3-[4-(1,2-Diphenylbut-1-enyl)phenyl]acrylic Acid: A Non-Steroidal Estrogen with Functional Selectivity for Bone over Uterus in Rats

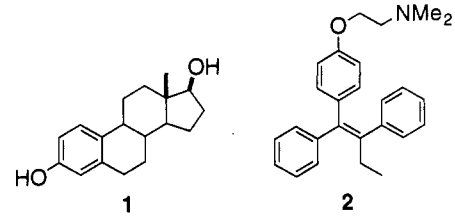
Timothy M. Willson,*,† Brad R. Henke,† Tanya M. Momtahen,† Paul S. Charifson,⊥ Kenneth W. Batchelor,‡ Dennis B. Lubahn,‡ Linda B. Moore,‡ Beverly B. Oliver,‡ Howard R. Sauls,§ James A. Triantafillou,§ Steven G. Wolfe,§ and Philip G. Baer§

Glaxo Research Institute, Five Moore Drive, Research Triangle Park, North Carolina 27709

Received March 7, 1994

The female sex hormone estradiol (1) has a variety of beneficial and detrimental effects in women.[1] The triphenylethylene class of non-steroidal estrogens (e.g., tamoxifen, 2) shows tissue-dependent expression of estrogen agonist and antagonist activity and may represent a significant advance over conventional hormone replacement therapy with 1 for prevention of osteoporosis and cardiovascular disease in postmenopausal women[2] (Figure 1). The estrogen receptor is a ligand-activated transcription factor that belongs to the steroid/retinoid family of DNA-binding intracellular receptors (ICR). Studies with deletion and point-mutated receptors have revealed two independent transcription activation domains (AF-1 and AF-2, Figure 2) within the receptor that allow the expression of cell- and promoter-specific agonist activity in transient cotransfection experiments *in vitro*.[3] The translation of these observations to the design of ligands for ICRs that show tissue-specific expression of functional activity is at the forefront of modern endocrinology.[4] For this purpose, we formulated the hypothesis that the tissue-selective profile of 2 was due to induction of a unique receptor conformation[5] in which the antagonist activity in some tissues was due to disruption of AF-2, mediated by a H-bond interaction[6] with the receptor protein in the region of the putative AF-2 α-helix,[3c,7] and the agonist activity in other tissues was a result of a functional AF-1 domain.[3b,d] Combining this hypothesis with analysis of the *in vitro* and *in vivo* pharmacology of non-steroidal estrogens,[8] it was proposed that the stilbene portion of 2 was required for AF-1 activity leading to agonist activity in bone, and the ethanolamine side chain was responsible for blocking AF-2 activity leading to antagonism in the uterus. We report here on the use of this hypothesis to identify triphenylethylene estrogens that show full agonist activity in bone through inhibition of bone loss in ovariectomized rats but which are antagonists in the rat uterus with minimal residual agonist activity.

We elected to synthesize analogs of the triphenylethylene 2 in which the ethanolamine side chain was replaced by alternate H-bond acceptor groups and the degree of conformational freedom was reduced. Following the general synthetic strategy of Miller[9] for synthesis of (*Z*)-tamoxifen, bromide 3 was coupled with arylboronic acid



1 2

Figure 1.

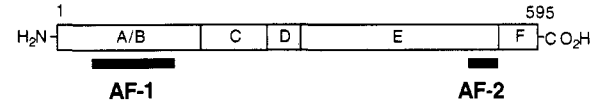


Figure 2. Structure of the human estrogen receptor. Key: A/B, N-terminal domain; C, DNA-binding domain; D, hinge region; E, ligand binding domain; F, C-terminal domain; see ref 4.

