Investigation of the Configurational and Conformational Influences on the Hormonal Activity of 1,2-Bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines and of Their Platinum(II) Complexes. 1. Synthesis, Estradiol Receptor Affinity, and Estrogenic Activity of Diastereomeric [*N*-Alkyl- and *N,N'*-Dialkyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) Complexes

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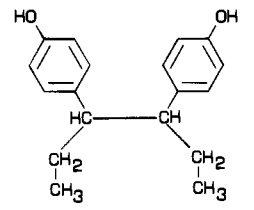
N-Monoalkylated (Et) and N,N'-dialkylated (Me and Et) 1,2-bis(2,6-dichloro-4-hydroxyphenyl)-ethylenediamines and their dichloroplatinum(II) complexes were synthesized, and their configuration and conformational behavior were ^{1}H-NMR spectroscopically clarified. The latter was brought in relation to their relative binding affinity (RBA) to the estrogen receptor as well as to their estrogenic potency. In contrast to the *RR/SS*-configurated diamines, the *R/S*-configurated ones showed marked estrogenic properties which correlate with the RBA's. In the related *R/S*-configurated complexes the estrogenic activity is determined by the same structural requirements as in the diamine series. However, a correlation between RBA's and estrogenic potencies is missing. The connection between spatial structure and activity is discussed by use of a drug–receptor model recently proposed by Höltje and Dall.[1]

Introduction

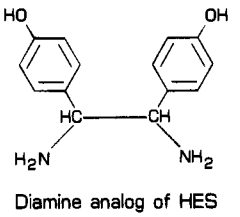
Since the discovery of the powerful antitumor activity of cisplatin,[2] a lot of platinum complexes of the second and third generation have been synthesized to get less toxic but more effective and more selectively acting drugs. Such compounds can be designed by combination of the cytotoxic $PtCl_2$ residue, which is the active moiety in cisplatin (i.e., *cis*-diamminedichloroplatinum(II)), with an appropriate carrier ligand. In the hormone-sensitive mammary carcinoma (MC), which cannot be cured by cisplatin treatment, the estrogen receptor (ER) is a useful target for a new type of platinum complex. This tumor contains the ER in higher concentrations than normal, nonlactating breast tissue.[3,4] In these new dichloroplatinum(II) complexes, the central platinum atom is linked to an ER-affinic diamine ligand, by which a more specific potency against the ER-containing breast cancer cell should be achieved. In our group the synthetic estrogen hexestrol (HES, see Chart 1), which possesses a receptor binding affinity (RBA) of 27 compared to estradiol (RBA = 100),[5] was used as a model for the construction of ER-affinic ligands.

By exchange of the ethyl side chains by amino groups, HES can be transformed into a compound suitable for coordination to platinum(II). However, after this structural modification HES has lost its high affinity to ER as well as its marked estrogenic activity.[6] In accordance with our supposition that the two polar NH_2 groups cause a weakening of the hydrophobic interaction with the ER, we introduced two chlorine atoms into the 2,6-positions of the aromatic rings[7] to increase the lipophilic character of the diamine. The new compound 1,2-bis-(2,6-dichloro-4-hydroxyphenyl)ethylenediamine (*meso*-**1**, see Chart 1) as well as its dichloroplatinum(II) complex (*meso*-**1**–$PtCl_2$) proved to be "true" estrogens. Their RBA's to the ER, however, were low compared to that of HES (*meso*-**1**, 0.45; *meso*-**1**–$PtCl_2$, 0.3). Nevertheless, *meso*-**1**–$PtCl_2$ showed a marked activity on the hormone sensitive, DMBA-induced MC of the SD rat, which was superior to that of cisplatin. The estimation of platinum in the tumor revealed a 22-fold enrichment compared to the skeletal muscle.[7] In a former paper,[8] the attempt to optimize the RBA of the ligand *meso*-**1**

Chart 1

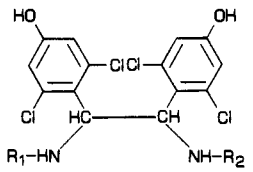


Hexestrol (HES)

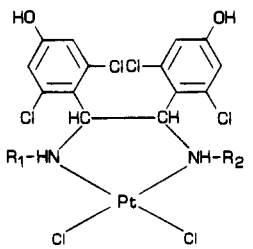


Diamine analog of HES

compd.	R1	R2
meso-1	H	H
D,L-1	H	H
erythro-2	Et	H
threo-2	Et	H
meso-3	Et	Et
D,L-3	Et	Et
meso-4	Me	Me
D,L-4	Me	Me



meso-1, meso-3, meso-4
D,L-1, D,L-3, D,L-4
erythro-2, threo-2



meso-1-$PtCl_2$, meso-3-$PtCl_2$, meso-4-$PtCl_2$
D,L-1-$PtCl_2$, D,L-3-$PtCl_2$, D,L-4-$PtCl_2$
erythro-2-$PtCl_2$, threo-2-$PtCl_2$

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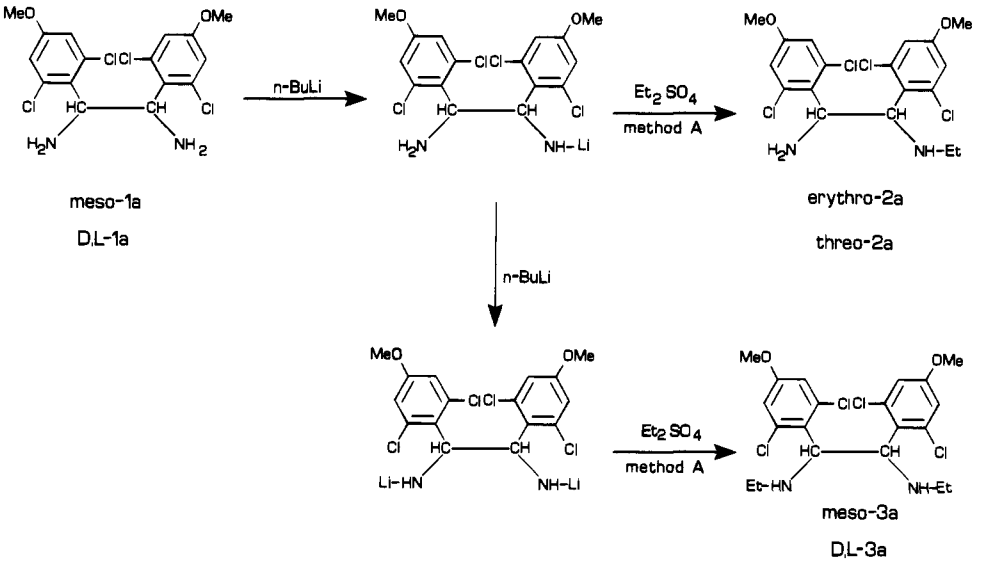
Scheme 1



