Antiestrogenically Active 1,1,2-Tris(4-hydroxyphenyl)alkenes without Basic Side Chain: Synthesis and Biological Activity

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C2-Alkyl substituted derivatives of the 1,1,2-tris(4-hydroxyphenyl)ethene **3a** (alkyl = Me (**3b**), Et (**3c**), Prop (**3d**), But (**3e**)) were synthesized by reaction of 1,2-bis(4-methoxyphenyl)ethanone with the appropriate alkyl halide, followed by a Grignard reaction with 4-methoxyphenylmagnesium bromide, dehydration with phosphoric acid or hydrobromic acid, and ether cleavage with BBr_3. The compounds were tested for estrogen receptor (ER) binding affinity in a competition experiment with radio labeled estradiol ([^{3}H]-E2) and for gene activation on the ER-positive MCF-7-2a cell line. All compounds showed high receptor binding affinity (RBA-value: **3b** (52.1%) > **3a** (45.5%) > **3c** (29.6%) > **3d** (4.03%) > **3e** (0.95%)). The tests on hormone dependent MCF-7-2a breast cancer cells, stably transfected with the plasmid ERE_{wtc}luc, revealed that all 1,1,2-tris(4-hydroxyphenyl)ethenes antagonized the effect of 1 nM estradiol (E2). The compounds **3b** (IC_{50} = 15 nM) and **3c** (IC_{50} = 10 nM) were equal in their effects to 4-hydroxytamoxifen (4OHT) (IC_{50} = 7 nM). Agonistic effects were low. Only **3a** and **3b** activated the luciferase expression (relative activation at 1 μM: **3a** 60%; **3b** 35%). Despite their highly antagonistic potency, the 1,1,2-tris(4-hydroxyphenyl)ethenes showed only low cytotoxic properties on the hormone sensitive MCF-7 cell line.

Introduction

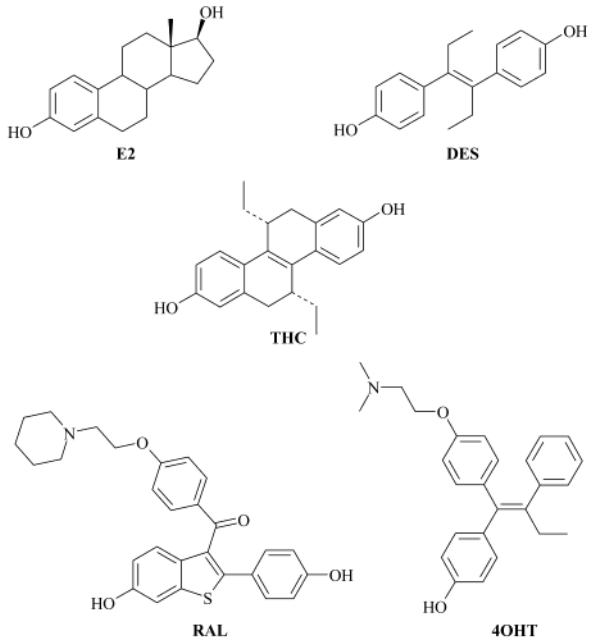
The use of the nonsteroidal antiestrogen tamoxifen (TAM) is the therapy of choice for patients with estrogen receptor (ER)-positive breast cancer.[1,2] Furthermore, TAM is the first drug, which is able to reduce the incidence of breast cancer in high risk women.[3] Besides its antiestrogenic effect on breast cancer cells, it exhibits significant estrogen-like properties in some estrogen target tissues such as bone, uterus, and liver.[4] These ambiguous effects led to the classification as a selective estrogen receptor modulator (SERM).[5]

4OHT, the active metabolite of TAM,[6] possesses an 8 times higher relative binding affinity (RBA) than TAM, and an about 100 times higher antiestrogenic potency in hormone dependent MCF-7-2a breast cancer cells, stably transfected with the plasmid ERE_{wtc}luc.[7] Its pharmacological properties determined in this transcription assay are related to the ability to compete with E2 for binding sites in the ligand-binding domain (LBD) of the ERα, because the MCF-7-2a cell line was established from ERα-positive MCF-7 breast cancer cells.

The binding mode of agonists (E2 or diethylstilbestrol (DES), see Chart 1) and antagonists (4OHT or raloxifene (RAL), see Chart 1) at ERα can be deduced from the crystal structure analyses of the LBD cocrystallized with these drugs.[8,9]

Although agonists and antagonists occupy the same binding cavity, the LBD/drug conjugates differ in their conformations. E2 and DES are completely protected from the external environment by helix 12. The right position of helix 12 is a prerequisite for transcriptional activation and generates a competent activation function 2 (AF2) which is capable to interact with coactivators.

Chart 1. Chemical Structures of the ER-Ligands Estradiol (E2), Diethylstilbestrol (DES), Tetrahydrochrysene (THC), Raloxifene (RAL), and 4-Hydroxytamoxifen (4OHT).



4OHT and RAL are bound similarly in the LBD, but their side chains are located in a narrow side pocket displacing helix 12 from the binding cavity. The reorientation blocks coactivator binding in the AF2 region. Silencing AF2 is therefore the primary mechanism of action for nonsteroidal antiestrogens.[10]

To achieve this conformational change of ERα, the side chains of 4OHT and RAL are anchored by a direct hydrogen bond between Asp 351 and the piperazine or the dimethylaminoethoxy nitrogen. Jordan et al.[11] hypothesized that alteration in the charge of this amino

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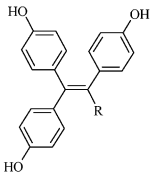
Table 1. Biological Properties of the Compounds **3a**−**e**.

compound	formula	R	RBA [%]	agonistic effect (at 1μM)	antagonistic effect (IC_{50}[nM])	cytotoxicity T/C_{corr} [%] (at 5μM)
3a		H	45.5	60	200	70
3b		Me	52.1	35	15	88
3c		Et	29.6	2	10	97
3d		Pr	4.03	0	70	102
3e		Bu	0.95	0	240	114
4OHT			15.6	0	7	25
TAM			1.8	0	500	15

acid and changes in the interaction with the side chain of an antiestrogen are critical for the estrogenity of the ER/drug complexes. Therefore, they interpreted the estrogen-like side effects of 4OHT by an insufficient shielding of the charge of Asp 351.