Table 1. Estrogen Agonist Activity of Triphenylethylene Analogs in Ishikawa Cells[a]

no.	R	EC_{50} (nM)[b]	E_{max} (%)[c]
1	estradiol	0.01	100
2	tamoxifen	33	16.5 ± 0.6
6	CO_2H	58	3.8 ± 0.9
7	$CONEt_2$	2.3	11.9 ± 1.2
8	CO(morpholino)	6.9	14.8 ± 2.4
9	$CONH(CH_2)_3OMe$	11	14.0 ± 1.5
10	CON(cyclohexyl)$_2$	70	19.4 ± 2.0
11	$CON[(CH_2)_2NMe_2]Et$	4.6	16.5 ± 1.7
12	$CONH(CH_2)_3OH$	17	15.3 ± 2.4
13	CON(*n*-octyl)Me	12	6.3 ± 1.2
14	CONHEt	18	11.8 ± 1.9
15	$CONH_2$	8.6	8.9 ± 1.4

[a] Low passage (<30) subconfluent Ishikawa-Var I cells were removed from maintenance growth conditions and resuspended in phenol red-free DMEM-F12 (Irvine) containing 5% charcoal-stripped FBS (Irvine) and 2 mM glutamine at a concentration of 58 500 cells/mL. Cells were plated at a density of 13 000 cells/cm^2 and placed in an incubator (37 °C, 5% CO_2) for 3 days. Cells were harvested and resuspended in phenol red-free DMEM-F12 containing 1% charcoal-stripped FBS, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin to a concentration of 83 000 cells/mL. Cells were seeded at a density of 8300 cells/well in 96-well plates and allowed to attach overnight. Controls or appropriate drug treatments at 2× concentrations were added in 0.1 mL of medium containing 0.2% DMSO. Plates were incubated for 2 days, media was aspirated, and plates were washed once with 300 μL of 0.9% sterile saline. Plates were frozen at −70 °C and then warmed to room temperature. The attached cells were assayed for alkaline phosphatase activity by addition of 200 μL of 5 mM *p*-nitrophenyl phosphate in 1 M diethanolamine, pH 10.4, containing 0.1% (w/v) Triton X-100, incubation at 37 °C for 30 min, and measurement of absorbance at 405 nm on a Molecular Devices ThermoMax plate reader. [b] Concentration of compound required to induce 50% of the maximum stimulation of alkaline phosphatase activity, n = 4–6. [c] Maximum stimulation of alkaline phosphatase activity expressed as a percent of the maximum induced by 10 nM estradiol ± standard error, n = 4–6.

4 under palladium catalysis to yield the aldehyde 5 as a single isomer (Scheme 1). Horner–Emmons reaction of aldehyde 5 followed by saponification yielded acid 6. This compound proved to be an interesting compound in its own right as well as a versatile intermediate for synthesis of a series of α,β-unsaturated amides 7–15.

The primary *in vitro* assay measured the estrogenic activity of the compounds in human uterine Ishikawa cells.[10,11] Compounds were tested as estrogen agonists and antagonists by their effect on the estrogen-regulated induction of alkaline phosphatase activity following a 2-day incubation with estrogen-depleted cells. The maximal residual agonist activity (E_{max}) was expressed as a percent of the maximum induction by 10 nM estradiol (1) (Table

* Address correspondence to: Timothy M. Willson, Glaxo Research Institute, 3-4175, Five Moore Drive, Research Triangle Park, NC 27709. Internet: tmw20653@usav01.glaxo.com.
† Department of Medicinal Chemistry.
‡ Department of Cellular Biochemistry.
§ Department of Pharmacology.
⊥ Department of Bioanalytical and Structural Chemistry.

