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Identification of New Triarylethylene Oxyalkanoic Acid Analogues as Bone Selective Estrogen Mimetics

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Abstract—Previously, the estrogen receptor (ER) ligand 4-[1-(*p*-hydroxyphenyl)-2-phenylethyl]phenoxyacetic acid (**5**) was found to have differential bone loss suppressive effects in the ovariectomized (OVX) rat approaching those of selective ER modulators (SERMs) such as tamoxifen. In an effort to improve efficacy, analogues of this compound were prepared which incorporated features designed to reduce polarity/ionizability. Thus, the acetic acid side chain of **5** was replaced by *n*-butanoic acid and 1*H*-tetrazol-4-ylmethyl moieties, to give **8** and **10**, respectively. Also, the phenolic hydroxyl of **5** was replaced, giving deoxy analogue **9**. We also developed new methods for the synthesis of triarylethylene variants of **5** and **9**, namely 4-{[1-(*p*-hydroxyphenyl)-2-phenyl-1-butenyl]phenoxy}-*n*-butanoic acid (**6**) and its des-hydroxy counterpart (**7**), because the former of these had in vitro antiestrogenic effects characteristic of known SERMs. In the OVX rat, **6** and **7** were as effective as 17β-estradiol in suppressing serum markers of bone resorption/turnover, namely osteocalcin and deoxypyridinoline, but had only 30% of the uterotrophic efficacy of 17β-estradiol. This study has thus identified two triarylethylene oxybutyric acids, 6 and 7, that have differential bone/uterus effects like those of known SERMs.

Introduction

Estrogens are the endogenous ligands for the estrogen receptor (ER). They exert a direct effect on the female reproductive organs (uterus, ovaries, and breast) as well as several other ER-containing tissues such as those of the skeletal and cardiovascular systems. The decreased ovarian production of estrogen characteristic of menopause leads to numerous health complications in both menopausal and postmenopausal women. Estrogen replacement therapy (ERT) is widely used to alleviate some of the discomforts associated with menopause and to prevent osteoporosis.[1] However, ERT is associated with an increased risk of breast and endometrial cancer.[2]

As an alternative to ERT, selective estrogen receptor modulators (SERMs) offer the advantage of acting as estrogen agonists in extra-reproductive tissue (liver, bone, and brain) while having no effect or antagonizing the effects of estrogen in reproductive tissue (uterus, breast). The first SERM to be used clinically, tamoxifen (**1**) is widely used for the prevention and treatment of breast cancer due to its antiestrogenic effect on ER-positive breast cancer cells.[3] But **1** was a full estrogen mimetic in the skeletal system, thus protecting against bone loss in postmenopausal women.[4] A newer SERM, raloxifene (**2**) was recently introduced for the prevention of postmenopausal osteoporosis. Although **2** has some advantages over **1** and ERT,[5a,6] both **1** and **2** possess significant therapeutic drawbacks. For example, neither of these SERMs prevents, and in fact might actually intensify, a common disquieting symptom of estrogen deficiency known as hot flashes or hot flushes.[7] Thus, identification of novel SERMs remains a priority (Fig. 1).

The OVX rat has become a widely used animal model for determining selective estrogen activity of ER ligands. Specifically, it is used as a model of osteopenia associated with estrogen deficiency.[8] OVX rats treated with 17β-estradiol (E2), ethynyl estradiol (EE2), **1** or **2** show a decreased rate of bone turnover and maintenance of normal bone mass compared to untreated animals.[5,9,10] Similar results are seen clinically with postmenopausal patients on ERT[11] or taking **1**[4] or **2**[12] for extended periods of time. The OVX rat also serves as a model for measuring uterotrophic effects of ER ligands.

Estrogens and SERMs exert their bone protecting effects by reducing the rate of bone turnover (formation and resorption). The degree to which these processes are occurring can be monitored by determining the levels of osteocalcin (OC) and deoxypyridinoline (Dpd).[13] OC is

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a polypeptide produced by osteoblasts during bone formation, and a significant percentage is released into the blood.[14] Dpd is released into the blood during breakdown of bone collagen, which accompanies resorption.[15] In the OVX rat, the degree to which ER ligands are able to suppress serum OC and Dpd parallels their ability to suppress bone density loss and maintain other bone histomorphometric parameters.[8,16,17] Thus, assessment of these serum markers can be used to identify bone protective ER ligands subsequent to more time-consuming bone densitometric and microanatomical studies.

