(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxyphenyl)-2-imidazolines: Ligands for the Estrogen Receptor with a Novel Binding Mode

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(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxyphenyl)-2-imidazolines **1**−**7** were synthesized by the reaction of the methoxy-substituted (1*R*,2*S*)/(1*S*,2*R*)-1,2-diarylethylenediamines **1b**−**7b** with triethyl orthoformate and subsequent ether cleavage with BBr_3. All compounds were tested for estrogen receptor (ER) binding in a competition experiment with [^{3}H]-estradiol and for gene activation in a luciferase assay using ER positive MCF-7-2a breast cancer cells stably transfected with the plasmid ERE_{wtc}luc. The relative binding affinities of the 2-imidazolines were very low (RBA < 0.1%). Nevertheless, **4**−**7** possessed full agonistic activity in the luciferase assay. The relative transcription potency increased in the order **5** (2,2′-I) < **6** (2,6-Cl_2, 2′-F) < **4** (2,2′-Cl) < **7** (2,6-Cl_2, 2′-Cl). These data together with spectroscopic and molecular modeling studies were used to investigate the preferred binding mode adopted by the imidazoline ligands. The 1,2-diarylethane pharmacophor takes a *Z*-stilbene-like structure with pseudoaxially oriented phenyl rings at the planar heterocyclic ring. Because of this unusual spatial structure, the (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazolines have to be assigned to a second class of estrogenically active compounds (type II estrogens). In contrast to type I estrogens, e.g., estradiol (E2), diethylstilbestrol (DES), and meso-hexestrol (HES), which are connected to His 524 in the binding site, type II estrogens are very likely H-bonded to Asp 351 in a hydrophobic side pocket.

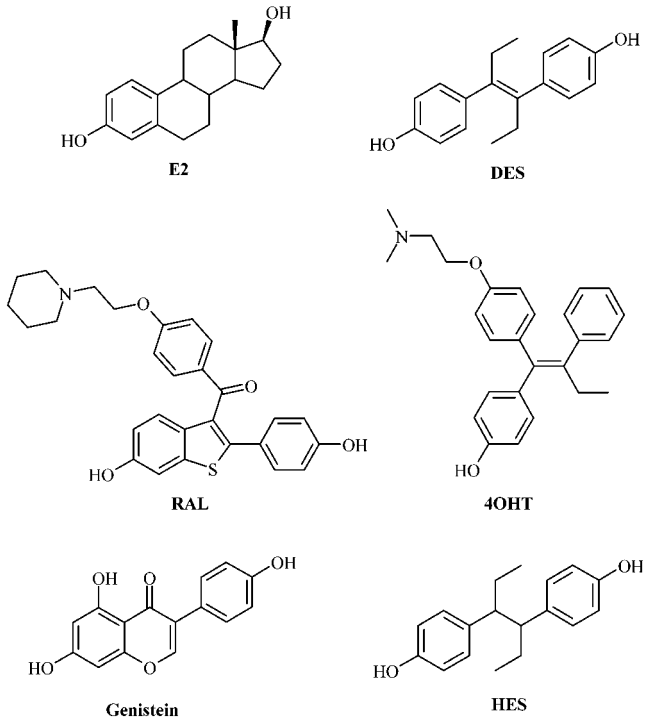
Introduction

Steroidal estrogens not only play an important role in the growth, differentiation, and function of male and female reproduction systems but also influence positively bone maintenance, the central nervous system, and the cardiovascular system.[1−4] Most of the effects are mediated by two intranuclear proteins: the estrogen receptors α (ERα) and *β* (ER*β*).

The binding of estrogens to ERα or ER*β* leads to a conformational change of the receptor molecule, dimerization of ER/drug conjugates, and gene activation after interaction with the "estrogen response elements" (ERE) at the DNA. The ligand-binding domain (LBD) is an essential part of the ER and was characterized during the past decades in many structure−activity relationship (SAR) studies on hormonally active compounds. SAR studies date back more than 6 decades to the early work of Dodds et al.[5,6] Meanwhile, a lot of steroidal and nonsteroidal estrogens were synthesized and evaluated for ER binding. On the basis of these results, the criteria for hormonal activity were derived:[7] (i) H-bonding ability of hydroxyl groups mimicking the 3-OH and the 17*β*-OH of estradiol (E2); the O−O distance between both groups should be in the range 10.9−12.5 Å; (ii) precise steric hydrophobic centers mimicking steric 7α and 11*β* substituents at E2; (iii) hydrophobicity; (iv) a ring structure.

The recently published X-ray structures[8−10] of the LBD of ERα and ER*β* cocrystallized with E2, raloxifene (RAL), 4-hydroxytamoxifen (4OHT), DES, or genistein (for structures, see Chart 1) confirm the above-mentioned structural parameters and allow insight into the binding mode of these drugs.

Chart 1



H bridges to Glu 353, Arg 394, and His 524 and van der Waals contacts to aliphatic residues of amino acids fix agonists in the LBD of ERα. A comparable anchorage of estrogens takes place in the LBD of ER*β* (H bridges to Glu 305, Arg 346, His 475). RAL and 4OHT as

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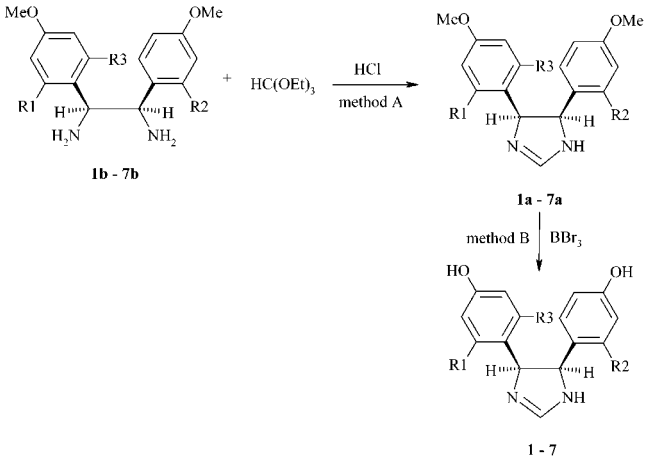
Scheme 1



R_1	R_2	R_3	compound
H	H	H	**1b, 1a, 1**
CH_3	CH_3	H	**2b, 2a, 2**
F	F	H	**3b, 3a, 3**
Cl	Cl	H	**4b, 4a, 4**
I	I	H	**5b, 5a, 5**
Cl	F	Cl	**6b, 6a, 6**
Cl	Cl	Cl	**7b, 7a, 7**

antagonistically active compounds occupy the same binding cave, and the basic side chain is located in a narrow side pocket and is connected to Asp 351 by an H bond.

In recent years, attempts were made to synthesize compounds with high selectivity to one of the ER subtypes or with unusual binding mode to get "selective estrogen receptor modulators" (SERMs). In particular, ring-sized fused and nonfused carbocyclic and heterocyclic systems,[11−14] e.g., benzothiophene,[15] pyrazoles,[16] furanes,[17,18] and related compounds, are the subjects of intensive studies.

In previous investigations,[19] we identified (*R*,*S*)/(*S*,*R*)-configurated 2,3-diarylpiperazine, 4,5-diaryl-2-imidazoline, and 4,5-diarylimidazole with a 2-Cl,4-OH/2,6-Cl_2,4-OH substitution pattern in the aromatic rings as new lead structures for the design of SERM. These compounds induced gene activation of the ER, although they competed only slightly with E2 for the binding site. Therefore, we assigned them to a second class of estrogens (type II estrogens).

In the first SAR study on (2*R*,3*S*)/(2*S*,3*R*)-2,3-diarylpiperazines,[20] we determined characteristic structural parameters for the activation of the ER, and we report now studies using the (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline **7** as the lead structure.