0022-2623/94/1837-1550$04.50/0

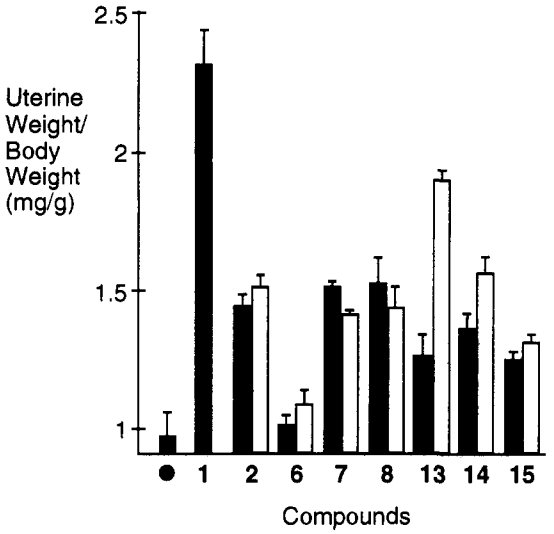
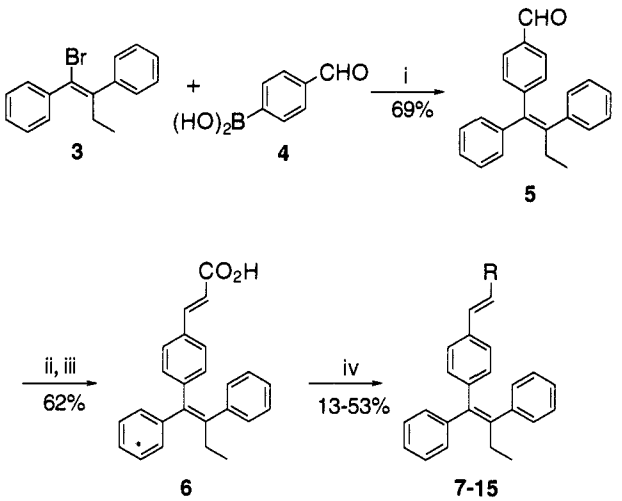


Figure 3. Uterotrophic activity of triphenylethylene analogs in immature rats. Groups of five 21 day old female SD rats (30–35 g) were weighed, and the average weights were recorded for each treatment group. 10× stock solutions of the triphenylethylene analogs in ethanol were diluted with 0.5% methyl cellulose, and 10 μmol/kg was dosed by gavage to the animals. Estradiol (1) was dissolved in sesame oil and 100 nmol/kg dosed by subcutaneous injection. Animals were dosed for 3 days and sacrificed on day 4 by CO_2 asphyxiation. The body weights were obtained, and uteri were removed, blotted, and weighed. Data is expressed as uterine weight/body weight ± standard error. Solid bars represent data from animals dosed with test compound alone. Open bars represent data from animals dosed with test compounds 6 h prior to a dose of estradiol. The solid circle (●) represents animals dosed with vehicle control.

Scheme 1[a]



[a] Reagents: (i) $Pd(PPh_3)_4$, Na_2CO_3, DME; (ii) $(MeO)_2P(O)CH_2CO_2Me$, $NaN(TMS)_2$, THF; (iii) 0.2 M KOH, MeOH, THF; (iv) amine, EDC, HOBT, Et_3N, CH_2Cl_2.

1). This assay provided a precise measure of the partial agonist activity of triphenylethylene estrogens and correlated well with the uterine agonist activity seen in rats.[12] The data showed that the receptor tolerated a large variation in the size and lipophilicity of the amide substituents and that compound potency did not correlate with relative efficacy. Acid **6**, lipophilic amide **13**, and primary amide **15** showed significantly lower E_{max} compared to **2**. Molecular modeling studies revealed that the carbonyl group of these ligands can function as an H-bond acceptor to mimic a low-energy gauche conformation of the ethanolamine side chain found in **2**.[13]