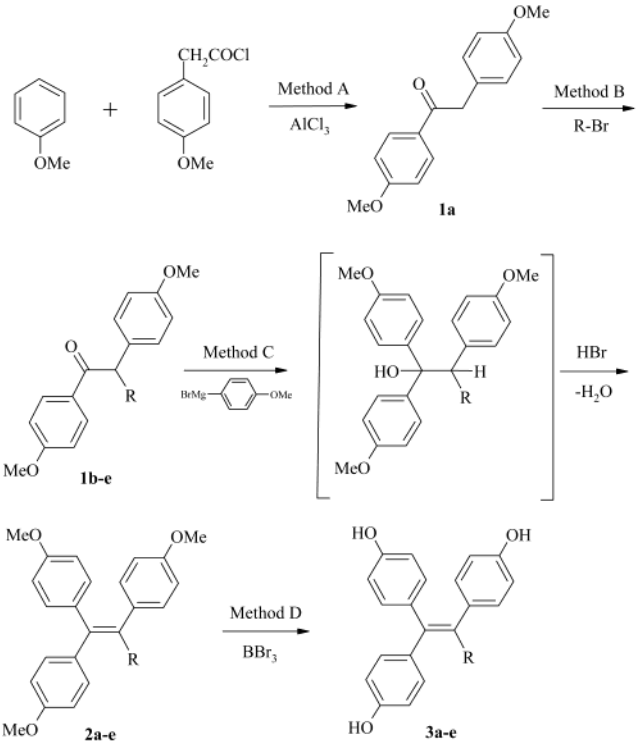
RAL and 4OHT are bound in the LBD by extensive hydrophobic contacts and H-bridges. While RAL contacts in the LBD comparable to E2 Glu 353, Arg 394, and His 524, the latter H-bond cannot be present in the case of 4OHT due to a missing OH group in the C2-phenyl ring.

The role of His 524 on the biological effects caused after binding of agonists or antagonists in the LBD of the human ERα were recently described by Aliau et al.[12] They deduced from their results that this amino acid exerts only a negligible or moderate role on the pharmacological properties of antagonists such as 4OHT or RAL, as far as interactions of Asp 351 with the basic side chain of these ligands exist.

Our findings, however, indicate that in the class of 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes the basic side chain is not a prerequisite for high binding affinity and antagonistic effects. The hormonal properties depend only on the length of the C2-alkyl chain.

To continue our structure−activity relationship study, we synthesized 1,1,2-tris(4-hydroxyphenyl)ethenes and studied the influence of the hydroxy group in the C2-phenyl ring on the biological effects, because an additional anchorage at His 524 in the LBD is possible for these compounds. The influence of this interaction is discussed in relation to the previously published data of 1,1-bis(4-hydroxyphenyl)-2-phenylethenes.[7]

Scheme 1. Synthesis of 1,1,2-Tris(4-hydroxyphenyl)alkenes



compound	R	compound	R
1a, 2a, 3a	H	**1d, 2d, 3d**	C_3H_7
1b, 2b, 3b	CH_3	**1e, 2e, 3e**	C_4H_9
1c, 2c, 3c	C_2H_5		

Results

Chemistry. The 1,1,2-tris(4-hydroxyphenyl)ethene derivatives **3a**−**e** were synthesized according to the method of Dodds et al.[13] starting from 1,2-bis(4-methoxyphenyl)ethanone **1a**, which was alkylated with the appropriate alkyl halide under the influence of potassium *tert*-butanolate to give the alkyl substituted 1,2-bis(4-methoxyphenyl)ethanones **1b**−**e** (Scheme 1).

Compounds **1a**−**e** were converted into the corresponding carbinols by use of a Grignard reaction with 4-methoxyphenylmagnesium bromide. Dehydration with either phosphoric acid or hydrobromic acid in THF yielded the C2-alkyl substituted 1,1,2-tris(4-methoxyphenyl)ethenes **2a**−**e**, which were purified by column chromatography on silica gel with diethyl ether/ligroine. The conversion into the hydroxy derivatives **3a**−**e** was performed with BBr_3. The identity of the compounds was established after recrystallization from CH_2Cl_2 by ^1H NMR, IR, and mass spectroscopy.

Pharrmacology. The affinity to the ER was determined in a competition experiment with [^{3}H]-E2 using calf uterine cytosol.[14] We used this ER resource to relate to data published earlier.

As listed in Table 1, all compounds displaced effectively E2 from its binding site. The binding curves were parallel to that of E2 and indicated a competitive inhibition. The 1,1,2-tris(4-hydroxyphenyl)ethene **3a** and the 1,1,2-tris(4-hydroxyphenyl)prop-1-ene **3b** showed the highest relative binding affinity (RBA). Elongation of the C2-alkyl side chain decreased the RBA in the

