sample absorbance with those of triglyceride standards, run in parallel.

Biotransformation of 9. Metabolism of **9** with 9000 x g in supernatant (9S) fraction prepared from pooled livers of vehicle-treated OVX rats was carried out as follows. Triplicate incubations were run in 12 x 75 mm polypropylene tubes. The standard incubation mixture (1.0 mL) contained 20 mM potassium phosphate buffer, pH 7.05, 90 mM potasssium chloride, 5 mM magnesium chloride, 0.4 mM NADP, 6.5 mM glucose 6-phosphate, and 9S fraction equivalent to 50 mg of wet liver. Each incubation was started by addition of **9** in 20 μL of DMF to give a final concentration of 0.1 mM (38 μg/mL). In control incubations, either the cofactor mixture (NADP and glucose-6-phosphate), or **9**, was omitted. Incubations were shaken at 70 cycles/min at 37 °C for 20 min, and then to each was added 0.1 mL of 50 mM EDTA disodium salt. Each mixture was vortexed and poured into 3 mL of methanol. The mixture was shaken for 5 min and then centrifuged for 10 min at 450 x g. The supernatant was concentrated at 40 °C to low volume under a stream of compressed nitrogen gas, and the aqueous concentrate was lyophilized. The residue was dissolved in 1 mL of water and the mixture was shaken for

5 min with 3 mL of ether. The mixture was centrifuged for 10 min at 450 x g. The ether layer (2.0 mL) was concentrated as before. The residue was reconstituted in 100 uL of HPLC mobile phase and subjected to high performance liquid chromatography (HPLC). Column: 4.6 x 250 mm stainless steel, packed with 10 μm Whatman ® Partisil ® ODS-3 (Mitchell modification); mobile phase: MeOH - 40 mM sodium phosphate buffer, pH 2.45 (67/33, v/v), 1.0 mL/min; UV detection at 277 nm; 20 μL flushed loop injection. Retention times (relative % area) for the geometric isomers of **10** were 32.7 min (47%) and 35.2 min (53%).

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