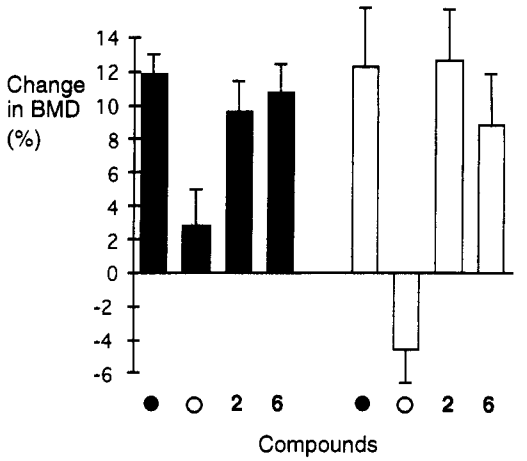


Figure 4. Changes in bone mineral density in ovariectomized rats in lumbar spine and tibia. BMD was measured by dual-energy X-ray absorptiometry using a Hologic QDR-2000 bone densitometer using a regional high-resolution software package with default scan length, width, line spacing and point resolution of 2, 0.75, 0.01, and 0.005 in., respectively. Ninety day old SD rats were divided into groups of 6; 3 groups were surgically ovariectomized. At 2 days postovariectomy, animals were dosed by gavage with either 10 μmol/kg **2** or **6** in 0.5% methyl cellulose or vehicle once a day for 28 days. One group of animals was sham-operated, and 2 days postovariectomy dosed with vehicle once a day for 28 days. At 0, 14, and 28 days, rats were anesthetized with isoflurane and placed in the supine position with their spines parallel to the long axis of the densitometer table. The lumbar spine was scanned using the pelvic bones as a landmark. To scan the right tibia, the leg was taped in position parallel to the long axis of the table and scanned up to the junction with the femur. Analysis of the lumbar spine was accomplished by dividing vertebra and intervertebral spaces with normal analysis software and including only target vertebra in the global region of interest. The right tibia was analyzed with subregional high-resolution software, focusing on the 3–5-mm distal from the growth plate previously identified as a region of accelerated bone loss due to ovariectomy (ref 15). Data at 14 and 28 days did not differ significantly; data at 28 days is presented. Solid and open bars represent data from lumbar spine and right tibia, respectively, expressed as the percent change in the BMD relative to the animals at day 0 of drug treatment ± standard error. The closed (●) and open (○) circles represent data from vehicle-treated sham-operated and ovariectomized controls, respectively.

Compounds with low E_{max} values were tested *in vivo* for their stimulation of uterine weight gain following oral dosing at 10 μmol/kg to 21 day old immature rats for 3 days[14] (Figure 3). In this assay, a partial agonist can antagonize estradiol no lower than the level of its own agonist activity, and only a full antagonist can reach the level of the vehicle control. Lipophilic amide **13** demonstrated poor *in vivo* bioactivity at this dose since its agonist and antagonist values did not converge. Of the remaining compounds, acid **6** tested as an estrogen antagonist in the rat uterus with significantly less residual agonist activity compared to **2**, reflecting its superior *in vitro* profile and demonstrating the importance of the interaction of the side-chain H-bond acceptor in controlling the expression of estrogen agonist activity in the uterus. Finally, to evaluate the functional profile of these compounds in bone, tamoxifen (**2**) and acid **6** were evaluated in 90 day old estrogen-deficient ovariectomized rats for their ability to inhibit loss of bone mineral density (BMD) in the lumbar spine and the tibia[15] (Figure 4). An orally administered dose of 10 μmol/kg of either **2** or **6** demonstrated full agonist activity, maintaining BMD at the levels of the sham-operated rats for the duration of the 28-day study.

Preliminary biochemical data demonstrated that the mechanism of action was through inhibition of bone resorption,[16] consistent with their activity as estrogen agonists in bone.

The identification of **6** as an analog of **2** with reduced uterine agonist activity that maintained its full agonist activity in bone adds credence to our hypothesis that triphenylethylenes code for expression of AF-1 and AF-2 activity through separate parts of the molecule. Studies to assess the activity of **2** in assays of AF-1 and AF-2 activity with mutated estrogen receptors are in progress and will be reported at a later date.

