Investigations on Estrogen Receptor Binding. The Estrogenic, Antiestrogenic, and Cytotoxic Properties of C2-Alkyl-Substituted 1,1-Bis(4-hydroxyphenyl)-2-phenylethenes

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C2-Alkyl-substituted 1,1-bis(4-hydroxyphenyl)-2-phenylethenes were synthesized and assayed for estrogen receptor binding in a competition experiment with radiolabeled estradiol ([^{3}H]-**E2**) using calf uterine cytosol. The relative binding affinity decreased with the length of the side chain R = H (**3a**: 35.2%) > Me (**3b**: 32.1%) > Et (**3c**: 6.20%) ≈ CH_2CF_3 (**3d**: 5.95%) > n-Pr (**3e**: 2.09%) > Bu (**3f**: 0.62%). Agonistic and antagonistic effects were evaluated in the luciferase assay with MCF-7-2a cells stably transfected with the plasmid ERE_{wtc}luc. All compounds showed high antiestrogenic activity without significant agonistic potency. The comparison of the IC_{50} values for the inhibition of **E2** (1 nM) documented the dependence of the antagonistic effects on the kind of the side chain: **3a** (IC_{50} = 150 nM), **3b** (IC_{50} = 30 nM), and **3f** (IC_{50} = 500 nM) were weak antagonists, while **3c** (IC_{50} = 15 nM), **3d** (IC_{50} = 9 nM), and **3e** (IC_{50} = 50 nM) were full antiestrogens and antagonized the effect of **E2** completely. The most active compound **3d** possessed the same antagonistic potency as 4-hydroxytamoxifen (**4OHT**: IC_{50}= 7 nM) without bearing a basic side chain. **3d** as well as all other 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes were not able to influence the proliferation of hormone dependent MCF-7 cells despite the antagonistic mode of action. In this assay tamoxifen (**TAM)** and **4OHT** reduced the cell growth concentration dependent up to T/C_{corr} = 15% and 25%, respectively.

Introduction

The estrogen receptor (ER) as a ligand inducible transcription factor mediates the physiological effects of endogenous estrogens.[1] Besides diverse positive effects (e.g., on bone density maintenance,[2] regulation of blood lipid profile,[3] and brain function[4]) estrogens play a predominant role in breast cancer growth and development.[5] Therefore, many efforts have been devoted to the blockade of estrogen formation and action and led to the introduction of antiestrogens in the therapy of breast cancer. Tamoxifen (**TAM**), as a nonsteroidal antiestrogen,[6] has become a widely used drug for a first line endocrine therapy for all stages of breast cancer in pre- and postmenopausal women.[7] The estrogen-like effects in certain tissues led to the classification as a selective estrogen receptor modulator (SERM). Its pharmacological properties are related to the ability to compete in target tissues with estradiol (**E2**) for binding sites in the ligand-binding domain (LBD) of the ER.

TAM itself acts as a prodrug, which is activated by hydroxylation of the para-position of the 1-phenyl ring. The resulting 4-hydroxytamoxifen (**4OHT**) possesses an 8 times higher binding affinity to the ER and acts as a pure antiestrogen in hormone dependent tumor cells. Ligand recognition is achieved in both cases by a combination of specific hydrogen bonds and van der Waals interactions leading to a reorientation of the 12 helical units of the LBD.[8] Nevertheless, binding of **4OHT** induces a conformation of the LBD that differs in both secondary and tertiary structural organization from that driven by **E2** binding. Especially the orientation of helix 12 is different in both structures. While helix 12 protects the LBD nearly completely from the environment after binding of agonists, it is reoriented in the **4OHT** complex and occupies the part of the coactivator binding groove formed by the residues of helices 3, 4, and 5 and the turn connecting helices 3 and 4. The binding of the antiestrogens **4OHT** and raloxifen (**RAL**)[9] in the LBD is very similar and indicates that the orientation of the basic side chains in a narrow side pocket of the LBD effects the alternative conformation of helix 12, which inhibits coactivator recruitment and ultimate transcription regulation and leads to antiestrogenic effects.

Interestingly, Schneider and co-workers[10−13] showed that, depending on the number and position of *O*-acyl groups, the 1,1,2-triarylethenes induce in the immature mouse uterine weight test low but significant antiestrogenic effects up to 50%, however, in the low dose of 1 μg. In higher dosage, the estrogenic potency was dominant. To get more information about the antagonistic effects in hormone-dependent tumor cells, we synthesized 1,1-bis(4-hydroxyphenyl)-2-phenylethene analogues and studied their agonistic and antagonistic potency on the molecular level in a luciferase assay on MCF-7-2a cells stably transfected with the plasmid ERE_{wtc}luc.

Chemistry

The 1,1-bis(4-hydroxyphenyl)-2-phenylethenes **3a**−**f** were synthesized according to the method of Dodds et