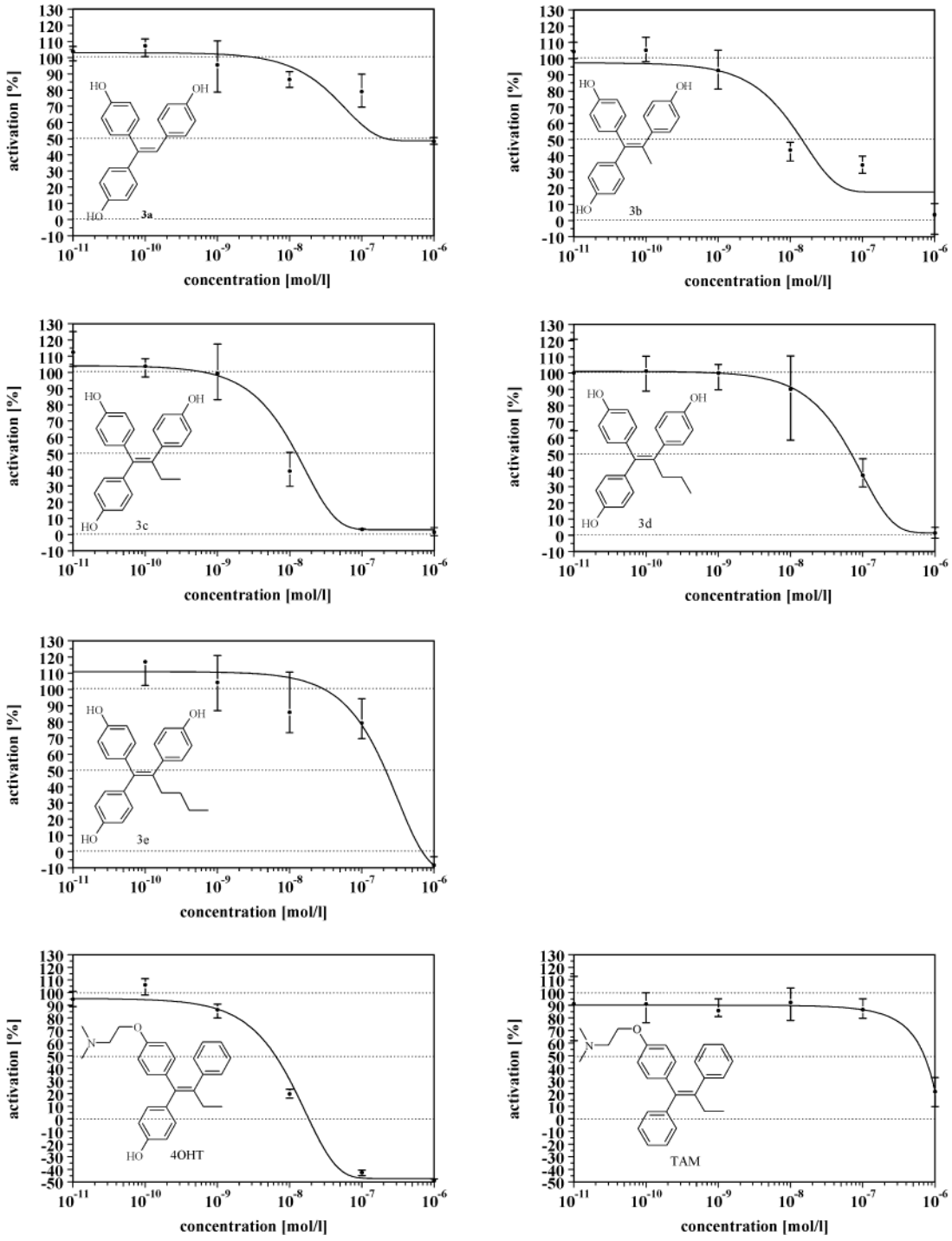


Figure 1. Luciferase expression in MCF-7-2a cells, stably transfected with the reporter plasmid ERE_{wtc}luc treated with a combination of E2 (1 nM) and the 1,1,2-tris(4-hydroxyphenyl)alkenes **3a**−**e**, tamoxifen (TAM), or 4-hydroxytamoxifen (4OHT). The effect of E2 (1 nM) is about 100%.

series:

H (45.5%) ≈ Me (52.1%) > Et (29.6%) > Prop (4.03%) > But (0.95%)

The gene activation resulting from the ER binding was evaluated in a luciferase assay using MCF-7-2a cells.[15] These ER-positive human breast cancer cells are stably transfected with the reporter plasmid ERE_{wtc}luc. The binding of ER/drug dimers at the estrogen response elements (ERE) of the plasmid activates the luciferase gene. The quantification of the luciferase expression allows a prediction of the agonistic potency; the inhibition of the E2 induced activation correlates with the antagonistic effects of the compounds.

All 1,1,2-tris(4-hydroxyphenyl)ethenes were antagonistically active on the MCF-7-2a cell line (see Table 1) and inhibited the effect of 1 nM E2 dependent on the length of the alkyl side chain (IC_{50} for the inhibition of 1 nM E2: R = But (240 nM) > H (200 nM) > Prop (70 nM) > Me (15 nM) > Et (10 nM).

The concentration activation curves (see Figure 1) document that **3a** and **3b** are partially antagonistically

active while **3c**−**e** are true antiestrogens, which antagonize completely the E2 effect. As most active compound, **3c** showed the same antagonistic potency as 4OHT (IC_{50} = 7 nM) and was almost a 100 times more active than TAM (IC_{50} = 500 nM). The agonistic activity of the partial antiestrogens **3a** and **3b** was low. They activated the luciferase expression in a concentration of 1 μM to 60% (**3a**) and 25% (**3b**), respectively (see Table 1).

To assess the relevance of the antiestrogenic properties for the proliferation of human breast cancer cells, we tested TAM, 4OHT**,** and the 1,1,2-tris(4-hydroxyphenyl)alkenes **3a**−**e** on the MCF-7 cell line (Table 1). Interestingly, the inhibition of cell proliferation did not correlate with the antagonistic potency. TAM with only low antiestrogenicity inhibited the cell proliferation in a concentration of 5 μM to T/C_{corr}= 15%, while the pure antagonists 4OHT (T/C_{corr} = 25%) and **3c** (T/C_{corr} = 97%) were less active. A weak cytotoxic effect (T/C_{corr} = 67%) was observed for **3c** only in a higher concentration of 10 μM. The other 1,1,2-tris(4-hydroxyphenyl)ethenes showed also only modest effects on the MCF-7 cell line (**3a** and **3b**) or were inactive (**3d** and **3e**, see Table 1).

Discussion

The binding of agonists and antagonists in the LBD of ERα causes different conformations of the ER. It is obvious from the crystal structures of the LBD cocrystallized with either E2, DES, RAL, or 4OHT that the position of helix 12 plays the major role in transcriptional activation. In the ER/E2 or ER/DES complex helix 12 is oriented over the binding cave and allows coactivator binding, which is a prerequisite for agonistic activity. 4OHT is bound within the same pocket that recognizes DES and E2. Its 4-hydroxyphenyl ring is analogously to E2 and DES H-bound to Glu 351 and Arg 394, while its 4-dimethylaminoethoxyphenyl residue is oriented out of the binding cavity in a narrow side pocket. This arrangement displaces helix 12 from the binding pocket and blocks AF2 activity. Therefore, the basic side chain of 4OHT is held responsible for its antagonistic properties. However, we demonstrated that the antagonistic effect of 4OHT in hormone dependent MCF-7-2a cells[7] is mainly caused by the 1,1-bis(4-hydroxyphenyl)-2-phenylethene moiety.

In continuation of this study, we focused our attention on C2-alkyl substituted 1,1,2-tris(4-hydroxyphenyl)-alkenes. These compounds can be anchored at His 524, which seems to be also of high relevance for the ER binding of antiestrogens as demonstrated for RAL.[16]

A contact of His 524 by an additional 4-OH group in the 2-phenyl ring of the 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes **4a**−**e** increased RBAs (see Figure 2). The most pronounced effect was observed for the 1,1-bis(4-hydroxyphenyl)-2-phenylbut-1-ene **4c** (**4c**: RBA = 6.2%; **3c**: RBA = 29.6%). A still higher receptor affinity was measured for the ethylene and propylene derivatives **3a** and **3b** which displaced [^{3}H]-E2 in amounts of 45.5 and 52.1% from its binding site.

In the luciferase assay with MCF-7-2a cells only **3a** and **3b** induced low, but significant gene activation, which lowers the antagonistic effects of both compounds in higher concentrations (see Figure 1). **3c**, **3d**, and **3e** showed the hormonal profile of true antiestrogens.