Results

Synthesis. 2-Imidazolines can be obtained by various condensation reactions of 1,2-diarylethylenediamines under preservation of the configuration. The best results were achieved following the suggestions of Martin et al.[21] (Scheme 1).

The 1,2-diarylethylenediamines **1b**−**7b** were dissolved in an excess of triethyl orthoformate, treated with catalytical amounts of concentrated HCl and heated to 90−100 °C for 24 h. The temperature and the time of reaction must be optimized if the number of substituents in the ortho positions of the aromatic rings increases. For example, the (1*R*,2*S*)/(1*S*,2*R*)-1-(2-chloro-4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)ethylenediamine **7b** has to be refluxed for 3 days. Subsequently, the solutions were adjusted to pH 8−9 with NaOH (20%) and the free bases were extracted with $CHCl_3$. The crude products, purified by chromatography on silicagel or by recrystallization, were ether-cleavaged with BBr_3.

Structural Characterization. The 2-imidazolines **1**−**7** were characterized by means of NMR spectroscopical and theoretical methods. Although the molecules are formally asymmetric because of the typical amidine structure, in the ^{1}H NMR spectra of the 4,5-bis(4-hydroxy/methoxyphenyl)-2-imidazolines **1**/**1a** to **5**/**5a** only one set of signals for the aromatic protons and a singlet resonance of the benzylic protons (see Figure 1A and Material and Methods) are observed. The same effect showed the ^{13}C spectrum of **1a** (Figure 1B) with seven signals. Such spectra can only be realized in the case of a symmetrical heterocyclic ring with partially sp^2-hybridized nitrogens and a delocation of the proton between both atoms. Consequently, the planar N−C═N moiety forces the 2-imidazoline ring into a planar structure.

The arrangement of the aromatic rings can be deduced from the ^{1}H NMR spectra of the 2-imidazolines **6**/**6a** and **7**/**7a** by coupling constant analysis. The nonequivalent benzylic protons are diastereotopically split with coupling constants of about 12.0−12.4 Hz (**6**, ^3J = 12.1 Hz; **7**, ^3J = 12.0 Hz; for the OMe derivatives **6a** and **7a**, ^3J = 12.4 Hz) because of different substituted aromatic rings. Although the Karplus relationship can be strictly applied only to open-chain compounds and six-membered rings,[22] it nevertheless provides insight into the conformational behavior of five-membered rings.[23]

The (4*S*,5*R*)/(4*R*,5*S*)-configurated 4,5-diaryl-2-imidazolines can adapt conformations with an arrangement of the benzylic protons at a dihedral angle between 0° and 60°. The coupling constant of about 12 Hz correlates with an angle of 0−15°, which confirms the proposed planar structure of the 2-imidazoline ring.

The pseudoaxial orientation of the phenyl rings causes a restricted rotation of the 2,6-dichloro-4-hydroxy-substituted aromatic ring and a splitting of its resonance in the ^{1}H NMR spectra of the 2-imidazolines **6** and **7**.[24] However, the broadening of the signals at room temperature indicates the beginning of dynamic effects.

This spatial structure correlates very well with the theoretical structure determined by energy calculations. All 2-imidazolines **1**−**7** take low-energy conformations in which the aromatic rings are arranged at a dihedral angle of 2.8−8.2°. Consequently, the O−O distances amount to 5.1−5.6 Å (see, for example, the low-energy structure of **1** presented in Figure 2)

Biological Properties. A competition experiment with [^{3}H]-E2 and calf uterine cytosol was used for the determination of the binding affinity to the ER (RBA).[25] The results listed in Table 1 show that the 2-imidazolines competed only slightly with E2 for ER binding. Compounds **1**−**3** are completely inactive in a concentra-

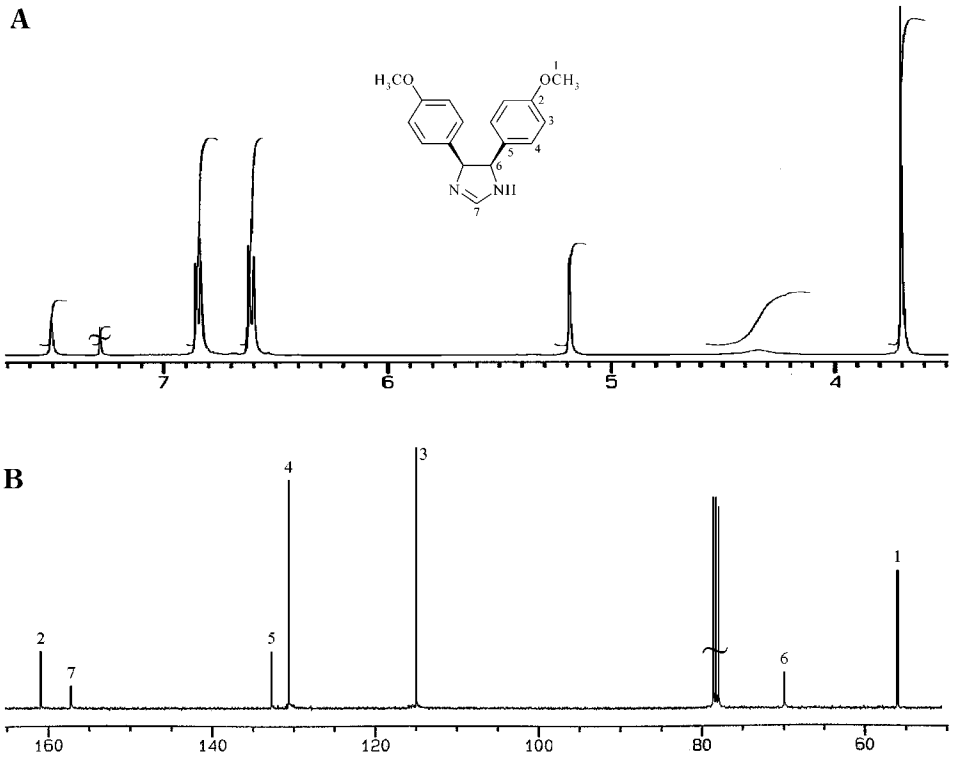


Figure 1. ^{1}H NMR spectrum (A) and ^{13}C NMR spectrum (B) of 2-imidazoline **1a** in $CDCl_3$.

Table 1. Binding Affinity to Calf Uterine Cytosol and Activation of Luciferase Expression in MCF-7-2a Cells

			binding affinity to calf uteri cytosol[a]		luciferase expression in MCF-7-2a cells			
					relative activation [%] at 1 μM		EC_{50} [μM]	RTP[d] [%]
	R_1	R_2	displaced [^{3}H]-E2 at 20 μM [% of control]	RBA[b] [%]	OH derivatives	OCH_3 derivatives	OH derivatives	OH derivatives
1	4-OH	4-OH	1.7	0.00	0	9		
2	2-CH_3, 4-OH	2-CH_3, 4-OH	0.0	0.00	1	1		
3	2-F, 4-OH	2-F, 4-OH	20.9	0.00	0	−2		
4	2-Cl, 4-OH	2-Cl, 4-OH	44.0	<0.02	67	2	0.380	0.13
5	2-I, 4-OH	2-I, 4-OH	42.9	<0.02	38	5	1.800	0.03
6	2-F, 4-OH	2,6-Cl_2, 4-OH	40.1	<0.02	74	4	0.620	0.08
7	2-Cl, 4-OH	2,6-Cl_2, 4-OH	70.3	0.08	112	6	0.065	0.75
8-PtI_2				0.43[c]	81		0.120	0.41
E2				100	100		0.000 49	

[a] IC_{50}(E2**)** = 0.000 37 μM; IC_{50} (**7**) = 4.75 μM. [b] Relative binding affinity: % RBA = [IC_{50}(E2)/IC_{50}(ligand)] × 100. [c] See ref 29; [d] Relative transcriptional potency: % RTP = [EC_{50}(E2)/EC_{50}(ligand)] × 100.