Most members of the steroid/retinoid receptor family have been shown to possess multiple transcription activation domains[17] and contain regions of predicted structural homology with the estrogen receptor within their ligand binding domains;[3c] therefore, the application of these design principles to the synthesis of tissue- and promoter-selective ligands for these receptors remains a promising area of future research.

Acknowledgment. We gratefully acknowledge Dr. Erlio Gurpide (Mt. Sinai School of Medicine) for providing the Ishikawa-Var I cell line and Dr. Ken Korach (National Institute of Environmental Health Sciences) for stimulating discussions on our AF-1/AF-2 hypothesis. We also thank Dr. Bob Dougherty and Mr. Rob Byerly (Department of Cellular Biochemistry, Glaxo Research Institute) for conducting estrogen receptor binding assays.

Supplementary Material Available: General experimental and spectral and analytical data for final products and a computer-generated overlay of low-energy conformations of **2** and **7** (5 pages). Ordering information is given on any current masthead page.

References

(1) For a recent review of the effects of long-term administration of estrogens to women, see: Henderson, B. E.; Ross, R. K.; Pike, M. C. Hormonal Chemoprevention of Cancer in Women. *Science* **1993**, *259*, 633–638.

(2) (a) Love, R. R.; Mazess, R. B.; Barden, H. S.; Epstein, S.; Newcomb, P. A.; Jordan, V. C.; Carbone, P. P.; DeMets, D. L. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N. Engl. J. Med.* **1992**, *326*, 852–856. (b) Ward, R. L.; Morgan, G.; Dalley, D.; Kelly, P. J. Tamoxifen reduces bone turnover and prevents lumbar spine and proximal femoral bone loss in early postmenopausal women. *Bone Miner.* **1993**, *22*, 87–94. (c) Love, R. R.; Weibe, D. A.; Newcomb, P. A.; Cameron, L.; Leventhal, H.; Jordan, V. C.; Feyzi, J.; DeMets, D. L. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. *Ann. Int. Med.* **1991**, *115*, 860–864. (d) Catherino, W. H.; Jordan, V. C. A Risk-Benefit Assessment of Tamoxifen Therapy. *Drug Safety* **1993**, *8*, 381–397.

(3) (a) Tora, L.; White, J.; Brou, C.; Tasset, D.; Webster, N.; Scheer, E.; Chambon, P. The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **1989**, *59*, 477–487. (b) Berry, M.; Metzger, D.; Chambon, P. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO J.* **1990**, *9*, 2811–2818. (c) Danielian, P. S.; White, R.; Lees, J. A.; Parker, M. G. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J.* **1992**, *11*, 1025–1033. (d) Tzukerman, M. T.; Esty, A.; Santiso-Mere, D.; Danelian, P.; Parker, M. G.; Stein, R. B.; Pike, J. W.; McDonnell, D. P. Human estrogen receptor transactivation capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol. Endocrinol.* **1994**, *8*, 21–30.

(4) Gronemeyer, H. Transcription activation by estrogen and progesterone receptors. *Annu. Rev. Genet.* **1991**, *25*, 89–123.

(5) For the role of the ligand in affecting receptor conformation and nuclear translocation, see: (a) Hansen, J. C.; Gorski, J. Conformational transitions of the estrogen receptor monomer. *J. Biol. Chem.* **1986**, *261*, 13990–13996. (b) Emmas, C. E.; Fawell, S. E.; Hoare, S. A.; Parker, M. G. Effect of ligand binding and DNA binding on the structure of the mouse oestrogen receptor. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 291–299.

(6) For the importance of the side chain as an H-bond acceptor, see: (a) Murphy, L. C.; Sutherland, R. L. Differential effects of tamoxifen and analogs with nonbasic side chains on cell proliferation *in vitro*. *Endocrinology* **1985**, *116*, 1071–1078. (b) Robertson, D. W.; Katzenellenbogen, J. A.; Hayes, J. R.; Katzenellenbogen, B. S. Antiestrogen basicity-activity relationships: a comparison of the estrogen receptor and antiuterotrophic potencies of several analogues of (*Z*)-1,2-diphenyl-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1-butene (tamoxifen, Nolvadex) having altered basicity. *J. Med. Chem.* **1982**, *25*, 167–171.