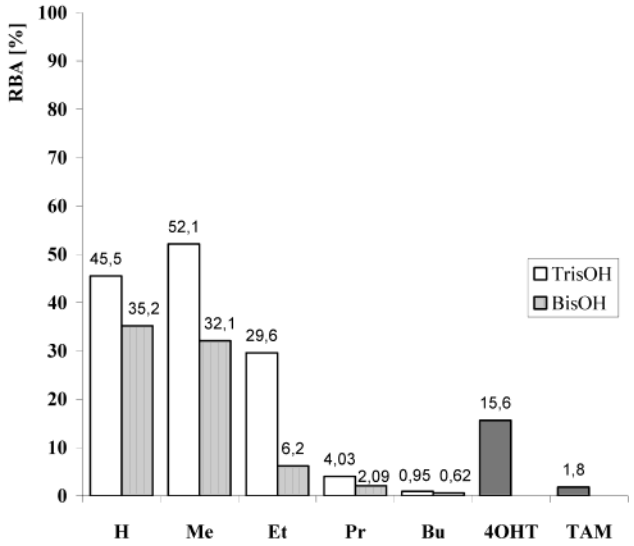


Figure 2. Comparison of the RBA-values of 1,1,2-tris(4-hydroxyphenyl)alkenes **3a**−**e** and 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes **4a**−**e**. RBA of E2 = 100%.

As one of the compounds described in this paper, **3b** has been tested on its influence on the prolactin synthesis in pituitary cell cultures of immature rats by Jordan et al.[17] They showed that **3b** induced prolactin synthesis to about 50% of the maximum value of E2. The prolactin increasing effect of E2 was competitively inhibited in a dose-dependent manner. These findings are in good agreement with our results.

The comparison of the results of the **3a**−**e** series with those of the **4a**−**e** series shows that the importance of a 4-OH group in the 2-phenyl ring is low for the antagonistic potential (see Figure 3). The most active compound **3c** (IC_{50} = 10 nM) is a pure antiestrogen without estrogenic side effects. The IC_{50} value for the antagonism of 1 nM E2 is comparable to that of 4OHT (IC_{50} = 7 nM) and **4c** (IC_{50} = 15 nM).

This demonstrates clearly that only the C2-alkyl substituent determines the extent of the pharmacological effects in vitro. A basic side chain and an anchorage at His524 is not necessary for antiestrogenic effects of 1,1,2-triarylalkene derivatives.

Our results contradict the mechanistical conception deduced from the crystal structures of the LBD/4OHT and LBD/RAL complexes. It has been suggested that the capability of Asp 351 to form an H-bond with the tertiary amine fragments present at the end of the side chains is a prerequisite for the antiestrogenicity of 4OHT and RAL.[18] Interestingly, Anghel et al.[19] demonstrated that in HeLa cells transiently transfected with the expression vectors for mutants of ERα in which Asp 351 is exchanged by Gly, Ala, or Val, no diminution of the antagonistic activity of 4OHT and RAL takes place. It is likely that the hydrogen bonds formed by the hydroxy group of 4OHT and the numerous hydrophobic interactions with the steroid-like skeleton contribute most to the interaction between the ERα and antiestrogens.

A recently published theory of a novel mode of estrogen receptor antagonism is very helpful for the interpretation of these results. Shiau et al.[20] studied the effects of the *R*,*R*-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC, see Chart 1) on ERα and ERβ.

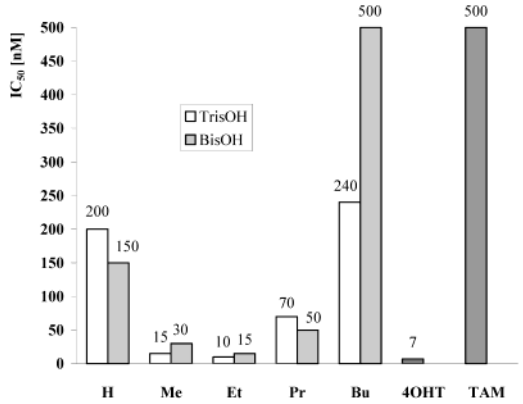


Figure 3. Comparison of the E2-antagonistic potency of 1,1,2-tris(4-hydroxyphenyl)alkenes **3a**−**e** and 1,1-bis(4-hydroxyphenyl)-2-phenylalkenes **4a**−**e**.

THC acts as an ERα agonist and an ERβ antagonist.[21,22] THC lacks a bulky side chain and in the crystal structure, helix 12 is not sterically precluded from adopting the agonist-bound conformation. It is postulated, that THC antagonizes ERβ by stabilizing nonproductive conformations of key residues in the LBD, which result in disfavoring the agonist-bound orientation of the helix 12 and the stabilization of an inactive conformation of helix 12. This mode of action of THC is called "passive antagonism" and could also be used for the interpretation of the results of our studies, since both THC and OH substituted 1,1,2-triphenylethenes are antagonists without a basic side chain.

However, passive antagonism was until yet described exclusively for THC at ERβ. The results of our transcription assay contribute mainly to an interaction with ERα. Therefore, further investigations using assays to assess the effects of 1,1,2-triphenylethenes on ERα and ERβ gene regulation will be necessary to confirm our supposition.

Although the 1,1,2-tris(4-hydroxyphenyl)alkenes are antiestrogens, the influence on MCF-7 breast cancer cells is very low. In contrast to TAM, which reduced the growth of the tumor cells in a concentration of 5 μM to a $T/C_{corr} = 15\%$ none of the compounds is able to inhibit cell proliferation in an equimolar concentration. In vivo, however, these compounds seem to be antitumor active. The 1,1,2-tris(4-acetoxyphenyl)but-1-ene, which should be quickly transformed into **3c**, reduced the tumor growth of the hormone dependent MXT mammary carcinoma of the mouse in a dose of 9.2 mg/kg to T/C = 2% (TAM: T/C = 9%).[23]

Although the mode of action is unknown, the enhanced uterus weight of the mice, detected at the end of the test, indicates that the antibreast cancer effects cannot be caused by inhibition of the tumor growth stimulating effect of endogenous estrogens. Rather a mechanism that involves also other cells of the host e.g. such of the immune system must be taken into consideration.[24,25] This hypothesis is supported by the investigations of Curran et al.[26] Their data suggest that ERβ or possibly a novel receptor are involved in mediating estrogen action on natural killer cell activity. This could raise the potential for therapeutic modulation with selective estrogen receptor modulators.

In conclusion, our data demonstrate that 1,2-bis(4-hydroxyphenyl)-2-phenylbut-1-ene and its derivatives described in this paper possess an antagonistic profile. A basic dimethylaminoethoxy side chain or an additional 4-hydroxy group in the C2-phenyl ring, which allow an anchorage at His 524, change the antagonistic properties in MCF-7-2a cells only marginally. Since a basic side chain which displaces the helix 12 from the binding cave and stabilizes this antagonistic conformation by interaction with Asp 351 is held responsible for an "active" antagonism, we postulate a mode of action analogously to the "passive" antagonism described by Shiau et al.[20] In this connection, studies on the antiestrogenicity of the novel compounds by measuring a profile of estrogen responsive gene including both classical ERE and tethered or nonclassical estrogen responsive pathways are of great interest.