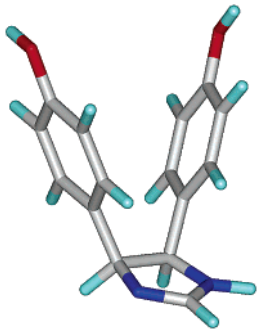


Figure 2. Calculated low-energy structure of 2-imidazoline **1**.

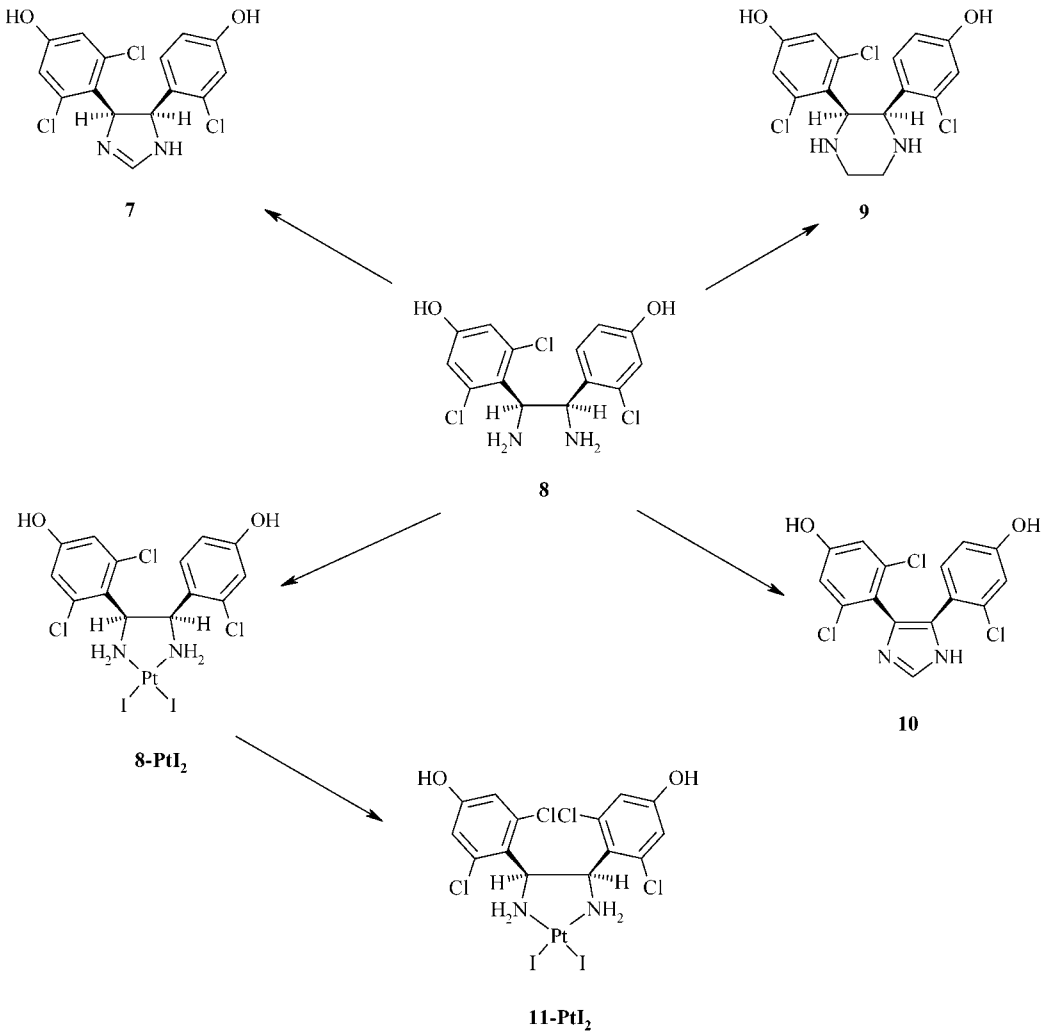
tion of 20 μM, while compounds **4**−**6** displaced [^{3}H]-E2 to 40−45% (RBA < 0.02%) and **7** to 70% from its binding site (IC_{50} = 4.75 μM; RBA = 0.08%).

The gene activation resulting from ER binding was proved in a luciferase assay with MCF-7−2a cells.[26] These estrogen receptor containing breast cancer cells are stably transfected with the plasmid ERE_{wtc}luc. This plasmid contains the "estrogen response element" (ERE) of the DNA as enhancer sequence and a reporter sequence, which codes for luciferase. The binding of ER/drug conjugate dimers leads to expression of luciferase, which correlates very well with the estrogenic potency of the drug.[27]

The 2-imidazolines induced luciferase expression, which depends on the substitution pattern in the aromatic rings. Compounds **1**−**3** with 2,2′-H, 2,2′-F, and 2,2′-CH_3 substituents were inactive, while **4** (2,2′-Cl), **5** (2,2′-I), and **6** (2-F,4-OH/2,6-Cl,4-OH) activated the luciferase gene at a concentration of 1 μM to 67%, 38% and 74%, respectively. **7** was the most active compound in this series and attained the effect of E2 at 0.52 μM.

O-methylation led in all cases to a loss of activity. This indicates clearly that both hydrophobic ortho substituents and free 4-OH groups in the aromatic rings have to be present to cause gene expression. The (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazoline **1** and

Chart 2



(4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)-2-imidazoline **7a** are likewise inactive (see Table 1).

Discussion

The compounds described in this paper were derived from antitumor active [1,2-diarylethylenediamine]platinum(II) complexes.[23,28] We have already shown in an earlier SAR study that the substitution pattern in the aromatic rings and the configuration at the benzylic C atoms determine the mode of action. The [(1*R*,2*S*)/(1*S*,2*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]diiodoplatinum(II) complex **8**-PtI_2 possesses the hormonal profile of a true estrogen, while its diastereomeric congener is hormonally inactive. This discrepancy results from a different orientation of the 1,2-diarylethane at the five-membered chelate ring.[29]

To get more information about the significance of the conformational behavior of the 1,2-diarylethane pharmacophor[30] for hormonal activity, we synthesized heterocyclic derivatives of the (1*R*,2*S*)/(1*S*,2*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine **8** (for structures, see Chart 2). (2*R*,3*S*)/(2*S*,3*R*)-2-(2-Chloro-4-hydroxyphenyl)-3-(2,6-dichloro-4-hydroxyphenyl)piperazine **9**, (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline **7**, and 4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)imidazole **10** were comparatively tested.[19] The 2-imidazoline **7** was selected as most active compound and used as lead structure in this SAR study.

The derivatives of **7** displaced E2 from its binding site in only small amounts (RBA < 0.02%) but activated the luciferase expression in ER-positive MCF-7-2a cells stably transfected with the plasmid ERE_{wtc}luc dependent on hydrophobic substituents in the ortho positions of the aromatic rings. The activity−concentration curves of the most active 2-imidazolines **4**−**7** are shown in Figure 3. The effect of E2 was reached by **7** at a concentration of 0.52 μM and by **4**−**6** at about 10 μM. The determination of the maximum activity of compounds **1**−**3** ($\gg$10 μM) was limited by insufficient water solubility in this concentration range.

Interestingly, the 2,2′-I-substituted 2-imidazoline **5** is less active than its 2,2′-Cl analogue **4**. This indicates clearly that steric effects diminish van der Waals interactions.