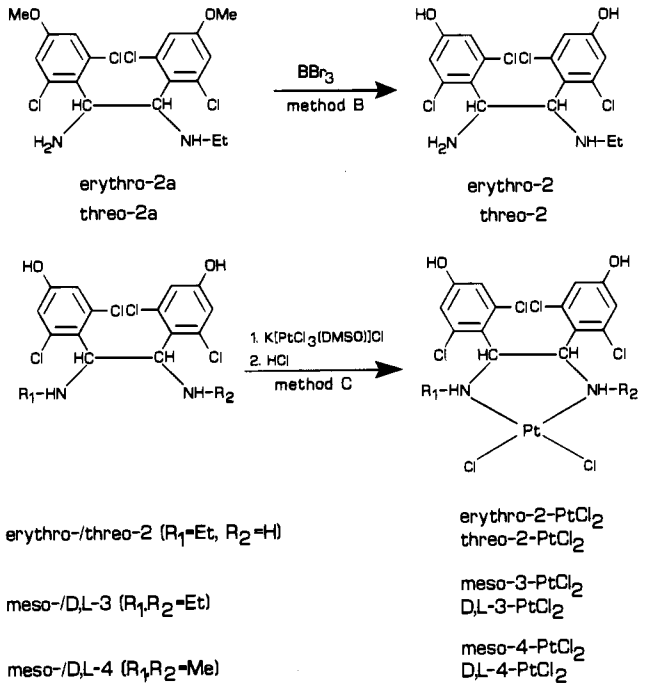
by N,N′-disubstitution with alkyl groups was described. The resulting compounds showed an increase of affinity to the ER as a function of chain length. Therefore, we tried to enhance the RBA of *meso*-1−$PtCl_2$ in the same way. In this paper we describe the synthesis of [*N*-ethyl-, *N,N′*-dimethyl-, and *N,N′*-diethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (formula, see Chart 1), their structural characterization, their ER affinity, and their estrogenic activity.

Results

Synthesis of 1,2-Diarylethylenediamines and of Their Platinum Complexes. The synthesis of diastereomeric *N,N′*-dialkyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines (*meso*- and D,L-**3**; *meso*- and D,L-**4**) was carried out in accordance with the method described by von Angerer[8] by using a reductive dimerization of the methoxybenzaldehyde alkylimines with activated aluminum. Subsequent separation of the resulting mixtures of *meso*- and D,L-*N,N′*-dialkyl-1,2-bis(2,6-dichloro-4-methoxyphenyl)ethylenediamines (*meso*- and D,L-**3a**; *meso*- and D,L-**4a**) by fractional crystallization of the hydrochlorides and ether cleavage with BBr_3 led to the hydroxy derivatives *meso*- and D,L-**3** and *meso*- and D,L-**4**, respectively.

For the synthesis of *erythro*- and *threo*-*N*-ethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines (*erythro*- and *threo*-**2**) a new synthetic route was developed (see Scheme 1). As educts the diastereomeric 1,2-bis(2,6-dichloro-4-methoxyphenyl)ethylenediamines (*meso*- and D,L-**1a**), which were available from the stereoselective *meso-meso*- and D,L-D,L-diaza-Cope rearrangement reactions of *meso*- and D,L-*N,N′*-bis(2,6-dichloro-4-methoxybenzylidene)-1,2-bis(2-hydroxyphenyl)ethylenediamines, were used.[9] In a first reaction step, *meso*-**1a** and D,L-**1a**, respectively, were treated with *n*-butyllithium to achieve a nucleophilic center on one amino group by NH/NLi exchange. The resulting *N*-lithium compounds were easily transferred into the *N*-ethyl derivatives by reaction with diethyl sulfate. Analysis of the resulting product mixtures indicated the presence of three compounds which were identified as educt, N-monoethylated, and N,N′-diethylated ethylenediamines. Best yields of the monoethyl derivatives were obtained if a 1.5-fold excess of *n*-butyllithium was used for the N-metalation. Separation of *erythro*- and *threo*-**2a** from their product mixtures was possible by column chromatography on SiO_2. Ether cleavage of *erythro*- and *threo*-**2a** to give *erythro*- and *threo*-**2** was done with BBr_3 (see Scheme 2).

Scheme 2



The dichloroplatinum(II) complexes *erythro*- and *threo*-**2**−$PtCl_2$, *meso*- and D,L-**3**−$PtCl_2$, as well as *meso*- and D,L-**4**−$PtCl_2$ were synthesized by reacting the corresponding ligands with K[$Pt(Cl)_3$(DMSO)] prepared by treatment of K_2PtCl_4 with an equimolar amount of DMSO.[10] Coordination of the ethylenediamines yielded [chloro(DMSO)enPt]Cl as intermediates. The respective dichloroplatinum(II) complexes could be obtained by thermal decomposition of the separated [chloro(DMSO)-enPt]Cl complexes or in a simpler, faster, and more specific preparation route by direct substitution of the coordinated DMSO with Cl^-. For this purpose, HCl was