Materials and Methods

Chemistry. IR spectra (KBr pellets): Perkin-Elmer model 580 A. ^{1}H NMR spectra: ADX 400 spectrometer at 400 MHz (internal standard: TMS). Elemental analyses: Microlaboratory of Free University of Berlin. Based on the C, H, and N analyses, all compounds were of acceptable purity (within 0.4% of the calculated values).

Method A: 1,2-Bis(4-methoxyphenyl)ethanone (1a). 4-Methoxyphenylacetyl chloride (16.6 g, 9.0 mmol) was added dropwise to a suspension of aluminum chloride (14.4 g, 10.8 mmol) and anisole (10 mL, 9.2 mmol) in 20 mL of dry 1,2-dichloroethane under cooling. After heating to reflux for 2 h, 50 mL of water were 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 110 °C). Yield: 15.2 g (59.2 mmol, 67%): ^{1}H NMR ($CDCl_3$): δ = 3.77 (s, 3H, OCH_3); 3.85 (s, 3H, OCH_3); 4.16 (s, 2H, CH_2); 6.85 (AA′BB′ ^3J = 8.5 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.91 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.17 (AA′BB′ ^3J = 8.5 Hz, 2H, Ar*H*-2, Ar*H*-6); 7.98 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-2, Ar′*H*-6). IR (KBr; cm^{-1}): 3022 w (ArH); 2965 m (CH_2); 2939 w (CH_2); 2841 m (OCH_3); 1676 s (C═O); 1598 s (C═C); 1512 s (C═C). MS (EI, 100 °C): *m*/*z* (%) = 256 $[M]^{+\bullet}$ (6.04), 135 H_3COPh-CO^+ (100).

Method B: 1,2-Bis(4-methoxyphenyl)propanone (1b). To a suspension of **1a** (2.56 g, 10.0 mmol) and potassium *tert*-butanolate (1.35 g, 12.0 mmol) in freshly distilled dry diethyl ether was added carefully (1.42 g, 10.0 mmol) methyl iodide. After the addition was completed, the mixture was refluxed for 6 h. The organic layer was separated after addition of 20 mL of water, dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography with diethyl ether/ligroine 1:5. Yield: 1.83 g (6.76 mmol, 68%) of a light brown oil. ^{1}H NMR ($CDCl_3$): δ = 1.48 (d, 3H, CH_3); 3.75 (s, 3H, OCH_3); 3.81 (s, 3H, OCH_3); 4.58 (t, 1H, C*H*); 6.82 (AA′BB′ ^3J = 8.0 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.85 (AA′BB′ ^3J = 8.2 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.19 (AA′BB′ ^3J = 8.0 Hz, 2H, Ar*H*-2, Ar*H*-6); 7.93 (AA′BB′ ^3J = 8.2 Hz, 2H, Ar′*H*-2, Ar′*H*-6). IR (film; cm^{-1}): 2968 s (CH_2); 2932 s (CH_2); 2837 m (OCH_3); 1673 s (C═O); 1601 s (C═C); 1511 s (C═C). MS (EI, 60 °C): *m*/*z* (%) = 270 $[M]^{+\bullet}$ (4.4); 135 H_3CO-Ph-CO^+ (100).

1,2-Bis(4-methoxyphenyl)butanone (1c). From **1a** (2.55 g, 10.0 mmol), potassium *tert*-butanolate (1.35 g, 12.0 mmol) and ethyl iodine (1.56 g, 10.0 mmol) in diethyl ether. Purification was carried out by column chromatography with diethyl ether/ligroine 1:3. Yield: 2.22 g (7.8 mmol, 78%) of a colorless oil. ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 3H, CH_3); 1.81 (m, 1H, ROC-CHRCH_2); 2.15 (m, 1H, ROC-CHRCH_2); 3.75 (s, 3H, OCH_3); 3.82 (s, 3H, OCH_3); 4.34 (t, 1H, C*H*); 6.82 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.86 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.29 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-2, Ar*H*-6); 7.95 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-2, Ar'*H*-6). IR (film; cm^{-1}): 2963 s (CH_2); 2934 s (CH_2); 2838 m (OCH_3); 1671 s (C═O); 1601 s

(C═C); 1510 s (C═C). MS (EI, 80 °C): *m/z* (%) = 284 $[M]^{+\bullet}$ (6.6); 135 H_3CO-Ph-CO^+ (100).

1,2-Bis(4-methoxyphenyl)pentanone (1d). From **1a** (5.12 g, 20 mmol), potassium *tert*-butanolate (3.47 g, 30.9 mmol) and bromopropane (3.66 g, 29.7 mmol) in THF. The crude product was purified by column chromatography with diethyl ether/ligroine 1:5. Yield: 3.25 g (10.3 mmol, 54%) of a yellow oil. ^{1}H NMR ($CDCl_3$): δ = 0.91 (t, 3H, C*H*$_3$); 1.27 (m, 2H, C*H*$_2$CH$_3$); 1.77 (m, 1H, ROC-CHRC*H*$_2$); 2.15 (m, 1H, ROC-CHRC*H*$_2$); 3.75 (s, 3H, OC*H*$_3$); 3.82 (s, 3H, OC*H*$_3$); 4.46 (t, 1H, C*H*); 6.81 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.86 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.21 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-2, Ar*H*-6); 7.95 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-2, Ar′*H*-6). IR (film; cm^{-1}): 2957 s (CH_2); 2934 s (CH_2); 2869 m (OCH_3); 2838 m (OCH_3); 1671 s (C═O); 1601 s (C═C); 1510 s (C═C). MS (EI, 60 °C): *m/z* (%) = 163 [H_3CO-Ph-$CH^+C_2H_5$] (18.9); 135 H_3CO-Ph-CO^+ (100).