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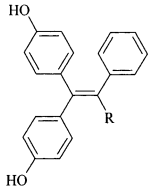
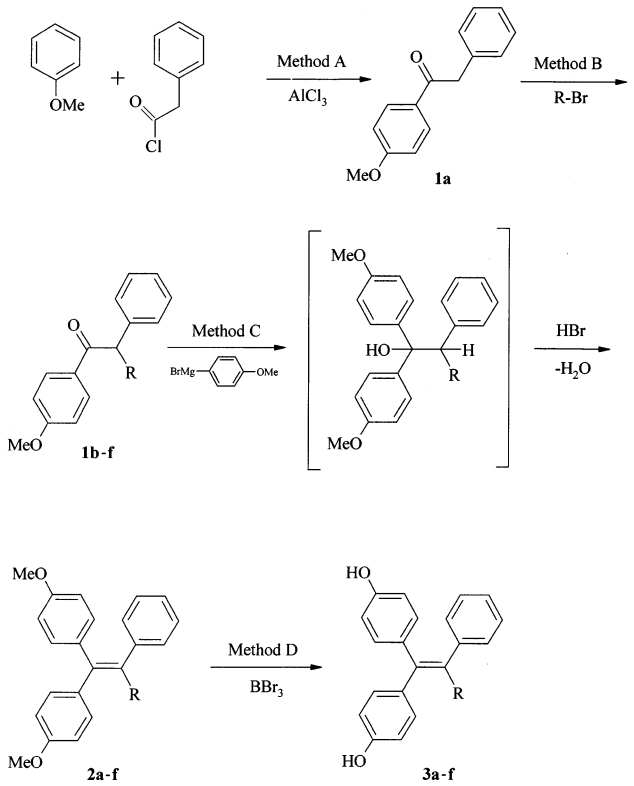
Table 1. Biological Properties of Compounds **3a**−**f**

compounds	formula	R	RBA[%]	estrogenic activity [%] (1μM)	antiestrogenic activity IC_{50} (nM)	cytotoxicity T/C_{corr}[%](5μM)
3a		H	35.2	25	150	92
3b		Me	32.1	10	30	90
3c		Et	6.20	0	15	99
3d		CH_2CF_3	5.95	0	9	68
3e		n-Pr	2.09	5	50	98
3f		Bu	0.62	0	500	87
TAM			1.80	-19.4	500	15
4OHT			15.6	-17.7	7	25

Scheme 1. Synthesis of 1,1-Bis(4-hydroxyphenyl)-2-phenylethenes



Compound	R
1a,2a,3a	H
1b,2b,3b	CH_3
1c,2c,3c	C_2H_5
1d,2d,3d	CH_2CF_3
1e,2e,3e	C_3H_7
1f,2f,3f	C_4H_9

al.[14] starting with 1-(4-methoxyphenyl)-2-phenylethan-1-one **1a** which was obtained by a Friedel−Crafts acylation of phenylacetyl chloride and anisole (Scheme 1, method A). Subsequently, **1a** was reacted under the influence of potassium *tert*-butanolate with the appropriate alkyl bromide to obtain the C2-alkyl-substituted 1-(4-methoxyphenyl)-2-phenylethanones **1b**−**f** (Scheme 1, method B). The following Grignard reaction of **1a**−**f** with 4-methoxyphenylmagnesium bromide yielded the corresponding carbinols, which were dehydrated to the C2-alkyl-substituted 1,1-bis(4-methoxyphenyl)-2-phenylethenes **2a**−**f** by using either phosphoric acid or hydrobromic acid in THF (Scheme 1). Compounds **2a**−**f** were converted to the hydroxy derivatives **3a**−**f** by ether cleavage with BBr_3 (Scheme 1, method D). The characterization of all compounds was performed by ^1H NMR, IR, and mass spectroscopy.

Results and Discussion

The affinity to the ER was determined in a competition experiment with [^{3}H]-**E2** using calf uterine cytosol.[15] As listed in Table 1, all 1,1,2-triarylethenes effectively displaced **E2** from its binding site. The binding curves were parallel to that of **E2** so that a competitive inhibition can be assumed. The relative binding affinity (RBA) showed a clear dependence on the C2-alkyl chain and decreased with the length of the side chain in the series H (35.2%) < Me (32.1%) < Et (6.20%) ≈ CH_2CF_3 (5.95%) < n-Pr (2.09%) < Bu (0.62%).

Not only the C-2 substituents but also the substituents on the C-1 aryl rings influenced the ER binding. **4OHT** as 4-OH derivative of **TAM** possesses an 8 times higher affinity to the ER (**4OHT**: RBA = 15.6%; **TAM**: RBA = 1.8%). The comparison of the RBA of **4OHT** with that of **3c** (RBA = 6.20%) shows that the dimethylaminoethoxy side chain increases the RBA by the factor 3.

Because the RBA value does not directly reflect biological effects such as estrogenic or antiestrogenic activity, the compounds **3a**−**f** were evaluated in more detail in a luciferase assay using MCF-7−2a cells.[16] These ER positive human breast cancer cells are stably transfected with the reporter plasmid ERE_{wtc}luc. After binding of a hormonally active drug, the ER/drug conjugates dimerize and are able to interact with the estrogen response elements (ERE) of the plasmid, leading to the activation of the luciferase gene. Therefore, the quantification of the luciferase expression allows not only a prediction of the agonistic but also of the antagonistic potencies of drugs.

Among the assayed 1,1,2-triarylethenes (**3a** to **3f**), only **3a** and **3b** exerted low estrogenic effects in the luciferase assay (relative activation at 1 μM: **3a** 25%; **3b** 10%, see Table 1). On the other hand, all compounds were antagonists and inhibited the effect of 1 nM **E2** (see Table 1) dependent on the length of C2-alkyl chain (IC_{50} for the inhibition of 1nM **E2**: R = H (150 nM) > Me (30 nM) > Et (15 nM) > CH_2CF_3 (9 nM) < n-Pr (50 nM) < Bu (500 nM)).