Table 1. ^{1}H-NMR Data[a] of N-Monoalkylated and N,N'-Dialkylated [1,2-Bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II)

compd	CH_3	CH_2[b]	CH	NH	Ar-H	OH
erythro-**2**-$PtCl_2$	1.39 (t, ^3J = 7.0 Hz, 3H)	2.54–2.68 (m, 1H) 3.44–3.49 (m, 1H)	5.06–5.16 (m, 1H) 5.16–5.27 (m, 1H)	5.36–5.48 (m, 1H) 6.08–6.18 (m, 1H) 6.66–6.75 (m, 1H)	6.81 (d, ^4J = 2.5 Hz, 1H) 6.84 (s, 2H) 6.93 (d, ^4J = 2.5 Hz, 1H)	11.23 (br, 2H)
threo-**2**-$PtCl_2$	1.61 (t, ^3J = 7.0 Hz, 3H)	2.36–2.50 (m, 1H) 3.30–3.46 (m, 1H)	5.32–5.47 (m, 1H) 5.47–5.66 (m, 1H)	4.92–5.08 (m, 1H) 5.90–6.04 (m, 1H) 6.88–7.00 (m, 1H)	6.77 (d, ^4J = 2.5 Hz, 1H) 6.82 (d, ^4J = 2.5 Hz, 1H) 6.99 (d, ^4J = 2.5 Hz, 1H) 7.01 (d, ^4J = 2.5 Hz, 1H)	11.16 (br, 2H)
meso-**3**-$PtCl_2$	1.45 (t, ^3J = 7.0 Hz, 6H)	2.60–2.84 (m, 1H) 3.41–3.63 (m, 1H)	5.00–5.08 (m, 2H)	6.22 (br, 2H)	6.86 (d, ^4J = 2.5 Hz, 2H) 6.93 (d, ^4J = 2.5 Hz, 2H)	11.06 (br, 2H)
D,L-**3**-$PtCl_2$	1.53 (t, ^3J = 7.0 Hz, 6H)	2.27–2.45 (m, 1H) 3.30–3.56 (m, 1H)	5.31–5.46 (m, 2H)	6.03 (br, 2H)	6.83 (d, ^4J = 2.5 Hz, 2H) 7.08 (d, ^4J = 2.5 Hz, 2H)	11.20 (br, 2H)
meso-**4**-$PtCl_2$	2.73 (d, ^3J = 5.8 Hz, 6H)		5.00–5.09 (m, 2H)	6.22 (br, 2H)	6.85 (d, ^4J = 2.5 Hz, 2H) 6.92 (d, ^4J = 2.5 Hz, 2H)	11.12 (br, 2H)
D,L-**4**-$PtCl_2$	2.73 (d, ^3J = 5.8 Hz, 6H)		5.35–5.47 (m, 2H)	6.05 (br, 2H)	6.82 (d, ^4J = 2.5 Hz, 2H) 7.00 (d, ^4J = 2.5 Hz, 2H)	10.50 (br, 2H)

[a] The spectra were taken at 250 MHz in [D_7]DMF with TMS as internal standard, the chemical shifts are given as δ values. [b] Detected after addition of D_2O.

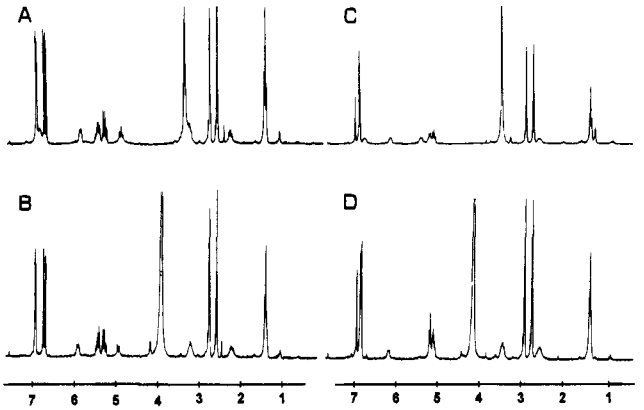


Figure 1. 250 MHz ^{1}H-NMR spectrum in [D_7]DMF of (A) *threo*-**2**-$PtCl_2$, (B) *threo*-**2**-$PtCl_2$ NH partial displaced by deuterium, (C) *erythro*-**2**-$PtCl_2$, (D) *erythro*-**2**-$PtCl_2$ NH partial displaced by deuterium.

added to the reaction mixture. After heating for 1 h to 50–60 °C, the dichloroplatinum(II) complexes precipitated and were separated by filtration.

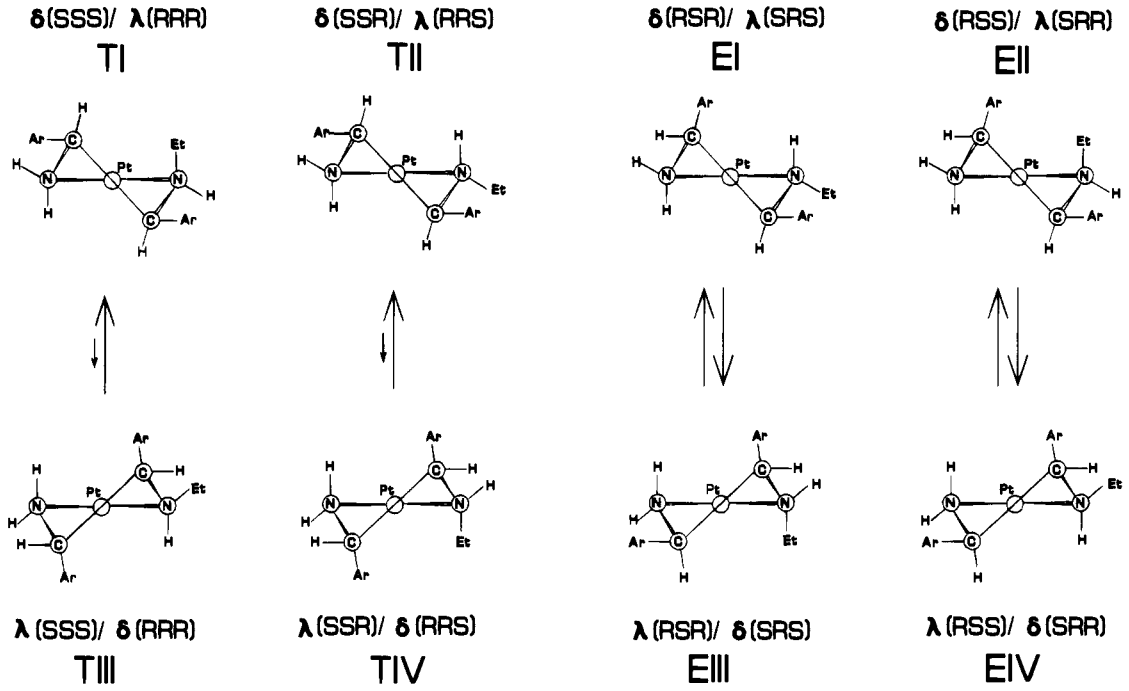
Spectroscopical Characterization of the Dichloroplatinum(II) Complexes and Discussion of Their Spatial Structures. The structural characterization of the platinum complexes reported in this paper was done by IR and ^{1}H NMR spectroscopy. In their IR spectra *erythro*- and *threo*-**2**-$PtCl_2$, *meso*- and D,L-**3**-$PtCl_2$ as well as *meso*- and D,L-**4**-$PtCl_2$ showed Pt–Cl stretching vibrations in the region between 310 and 330 cm^{-1}, characteristic for dichloroplatinum(II) complexes.[11] Contamination with PtCl(DMSO) compounds, intermediates of the coordination reaction, could be excluded, since the IR spectra did not exhibit (ν S–O) bands in the region between 1110 and 1140 cm^{-1} and the ^{1}H-NMR spectra did not show a DMSO signal at δ = 3.54 typical for coordinated DMSO (see Figure 1).[10,12]