1,2-Bis(4-methoxyphenyl)hexanone (1e). From **1a** (5.09 g, 19.9 mmol), potassium *tert*-butanolate (3.41 g, 30.4 mmol) and bromobutane (4.18 g, 30.5 mmol) in 25 mL of THF. The crude product was purified by column chromatography with ether/ligroine 1:5. Yield: 3.16 g (10.1 mmol, 51%) of a yellow oil. ^{1}H NMR ($CDCl_3$): δ = 0.86 (t, 3H, C*H*$_3$); 1.13−1.39 (m, 4H, 2 × C*H*$_2$); 1.77 (m, 1H, ROC-CHRC*H*$_2$); 2.15 (m, 1H, ROC−CHRC*H*$_2$); 3.75 (s, 3H, OC*H*$_3$); 3.82 (s, 3H, OC*H*$_3$); 4.43 (t, 1H, C*H*); 6.81 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.86 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 7.21 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-2, Ar*H*-6); 7.95 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar′*H*-2, Ar′*H*-6). IR (Film; cm^{-1}): 3003 w (ArH); 2955 s (CH_2); 2932 s (CH_2); 2859 m (OCH_3); 2840 m (OCH_3); 1671 s (C═O); 1601 s (C═C); 1510 s (C═C). MS (EI, 110 °C): *m/z* (%) = 312 $[M]^{+\bullet}$ (4.7); 135 [H_3CO-Ph-CO^+] (100).

Method C: 1,1,2-Tris(4-methoxyphenyl)ethene (2a). A solution of **1a** (1.3 g, 5.05 mmol) in dry THF was added dropwise to a solution of 4-methoxyphenylmagnesium bromide (1.41 g, 7.5 mmol) in THF, which was generated before from 4-bromoanisole and Mg (0.19 g, 7.8 mmol). The mixture was refluxed for 12 h and then decomposed with ice and acetic acid (6 N). The solvent was removed under reduced pressure and the remaining aqueous layer was extracted with diethyl ether. The combined organic extracts were washed with saturated $NaHCO_3$ solution and water, dried over Na_2SO_4, and the solvent was removed to give the crude carbinol. After dissolution in dry THF it was added dropwise to ice cold 47% HBr. The mixture was warmed to room temperature and stirred for 2 h, then poured onto ice, and extracted with dichloromethane. The organic extracts were washed with $NaHCO_3$ solution and water. After the extracts were dried over Na_2SO_4, the solvent was removed and the remaining product was purified by column chromatography on silica gel with diethyl ether/ligroine 1:1. Yield: 0.99 g (2.86 mmol, 57%) of a colorless solid (mp 93−95 °C). ^{1}H NMR ($CDCl_3$): δ = 3.75 (s, 3H, OC*H*$_3$); 3.81 (s, 3H, OC*H*$_3$); 3.84 (s, 3H, OC*H*$_3$); 6.68 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.78 (s, 2H, C*H*); 6.84 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.87 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.96 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar″*H*-3, Ar″*H*-5); 7.12 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.24 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar″*H*-2, Ar″*H*-6). IR (KBr, cm^{-1}): 3001 w (ArH); 2957 w (CH_2); 2930 w (CH_2); 2836 w (OCH_3); 1605 s (C═C); 1571 m (C═C); 1511 s (C═C). MS (EI, 140 °C): *m/z* (%) = 346 $[M]^{+\bullet}$ (100).

1,1,2-Tris(4-methoxyphenyl)prop-1-ene (2b). The Grignard reagent was synthesized from magnesium (0.25 g, 10.1 mmol) and 4-bromoanisole (1.89 g, 10.1 mmol) in 15 mL of dry THF. 1,2-Bis(4-methoxyphenyl)propanone **1b** (1.82 g, 6.7 mmol) in 20 mL abs. THF were added and the mixture was refluxed for 12 h. After 2 h of hydrolysis with 27 mL of HBr, the crude product was purified by column chromatography with diethyl ether/ligroine 1:10. Yield: 1.84 g (5.12 mmol, 76%) of pink crystals (mp 116 °C). ^{1}H NMR ($CDCl_3$): 2.10 (s, 3H, C*H*$_3$); 3.70 (s, 3H, OC*H*$_3$); 3.75 (s, 3H, OC*H*$_3$); 3.82 (s, 3H, OC*H*$_3$); 6.58 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.69 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.79 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.87 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar″*H*-3, Ar″*H*-5); 7.06 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.14 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar″*H*-2, Ar″*H*-6). IR (KBr, cm^{-1}): 2955 w (CH_2); 2910 w (CH_2); 2836 w (OCH_3); 1607 s (C═C); 1573 w (C═C); 1511 s (C═C). MS (EI, 140 °C): *m/z* (%) = 360 $[M]^{+\bullet}$ (100).

1,1,2-Tris(4-methoxyphenyl)but-1-ene (2c). Magnesium (0.26 g, 10.6 mmol) and 4-bromoanisole (1.98 g, 10.6 mmol) were reacted in dry THF to give the Grignard reagent. **1c** (2.03 g, 7.0 mmol) was added and the mixture was refluxed for 12 h. Hydrolysis was carried out by heating the crude carbinol in 10 mL of H_3PO_4 for 2 h. The compound was purified by column chromatography with diethyl ether/ligroine 1:3. Yield: 2.07 g (5.53 mmol, 79%) of a colorless solid (mp 90 °C). ^{1}H NMR ($CDCl_3$): 0.92 (t, 3H, C*H*$_3$); 2.45 (q, 2H, C*H*$_2$); 3.69 (s, 3H, OC*H*$_3$); 3.76 (s, 3H, OC*H*$_3$); 3.82 (s, 3H, OC*H*$_3$); 6.56 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.71 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.78 (AA′BB′ ^3J = 8.9 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.87 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar″*H*-3, Ar″*H*-5); 7.02 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.14 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar″*H*-2, Ar″*H*-6). IR (KBr, cm^{-1}): 3061 w (ArH); 3011 w (ArH); 2953 m (CH_2); 2833 m (OCH_3); 1608 s (C═C); 1573 w (C═C); 1509 s (C═C). MS (EI, 150 °C): *m/z* (%) = 374 $[M]^{+\bullet}$ (100).

1,1,2-Tris(4-methoxyphenyl)pent-1-ene (2d). Magnesium (0.4 g, 16.3 mmol) and 4-bromoanisole (2.99 g, 16.0 mmol) were reacted in THF to give the Grignard reagent. 1,2-Bis(4-methoxyphenyl)pentanone **1d** (3.25 g, 10.9 mmol) in 20 mL of dry THF was added and heated for 14 h. Decomposition with 35 mL of HBr (47%) and column chromatography with diethyl ether/ligroine 1:3 gave 1.8 g (4.64 mmol, 43%) of colorless crystals (mp 91 °C). ^{1}H NMR ($CDCl_3$): 0.81 (t, 3H, C*H*$_3$); 1.26 (m, 2H, *CH*$_2$CH$_3$); 2.38 (m, 2H, ═CR−C*H*$_2$); 3.69 (s, 3H, OC*H*$_3$); 3.76 (s, 3H, OC*H*$_3$); 3.82 (s, 3H, OC*H*$_3$); 6.55 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.67 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.79 (AA′BB′ ^3J = 8.8 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.87 (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); 7.15 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar″*H*-2, Ar″*H*-6). IR (KBr, cm^{-1}): 3029 w (ArH); 2953 m (CH_2); 2836 w (OCH_3); 1607 s (C═C); 1573 w (C═C); 1510 s (C═C). MS (EI, 130 °C): *m/z* (%) = 388 $[M]^{+\bullet}$ (100).