The concentration activation curves (see Figure 1) document that **3a**, **3b**, and **3f** are weak antagonists,

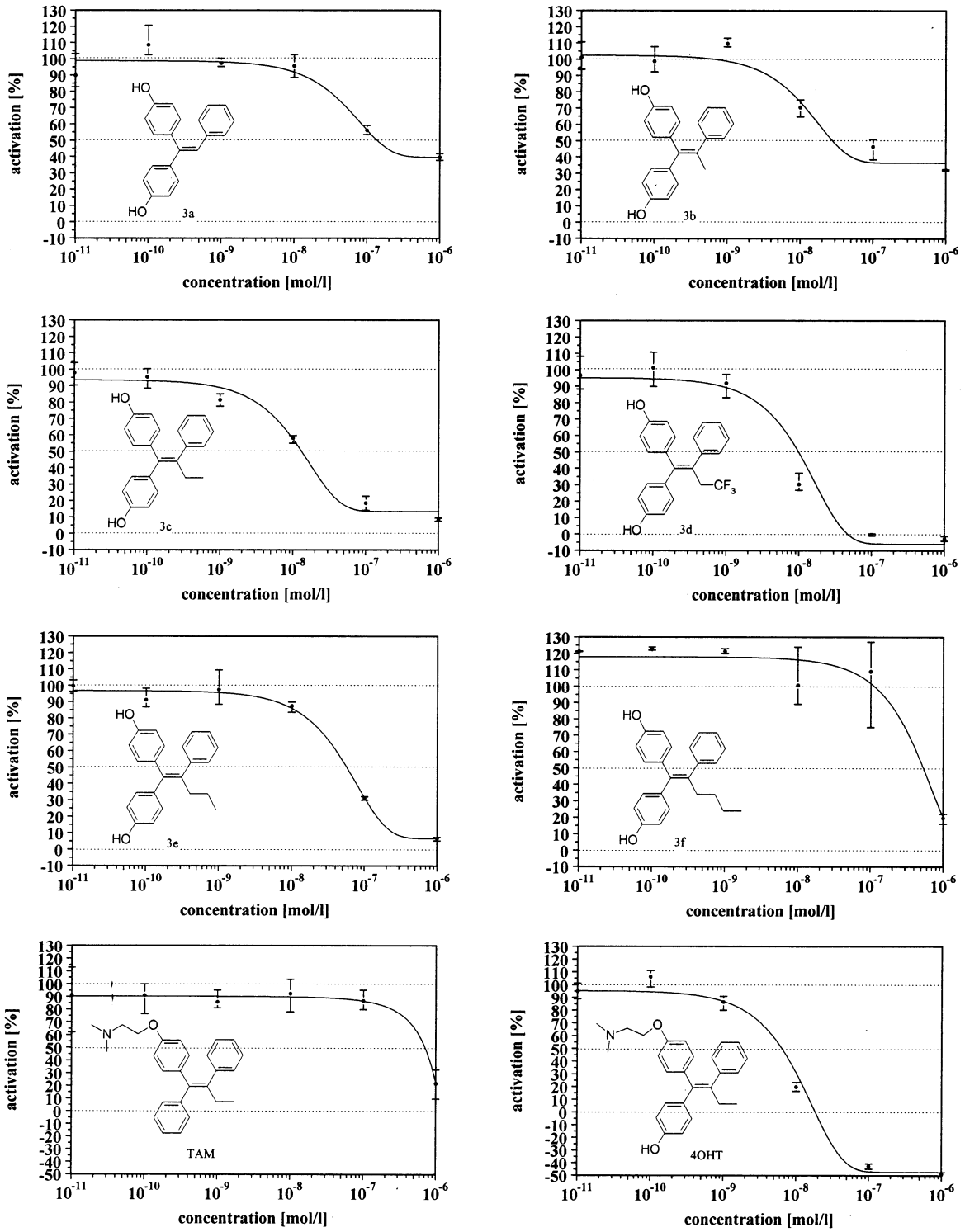


Figure 1. Luciferase expression in MCF-7-2a cells, stably transfected with the reporter plasmid $ERE_{wtc}luc$ treated with a combination of **E2** (1nM) and the 1,2-bis(4-hydroxyphenyl)-2-phenylalkenes **3a**−**f**, tamoxifen (**TAM**), or 4-hydroxytamoxifen (**4OHT**).

while **3c, 3d**, and **3e** are true antiestrogens and antagonized the **E2** effect completely (see Table 1 and Figure 1). As most active compound **3d** (IC_{50} = 9 nM) showed the same antagonistic potency as **4OHT** (IC_{50} = 7 nM) and was almost 50 times more active than **TAM** (IC_{50} = 500 nM). Interestingly, none of the 1,1-bis(4-hydroxyphenyl)-2-phenylethenes bears a basic side chain which is held responsible for antagonistic effects.

Shiau et al.[8] detected in their X-ray studies a direct hydrogen bond between the amino acid Asp351 and the nitrogen of the dimethylamino group of **4OHT**. This binding displaces helix 12 from the binding cavity and prevents the formation of a transcriptionally competent conformation of the activation function 2 (AF2).