Coordination of the ligands to platinum was also confirmed by ^{1}H-NMR spectroscopy. Binding to platinum blocks rotation around the C–N axes, whereby the protons of the NH_2 groups become diastereotopic due to the neighborhood of a chiral benzylic C atom with different signals for the axially and equatorially arranged protons. Therefore, in the range between 4 and 7 ppm, the ^{1}H-NMR spectra of *threo*- and *erythro*-**2**-$PtCl_2$ (Figure 1A + C, Table 1) contain three NH resonances and two resonances for the nonequivalent benzylic protons, respectively. In the five-membered chelate ring the NHEt nitrogen becomes a stable chiral center and the CH_2 protons of the *N*-ethyl groups suffer a diastereotopic splitting. Furthermore, the large difference in the chemical shift of $\Delta\delta$ = 0.9–1.0 between the two protons of the CH_2 group compared to that of the free ligands of $\Delta\delta$ < 0.2 hints at a restricted rotation of the ethyl group around the C–N axis in the complex due to the neighboring aromatic rings, whose rotation around the benzylic axis is hindered, too (the proton signals of the nonequivalent aromatic rings are split into AB systems).

Upon coordination to platinum, the prochiral center at the NHEt nitrogen of the ligands *erythro*- and *threo*-**2** gives rise to two pairs of enantiomers. Supposed that the five-membered chelate ring shows no interconversion, four racemic isomers are possible for *erythro*-**2**-$PtCl_2$ and *threo*-**2**-$PtCl_2$, respectively (see Chart 2).

However, the ^{1}H-NMR spectrum of *threo*-**2**-$PtCl_2$ revealed that only one of the four possible racemates was formed in the course of the binding of the diamines to platinum. Insights into the spatial structure of this compound were given by the resonances resulting from the coupling of the benzylic protons with each other and with the amino protons. To achieve a total exchange of the three N-standing protons in *threo*-**2**-$PtCl_2$ by deuterium it was necessary to heat the [D7]DMF solution after addition of D_2O to 60 °C for 3 h. In the spectrum of the N-deuterated *threo*-**2**-$PtCl_2$ the benzylic protons appear as an AB system with a coupling constant of 12.1 Hz, indicating a preferred conformation of the five-membered chelate ring with bisequatorially arranged aromatic rings. At room temperature, addition of D_2O leads only to a disappearance of the NH resonance at δ = 6.88–7.0 (NH^{eq}) and to a decrease of the multiplicity of the NH signal at δ = 4.92–5.08 (NH^{ax}) to a doublet with $^3J_{NH-CH}$ = 10.8 Hz (Figure 1B). This coupling constant indicates a dihedral angle of about 180° to the benzylic proton and therefore a location of the nonexchanged H in axial position. Due to the NH/ND exchange of NH^{eq} the splitting of the benzylic proton at δ = 5.47–5.66 is reduced demonstrating its vicinity to the NH_2 group. The signal of the second benzylic proton at δ = 5.21–5.47 is split by two couplings ($^3J_{CH-CH}$ = 12.1 Hz, $^3J_{CH-NH}$ = 9.9 Hz), which is in accordance with dihedral angles of about 180° between these protons. Such a structure is only realized in isomer TII (see Chart 2).Thus *threo*-**2**-$PtCl_2$ exists in a conformation with all bulky substituents (Ar and

Chart 2



Et) on the chelate ring in the energetically preferred equatorial position. An interconversion of TII into TIV does not take place.