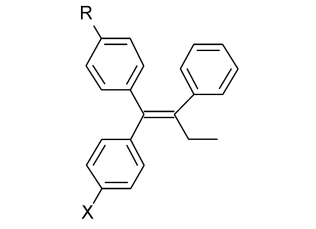
Analogues of **1** bearing acidic side-chain substituents have also been found to have selective estrogenic activity. Arylacrylic acid (**3**) was found, like **1** and **2**, to prevent against bone loss in OVX rats while displaying dominant estrogen antagonist activity in the uterus.[18] The oxyacetic acid side-chain analogues of **1** (**4** and **5**) were found to be estrogen agonists in MCF-7 human breast cancer cells,[19,20] a line of estrogen responsive cells used to assess potency and efficacy of ER ligands. Nevertheless, **5** was determined to be a selective estrogen in the OVX rat, having bone protecting effects approaching those of **1** while displaying no uterotrophic effects.[8] Extension of the acidic side-chain-substituent of **5** resulted in oxybutyric acid analogue **6**. In MCF-7 cells, **6** was found to be a full estrogen antagonist with greater potency than **1**.[21] These findings show how small structural modifications in these acidic side-chain analogues can have a profound effect on their agonist/antagonist balance, and they suggest that **6**, like **3**, might be a SERM.

Accordingly, we wanted to determine whether **6**, like **5**, was capable of selective bone-protective estrogenicity in the OVX rat. Also, because **5** had less bone-protective efficacy in the OVX rat than did E2, we prepared and evaluated **8** and **10**, in which **5**'s highly ionizable oxyacetic acid side chain was replaced with less acidic oxybutyric acid or oxymethyltetrazole moieties. And, because **3** and **4** were suggested to be 'active metabolites' produced by facile 4-hydroxylation of their nonphenolic precursors in the OVX rat,[18b,22] we chose to prepare and evaluate the bone-protective and uterotrophic effects of **9** and **7**, the putative precursors, in turn, of **5** and **6**.

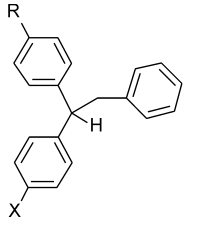
Results

Synthesis

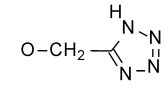
Scheme 1 outlines the approach used to prepare compounds **6**–**10**. The triarylethylene backbone of these compounds was obtained through application of the McMurry olefination reaction using low-valent titanium[23] to cross-couple substituted benzophenones (**11a**–**c**) with propiophenone or benzaldehyde. The desired intermediates (**12a**–**d**) were separated from self-coupling byproducts by column chromatography when needed. Introduction of the side chain of **6**, **7**, and **13**–**15** was carried out by *O*-alkylation of monophenols **12a**–**d**. Reaction of **15** with sodium azide and ammonium chloride[24] followed by debenzylation/hydrogenation, afforded tetrazole **10**.



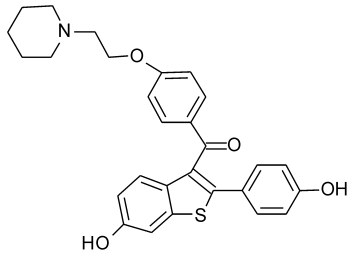
	R	X
1	$OCH_2CH_2N(CH_3)_2$	H
3	CH=CH-COOH	OH
4	OCH_2COOH	OH [a]
6	$OCH_2CH_2CH_2COOH$	OH [a]
7	$OCH_2CH_2CH_2COOH$	H