The group of Jordan also determined the relevance of an H-bridge between the basic side chain of **4OHT** and **RAL** and Asp351.[17] They studied the TGFα expression in MDA-MB-231 cells stably transfected with cDNA for wild-type ER (Asp351) as well as for the mutants Asp351Tyr and Asp351Gly. The exchange of Asp351 by

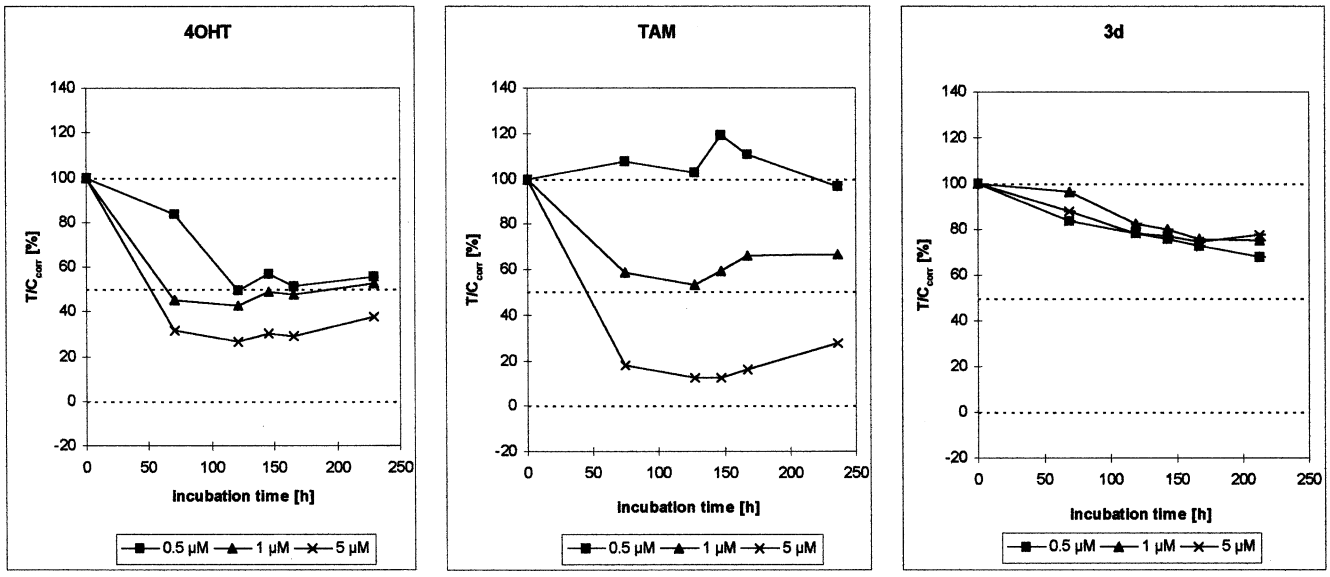


Figure 2. Effects of 4-hydroxytamoxifen (**4OHT**), tamoxifen (**TAM**), and the 1,1-bis(4-hydroxyphenyl)-2-phenyl-4,4,4-trifluorobut-1-ene (**3d**) on the MCF-7 breast cancer cell line.

Asp351Tyr enhanced the estrogenic properties of **4OHT** and changed also the pharmacology of **RAL** by converting it from antiestrogen to estrogen. Substitution of glycine for aspartate (Asp351Gly) resulted in the conversion of the ER/**4OHT** complex from estrogen-like to antiestrogen. The authors propose that the side chain of an antiestrogen either neutralizes or displaces the charge at Asp351 thereby removing a charged site for the opportunistic binding of a novel coactivator.

Previously, Shiau et al.[18] have suggested that it is not just the side chain that is responsible for the antagonistic effect of **TAM**. The bulk causes also a conformational change that is inappropriate for full coactivator binding. This is in accordance with our results because none of the antiestrogenically active 1,1,2-triarylethenes bears a side chain. The antiestrogenic properties depend only on the C2-alkyl chain.

The structural analogy of the compounds to **4OHT** investigated in this study allows the assumption of an analogous orientation in the LBD. One of the 4-hydroxyphenyl rings is H-bound to Glu351 and Arg394 and the other is positioned in the narrow side pocket and is oriented toward Asp351. In a structure activity study we determined a similar orientation of the pharmacophoric 1,2-diarylethane/ethene moiety in estrogenically active 2,3-diarylpiperazines, 4,5-diarylimidazolines and 4,5-diarylimidazoles and suppose that Asp351 is also an appropriate anchor for estrogens.[19] Therefore, the missing agonistic properties and the antagonistic effects of 1,1,2-triarylethenes must be the consequence of the interaction of the C2-phenyl and the C2-alkyl chain in the LBD.

The structural requirements for the pharmacological activity of nonsteroidal antiestrogens were already studied two decades ago. The inhibition of the **E2**-stimulated prolactin synthesis in primary cells of immature rat pituitary glands by antiestrogens were used as in vitro parameter.[20−22] Three categories of active compounds were identified: full estrogens, partial agonists with antiestrogenic actions against the effects of 0.1nM **E2**, and full antagonists which antagonize the effect of **E2** completely.

In this test, **4OHT** showed high antiestrogenic potency and turned out to be a partial agonist (**3c**) with antiestrogenic properties after deletion of the side chain. Interestingly, the effects did not change after O-acylation. A similar relationship was found for cyclophenyl, so it can be deduced that 1,1-bis(4-hydroxyphenyl)-alkene derivatives are partial agonists in pituitary cells.

The antitumor activity and the hormonal profile of **3c** and its *O*-acyl derivative were tested in vivo by Schneider et al.[13,23,24] Both compounds caused a growth inhibition of $T/C = 10-15\%$ on the hormone-dependent MXT-M 3.2 mammary tumor of the BDF1 mouse in a dose of 20 μM/kg. In the immature uterine weight test, however, they possessed significant estrogenic but no significant antiestrogenic properties. Therefore, the mammary tumor-inhibiting effects cannot be caused by the antagonism of tumor growth stimulating endogenous estrogens. Rather a mode of action must be taken into consideration described by Schlemmer et al.[25−28] They proposed an indirect mode of action for an estrogenic active platinum complex due to its hormonal properties, which involves other cells of the host as well, e.g., cells of the immune system.