A clear structure−activity correlation is possible using the concentrations to cause the 50% effect (EC_{50}) of E2. The EC_{50} value increased in the order **7** (0.065 μM) < **4** (0.38 μM) < **6** (0.62 μM) < **5** (1.8 μM).

The potency in the transcription assay can be expressed as a relative transcription potency value RTP = [EC_{50}(E2)/EC_{50}(ligand)] × 100%, which should cor-

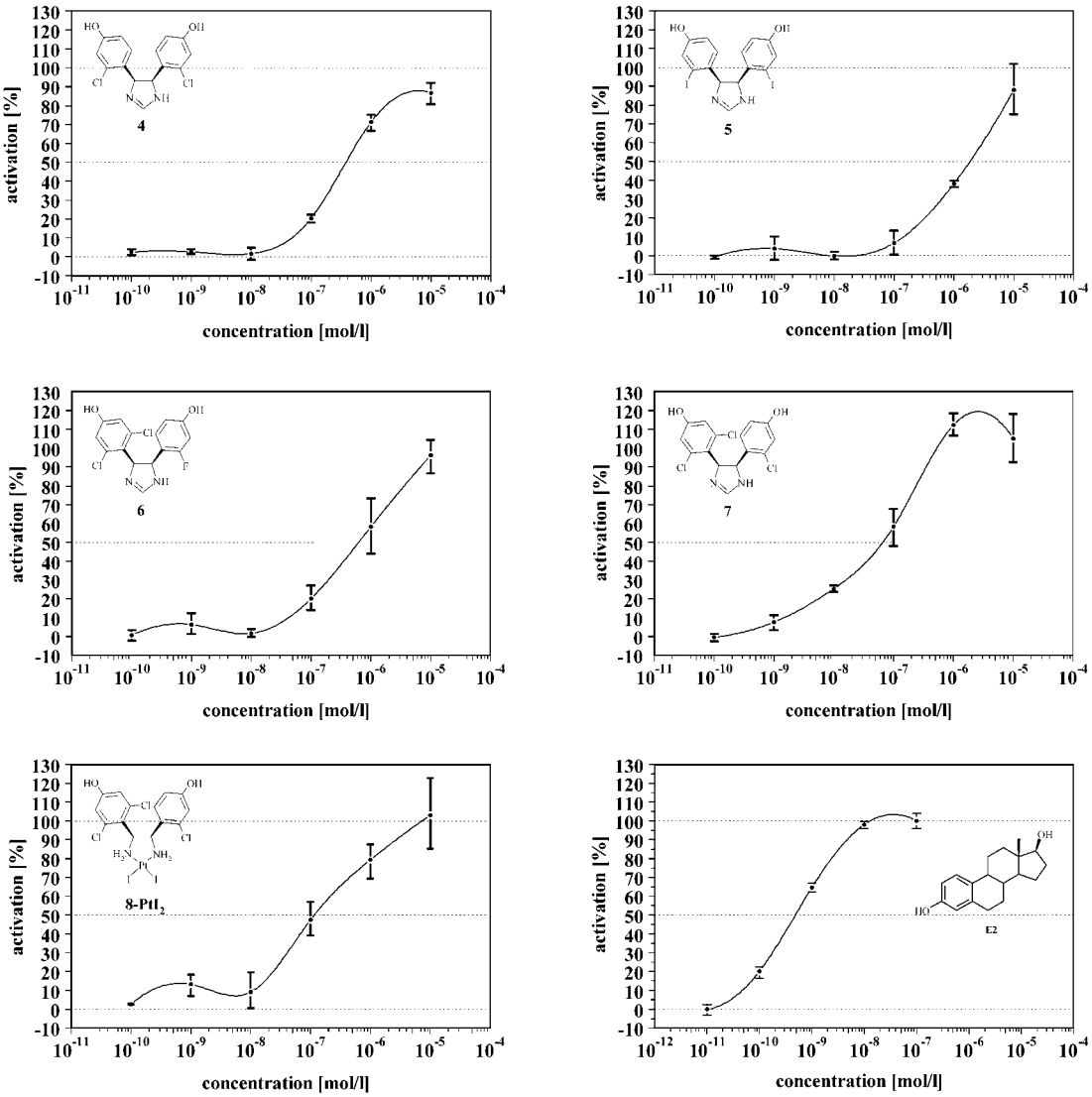


Figure 3. Activity−concentration curves of 2-imidazolines **4**−**7**, the platinum(II) complex **8**-PtI_2, and estradiol (E2).

relate with the RBA value.[31] However, the RTP values of the 2-imidazolines **4**−**7** are up to 10-fold higher than the RBA values (see Table 1) and confirm our assumption that the 4,5-diaryl-2-imidazolines as type II estrogens interact in a different way in the LBD as is known from E2.

A comparison of the data of the 2-imidazoline **7** with those of the related platinum complex **8**-PtI_2 and the piperazine **9** shows that **7** with the high RTP = 0.75% appears to be best attached to the LBD.

The aromatic rings of **8**-PtI_2 and **9** are synclinally arranged, while they are pseudoaxially oriented in **7**. An interaction with the ER comparable to synthetic (e.g. DES) or steroidal (e.g. E2) type I estrogens is impossible for each of the compounds.[19]

The binding mode of type II estrogens is still speculative, while the interactions of type I estrogens and antiestrogens are already known from X-ray structure analysis, for example, of ER/E2 and ER/RAL conjugates (Figure 4).

Figure 4 presents a theoretical binding mode[19,20] of the (4*R*,5*S*)/(4*S*,5*R*)-2-(2-chloro-4-hydroxyphenyl)-3-(2,6-dichloro-4-hydroxyphenyl)piperazine **9** and the (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline **7**. The molecules are positioned in the LBD in such a way that a phenolic ring is positioned in the hydrophobic side pocket and is connected to Asp 351.

The significance of Asp 351 for the estrogenic as well as the antiestrogenic properties of 4OHT and other compounds has been verified by the group of Jordan.[32−35] They used a TGF-α assay to study the interaction of estrogens and antiestrogens with cDNA of wild-type ER (Asp351 ER) and various mutants. In this assay, RAL shows the profile of a true estrogen if the MDA-MB-231 breast cancer cells are stably transfected with cDNA for the mutant Asp351Tyr ER (natural point mutation Tyr 351 instead of Asp 351). The substitution of glycine for aspartate at position 351 (Asp351Gly ER) changed the activity of the ER/4OHT complex from estrogen-like to completely antiestrogenic.