5	OCH_2COOH	OH
8	$OCH_2CH_2CH_2COOH$	OH
9	OCH_2COOH	H



10 OH



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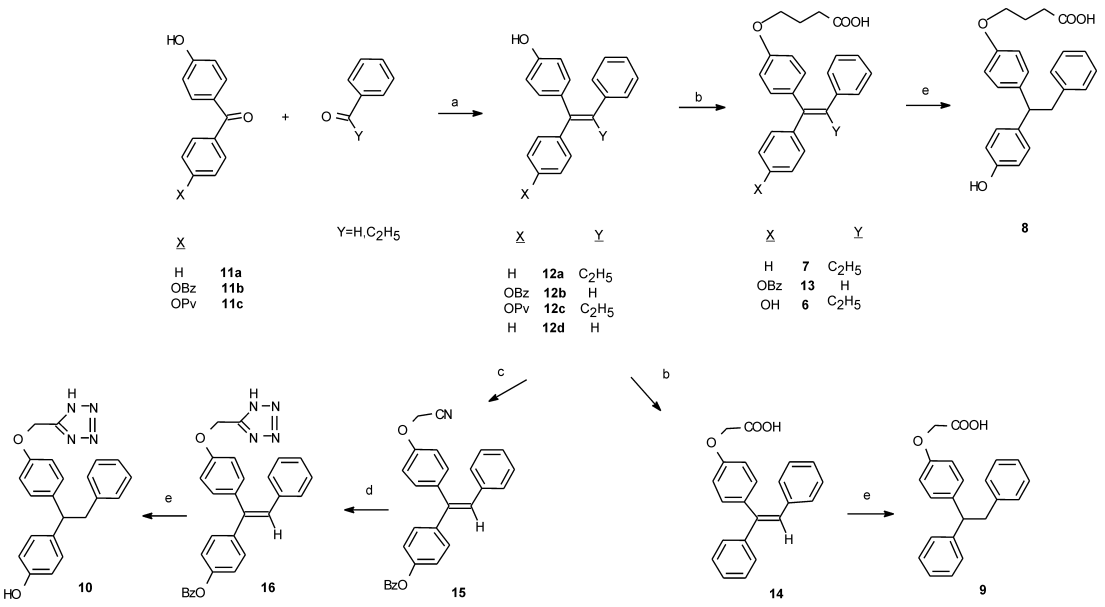
[a] 1:1 mixture of *Z* isomer (shown) and its *E* counterpart.

Figure 1. Structure of selected ER ligands.



Scheme 1. (a) Ti, THF; (b) $Br(CH_2)_3COOC_2H_5$ or $BrCH_2COOC_2H_5$, K_2CO_3, acetone, then NaOH, aqueous dioxane; (c) $BrCH_2CN$, K_2CO_3, acetone; (d) NaN_3, DMF; (e) H_2, 10% Pd/C, THF/MeOH.

Previously, synthesis of **6** was carried out starting with **11b** and propiophenone, followed by a debenzylation step.[21] The product so obtained was difficult to purify and the overall yield (4%) was not satisfactory. Use of a pivaloyl (Pv) instead of a benzyl (Bz) phenolic protecting group resulted in improvement in overall yields to 28% after crystallization. Removal of the excess 4-bromobutyric acid and pivalic acid from the final product was accomplished by differential liquid–liquid extraction of **6**, taking advantage of **6**'s greater relative lipophilicity. The starting benzophenone (**11c**) was prepared by a modification of the previously reported alkylation procedure.[25]

The configuration of final compounds (*E*/*Z* isomerism, when relevant) was determined by the relative chemical shift of the side chain *O*-methylene protons in NMR spectra. For example, the spectrum of **7** contained a single *O*-methylene triplet centered at 3.87. This was assigned to the *Z* configuration of **7** (as shown in Scheme 1) based on correlation with *O*-methylene shifts of corresponding geometric isomers of **1** as well as other acidic-side chain analogues.[21] Similarly, the spectrum of **6** contained two *O*-methylene triplets of about equal intensities, centered at 3.87 and 4.03, corresponding to the *Z* and *E* isomers of **6**, respectively.

Differences in ionizability (acidity) of **6**–**8** and **10**, compared to **5**, were estimated from the pK_as of their respective phenoxy-substituted variants.[26] The pK_as 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, **10** was 2.3 times less acidic than **5**; and **6**–**8** were each calculated to be 20-fold less acidic than **5**. Furthermore, the side chain acidity of **6**–**8** was suggested to be comparable to that of **3** whose pK_a, extrapolated from that of *trans*-cinnamic acid,[26a] was around 4.40.

ER Affinities

In the present series of acidic triarylethylenes, compound **6** exhibited the highest ER affinity, considerably greater than that of its nonphenolic analogue (**7**) and about one-tenth that of E2 (Table 1). Replacement of the oxyacetic acid side chain in **5** with an oxybutyric acid moiety (**8**) resulted in about a 70% increase in affinity; **5**'s oxymethylenetetrazole analogue **10** had about 25% the ER affinity of **5**.

Estrogenic effects

In the OVX rat, **6** and its nonphenolic counterpart **7** exhibited similar bone protective efficacies, which were equivalent to that of E2 (Table 2). But the uterotrophic efficacy of either **6** or **7**, measured with respect to vehicle treated controls, was only about 25–30% that of E2 or EE2. The observed efficacy of **6** in bone and uterus was independent of its route of administration.