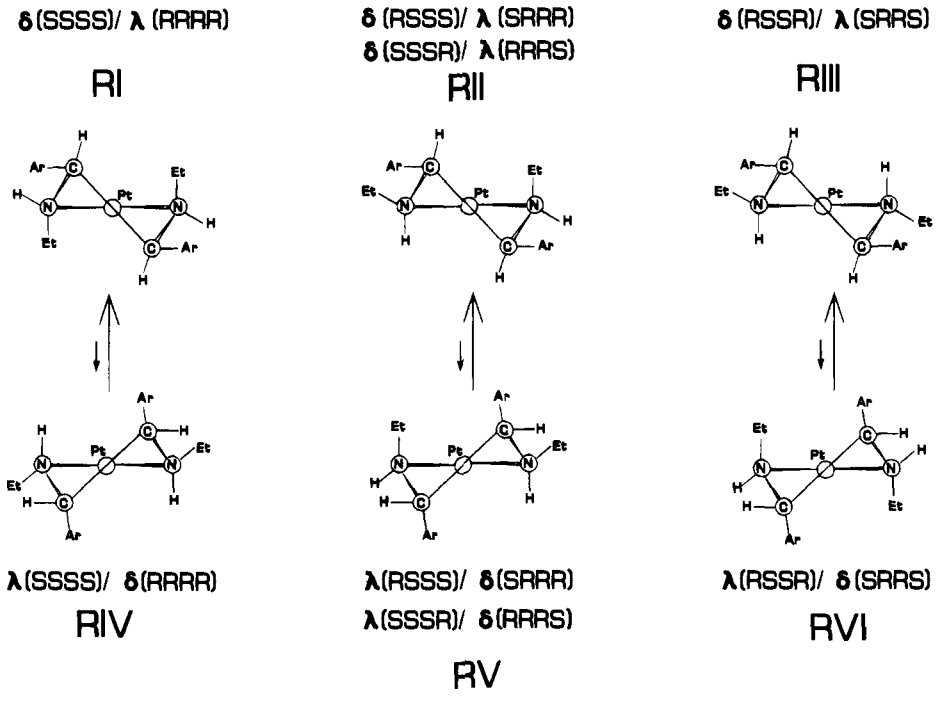
In contrast to this *erythro*-**2**−$PtCl_2$ is present in an equilibrium of two conformers, EI/EIII (see Chart 2). At room temperature both NH_2 protons are exchanged by deuterium after addition of D_2O (Figure 1D), whereas exchange of the NHEt proton requires heating to 60 °C for 4 h. From the coupling of the NHEt proton with the benzylic proton in the bisdeuterated *erythro*-**2**−$PtCl_2$ ($^3J_{NH-CH}$ = 4.8 Hz; see Figure 1D) an assignment to a single isomer (EI, etc.) is impossible. This coupling constant could be caused by a mean angle between 60 and 180° (EI ↔ EIII) as well as between −60 and 60° (EII ↔ EIV). The 3-fold deuterated *erythro*-**2**−$PtCl_2$ shows an AB spectrum for the benzylic protons with $^3J_{CH-CH}$ = 7.9 Hz, also indicative of a fast interconversion of the five-membered chelate ring. An assignment of *erythro*-**2**−$PtCl_2$ to EI ↔ EIII was possible by comparison of the chemical shift of the NEt signal with that of diastereomeric [*erythro*-*N*-ethyl-1,2-bis(3-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) of known absolute configuration. In recent studies we obtained two diastereomers by coordination of *erythro*-*N*-ethyl-1,2-bis(3-hydroxyphenyl)ethylenediamine to dichloroplatinum(II), which could be separated by virtue of their different solubilities in H_2O. H,H-NOESY experiments revealed absolute configurations for diastereomers I and II, which correspond to those of EIV and EI, respectively (see Chart 2). Contrary to diastereomer I, diastereomer II exists in a δ ↔ λ equilibrium (compare EI ↔ EIII in Chart 2). Investigations on the diastereomeric [*erythro*-*N*-ethyl-1,2-bis(3-hydroxyphenyl)ethylenediamine]-dichloroplatinum(II) complexes[13] have shown that the chemical shift of the CH_3 resonance of the ethyl group depends on the configurations at the N atom (NHEt group) and at the neighboring benzylic C atom. The diastereomer I (λ(*RSS*)/δ(*SRR*)) shows its CH_3 resonance at δ = 0.98 and the diastereomer II (δ(*RSR*)/λ-(*SRS*)) at δ = 1.37. The latter correlates very well with the CH_3 resonance found for *erythro*-**2**−$PtCl_2$ (δ = 1.39, see Table 1), so this complex can be assigned to EI/EIII. The ^{1}H-NMR spectra of the N,N′-dialkylated complexes *meso*- and D,L-**3**−$PtCl_2$ as well as *meso*- and D,L-**4**−$PtCl_2$ suggest the presence of a single isomer or of two isomers in a δ ↔ λ equilibrium. The benzylic protons of *meso*- and D,L-**3**−$PtCl_2$ are split into an AA′XX′ system with couplings between $NH/CH_{benzylic}$ and $CH_{benzylic}/CH_{benzylic}$ (see Table 1).

After exchange of the N-standing protons by deuterium (60 °C, 3 h), the benzylic protons show a common singlet with unresolved ^{195}Pt satellites of $^3J_{Pt-H}$ = 45 Hz (*meso*-**3**−$PtCl_2$) and $^3J_{Pt-H}$ = 25 Hz (D,L-**3**−$PtCl_2$). These couplings indicate that D,L-**3**−$PtCl_2$ exists preferentially in a conformation with bisequatorially arranged aromatic rings, while the five-membered chelate ring of *meso*-**3**−$PtCl_2$ undergoes a rapid interconversion.[14,15] The methylene protons of the ethyl chains are diastereotopically split, comparable to those of *erythro*-**2**−$PtCl_2$ and *threo*-**2**−$PtCl_2$ (due to the presence of chiral centers on the nitrogens). The lack of coupling between the benzylic protons and the existence of common resonances for both ethyl and both amino protons, respectively, make believe that D,L- and *meso*-**3**−$PtCl_2$ are symmetric molecules with identical environments at both benzylic C atoms and at both N atoms. Among the theoretically possible isomers of D,L-**3**−$PtCl_2$, RI and RIII (see Chart 3) represent in fact symmetric molecules, while the *meso*-**3**−$PtCl_2$ isomers MI/MV and MII/MVI (see Chart 4) simulate symmetry by the interconversion of the five-membered chelate ring.

Since the chemical shift of CH_3 in *meso*-**3**−$PtCl_2$ (δ = 1.45) is comparable to that in *erythro*-**2**−$PtCl_2$ (δ = 1.39), identical configurations and environments on the nitrogens can be assumed. Thus the structure of *meso*-**3**−$PtCl_2$ is assigned to MI/MV and, in analogy, that of D,L-**3**−$PtCl_2$ to RIII.