This is supported by the results on the MCF-7 cell line. **3c** and its derivatives were inactive, independent of their antiestrogenic properties determined in these cells (Table 1). Only the CF_3 derivative **3d** caused low antiproliferative effects ($T/C = 70\%$, see Figure 2). On the other hand, **TAM** with only low antiestrogenic potency inhibited the cell proliferation to $T/C_{corr} = 15\%$ in a concentration of 5μM, while **4OHT** was less active ($T/C_{corr} = 25\%$).

Conclusion

In this study we demonstrated that the removal of the dimethylaminoethoxy side chain of 4-hydroxytamoxifen did not decrease the antagonistic effects on the MCF-7-2a cell line. All 1,1-bis(4-hydroxyphenyl)-2-phenylethenes derived from **4OHT** showed antiestrogenic potency without estrogenic side effects. These properties do not influence in vitro the proliferation of

hormone dependent tumor cells. The antitumor effects of **3c** and related compounds should follow the mechanism proposed by Schlemmer et al., because growth-inhibiting properties are only observed in vivo.

Experimental Section

General Procedures. IR spectra (KBr pellets): Perkin-Elmer Model 580 A. ^{1}H NMR: ADX 400 spectrometer at 400 MHz (internal standard: TMS). Elemental analyses: Microlaboratory of Free University of Berlin; on the basis of the C, H, and N analyses, all compounds were of acceptable purity (within 0.4% of the calculated values). Liquid Scintillation Counter: 1450 Microbeta Plus (Wallac, Finland). Microplate Photometer: Labsystems Multiscan Plus (Labsystems, Finland). Microlumat: LB 96 P (EG & G Berthold, Germany).

Syntheses. Methods A to D are representatives for the compounds reported in Scheme 1. The compounds **1b** to **1e** and **2a** to **2e** were already described by Schneider et al.[13]

Method A: 1-(4-Methoxyphenyl)-2-phenylethanone (1a). Phenylacetyl chloride (9.28 g, 6.0 mmol) was added dropwise to a suspension of aluminum chloride (9.71 g, 7.28 mmol) and anisole (6.5 g, 6.01 mmol) in 20 mL of dry 1,2-dichloroethane under cooling. After heating to reflux for 2 h, 50 mL of water was added, and the organic layer was separated, washed with water, and dried over Na_2SO_4. After removal of the solvent, the crude product was recrystallized from diethyl ether/ligroine to give **1a** as colorless crystals (mp 68−69 °C). Yield: 10.43 g (4.61 mmol, 76.8%). ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 3H, OC*H*$_3$); 4.23 (s, 2H, C*H*$_2$); 6.92 (AA′BB′, ^3J = 8.9 Hz, 2H, Ar*H*-3, Ar*H*-5); 7.27 (m, 5 H, Ar′*H*), 7.99 (AA′BB′, ^3J = 8,9 Hz, 2H, Ar*H*-2, Ar*H*-6). IR (KBr, cm^{-1}): 3063 w (ArH); 3029 w (ArH); 2973 w (CH_2); 2932 w (CH_2); 2904 w (OCH_3); 1678 s (C=O); 1602 s (C=C); 1576 m (C=C); 1507 m (C=C). MS (EI, 50 °C): *m*/*z* (%) = 226 (1.6) [M]$^{+\bullet}$; 135 (100).

Method B: 1-(4-Methoxyphenyl)-2-phenylhexanone (1f). Butyl bromide (0.49 g, 3.5 mmol) was added carefully to a suspension of **1a** (1.0 g, 3.5 mmol) and potassium *tert*-butylate (0.39 g, 3.5 mmol) in dry diethyl ether and heated to reflux for 6 h. The organic layer was separated after addition of 20 mL of water and was washed with 0.5 N sodium thiosulfate solution and water, dried over Na_2SO_4, and evaporated. The crude product was purified by column chromatography on silica gel with diethyl ether/ligroine 1:2 to obtain a colorless oil. Yield: 0.79 g (2.79 mmol, 79.7%) of a colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.85 (t, 3H, C*H*$_3$); 1.14−1.38 (m; 4H, 2 × C*H*$_2$); 1.81 (m, 1H, R$_2$C=CRC*H*$_2$); 2.17 (m; 1H; R$_3$C−CHPhC*H*$_2$); 3.82 (s, 3H, OC*H*$_3$); 4.49 (t, 1H, C*H*); 6.86 (AA′BB′, ^3J = 8.9 Hz, 2H, Ar*H*-3, Ar*H*-5); 7.18 (m, 1H, Ar′*H*-4); 7.29 (m, 4H, Ar*H*-2, 3, 5, 6); 7.96 (AA′BB′, ^3J = 8.9 Hz, 2H, Ar*H*-2, Ar*H*-6). IR (Film; cm^{-1}): 3061 w (ArH); 3025 w (ArH); 2955 s (CH_2); 2930 s (CH_2); 2869 m (OCH_3); 1672 s (C=O); 1601 m (C=C); 1575 m (C=C); 1509 s (C=C). MS (EI, 80 °C): *m*/*z* (%) = 282 [M]$^{+\bullet}$ (0.4); 135 (100).