Compound **10**, the 'tetrazole' analogue of **5**, had only a weak uterotrophic effect. Unfortunately, it had no effect on serum OC, and its ability to reduce serum total Dpd was of only borderline ($P<0.10$) significance. Neither **8**

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

compd	1	2	5	6	7	8	9	10	E2
RBA[a]	6.7	94	0.91	10.3	0.35	1.57	[b]	0.26	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 RBA value is the average of three separate determinations in which calculated individual values differ by $<10\%$.

[b]At 10 μM, **9** displaced 54% of specifically bound [^{3}H]E2.

Table 2. Skeletal and reproductive tract effects of acidic triarylethylene derivatives in the OVX rat

Compd	Dosing route[a]	Uterine wet wt., mg	Serum OC, ng/mL	Serum total Dpd, pmol/mL
6	sc; po	167 (25[b])[cd]; 184 (17)[cd]	76 (4)[c]; 62 (8)[cd]	7.4 (0.8)[c]; 12.3 (3.9)[c]
7	po	195 (30)[c,d]	59 (5)[c,d]	10.7 (1.2)[c]
8	sc	75 (13)	92 (10)	12.1 (2.3)
9	sc	72 (8)	96 (6)	11.8 (1.7)
10	sc	123 (14)[c]	93 (9)	8.7 (1.5)
E2	sc	355 (73)[c]	65 (12)[c]	6.3 (1.4)[c]
EE2	po	428 (53)[c]	43 (4)[c]	11.0 (2.3)[c]
vehicle	sc; po	87 (14); 102 (10)	93 (9); 84 (7)	11.3 (1.7); 18.3 (2.9)

[a]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.
[b]Standard deviations are in parentheses.
[c]$P < 0.05$ with respect to relevant vehicle-treated control.
[d]$P < 0.05$ with respect to relevant estrogen-treated control.

nor **9**, the oxybutyric acid and nonphenolic analogues of **5**, exhibited any observable effect on uterine weight or the serum markers of bone resorption.

Biotransformation of 7

Components with HPLC retention times of 32.7 and 35.2 min, inferred to correspond to the *Z* and *E* isomers of **6**, respectively (see Discussion), were observed in extracts obtained from incubation of **7** with rat liver 9S fraction. These were observed only when both **7** and the cofactor (NADPH) for oxidative drug metabolism were present in incubation mixtures. These retention times corresponded to those of authentic **6**, and the chromatographic peak intensity of each of these extract components was increased by dilution with **6**. However, the respective relative peak areas of these extract components (70 and 30%) differed from those in **6** (47 and 53%).

The observed conversion rate of **7** to components chromatographically identical to **6** in cofactor-enriched incubations with 9S liver fractions was 2.5% per 20 min.

Discussion

In the OVX rat, compounds **6** and **7** were comparable to established SERMs **1** and **2** in terms of degree of selective bone/uterus estrogenic efficacy.[10b,17] Thus, like **1** and **2**, these triarylethylene oxybutyric acids suppressed serum markers of bone resorption/turnover (Dpd and OC) 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. Administered orally, **7** was equivalent to **6** regarding the magnitude of its differential estrogenic effects, despite its low ER affinity (Table 1). This might be a consequence of in vivo enzymatic hydroxylation. In the present study, **7** was suggested to undergo regioselective 4-hydroxylation to **6** in the presence of liver enzymes from OVX rats. The rate of 4-hydroxylation of **7** was similar to that of **1** in the presence of rat liver enzymes.[27] And 4-hydroxy **1**, a major in vivo metabolite of **1**, had an RBA at least 50 times greater than **1** for rat uterine ER.[28] But **1** was also metabolized oxidatively in the rat to a host of other Phase 1 metabolites that, based on their ER affinities and accumulation in ER-containing tissues, might contribute to its observed effects.[28] Thus, unequivocal support for the proposition that **7** is a prodrug of **6** would seem to require systematic whole-animal biotransformation studies of **7**.