The structure of the complexes is not influenced by the length of the *N*-alkyl chain. Both the *N*,*N*′-diethyl (*meso*-and D,L-**3**−$PtCl_2$) and *N*,*N*′-dimethyl derivatives

Chart 3

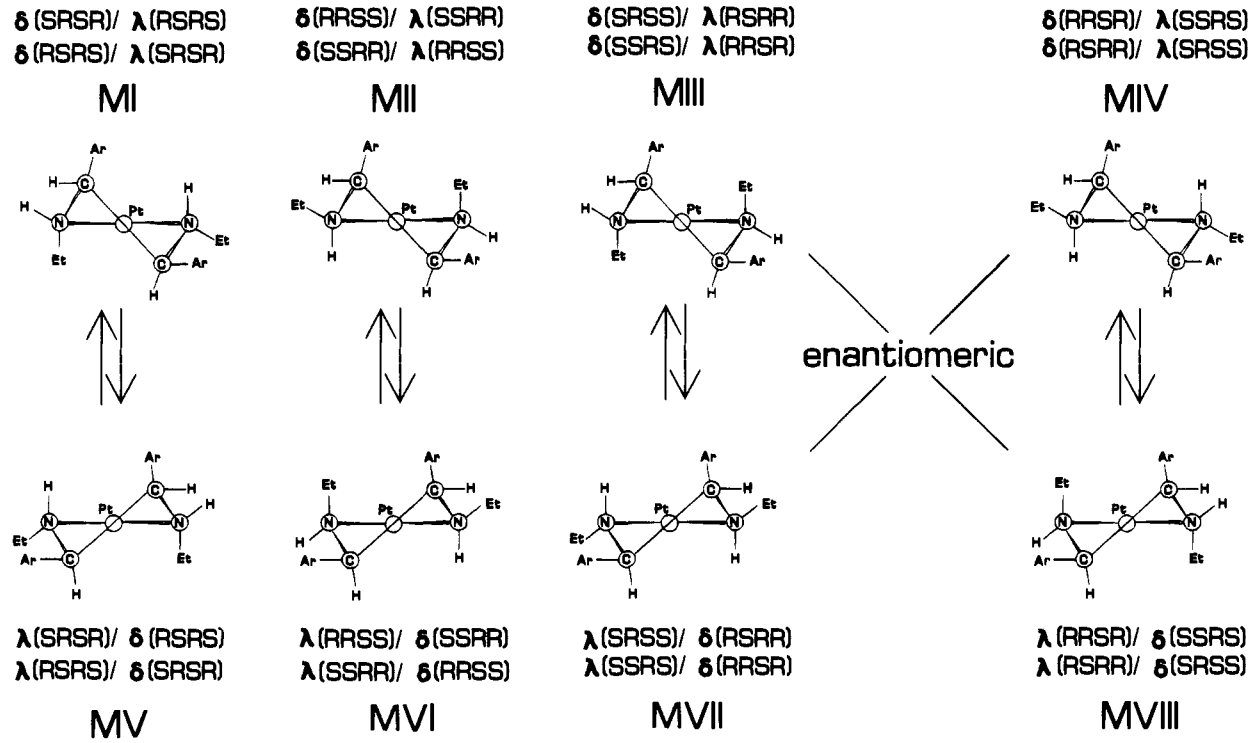


(*meso*- and D,L-**4**–$PtCl_2$) possess NH, CH, and aromatic resonances comparable in chemical shift and CH/CH and CH/NH couplings (see Table 1). Therefore, *meso*- and D,L-**4**–$PtCl_2$ can be assigned to structures analogous to MI/MIII and RIII, too.

The conformational behavior of the *RS*- and *SS/RR*-configurated ligands was investigated by use of the N-ethylated compounds *erythro*-**2** and *threo*-**2** as models. Due to the monoethyl substitution, the benzylic protons become nonequivalent, giving doublets in the spectra with coupling constants of 11.0 Hz (*erythro*-**2**) and 10.7 Hz (*threo*-**2**), respectively, which result from a dihedral angle between the vicinal protons of about 180°. Therefore, the arrangement of the aromatic rings is antiperiplanar in *erythro*-**2** and synclinal in *threo*-**2**. This means that during the coordination of the ligands to platinum the orientation of the phenyl residues in the *R/S*-configurated ligands is changed into synclinal, while it is conserved in the *RR/SS* representatives.

Estrogenic Properties and Discussion of the Structure–Activity Relationship. The relative binding affinity (RBA) to the calf uterine estrogen receptor (ER) was measured by an indirect, competitive binding

Chart 4



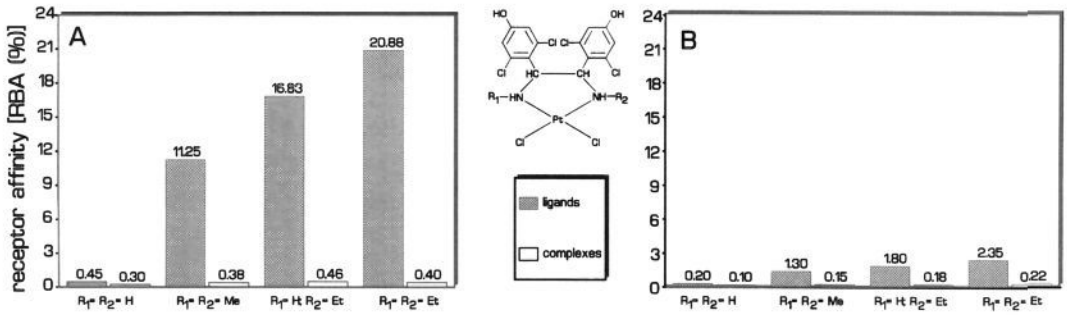


Figure 2. Receptor affinity of (A) *RS/SR*-configurated ligands and their complexes; (B) *SS/RR*-configurated ligands and their complexes.

assay with [^{3}H]estradiol ([^{3}H]E2; $RBA_{E2} = 100$) and the dextran−charcoal method.[16]

The RBA's of the *R/S*-configurated 1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines depend strongly on the substituents on the amino groups. The nonalkylated parent diamine (*meso*-**1**) showed only marginal affinity to ER with an RBA of 0.45 (see Figure 2A). N,N′-Dimethylation led to a compound (*meso*-**4**) with a relatively high RBA of 11.25, which can be further increased by prolongation of the two alkyl residues. The N,N′-diethyl derivative (*meso*-**3**) reached an RBA of 20.88. Already substitution of one amino group in the parent compound *meso*-**1** with an ethyl group resulted in a more ER-affinic diamine with an RBA of 16.83.