Investigations of several representative compounds confirmed the finding that the area around the amino acid 351 is important for the estrogenicity of ER/drug conjugates. An insufficient neutralization or shielding of the negative charge at Asp 351 by the side chains of antagonists changes the hormonal activity. Furthermore, most of the drugs cause different conformations

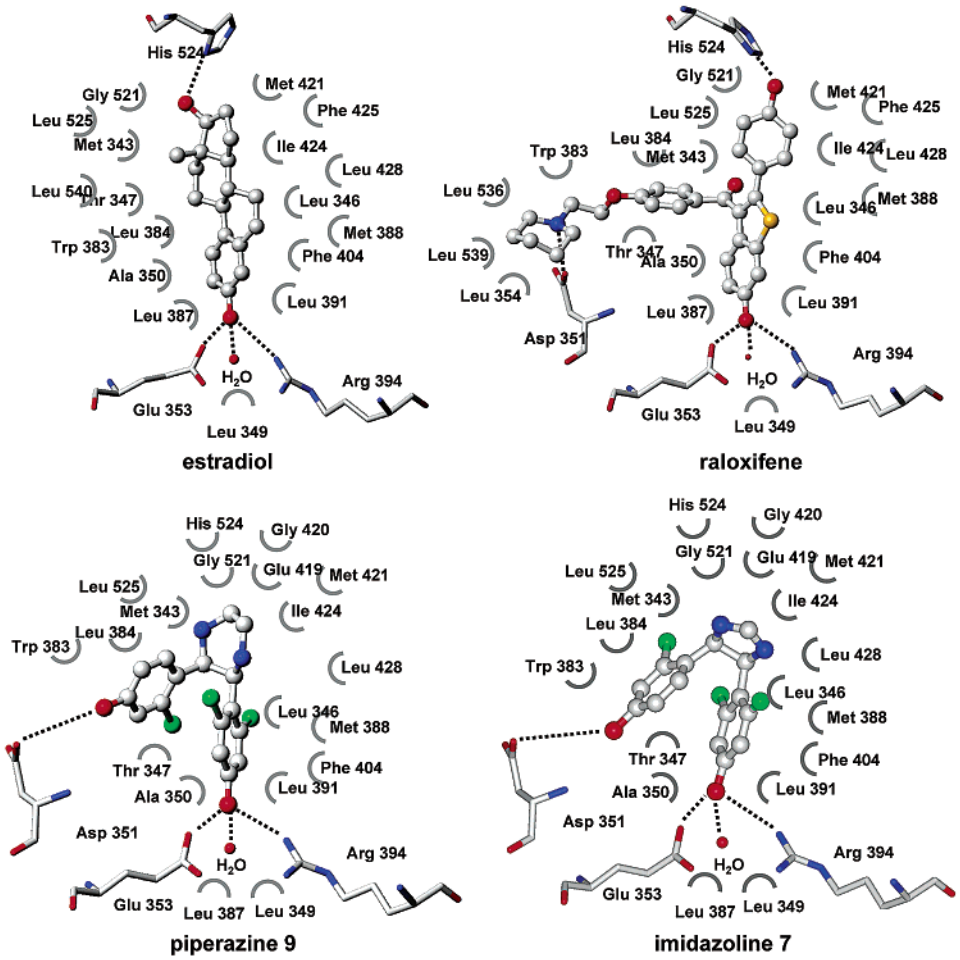


Figure 4. Comparison of the theoretical binding mode of piperazine **9** and 2-imidazoline **7** with that of estradiol and raloxifene.

of the ER, which influence the binding of coactivators. 4OHT does not increase the binding of coactivators with Asp351 ER despite the fact that the compounds produced estrogen-like actions at the TGF-α gene. Therefore, a novel site called AF2b for coactivator binding is suggested.[34]

On the basis of these data and the results of our structure activity studies, a refined model for the binding of type II estrogens can be proposed.

Classical type I estrogens such as E2 and DES are sealed within the LBD, and Asp 351 is in close contact with helix 12. The activation function 2 (AF2) is correctly positioned, and the coactivator binding site will synergize with AF1 to produce optimal estrogen-like action. Therefore, the estrogens have to be attached by H bridges to Glu 353, Arg 394, a water molecule, and His 524.

Type II estrogens of the (2*R*,3*S*)/(2*S*,3*R*)-2,3-bis(4-hydroxyphenyl)piperazine and the (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-hydroxyphenyl)-2-imidazoline series do not activate AF2 but use the novel AF2b site that includes acidic surface amino acids on a repositioned helix 12, an exposed aspartate at position 351, and AF1. The molecules have to be positioned in the LBD in a way that primary H bridges can be built to Glu 353, Arg 394, and the water molecule and an attachment to Asp 351 is guaranteed. Lipophilic parts of the molecule, especially ortho Cl substituents, are in van der Waals contact with lipophilic residues of amino acids and enhance the transcription potency.

The significance of a phenolic ring in contact with Asp 351 in order to get hormonal activity has already been shown for derivatives of the [(1*R*,2*S*)/(1*S*,2*R*)-1-(2-chloro-4-hydroxyphenyl)-2-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]diiodoplatinum(II) complex **8**-PtI_2, also assigned to type II estrogens.[23,24,36]

Exchange of the axially arranged 2-chloro-4-hydroxyphenyl ring by H results in the [1-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]diiodoplatinum(II) complex, which shows an excellent structural analogy to E2.[36] However, the complex induced neither luciferase expression in MCF-7-2a cells nor uterus growth in the uterotrophic test with juvenile NMRI mice. This indicates clearly that the 2-phenyl ring is essential for hormonal activity. To obtain estrogenically active complexes, the 2-phenyl ring has to be 2-F,4-OH,2-Cl, 4-OH- or 2,6-Cl_2,4-OH-substituted.

This tendency was confirmed in vitro. The relative activation of luciferase expression at 1 μM increased in the order 4-OH (12%) < 2-F,4-OH (57%) < 2-Cl,4-OH (81%) < 2,6-Cl_2,4-OH (92%) and shows the necessity of hydrophobic substituents in the ortho positions of the 2-phenyl ring in [1-(2,6-dichloro-4-hydroxyphenyl)-2-phenylethylenediamine]diiodoplatinum(II) complexes.

Additionally, dynamic effects must be taken into consideration. The [(1*R*,2*S*)/(1*S*,2*R*)-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]diiodoplatinum(II) complex **11**-PtI_2 undergoes a dynamic interconversion between a λ- and a δ-conformation, while **8**-PtI_2 exists in a stable conformation with an equatorially arranged

2,6-dichloro-4-hydroxyphenyl ring.[22] The spatial structure of **8**-PtI_2 agrees very well with that of 2,3-diphenylpiperazines, and the medial arrangement of the aromatic rings in **11**-PtI_2 during the conversion of the five-membered chelate ring is comparable to a pseudo-axial arrangement in 4,5-diaryl-2-imidazolines.

It can be concluded that the 1,2-diphenylethane moiety has to be arranged at a dihedral angle of 0−10° for a high gene expression. A stable synclinal position at a 40−60° angle, as realized in piperazines, reduced the activity distinctly. Since the different gene activation could also be the result of differing polarities of the core heterocyclic structure, we will evaluate in a further SAR study the effects of substituted imidazoles on the ER activation. In this class of compounds, the aromatic rings are fixed in a planar *Z*-stilbene-like structure.

Materials and Methods

General Procedures. The following instruments were used to collect various spectra: IR spectra (KBr pellets), Perkin-Elmer model 580 A; ^{1}H NMR, Bruker ADX 400 spectrometer at 400 MHz (internal standard, TMS); EI-MS spectra, CH-7A-Varian MAT (70 eV) and Kratos MS 25 RF (80 eV). Elemental analyses were carried out at the Microlaboratory of Free University of Berlin. All computational graphics were built using SYBYL 6.6 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144). Geometry optimization was carried out using the Tripos force field within the SYBYL program, running on an INDY workstation. The liquid scintillation counter was a 1450 Microbeta Plus (Wallac, Finland), and luminescence measurements were made with a Microlumat LB 96 P (EG & G Berthold, Germany).

Syntheses. (1*R*,2*S*)/(1*S*,2*R*)-1,2-diarylethylenediamines **1b** − **7b** were synthesized as described earlier.[23,28]

General Procedure for the Synthesis of 4,5-Diphenyl-2-imidazolines (Method A). A solution of each of the 1,2-diarylethylenediamines **1b**−**7b** (1.00 mmol) with 0.256 mL of HCl_{conc} and 2.17 mL of triethylorthoformate was heated to reflux. The reaction mixture was cooled to room temperature and combined with 20 mL of chloroform. It was extracted with 5 mL of 1 N NaOH and three times with 10 mL of H_2O. The organic layer was dried over Na_2SO_4, and the solvent was evaporated. Purification was performed by chromatography on silica gel or by recrystallization.