Although **7** was not subject to significant observable geometric isomerization, **6** (shown in Scheme 1 as the *Z* isomer) was always accompanied by its *E* isomer. Thus, hydrolysis of **6**'s *Z*-ester precursor (**12c**) under alkaline conditions (described below), or under mildly acidic conditions with prolonged reaction times at room temperature, gave **6** and its *E* isomer in ca. 1:1 ratios. Separate batches of **6**, prepared from **12c** by several repetitions of the current procedure, contained slightly varying amounts of **6**'s *E* isomer, as determined directly by ^{1}H NMR spectroscopy and confirmed by HPLC analysis. HPLC data signified, incidentally, that geometric isomerization of **6** in the chromatographic mobile phase was negligible. Also, 'metabolic' **6**, produced enzymatically from **7**, was found to have undergone isomerization to the extent of about 30%. Because of this tendency, no attempt was made to resolve **6** from its geometric isomer prior to biological evaluation. Configurationally stable geometric isomers of other triarylethylenes, such as **1** and its *E* isomer, exhibit contrasting ER affinities and estrogenic/antiestrogenic potencies and efficacies.[29,30] Thus, **6** might owe its observed ER affinity and bone protective and uterine effects in part to its *E* isomer.

We determined the interaction of **6**–**10** with human ERα (hERα), rather than with rat ER, because much recent information about the topography of hERα interactions with its ligands was available for comparison purposes. Compounds **7** and especially **9** each exhibited low affinity for hERα, presumably due to lack of an appropriately positioned phenolic substituent. Crystal structures of the hERα ligand binding domain (LBD) complexed with high affinity ligands E2 (or **2**) indicates the critical role of respective 3-hydroxy (6-hydroxy) groups in anchoring each of these ligands to ER by completing a hydrogen bonding network involving Glu 353 and Arg 394 of the hERa LBD.[31] Accordingly, affinity of phenolic ligand **6** was greater than that of **7**, whose

affinity is presumably supported only by hydrophobic and van der Waals interaction of its triarylethylene nucleus with amino acid residues lining the ER binding pocket that bear aliphatic chains or aromatic rings.

However, the hER RBA of **6** was less than that of high affinity ligands like **2**. Formal replacement of the butyric acid side chain of **6** with either a hydrogen or an *N*,*N*-dimethylaminoethyl chain gives **1** 'bisphenol' and 4-hydroxy **1**. These had respective hER RBAs of 76 (determined as a standard in the current study) and 123.[30] The higher ER affinity of 4-hydroxy **1** is due to the ability of its (protonated) side chain to interact by ionic bonding with Asp 351 of hERα LBD, in addition to participation of its 4-hydroxyl group in the hydrogen bonding triad specified above.[31,32] The reduced ER affinity of **1** bisphenol relative to 4-hydroxy **1** would therefore seem to be a consequence of the former's inability to make contact with Asp 351. The further reduction in ER affinity seen in **6**, considered in light of the above observations, indicates that the butyrate side chain of **6** not only is incapable of interaction with a complementary amino acid residue in the hERα LBD, but also interferes sterically with the ligand binding process.

The molecular basis for tissue-selective estrogenic effects expressed by **1**, **2** and other SERMs arises from the distinct conformation they induce in the LBD of the ER, compared to that induced by E2.[33] Inspection of crystal structures of SERM-liganded and E2-liganded hERα LBD indicates the former to have a more 'open' conformation than the latter, due to an inability of helix 12 of the LBD to fold down completely and enclose the ligand.[31,32] Such complexes are evidently less able to activate DNA estrogen response elements (EREs) in uterine tissue, but are nearly as effective as E2-ER complexes in activation of functionally distinct EREs[34] such as those in osteoblasts and osteoclasts, cells responsible for bone remodelling. In light of the analysis in the preceding paragraph, **6** interacts differently with the ER, especially with regard to positioning of its side chain, than do **1** and **2**. However, our results indicating a similarity of differential bone/uterus estrogenic efficacy of **6** compared to these SERMs suggest that the conformation of the ER LBD liganded with **6** does not differ greatly from that arising from interaction with **1** or **2**. However, arylacrylic acid **3** inhibited the (partial) agonist effects of 4-hydroxy **1**, and **2**, in an estrogen-responsive line of liver cells transfected with hER.[18b] This unique antagonist activity profile suggests a divergence in the way that triarylethylene carboxylic acids interact with the ER, compared to established SERMs **1** and **2**.