In the racemate series the effect of N-monoalkylation or N,N′-dialkylation is less pronounced (see Figure 2B). The *N,N′*-diethyl derivative (D,L-**3**) showed the best affinity to the ER with RBA = 2.35, which is, however, about 10-fold lower than that of the *meso*-configurated counterpart (*meso*-**3**). A much weaker ER affinity of D,L- than of *meso*-configurated *N,N′*-dialkyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines has also been reported by von Angerer.[8] The coordination of the diamines to dichloroplatinum(II) reduced the affinity to the ER drastically. The *R/S*- as well as the *RR/SS*-configurated complexes showed, in spite of the different substituents on the amino groups, similarly low RBA's (*R/S*, 0.30−0.46; *RR/SS*, 0.10−0.22; see Figure 2A,B).

In the class of estrogens, a correlation between the RBA's and the estrogenic potencies is observed. Compounds with high RBA's cause stronger estrogenic effects than those with small RBA's. The same is true for the *R/S*-configurated 1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines, which proved to be "true" estrogens in the mouse uterine weight test. The estrogenicity rises continously in this series beginning with the parent compound *meso*-**1** to the *N,N′*-dimethyl derivative (*meso*-**4**) and further to the *N,N′*-diethyl derivative (*meso*-**3**). The N-monoethylated diamine (*erythro*-**2**) is similarly active like the N,N′-dimethylated diamine (*meso*-**4**). In accordance with their higher RBA's *meso*-**1**, *meso*-**3**, and *meso*-**4** are considerably stronger estrogenic than their *RR/SS* isomers (compare Table 2).

In the *RR/SS*-configurated diamine series, too, an increase of the estrogenic properties with elongation of the alkyl chains is recognizable (compare D,L-**4** with D,L-**3** in Table 2). However, the N-unsubstituted diamine (D,L-**1**) and its *N,N′*-dimethyl derivative (D,L-**4**) are only marginally active. In analogy to the similarly potent pair *erythro*-**2**/*meso*-**4** the N-monoethylated diamine (*threo*-**2**) is expected to be only marginally active like the *N,N′*-dimethyl derivative (D,L-**4**). Therefore, we excluded *threo*-**2** from an in vivo testing. The only compound which equals the 100% effect of estrone and even surpasses it at the high dose of 100 nmol/animal is the N,N′-diethylated diamine (D,L-**3**).

Table 2. Estrogenic Effect[a] of N-Substituted and N,N′-Disubstituted 1,2-Bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamines

dose (nmol/animal)	*meso*-**1**[b]	D,L-**1**[b]	*erythro*-**2**[c]	*meso*-**3**[d]	D,L-**3**[d]	*meso*-**4**[e]	D,L-**4**[e]
0.1	0	0	22	61	0	0	0
1	38	0	93	125	0	47	0
10	135	13	140	140	73	139	3
100	100	10	156	128	142	127	12
1000		21				47	72

[a] Mouse uterine weight test: compounds were administered at three consecutive days sc as solutions poly(ethylene glycol) 400/water (1.8% NaCl), 1:1. The uteri were removed 24 h after the last injection, estrogenic effect = $[(E_T - E_V)/(E_E - E_V)] \times 100$; E_T = effect of test compound; E_V = effect of vehicle; E_E = effect of estrone standard (0.4 μg); effect = [uterus dry weight (mg)/body weight (g)] × 100. [b] Data from ref 7. [c] *threo*-**2** was not tested for its estrogenic properties. [d] Data of the ligands from ref 8. [e] Data from ref 23.

After transformation of the diastereomeric diamines into their dichloroplatinum(II) complexes the estrogenic properties were unchanged though with this a conformational change of the *R/S*-configurated diamine ligands (but not of the *RR/SS*-configurated diamine ligands) takes place (compare Table 2 with Table 3). A correlation between estrogenicity and receptor affinity of the homologous complexes was not observable as was in the amine series. In the following we attempt to interpret these controversial experimental results.

In former publications[17,18] and also in the current study we could show that in diastereomeric 1,2-diphenylethylenediamines (*R/S* and *RR/SS*) the benzylic protons are in antiperiplanar arrangement. This implicates an antiperiplanar location of the phenyl rings in the *R/S* form and a synclinal one in the *RR/SS* form. The relatively high conformational stability of diastereomeric diamines which are substituted by two chlorine atoms in the 2,6-positions of their phenyl rings (compare our conformation studies in the 1,2-bis(2,6-dihalo-3-hydroxyphenyl)ethylenediamine series)[19] allows the assumption that the *R/S*- and *RR/SS*-configurated diamines **1**−**4** interact exclusively in these conformations with the ER.

In earlier studies of our group,[20] *meso-N,N′*-dimethyl-1,2-bis(4-hydroxyphenyl)ethylenediamine, which is closely related to hexestrol in terms of chemical and spatial structure, showed neither ER-affinity nor estrogenic

Table 3. Estrogenic Effect[a] of [N-Substituted and N,N'-Disubstituted 1,2-Bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II)

dose (nmol/animal)	*meso*-1–$PtCl_2$[b]	D,L-1–$PtCl_2$[b]	*erythro*-**2**–$PtCl_2$[c]	*meso*-**3**–$PtCl_2$	D,L-**3**–$PtCl_2$	D,L-**4**–$PtCl_2$[c,d]
0.1	0	0	40	94	6	0
1	45	0	87	189	30	0
10	90	4	130	123	78	0
100	96	6	110	80	110	28
1000		23			160	88

[a,b] See Table 2. [c] *threo*-**2**–$PtCl_2$ and *meso*-**4**–$PtCl_2$ were not tested for their estrogenic properties. [d] Data of the ligands from ref 8.