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-methoxyphenyl)-2-imidazoline (1a). 1a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1,2-bis(4-methoxyphenyl)ethylenediamine **1b**: 1.836 mmol (500 mg). The reaction mixture was heated to 90−100 °C for 10 h. Purification was attained by chromatography on silica gel (methanol) and subsequent recrystallization from $CHCl_3$. Yield 1.47 mmol (415 mg); 80%; colorless needles; mp 96−97 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−3300 m, br (NH); 2830 m (OCH_3); 1620 m; 1600 m; 1585 m; 1515 s; 1460 m; 1305 m; 1255 s; 1175 m; 1035 m; 880 m; 800 m. ^{1}H NMR ($CDCl_3$) δ: 3.70 (s, 6H, O*CH*$_3$); 4.34 (s, br, 1H, N*H*, exchangeable by D_2O); 5.19 (s, 2H, ArC*H*); 6.61 (AA′*BB*′, ^3J = 8.6 Hz, 4H, Ar*H*-3, Ar*H*-5); 6.84 (*AA*′BB′, ^3J = 8.6 Hz, 4H, Ar*H*-2, Ar*H*-6); 7.50 (s, 1H, N=C*H*−N). ^{13}C NMR$_{100\ MHz}$ ($CDCl_3$) δ: 55.1 (O*C*H$_3$); 68.8 (Ar*C*H); 113.2 (Ph−*C*-3, −*C*-5); 128.6 (Ph−*C*-2, -*C*-6); 130.7 (Ph−*C*-1); 154.8 (N=*C*H−N); 158.5 (Ph−*C*-4).

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-methoxy-2-methylphenyl)-2-imidazoline (2a). 2a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1,2-bis(4-methoxy-2-methylphenyl)ethylenediamine **2b**: 1.00 mmol (301 mg). Reaction time was 24 h. The crude product was dissolved in methanol and treated with etheral HCl. The solvent was evaporated, and the resulting hydrochloride was recrystallized from CH_2Cl_2/ether. Yield 0.894 mmol (310 mg); 89% as hydrochlorid; colorless powder; mp 266−269 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3005 m; 2836 w (OCH_3); 1626 s; 1610 s; 1580 m; 1524 m; 1504 s; 1457 m; 1373 w; 1305 m; 1254 s; 1199 m; 1165 m; 1115 m; 1041 m; 864 m; 845 m; 813 m. ^{1}H NMR ([D$_6$]-DMSO) δ: 2.15 (s, 6H, ArC*H*$_3$); 3.63 (s, 6H, O*CH*$_3$); 5.85 (s, 2H, ArC*H*); 6.56 (br, 4H, Ar*H*-3, Ar*H*-5); 6.90 (d, ^3J = 9.1 Hz, 2H, Ar*H*-6); 8.98 (s, 1H, N=C*H*−N); 10.78 (s, 2H, N*H*, exchangeable by D_2O).

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-fluoro-4-methoxyphenyl)-2-imidazoline (3a). 3a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1,2-bis(2-fluoro-4-methoxyphenyl)ethylenediamine **3b**: 1.00 mmol (308 mg). The reaction mixture was heated to reflux for 36 h. Recrystallization was from $CHCl_3$. Yield 0.914 mmol (291 mg), 91%; colorless powder; mp 149−150 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3117 m; 2837 w (OCH_3); 1629 s; 1601 s; 1508 s; 1465 w; 1442 m; 1317 m; 1266 m; 1192 m; 1176m; 1095 m; 1033 m; 938 m; 828 m. ^{1}H NMR ($CDCl_3$) δ: 3.68 (s, 6H, OCH_3); 5.51 (s, 2H, ArC*H*); 6.33 (dd, ^3J(H, F) = 11.6 Hz, ^4J = 2.3 Hz, 2H, Ar*H*-3); 6.43 (dd, ^3J = 8.3 Hz, ^4J = 2.3 Hz, 2H, Ar*H*-5); 6.97 (dd, ^3J = 8.3 Hz, ^{4}J(H, F) = 8.1 Hz, 2H, Ar*H*-6); 7.45 (s, 1H, N=C*H*−N).

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-chloro-4-methoxyphenyl)-2-imidazoline (4a). 4a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1,2-bis(2-chloro-4-methoxyphenyl)ethylenediamine **4b**: 0.733 mmol (250 mg). The reaction mixture was heated to reflux for 36 h. Recrystallization was from ether. Yield 0.715 mmol (251 mg), 98%; colorless powder; mp 44−47 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 2839 w (OCH_3); 1606 s; 1572 w; 1496 s; 1461 m; 1440 m; 1287 m; 1243 m; 1043 m; 869 m. ^{1}H NMR ($CDCl_3$) δ: 3.67 (s, 6H, OCH_3); 5.71 (s, 2H, ArC*H*); 6.55 (dd, ^3J = 8.7 Hz, ^4J = 2.5 Hz, 2H, Ar*H*-5); 6.67 (d, ^4J = 2.5 Hz, 2H, Ar*H*-3); 6.98 (d, ^3J = 8.7 Hz, 2H, Ar*H*-6); 7.48 (s, 1H, N=C*H*−N).

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-iodo-4-methoxyphenyl)-2-imidazoline (5a). 5a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1,2-bis-(2-iodo-4-methoxyphenyl)ethylenediamine **5b**: 0.672 mmol (352 mg). The reaction mixture was heated to reflux for 18 h. Purification was analogous to that of **2a**. Yield 0.421 mmol (240 mg), 63% as hydrochloride; gray powder; mp 266−269 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 2888 w; 2840 w (OCH_3); 1692 m; 1599 s; 1558 m; 1490 s; 1436 m; 1356 w; 1328 w; 1287 m; 1237 m; 1168 m; 1033m; 969 w; 840 m. ^{1}H NMR ([D$_6$]DMSO) δ: 3.68 (s, 6H, O*CH*$_3$); 5.83 (s, 2H, ArC*H*); 6.82 (dd, ^3J = 8.8 Hz, ^4J = 2.5 Hz, 2H, Ar*H*-5); 7.06 (d, ^3J = 8.8 Hz, 2H, Ar*H*-6); 7.25 (d, ^4J = 2.5 Hz, 2H, Ar*H*-3); 9.00 (s, 1H, N=C*H*−N); 10.95 (s, 2H, N*H*, exchangeable by D_2O).

(4*R*,5*S*)/(4*S*,5*R*)-4-(2,6-Dichloro-4-methoxyphenyl)-5-(2-fluoro-4-methoxyphenyl)-2-imidazoline (6a). 6a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1-(2,6-dichloro-4-methoxyphenyl)-2-(2-fluoro-4-methoxyphenyl)ethylenediamine **6b**: 1.00 mmol (360 mg). The reaction mixture was heated to reflux for 72 h. Purification was by chromatography on silica gel with $CHCl_3$/methanol (20:1). Yield 0.528 mmol (195 mg), 53%; colorless powder; mp 55−57 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3236 w (NH); 2838 w (OCH_3); 1618 s; 1555 m; 1507 m; 1466 m; 1434 m; 1385 w; 1280 s; 1154 m; 1112 M; 1040 m; 838 m. ^{1}H NMR ($CDCl_3$) δ: 3.69 (s, 3H, O*CH*$_3$); 3.70 (s, 3H, O*CH*$_3$); 5.63 (d, ^3J = 12.4 Hz, 1H, ArC*H*); 6.09 (d, ^3J = 12.4 Hz, 1H, ArC*H*); 6.29 (dd, ^3J(H, F) = 12.0 Hz, ^4J = 2.3 Hz, 1H, Ar*H*-3); 6.51−6.54 (m, 2H, Ar*H*-5, Ar′*H*); 6.75 (br, 1H, Ar′*H*); 7.38 (dd, ^3J = 8.7 Hz, ^4J(H, F) = 8.7 Hz, 1H, Ar*H*-6); 7.61 (s, 1H, N=C*H*−N).