The observed lack of systemic effects of **8** seems to be counter-intuitive. Its ER affinity was greater than that of **5**, shown previously to have differential bone protective activity.[8] And its polarity (acidity) was estimated to be comparable to those of **3**, **6**, and **7**, ER ligands shown previously[18] or in the current study to exhibit SERM-like effects. Efficacy of ER ligands is in part determined by drug metabolism. For example, although **2** had about six times greater ER affinity than **1**, its bone-protective and uterotrophic potencies in the OVX rat were similar.[6] This appears to be a consequence of the ease with which **2** undergoes inactivation by glucuronide conjugation,[35] a process to which **1** is not directly susceptible. Analogously, it is speculated that **8** might be a better substrate for metabolic conjugation than either **5** or **6**. However, the degrees to which **5**, **6**, and **8** undergo metabolic conjugation have not been assessed.

The goal of this study has been to modify the structure and polarity of **5** in order to identify putative bone-protective ER ligands with reduced uterine effects relative to conventional steroidal estrogens. The current results indicate that **6** and **7** are capable of such differential estrogenicity. Evaluation of the potency of these ER ligands in regard to these and other ER mediated effects in the OVX rat will be the subject of a subsequent report.

Experimental

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. Work up 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 **8**–**10**.

Starting materials

4-Hydroxy-4′-benzyloxy-benzophenone (**11b**) was available as reported.[20] 4-Hydroxy-4′-(trimethylacetoxy)-

benzophenone (**11c**) 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 THF (40 mL) was refluxed for 4 h. After cooling, the reaction was quenched with water (25 mL) and extracted with ethyl acetate (3×50 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 (**12a**) 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 (**11a**) (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 (**12b**) 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 (12c). Yield 69%. TLC (solvent 2), one spot, R_f 0.20. ^{1}H NMR d 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 (12d). Yield 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 12a–d. The synthesis of *Z*-4-[*p*-(1,2-diphenyl-1-butenyl)phenoxy]-*n*-butanoic acid (**7**) is typical. To a solution of **12a** (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% aq HCl was added until the mixture was slightly acidic. The suspension was extracted with ether (3×20 mL). After work up, 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_2CH_3), 2.03 (m, 2H, CH_2CH_2COOH), 2.42–2.52 (m, 4H, CH_2CH_3, CH_2COOH), 3.86 (t, 2H, $CH_2CH_2CH_2COOH$), 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^+), 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 (**13**) 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, CH_2CH_2COOH), 2.60 (t, 2H, CH_2COOH), 4.00 (t, 2H, $CH_2CH_2CH_2COOH$), 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 (6). A solution of **12c** (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 work up, the crude extract was shaken with 25 mL of ether and 3×20 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 (14). Solidified from $CHCl_3$-hexanes at 8 °C (56%). TLC (solvent 1), one spot, R_f 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 (15).** Synthesized by a similar procedure as described above. A solution of **12b** (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-(1*H*-tetrazol-2-ylmethoxy) phenyl]-2-phenyl-ethene (16).** A solution of **15** (1.0 g, 2.4 mmol) and NH_4Cl (0.38 g, 7.2 mmol) in 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% aq HCl (10 mL) was added. The mixture was extracted with ether (3×15 mL). The combined ether extracts were shaken with 10% aq 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 13, 14, and 16. To a solution of **13** (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 (**8**) (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_2COOH), 3.29 (d, 2H), 3.97 (t, 2H, $CH_2CH_2CH_2$COOH), 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 (**9**) (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_2COOH), 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-(1*H*-tetrazol-4-ylmethoxy)phenyl]-2-phenyl-ethane (10). 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 **6**–**10**, and standard ER ligands **1**, **2**, and **1**-'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] Test compounds (**6** and **8**–**10**) for subcutaneous (sc) dosing were administered in 5% benzyl alcohol–corn oil. For oral (po) dosing, compounds **6** and **7** 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 study period, each animal was euthanized under carbon dioxide. 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 aq KCl using a tissue homogenizer. The homogenate was centrifuged at 9000×*g* for 25 min at 4 °C. Aliquots (5 mL) of the supernatant were lyophilized and stored at −80 °C prior to use.

Assays for 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 immunoassay 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×*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.

Biotransformation of 7. Metabolism of **7** with 9000×*g* in supernatant (9S) fraction prepared from pooled livers of vehicle-treated OVX rats was carried out as follows. Triplicate incubations were run in 12×75 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 **7** 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 **7**, 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×*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×*g*. The ether layer (2.0 mL) was concentrated as before. The residue was reconstituted in 100 μL of HPLC mobile phase and subjected to high performance liquid chromatography (HPLC). Column: 4.6×250 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 **6** were 32.7 min (47%) and 35.2 min (53%).

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