Method C: 1,1-Bis(4-methoxyphenyl)-2-phenylhex-1-ene (2f). A solution of **1f** (0.5 g, 1.77 mmol) in 5 mL of dry THF was added dropwise to a solution of 4-methoxyphenylmagnesium bromide in THF, which was generated before from 4-bromoanisole (0.5 g, 2.66 mmol) and Mg (0.07 g, 2.66 mmol). The mixture was refluxed for 12 h and was then decomposed with ice and 6 N acetic acid. The solvent was narrowed down under reduced pressure, and the remaining aqueous solution was extracted with ether. The organic layers were combined, washed with saturated $NaHCO_3$ solution and water, dried over Na_2SO_4, and evaporated to dryness to obtain the carbinol. After dissolution in dry THF, it was added dropwise to ice cold HBr (47%). The mixture was stirred for 2 h, poured onto ice, and extracted with dichloromethane. The organic layer was washed with $NaHCO_3$ solution and water. After drying over Na_2SO_4, the solvent was evaporated, and the remaining product was purified by column chromatography on silica gel with diethyl ether/ligroine 1:5. Yield: 0.22 g (0.6 mmol, 33%) of a colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.78 (t, 3H, C*H*$_3$); 1.17−1.34 (m, 4H, 2 × C*H*$_2$); 2.43 (t, 2H, R$_2$C=CR−C*H*$_2$); 3.68 (s, 3H, OCH_3); 3.83 (s, 3H, OCH_3); 6.54 (AA′BB′, ^3J = 8.8 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.76 (AA′BB′, ^3J = 8.8 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.88 (AA′BB′, ^3J = 8.6 Hz, 2H, Ar*H*-3, Ar*H*-5); 7.07−7.18 (m, 7H; Ar*H*). IR (KBr, cm^{-1}): 3056 m (ArH); 3000 m (ArH); 2956 s (CH_2); 2930 s (CH_2); 2870 m (OCH_3); 2835 m (OCH_3); 1606 s (C=C); 1574 m (C=C); 1508 s (C=C). MS (EI, 80 °C): *m*/*z* (%) = 372 [M]$^{+\bullet}$ (54.38); 135 (100).

Method D: Ether Cleavage with BBr_3. A solution of 1 equiv of the appropriate methyl ether in dry dichloromethane was cooled to −52 °C. BBr_3 (6 equiv) in dry dichloromethane was added dropwise under nitrogen atmosphere. The mixture was warmed to room temperature and stirred for 3 days. Subsequently, dry methanol was added under cooling three times, and the solvent was removed respectively under reduced pressure to yield the 1,1-bis(4-hydroxyphenyl)-2-phenylethene.

1,1-Bis(4-hydroxyphenyl)-2-phenylethene (3a). From **2a** (0.15 g, 0.47 mmol). Purification was carried out by crystallization with chloroform/ligroine. Yield: 0.08 g (0.29 mmol, 61%) of colorless crystals; mp 134−137 °C. ^{1}H NMR (DMSO-d_6): δ 6.7−6.76 (m, 4H, Ar*H*); 6.81 (s, 1H, C*H*); 6.89 (AA′BB′, ^3J = 8,5 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.99 (AA′BB′, ^3J = 7.2 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.06−7.17 (m, 5H, (C2)Ar*H*); 9.5 (d, 2H, O*H*). IR (KBr; cm^{-1}): 3377 s (Ar−OH); 3077 (ArH); 3023 (ArH); 1606 m (C=C); 1512 s (C=C). MS (EI, 140 °C): *m*/*z* (%) = 288 [M]$^{+\bullet}$ (100). Anal. ($C_{20}H_{16}O_2$) C H.

1,1-Bis(4-hydroxyphenyl)-2-phenylprop-1-ene (3b). From **2b** (0.053 g, 0.16 mmol). Purification was performed by column chromatography on silica gel with ether and recrystallization from methanol/diethyl ether. Yield: 0.04 g (0.13 mmol, 81.3%) of a colorless powder (mp 135 °C). ^{1}H NMR (DMSO-d_6): δ 2.04 (s, 3H, C*H*$_3$); 6.41 (AA′BB′, ^3J = 8.4 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.60 (AA′BB′, ^3J = 8.4 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.74 (AA′BB′ ^3J = 8.4 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.97 (AA′BB′ ^3J = 8.4 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.03−7.17 (m, 5H, (C2)Ar*H*). IR (KBr; cm^{-1}): 3442 br (Ar−OH); 3073 m (ArH); 3026 m (ArH); 2976 m (CH_2); 1608 s (C=C); 1510 s (C=C). MS (EI, 90 °C): *m*/*z* (%) = 302.3 [M]$^{+\bullet}$ (6.5). Anal. ($C_{21}H_{18}O_2\cdot H_2O$) C H.

1,1-Bis(4-hydroxyphenyl)-2-phenylbut-1-ene (3c). From **2c** (0.75 g, 2.18 mmol). The crude product was purified by crystallization from chloroform/ligroine. Yield: 0.43 g (1.36 mmol, 62.4%) of light yellow crystals; mp 130−135 °C. ^{1}H NMR (MeOD-d_4): δ 0.9 (t, 3H, C*H*$_3$); 2.47 (q, 2H, C*H*$_2$); 6.39 (AA′BB′ ^3J = 8.5 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.65 (AA′BB′ ^3J = 8.5 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.76 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.01 (AA′BB′, ^3J = 8.6 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.06−7.17 (m, 5H, (C2)Ar*H*). IR (KBr, cm^{-1}): 3400 bs (OH); 3028 m (ArH); 2966 m (ArH); 2928 m (CH_2); 2870 m (CH_2); 1608 s (C=C); 1508 s (C=C). MS (EI, 130 °C): *m*/*z* (%) = 316 [M]$^{+\bullet}$ (100); 301 (38.9). Anal. ($C_{22}H_{20}O_2\cdot 0.5H_2O$) C H.