(4*R*,5S)/(4*S*,5*R*)-4-(2-Chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)-2-imidazoline (7a). 7a was obtained from (1*R*,2*S*)/(1*S*,2*R*)-1-(2-chloro-4-methoxyphenyl)-2-(2,6-dichloro-4-methoxyphenyl)ethylenediamine **7b**: 1.00 mmol (376 mg). The reaction mixture was heated to reflux for 72 h. Purification was by chromatography on silica gel with $CHCl_3$/methanol (20:1). Yield 0.779 mmol (301 mg), 78%; colorless powder; mp 63−66 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3237 w (NH); 2837 w (OCH_3); 1606 s; 1556 m; 1494 m; 1465 m; 1434 m; 1283 m; 1241 m; 1177 m; 1043 s; 840 m; 787 m. MS (EI, 150 °C), *m*/*z* (%): 384 (18) [M]$^{+\bullet}$; 350 (10); 204 (56); 170 (100); 154 (10); 119 (14); 75 (11). ^{1}H NMR ($CDCl_3$) δ: 3.69 (s, 3H, O*CH*$_3$); 3.71 (s, 3H, O*CH*$_3$); 5.70 (d, ^3J = 12.4 Hz, 1H, ArC*H*); 6.18 (d, ^3J = 12.4 Hz, 1H, ArC*H*); 6.52 (br, 1H, Ar*H*); 6.63 (d, ^4J = 2.5 Hz, 1H, Ar′*H*-3); 6.66 (dd, ^3J = 8.7 Hz, ^4J = 2.5 Hz, 1H, Ar′*H*-5); 6.73 (br, 1H, Ar*H*); 7.51 (d, ^3J = 8.7 Hz, 1H, Ar′*H*-6); 7.77 (s, 1H, N=C*H*−N).

General Procedure for Ether Cleavage with BBr_3 (Method B). A solution of the methyl ether (1.00 mmol) in 20 mL of dry CH_2Cl_2 was cooled to −60 °C. At this temperature,

BBr_3 (4.5 mmol) in 5 mL of dry CH_2Cl_2 was added under N_2 atmosphere. Then the reaction mixture was allowed to warm to room temperature and was stirred for further 48 h. After the reaction mixture was cooled with an ice bath, the surplus of BBr_3 was hydrolyzed three times with methanol and the phenolic product was dissolved in 0.1 N NaOH. The alkaline water phase was filtrated, the pH was adjusted to 8 with 2 N HCl, and the precipitate was collected by suction filtration and dried over P_2O_5. Subsequently, the crude product was purified by column chromatography or fractional crystallization.

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxyphenyl)-2-imidazoline (1). 1 was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(4-methoxyphenyl)-2-imidazoline **1a**: 0.921 mmol (260 mg). The reaction mixture was stirred for 24 h. The product was used without further purification. Yield 0.590 mmol (150 mg), 64%; purple powder; mp 207−208 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2600 m, br (OH); 1630 s; 1610 s; 1600 s; 1560 m; 1510 s; 1500 s; 1460 m; 1250 s; 11 75 s; 1110 m; 880 m; 850 m; 810 s. ^1H NMR ([D_6]DMSO) δ: 4.98 (s, 2H, ArC*H*); 6.40 (AA′*BB*′, ^3J = 8.5 Hz, 4H, Ar*H*-3, Ar*H*-5); 6.68 (*AA*′BB′, ^3J = 8.5 Hz, 4H, Ar*H*-2, Ar*H*-6); 7.39 (s, 1H, N=C*H*−N); 8.99 (s, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{15}H_{14}N_2O_2$) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(4-hydroxy-2-methylphenyl)-2-imidazoline (2). 2 was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis-(4-methoxy-2-methylphenyl)-2-imidazoline **2a**: 0.605 mmol (210 mg). The reaction mixture was stirred for 36 h. The product was purified by stirring in heated methanol and suction filtration. Yield 0.195 mmol (55 mg), 32%; colorless powder; mp 210−211 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2700 s, br (OH); 2925 w; 1610 s; 1501 m; 1458 m; 1295 m; 1261 m; 1206 m; 1164 w; 1104 w; 1033 m; 960 w; 869 m; 814 m. MS (EI, 200 °C), *m*/*z* (%): 282 (27) $[M]^{+\bullet}$; 136 (100); 120 (10); 109 (10); 91 (15). ^1H NMR ([D_6]DMSO) δ: 2.02 (s, 6H, ArCH_3); 5.16 (s, 2H, ArC*H*); 6.25−6.29 (m, 4H, Ar*H*-3, Ar*H*-5); 6.70 (d, ^3J = 9.0 Hz, 2H, Ar*H*-6); 6.94 (s, br, 1H, N*H*, exchangeable by D_2O); 7.39 (s, 1H, N=C*H*−N); 8.89 (s, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{17}H_{18}N_2O_2$) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-fluoro-4-hydroxyphenyl)-2-imidazoline (3). 3 was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis-(2-fluoro-4-methoxyphenyl)-2-imidazoline **3a**: 0.675 mmol (215 mg). The reaction mixture was stirred for 36 h. The product was purified by recrystallization from methanol. Yield 0.386 mmol (112 mg), 57%; colorless powder; mp 229−231 °C (dec). IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2800 s, br (OH); 2921 w; 1622 s; 1564 m; 1507 m; 1484 s; 1302 s; 1155 m; 1099 s; 965 m; 849 m. MS (EI, 240 °C), *m*/*z* (%): 290 (30) $[M]^{+\bullet}$; 140 (100); 124 (14); 96 (11). ^1H NMR ([D_6]DMSO) δ: 5.22 (s, 2H, ArC*H*); 6.22 (d, br, ^3J(H, F) = 10.1 Hz, 2H, Ar*H*-3); 6.31 (dd, ^3J = 8.4 Hz, ^4J = 1.6 Hz, 2H, Ar*H*-5); 6.77 (dd, ^3J = 8.4 Hz, ^4J(H, F) = 8.4 Hz, 2H, Ar*H*-6); 7.19 (s, br, 1H, N*H*, exchangeable by D_2O); 7.41 (s, 1H, N=C*H*−N); 9.51 (s, br, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{15}H_{12}F_2N_2O_2$) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4,5-Bis(2-chloro-4-hydroxyphenyl)-2-imidazoline (4). 4 was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis-(2-chloro-4-methoxyphenyl)-2-imidazoline **4a**: 1.45 mmol (510 mg). The reaction mixture was stirred for 36 h. The product was purified by recrystallization from methanol. Yield 0.805 mmol (260 mg), 55%; colorless powder; mp 243 °C (dec). IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2600 s, br (OH); 2890 w; 2771 w; 2649 w; 1608 s; 1486 s; 1457 s; 1248 s; 1041 m; 902 s; 860 m; 815 m. MS (EI, 200 °C), *m*/*z* (%): 322 (22) $[M]^{+\bullet}$; 156 (100); 140 (10). ^1H NMR ([D_6]DMSO) δ: 5.36 (s, 2H, ArC*H*); 6.45 (dd, ^3J = 8.5 Hz, ^4J = 2.2 Hz, 2H, Ar*H*-5); 6.53 (d, ^4J = 2.2 Hz, 2H, Ar*H*-3); 6.82 (d, ^3J = 8.5 Hz, 2H, Ar*H*-6); 7.26 (s, br, 1H, N*H*, exchangeable by D_2O); 7.45 (s, 1H, N=C*H*−N); 9.57 (s, br, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{15}H_{12}Cl_2N_2O_2$) C, H, N.