1,1,2-Tris(4-methoxyphenyl)hex-1-ene (2e). Magnesium (0.12 g, 4.8 mmol) and 4-bromoanisole (0.84 g, 4.5 mmol) were reacted in THF to form the Grignard reagent. 1,2-Bis(4-methoxyphenyl)hexanone **1e** (0.96 g, 3.1 mmol) was added and the mixture refluxed for 12 h. The carbinol was decomposed with 10 mL of 85% H_3PO_4 and the product isolated by column chromatography with diethyl ether/ligroine 1:5. Yield: 0.8 g (2 mmol, 65%) of a colorless oil. ^{1}H NMR ($CDCl_3$): 0.78 (t, 3H, C*H*$_3$); 1.17−1.34 (m, 4H, 2 × C*H*$_2$); 2.4 (t, 2H, C═CRC*H*$_2$); 3.69 (s, 3H, OC*H*$_3$); 3.76 (s, 3H, OC*H*$_3$); 3.82 (s, 3H, OC*H*$_3$); 6.56 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-3, Ar*H*-5); 6.71 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′*H*-3, Ar′*H*-5); 6.78 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar*H*-2, Ar*H*-6); 6.87 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar″*H*-3, Ar″*H*-5); 7.02 (AA′BB′ ^3J = 8.6 Hz, 2H, Ar′*H*-2, Ar′*H*-6); 7.14 (AA′BB′ ^3J = 8.7 Hz, 2H, Ar″*H*-2, Ar″*H*-6). IR (KBr, cm^{-1}): 3031 w (ArH); 2955 s (CH_2); 2934 s (CH_2); 2834 m (OCH_3); 1608 s (C═C); 1573 w (C═C); 1509 s (C═C). MS (EI, 90 °C): *m/z* (%) = 402 $[M]^{+\bullet}$ (100).

Method D: 1,1,2-Tris(4-hydroxyphenyl)ethene (3a). A solution of **2a** (0.34 g, 1.0 mmol) in dry dichloromethane was cooled to −52 °C. Under nitrogen, BBr_3 (2.25 g, 9.0 mmol) in dry dichloromethane was added dropwise. After 0.5 h the cooling was removed and the reaction mixture was stirred at room temperature for 3 days. After that, dry methanol was added under cooling and the solvents were removed under reduced pressure for at least three times. The crude product was recrystallized from CH_2Cl_2. Yield: 0.1 g (0.34 mmol, 34%) of a dark red solid (mp 168 °C). ^{1}H NMR ($CDCl_3$): δ = 6.6−7.2 (m, 13H, Ar*H*, C*H*); 8.2−8.4 (3s, 3H, ArO*H*). IR (KBr, cm^{-1}): 3441 br, s (OH); 3394 br, s (OH); 3073 m (ArH); 3025 m (ArH); 2936 m (CH_2); 1608 s (C═C); 1511 s (C═C). MS (EI, 160 °C): *m/z* (%) = 304 $[M]^{+\bullet}$ (73.4); 94 (100). Anal. ($C_{20}H_{16}O_3$) C H.

1,1,2-Tris(4-hydroxyphenyl)prop-1-ene (3b). From **2b** (0.36 g, 1.0 mmol). Yield: 0.2 g (0.6 mmol, 60%) of a pink

powder (mp 93−94 °C).^{1}H NMR ([D4]methanol)): $\delta = 2.04$ (s, 3H, CH_3); 6.43 (AA′BB′ $^3J = 8.7$ Hz, 2H, ArH-3, ArH-5); 6.56 (AA′BB′ $^3J = 8.6$ Hz, 2H, Ar′H-3, Ar′H-5); 6.64 (AA′BB′ $^3J = 8.7$ Hz, 2H, ArH-2, ArH-6); 6.74 (AA′BB′ $^3J = 8.5$ Hz, 2H, Ar″H-3, Ar″H-5); 6.93 (AA′BB′ $^3J = 8.6$ Hz, 2H, Ar′H-2, Ar′H-6); 6.99 (AA′BB′ $^3J = 8.5$ Hz, 2H, Ar″H-2, Ar″H-6). IR (KBr, cm^{-1}): 3418 br s (OH); 3309 br s (OH); 3030 m (ArH); 2868 w (CH_2); 1609 s (C═C); 1510 s (C═C). MS (EI, 270 °C): m/z (%) = 318 $[M]^{+\bullet}$ (100). Anal. ($C_{21}H_{18}O_3 \times 0.75\ H_2O$) C H.

1,1,2-Tris(4-hydroxyphenyl)but-1-ene (3c). From **2c** (1 g, 2.67 mmol). Yield: 0.7 g (2.02 mmol, 76%) of a beige powder (mp 94 °C). ^{1}H NMR ([D_6]DMSO): $\delta = 0.83$ (t, 3H, CH_3); 2.33 (q, 2H, CH_2); 6.40 (AA′BB′ $^3J = 8.6$ Hz, 2H, ArH-3, ArH-5); 6.54 (AA′BB′ $^3J = 8.5$ Hz, 2H, Ar′H-3, Ar′H-5); 6.58 (AA′BB′ $^3J = 8.6$ Hz, 2H, ArH-2, ArH-6); 6.72 (AA′BB′ $^3J = 8.4$ Hz, 2H, Ar″H-3, Ar″H-5); 6.86 (AA′BB′ $^3J = 8.5$ Hz, 2H, Ar′H-2, Ar′H-6); 6.93 (AA′BB′ $^3J = 8.4$ Hz, 2H, Ar″H-2, Ar″H-6); 9.13 (s, 1H, OH); 9.2 (s, 1H, OH); 9.38 (s, 1H, OH). IR (KBr, cm^{-1}): 3391 br s (OH); 3034 m (ArH); 2968 m (ArH); 2930 m (ArH); 2870 m (CH_2); 1608 s (C═C); 1510 s (C═C). MS (EI, 200°C): m/z (%) = 332 $[M]^{+\bullet}$ (25.5). Anal ($C_{22}H_{20}O_3 \times 0.75\ H_2O$) C H.