1,1-Bis(4-hydroxyphenyl)-2-phenyl-4,4,4-trifluorobut-1-ene (3d). From **2d** (0.40 g, 1 mmol). Yield: 0.204 g (0.55 mmol, 55%) of a reddish powder; mp 157−161 °C. ^{1}H NMR ($CDCl_3$): δ 3.5 (q, ^3J = 10.7 Hz, 2H, C*H*$_2$); 6.5−6.7 (m, 13H, Ar-*H*); 8.2 (s, 1H, Ar*OH*); 8.4 (s, 1H, Ar-*OH*). IR (KBr, cm^{-1}): 3600−3200 s (ArH); 2900 s (CH); 1600 m (C=O); 1510 s (C=O); 1205 s (C−F); 840 s. Anal. ($C_{22}H_{17}F_3O_2$) C H.

1,1-Bis(4-hydroxyphenyl)-2-phenylpent-1-ene (3e). From **2e** (0.1 g, 0.29 mmol). The crude product was purified by column chromatography on silica gel with ether and subsequent crystallization from chloroform/ligroine. Yield 0.075 g (0.23 mmol, 80.7%) of an orange powder; mp 142−145 °C. ^{1}H NMR (DMSO-d_6): δ 0.75 (t, 3H, C*H*$_3$); 1.45 (m, 2H, C*H*$_2$CH$_3$); 2.34 (t, 2H, C=CPh-C*H*$_2$); 6.39 (AA′BB′, ^3J = 8.5 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.60 (AA′BB′, ^3J = 8.5 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.74 (AA′BB′, ^3J = 8.4 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.96 (AA′BB′, ^3J = 8.5 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.06−7.18 (m, 5H, (C2)Ar*H*); 9.12 (s, 1H, O*H*); 9.37 (s, 1H, O*H*). IR (KBr, cm^{-1}): 3400 bs (OH); 2957 m (ArH); 2927 m (ArH); 2868 m (CH_2); 1608 s (C=C); 1508 s (C=C). MS (EI, 150 °C): *m*/*z* (%) = 330 [M]$^{+\bullet}$ (100); 301 (79.4). Anal. ($C_{23}H_{22}O_2\cdot 0.5H_2O$) C H.

1,1-Bis(4-hydroxyphenyl)-2-phenylhex-1-ene (3f). From **2e** (0.16 g, 0.41 mmol). The brown crude product was recrystallized from 2-propanol/dichloromethane. Yield: 0.1 g

(0.29 mmol, 71.5%) of a pale orange powder; mp 158 °C. ^{1}H NMR ($CDCl_3$): δ = 0.78 (t, 3H, C*H*$_3$); 1.23 (m, 4H, 2 × C*H*$_2$); 2.43 (t, 2H, C≡CPh-C*H*$_2$); 6.46 (AA′BB′, ^3J = 8.7 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.72 (AA′BB′, ^3J = 8.7 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.80 (AA′BB′, ^3J = 8.5 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.08−7.2 (m, 7H, Ar*H*). IR (KBr, cm^{-1}): 3401 br s (OH); 3059 w (ArH); 3026 w (ArH); 2956 s (ArH); 2926 s (CH_2); 2857 (CH_2); 1608 s (C≡C); 1508 (C≡C). MS (EI, 140 °C): *m*/*z* (%) = 344 $[M]^{+\bullet}$ (100); 301 (83.9). Anal. ($C_{24}H_{24}O_2 \cdot 0.5H_2O$) C H.

Biochemicals, Chemicals, And Materials. Dextran, 17β-estradiol, L-glutamine (L-glutamine solution: 29.2 mg/mL phosphate-buffered saline (PBS), and Minimum Essential Medium Eagle (EMEM) were purchased from Sigma (Munich, Germany); Dulbecco's Modified Eagle Medium without phenol red (DMEM) was from Gibco (Eggenstein, Germany); fetal calf serum (FCS) was from Bio whittaker (Verviers, Belgium); *N*-hexamethylpararosaniline (crystal violet), and gentamicin sulfate were from Fluka (Deisenhofen, Germany); glutardialdehyde (25%) was from Merck (Darmstadt, Germany); trypsin (0.05%) in ethylenediaminetetraacetic acid (0.02%) (trypsin/EDTA) was from Boehringer (Mannheim, Germany); penicillin−streptomycin gold standard (10000 IE penicillin/mL, 10 mg streptomycin/mL), and geneticin disulfate (geneticin solution: 35.71 mg/mL PBS) were from ICN Biomedicals GmbH (Eschwege, Germany); norit A (charcoal) was from Serva (Heidelberg, Germany); cell culture lysis reagent (5×) (diluted 1:5 with purified water before use) and the luciferase assay reagent were from Promega (Heidelberg, Germany); optiphase HiSafe3 scintillation liquid was from Wallac (Turku, Finland); NET-317-estradiol[2,4,6,7-^{3}H(N)] (17β-[^{3}H]estradiol) was from Du Pont NEN (Boston, MA); PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g of KH_2PO_4 (all purchased from Merck or Fluka) in 1000 mL of purified water. TRIS-buffer (pH = 7.5) was prepared by dissolving 1.211 g of trishydroxymethylaminomethane, 0.3722 g of Titriplex III, and 0.195 g of sodium azide (all from Merck or Fluka) in 1000 mL of purified water. Deionized water, produced by means of a Millipore Milli-Q Water System, resistivity > 18 MΩ. T-75 flasks, reaction tubes, 96-well plates, and six-well plates were purchased from Renner GmbH (Dannstadt, Germany).