***(4R,5S)/(4S,5R)*-4,5-Bis(4-hydroxy-2-iodophenyl)-2-imidazoline (5). 5** was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4,5-bis(2-iodo-4-methoxyphenyl)-2-imidazoline **5a**: 0.175 mmol (100 mg). The reaction mixture was stirred for 48 h. The product was purified by recrystallization from methanol/acetone. Yield 0.095 mmol (48 mg), 54%; colorless powder; mp 202−205 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2800 s, br (OH); 2902 w; 2756 w; 2647 w; 1704 m; 1599 s; 1486 s; 1438 s; 1359 w; 1235 s; 1027 m; 865 s; 813 m. MS (EI, 160 °C), *m*/*z* (%): 506 (29) $[M]^{+\bullet}$; 379 (4) $[M - I]^+$; 286 (25); 248 (100); 220 (39); 132 (23); 120 (17); 105 (43); 93 (21). ^1H NMR ([D_6]DMSO) δ: 5.17 (s, 2H, ArC*H*); 6.53 (dd, ^3J = 8.5 Hz, ^4J = 2.4 Hz, 2H, Ar*H*-5); 6.74 (d, ^3J = 8.5 Hz, 2H, Ar*H*-6); 7.00 (d, ^4J = 2.4 Hz, 2H, Ar*H*-3); 7.32 (s, br, 1H, N*H*, exchangeable by D_2O); 7.47 (s, 1H, N=C*H*−N); 9.49 (s, br, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{15}H_{12}I_2N_2O_2$) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2,6-Dichloro-4-hydroxyphenyl)-5-(2-fluoro-4-hydroxyphenyl)-2-imidazoline (6). 6 was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4-(2,6-dichloro-4-methoxyphenyl)-5-(2-fluoro-4-methoxyphenyl)-2-imidazoline **6a**: 0.488 mmol (180 mg). The reaction mixture was stirred for 72 h. Purification was by chromatography on silica gel with methanol. Yield 0.278 mmol (95 mg), 57%; colorless powder; mp 233−236 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2800 s, br (OH); 3089 w; 2922 w; 1700 w; 1622 s; 1603 s; 1512 s; 1445 s; 1278 s; 1187 m; 1155 w; 1100 m; 1058 m; 950 m; 850 m. MS (EI, 210 °C), *m*/*z* (%): 340 (27) $[M]^{+\bullet}$; 190 (75); 178 (40); 162 (40); 151 (18); 140 (100); 124 (23). ^1H NMR ([D_6]DMSO) δ: 5.30 (d, ^3J = 12.1 Hz, 1H, ArC*H*); 5.78 (d, ^3J = 12.1 Hz, 1H, ArC*H*); 6.18 (dd, ^3J(H, F) = 12.1 Hz, ^4J = 2.1 Hz, 1H, Ar*H*-3); 6.39 (dd, ^3J = 8.7 Hz, ^4J = 2.1 Hz, 1H, Ar*H*-5); 6.43 (s, 1H, Ar′*H*); 6.69 (s, 1H, Ar′*H*); 7.22 (dd, ^3J = 8.7 Hz, ^4J(H, F) = 8.8 Hz, 1H, Ar*H*-6); 7.26 (s, 1H, N=C*H*−N); 9.60 (br, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{15}H_{11}Cl_2FN_2O_2$) C, H, N.

(4*R*,5*S*)/(4*S*,5*R*)-4-(2-Chloro-4-hydroxyphenyl)-5-(2,6-dichloro-4-hydroxyphenyl)-2-imidazoline (7). 7 was obtained from (4*R*,5*S*)/(4*S*,5*R*)-4-(2-chloro-4-methoxyphenyl)-5-(2,6-dichloro-4-methoxyphenyl)-2-imidazolin **7a**: 0.415 mmol (160 mg). The reaction mixture was stirred for 72 h. The product was purified by recrystallization from methanol/$CHCl_3$. Yield 0.204 mmol (73 mg), 49%; colorless powder; mp 238−241 °C. IR (KBr), $\bar{\nu}$, cm^{-1}: 3600−2700 s, br (OH); 2889 w; 2639 w; 1608 s;1578 m; 1499 w; 1443 s; 1273 s; 1050 m; 948 m; 904 m; 858 m. MS (EI, 300 °C), *m*/*z* (%): 356 (27) $[M]^{+\bullet}$; 204 (10); 194 (72); 190 (94); 174 (10); 162 (57); 156 (100); 139 (23). ^1H NMR ([D_6]DMSO) δ: 5.33 (d, ^3J = 12.0 Hz, 1H, ArC*H*); 5.83 (d, ^3J = 12.0 Hz, 1H, ArC*H*); 6.45 (s, 1H, Ar*H*); 6.50 (d, ^4J = 2.1 Hz, 1H, Ar′*H*-3); 6.55 (dd, ^3J = 8.5 Hz, ^4J = 2.1 Hz, 1H, Ar′*H*-5); 6.68 (s, 1H, Ar*H*); 7.28 (s, 1H, N=C*H*−N); 7.37 (d, ^3J = 8.5 Hz, 1H, Ar′*H*-6); 9.68 (br, 2H, ArO*H*, exchangeable by D_2O). Anal. ($C_{15}H_{11}Cl_3N_2O_2$) C, H, N.

Biological Methods. 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); bovine calf serum (BCS) 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 (10 000 IE of penicillin/mL, 10 mg of 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 liquid scintillator 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); $CDCl_3$ and [D_6]DMSO were from Aldrich (Steinheim, Germany). PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$ 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, 0.372 24 g of Titriplex III, and 0.195 03 g of sodium azide (all from Merck or Fluka) in 1 L of purified water. Deionized water was produced by means of a Millipore Milli-Q water system;

resistivity was greater than 18 MΩ. T-75 flasks, reaction tubes, 96-well plates, and 6-well plates were purchased from Renner GmbH (Dannstadt, Germany).

Estrogen Receptor Binding Assay. The applied method was already described by Hartmann et al.[25] and used with some modifications. The relative binding affinity (RBA) of the test compounds to the estrogen receptor 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 six to eight appropriate concentrations (300 μL). They were incubated with 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 overnight. To stop the reaction, a total of 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 minus 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 that reduced the binding of the radioligand by 50% were determined.

$$\% \text{ RBA} = \frac{c_{[^3\text{H}]-\text{estradiol}} \text{ at 50 \% inhibition}}{c_{\text{sample}} \text{ at 50\% inhibition}} \times 100$$

Luciferase Assay. The pertinent in vitro assay was described earlier by Hafner et al.[26] One week before the start of the experiment, MCF-7-2a cells were cultivated in DMEM supplemented with L-glutamine, antibiotics, and dextran/charcoal-treated BCS (ct-BCS, 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 2.0 mL of medium per well) under growing conditions (see above). After 24 h, a total of 25 μL of a stock solution of the test compounds was added to achieve concentrations ranging from 10^{-5}−10^{-10} M, and the plates were incubated for 50 h. Before being harvested, the cells were washed twice with PBS and then a total of 200 μL of cell culture lysis reagent was added to each well. After 20 min of lysing at room temperature, the cells were transferred into reaction tubes and centrifuged. Luciferase was assayed using the Promega luciferase assay reagent. A total of 50 μL of each supernatant was mixed with 50 μL of the 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[36]) of each sample with the mass of luciferase. Estrogenic activity was expressed as the percent activation of a 10^{-8} M estradiol control (100%).

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