(7) For the site of interaction of the basic side chain with the receptor, see: Harlow, K. W.; Smith, D. N.; Katzenellenbogen, J. A.; Greene, G. L.; Katzenellenbogen, B. S. Identification of cysteine 530 as the covalent attachment site of an affinity-labeling estrogen (ketononestrol aziridine) and antiestrogen (tamoxifen aziridine) in the human estrogen receptor. *J. Biol. Chem.* **1989**, *264*, 17476–17485.

(8) For comprehensive reviews of the pharmacology of non-steroidal estrogens, see: (a) Jordan, V. C. Biochemical Pharmacology of Antiestrogen Action. *Pharmacol. Rev.* **1984**, *36*, 245–276. (b) Jordan, V. C.; Murphy, C. S. Endocrine pharmacology of antiestrogens as anti-tumor agents. *Endocrine Rev.* **1990**, *11*, 578–610.

(9) Miller, R. B.; Al-Hassan, M. I. Stereospecific synthesis of (*Z*)-tamoxifen *via* carbometalation of alknylsilanes. *J. Org. Chem.* **1985**, *50*, 2121–2123.

(10) Littlefield, B. A.; Gurpide, E.; Markiewicz, L.; McKinley, B.; Hochberg, R. B. A simple and sensitive microtitre plate estrogen bioassay based on stimulation of alkaline phosphatase activity in Ishikawa cells: estrogenic action of Δ^5 adrenal steroids. *Endocrinology* **1990**, *127*, 2757–2762.

(11) All compounds reported showed specific displacement of [^{3}H]-estradiol from the rat estrogen receptor with $IC_{50} < 5$ μM.

(12) (a) *Vide infra.* (b) Moore, L. B. Unpublished results.

(13) See the supplementary material.

(14) Wakeling, A. E.; Valcaccia, B.; Newboult, E.; Green, L. R. Non-steroid antiestrogens – receptor binding and biological response in rat uterus, rat mammary carcinoma and human breast cancer cells. *J. Steroid Biochem.* **1984**, *20*, 111–120.

(15) Ammann, P.; Rizzoli, R.; Slosman, D.; Bonjour, J. P. Sequential and precise *in vivo* measurement of bone mineral density in rats using dual-energy X-ray absorptiometry. *J. Bone Miner. Res.* **1992**, *7*, 311–316.

(16) (a) Black, D.; Farquharson, C.; Robins, S. P. Excretion of pyridinium cross-links of collagen in ovariectomized rats as urinary markers for increased bone resorption. *Calcif. Tissue Int.* **1989**, *44*, 343–347. (b) Baer P. G. Unpublished results.

(17) For example, progesterone receptor: (a) Meyer, M. E.; Pornon, A.; Jingwei, J. I.; Boquel, M. T.; Chambon, P.; Gronemeyer, H. Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. *EMBO J.* **1990**, *12*, 3923–3932. Androgen receptor: (b) Jenster, G.; van der Korput, H. A. G. M.; van Vroonhoven, C.; van der Kwast, T. H.; Trapman, J.; Brinkmann, A. O. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol. Endocrinol.* **1991**, *5*, 1396–1404. Retinoid receptors: (c) Nagpal, S.; Saunders, M.; Kastner, P.; Durand, B.; Nakshatri, H.; Chambon, P. Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. *Cell* **1992**, *70*, 1007–1019. Glucocorticoid receptor: (d) Hollenberg, S. M.; Evans, R. M. Multiple and cooperative *trans*-activation domains of the human glucocorticoid receptor. *Cell* **1988**, *55*, 899–906.