1,1,2-Tris(4-hydroxyphenyl)pent-1-ene (3d). From **2d** (0.39 g, 1.0 mmol). Yield: 0.21 g (0.58 mmol, 58%) of a brown solid (mp 90−94 °C). ^{1}H NMR ([D4]methanol): $\delta = 0.8$ (t, 3H, CH_3); 1.32 (m, 2H, CH_2); 2.35 (q, 2H, CH_2); 6.42 (AA′BB′ $^3J = 8.7$ Hz, 2H, ArH-3, ArH-5); 6.57 (AA′BB′ $^3J = 8.6$ Hz, 2H, Ar′H-3, Ar′H-5); 6.66 (AA′BB′ $^3J = 8.7$ Hz, 2H, ArH-2, ArH-6); 6.75 (AA′BB′ $^3J = 8.6$ Hz, 2H, Ar″H-3, Ar″H-5); 6.90 (AA′BB′ $^3J = 8.6$ Hz, 2H, Ar′H-2, Ar′H-6); 6.99 (AA′BB′ $^3J = 8.6$ Hz, 2H, Ar″H-2, Ar″H-6). IR (KBr, cm^{-1}): 3382 br s (OH); 3058 m (ArH); 2957 m (ArH); 2868 m (CH_2); 1608 s (C═C); 1510 s (C═C). MS (EI, 200 °C): m/z (%) = 346 $[M]^{+\bullet}$ (100). Anal ($C_{23}H_{22}O_3 \times 0.75\ H_2O$) C H.

1,1,2-Tris(4-hydroxyphenyl)hex-1-ene (3e). From **2e** (0.15 g, 0.37 mmol). Yield: 0.1 g (0.26 mmol, 71%) of a yellow powder (mp 105−106 °C). ^{1}H NMR ([D4]methanol): $\delta = 0.78$ (t, 3H, CH_3); 1.16 − 1.32 (m, 4H, 2 × CH_2); 2.38 (t, 2H, CH_2); 6.41 (AA′BB′ $^3J = 8.6$ Hz, 2H, ArH-3, ArH-5); 6.57 (AA′BB′ $^3J = 8.55$ Hz, 2H, Ar′H-3, Ar′H-5); 6.65 (AA′BB′ $^3J = 8.6$ Hz, 2H, ArH-2, ArH-6); 6.74 (AA′BB′ $^3J = 8.5$ Hz, 2H, Ar″H-3, Ar″H-5); 6.90 (AA′BB′ $^3J = 8.55$ Hz, 2H, Ar′H-2, Ar′H-6); 6.98 (AA′BB′ $^3J = 8.5$ Hz, 2H, Ar″H-2, Ar″H-6). IR (KBr, cm^{-1}): 3433 br s (OH); 3347 br s (OH); 3035 m (ArH); 2956 m (ArH); 2929 m (ArH); 2871 m (CH_2); 1610 s (C═C); 1510 s (C═C). MS (EI, 190 °C): m/z (%) = 360 $[M]^{+\bullet}$ (8.8); 317 (66.1). Anal (**(**$C_{24}H_{24}O_3 \times H_2O$) C H.

Biological Methods. (a) Materials and Reagents for Bioassays. 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): Gibco (Eggenstein, Germany); fetal calf serum (FCS): Bio Whittaker (Verviers, Belgium); *N*-hexamethylpararosaniline (crystal violet) and gentamicin sulfate: Fluka (Deisenhofen, Germany); glutardialdehyde (25%): Merck (Darmstadt, Germany); trypsin (0.05%) in ethylenediaminetetraacetic acid (0.02%) (trypsin/EDTA): Boehringer (Mannheim, Germany); penicillin−streptomycin gold standard (10 000 IE of penicillin/mL, 10 mg of streptomycin/mL) and geneticin disulfate (geneticin solution: 35.71 mg/mL PBS): ICN Biomedicals GmbH (Eschwege, Germany); norit A (charcoal): Serva (Heidelberg, Germany); cell culture lysis reagent (5×) (diluted 1:5 with purified water before use) and the luciferase assay reagent: Promega (Heidelberg, Germany); optiphase HiSafe3 scintillation liquid: Wallac (Turku, Finland); NET-317-estradiol[2,4,6,7-^{3}H(N)] (17β-[^{3}H]-estradiol): Du Pont NEN (Boston, Maryland); $CDCl_3$, [D_6]-DMSO and [D4]methanol: Aldrich (Steinheim, Germany); PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \times 2\ H_2O$ 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, 0.37224 g of Titriplex III, and 0.195 g of sodium azide (all from Merck or Fluka) in 1000 mL of purified water. Deionized water was produced by means of a Millipore Milli-Q Water System, resistivity > 18 MΩ. T-75 flasks, reaction tubes, 96-well plates and 6-well plates were purchased from Renner GmbH (Dannstadt, Germany). Liquid Scintillation Counter: 1450 Microbeta Plus (Wallac, Finland). Microplate Photometer: Labsystems Multiscan Plus (Labsystems, Finland). Microlumat: LB 96 P (EG & G Berthold, Germany).

(b) Cell Lines and Growth Conditions. The MCF-7-2a cell line and the MCF-7 cell 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.[27]

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).

(c) Estrogen Receptor Binding Assay. The applied method was already described by Hartmann et al.[14] 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). They were incubated and shaken 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. 500 μL of a dextran-charcoal-suspension in TRIS-buffer were added to each tube to stop the reaction. After the sample was shaken for 90 min at 4 °C and centrifugation 500 μL HiSafe3 was mixed with 100 μL 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). Using 4 μmol of 17β-estradiol as competing ligand nonspecific binding was calculated. 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, the molar concentrations of unlabeled estradiol and of the competitors were determined which reduced the binding of the radioligand by 50%.

$$\mathrm{RBA} = \frac{\mathrm{IC_{50}\ estradiol}}{\mathrm{IC_{50}\ sample}} \times 100\%$$

(d) Transcriptional Binding Assay, Luciferase Assay. The pertinent in vitro assay was described earlier by Hafner et al.[15] 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 of cell suspension and 2 mL of medium per well) at growing conditions (see above). After 24 h, 25 μL of a stock solution of the test compounds were added to achieve concentrations ranging from 10^{-5}−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. 50 μL 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[28]) of each sample

with the mass of luciferase. Estrogenic activity was expressed as % activation of a 10^{-8} M Estradiol control (100%).

To evaluate the antagonistic activity, the cells were incubated with the test compounds in concentrations from 10^{-6}–10^{-11} M along with a constant amount of estradiol (10^{-9} M). The concentration of the compound, which is necessary to reduce the effect of estradiol by 50%, is IC_{50}.

(e) Determination of the Cytostatic Activity in MCF-7 Human Breast Cancer Cells. The cytotoxicity assay in MCF-7 cells had been described previously by us.[7] 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 that 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 of 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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