Cell Lines and Growth Conditions. The MCF-7-2a cell line and the MCF-7cell line were kindly provided by Prof. Dr. E. v. Angerer, University of Regensburg (Germany). Both cell lines were maintained as a monolayer culture at 37 °C in a humidified atmosphere (95% air, 5% CO_2) in T-75 flasks. Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay.[29]

Growth media: MCF-7−2a cell line: phenol red free DMEM with penicillin−streptomycin 1%, L-glutamine 1%, FCS 5%, and geneticin solution 0.5%. MCF-7 cell line: L-glutamine containing EMEM supplemented with $NaHCO_3$ (2.2 g/L), sodium pyruvate (110 mg/L), gentamicin sulfate (50 mg/L), and FCS (100 mL/L).

Estrogen Receptor Binding Assay. The applied method was already described by Hartmann et al.[15] and used with some modifications. The relative binding affinity (RBA) of the test compounds to the ER was determined by the displacement of 17β-[^{3}H]estradiol from its binding site. For this purpose the test compounds were dissolved in ethanol and diluted with TRIS-buffer to 6−8 appropriate concentrations (300 μL) and were incubated while shaking with calf uterine cytosol (100 μL) and 17β-[^{3}H]estradiol (0.723 pmol in TRIS-buffer (100 μL); activity: 2249.4 Bq/tube) at 4 °C for 18−20 h. To stop the reaction, 500 μL of a dextran−charcoal-suspension in TRIS-buffer was added to each tube. After shaking for 90 min at 4 °C and centrifugation, 500 μL of HiSafe3 was mixed with 100 μL of supernatant of each sample, and the reactivity was determined by liquid scintillation spectroscopy. The same procedure was used to quantify the binding of 17β-[^{3}H]estradiol (0.723 pmol − control). Nonspecific binding was calculated using 2 nmol of 17β-estradiol as the competing ligand. On a semilog plot the percentage of maximum bound labeled steroid corrected by the nonspecifically bound 17β-[^{3}H]estradiol vs concentration of the competitor (log-axis) is plotted. At least six concentrations of each compound were chosen to estimate its binding affinity. From this plot those molar concentrations of unlabeled estradiol and of the competitors were determined which reduced the binding of the radioligand by 50%.

$$\mathrm{RBA} = \frac{\mathrm{IC}_{50}\ \mathrm{Estradiol}}{\mathrm{IC}_{50}\ \mathit{Sample}}\ \mu\mathrm{L}\ 100\ \%$$

Luciferase Assay. Estrogenic Activity. The pertinent in vitro assay was described earlier by Hafner et al.[16] One week before starting the experiment MCF-7-2a cells were cultivated in DMEM supplemented with L-glutamine, antibiotics, and dextran−charcoal-treated FCS (ct-FCS, 50 mL/L). Cells from an almost confluent monolayer were removed by trypsinization and suspended to approximately 2.2 × 10^5 cells/mL in the growth medium mentioned above. The cell suspension was then cultivated in six-well flat-bottomed plates (0.5 mL cell suspension and 2 mL medium per well) at growing conditions (see above). After 24 h, 25 μL of a stock solution of the test compounds was added to achieve concentrations ranging from 10^{-5} to 10^{-10} M, and the plates were incubated for 50 h. Before harvesting, the cells were washed twice with PBS, and then 200 μL of cell culture lysis reagent was added into each well. After 20 min of lysis at room-temperature, cells were transferred into reaction tubes and centrifuged. Luciferase was assayed using the Promega luciferase assay reagent. Fifty microliters of each supernatant was mixed with 50 μL of substrate reagent. Luminescence (in relative light units, RLU) was measured for 10 s using a microlumat. Measurements were corrected by correlating the quantity of protein (quantified according to Bradford[30]) of each sample with the mass of luciferase. Estrogenic activity was expressed as % activation of a 10^{-8} M estradiol control (100%).

Antiestrogenic Activity. The MCF-7-2a cells were treated as mentioned above. The cells were incubated with the test compounds in concentrations from 10^{-6} to 10^{-11} M along with a constant concentration of estradiol (10^{-9} M). The concentration of the compound, which is necessary to reduce the effect of estradiol by 50%, is IC_{50}.

Determination of Cytostatic Activity in MCF-7 Human Breast Cancer Cells. The cytotoxicity assay in MCF-7 cells had been described previously by Ruenitz et al.[31] Fixation, staining, and quantitation of the cells were carried out according to Gillies[32] and Kueng et al.[33] Cells from an almost confluent monolayer were harvested by trypsinization and suspended to approximately 7 × 10^4 cells/mL. At the beginning of the experiment, the cell suspension was transferred to 96-well microplates (100 μL/well). After cultivating them for 3 days at growing conditions the medium was removed and replaced by one containing the test compounds. Control wells (16/plate) contained 0.1% of DMF, which was used for the preparation of the stock solutions. The initial cell density was determined by addition of glutaric dialdehyde (1% in PBS; 100 μL/well). After incubation for 4−7 days, the medium was removed, and glutaric dialdehyde (1% in PBS; 100 μL/well) was added for fixation. After 15 min, the solution of the aldehyde was decanted and 180 μL PBS/well added. The plates were stored at 4 °C until staining. Cells were stained by treating them for 25 min with 100 μL of an aqueous solution of crystal violet (0.02%). After decanting, cells were washed several times with water to remove the adherent dye. After addition of 180 μL of ethanol (70%), plates were gently shaken for 4 h. Optical density of each well was measured in a microplate autoreader at 590 nm.

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