potency. Also the replacement of the two N-standing methyl groups in this compound by hydrophobic butyl residues does not lead to a hormonally active compound.[20] This indicates that the hydrophobic interaction of the two phenyl rings with lipophilic centers in the ER plays an essential role in the binding of nonsteroidal estrogens of the 1,2-diphenylethane series (e.g., HES). In the case of the *N,N'*-dialkyl-1,2-bis(4-hydroxyphenyl)ethylenediamines the two NH_2 groups seem to disturb the hydrophobic interaction with the receptor. Since in this class of estrogens (prototype: HES) only compounds with two OH groups at a distance of about 12 Å cause strongly agonistic effects, we must assume that these functional groups are of importance for the approach and for the proper orientation to the binding area of the ER (e.g., HES exists also preferentially in a conformation with antiperiplanar phenyl rings O–O distance = 12.1 Å).[21]

In this connection it is of interest that the reduction of the O–O distance in HES by transformation of both 4-standing OH groups into the 3- or 2-positions (3,3'-HES and 2,2'-HES) strongly decreased the estrogenic potency.[22] In contrast to 2,2'-HES, which is a very poor "true" estrogen, 3,3'-HES gives in mouse uterine weight test a dose–activity curve of an "impeded" estrogen. 3,3'-HES even shows marked antiestrogenic effects.

The free binding energy from the two H bridges seems to be not sufficient for a change of the three-dimensional receptor structure and thus for the triggering of the biological effect. (Concerning the mechanism of ER-mediated hormonal effects, see ref 23.) The successful transformation of *meso-N,N'*-dimethyl-1,2-bis(4-hydroxypheny)ethylenediamine into a "true" estrogen—comparable in its activity with HES—by introduction of chlorine atoms into the two 2,6-positions of the phenyl rings and by exchange of the two N,N'-standing Me groups by Et groups (i.e., *meso*-**3**) points also to the important role of the hydrophobic interaction, especially between the chlorinated phenyl rings and nonpolar regions in the receptor, for the binding affinity and the hormonal effect of the *R/S*-configurated 1,2-diphenylethylenediamines.

Further hints at the importance of the hydrophobic character of the 1,2-diphenylethane skeleton and the 4-standing OH groups for the estrogenic activity are as follows:

(i) The exchange of the 2,6-standing chlorine atoms in *meso*-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine (*meso*-**1**) by the less lipophilic fluorine or methyl residues (lipophilic constant π: Cl = 0.71, F = 0.14, and CH_3 = 0.56) led to a strong decrease of the estrogenic potency (estrogenic effect: F-der, inactive at 10–1000 nmol;[24] CH_3-der, 15 at 100 nmol and 46 at 1000 nmol).[25] The same is true for a partial exchange of the chlorine atoms in *meso*-**1** by fluorine or hydrogen—compare ref 17.

(ii) In nonsteroidal estrogens the introduction of halogen atoms in positions ortho to the OH groups, which hinders the formation of H bridges between drug and receptor, proved to be unfavorable for the binding of the drug to the ER.[26] In the *meso*-3,4-bis(3-halo-4-hydroxyphenyl)hexane series, for example, a decrease of the RBA's (F = 16.0; Cl = 1.6; Br = 0.255; I = 0.17; HES = 27.0) was observed with increase of the van der Waals radius of the halogen. Therefore, a substitution by halogens in the ortho positions of the OH groups of 1,2-bis(4-hydroxyphenyl)ethylenediamines was not further pursued.

The interaction of the *R/S*-configurated diamines 1–**4** with the ER can be described by a model developed by Höltje and Dall[1] for estradiol (E2) and nonsteroidal estrogens.

The authors stated that in their pharmacophoric conformations E2 and highly ER-affinic nonsteroidal estrogens like HES are very similar to each other in their van der Waals volumes as well as in their interaction patterns and that the essential contribution of the hydrophobic binding results from an interaction of the AB region in E2 (or of the respective molecule fragment in HES) with lipophilic centers of the ER situated above and below the drug plane. Van der Waals interactions as well as hydrogen bridges and/or electrostatic interactions coming from the two OH groups are also responsible for the high ER affinity of these drugs.

Surprisingly, the coordination of the *R/S*-configurated diamines 1–**3** to dichloroplatinum(II) does not lead to substantial changes in their estrogenic potencies, although in this process the aromatic rings alter their positions from antiperiplanar to synclinal. In this conformation the platinum-coordinated diamine ligands, which are the pharmacophores responsible for the binding to the ER, cannot be accepted by the drug–receptor model described by Höltje and Dall.[1] This finding induces us to assume that a second binding site exists in the ER, which accepts a 1,2-bis(4-hydroxyphenyl)ethane fragment in conformations with shorter O–O distances. The search for a compound containing such a fragment led us to (*Z*)-1,2-bis(4-hydroxyphenyl)-1-phenylbut-1-ene (*Z*-BAPB) described by Schneider[27] to be a highly active "true" estrogen. In this compound the oxygen atoms are 8.4 Å apart from each other, while in its *E*-isomer the O–O distance amounts to 12.2 Å corresponding to that in HES. The O–O distance of the *Z*-isomer (8.4 Å) is very close to that found in the new "true" estrogens *meso*-**1**–$PtCl_2$, *erythro*-**2**–$PtCl_2$ and *meso*-**3**–$PtCl_2$ (about 8 Å). Since these complexes exist in a $\delta \leftrightarrow \lambda$ equilibrium (compare Charts 2 and 4) at physiological temperatures, the fit to the binding area of the receptor is facilitated. *meso*-**1**–$PtCl_2$, *erythro*-**2**–$PtCl_2$, and *meso*-**3**–$PtCl_2$ possess strong estrogenic properties (see Table 2) comparable to that of *Z*-BAPB.

A further indication that *meso*-1−$PtCl_2$, *erythro*-**2**−$PtCl_2$, and *meso*-**3**−$PtCl_2$ interact with a binding site (i.e., binding site II) different from that of HES (i.e., binding site I or E2 binding site) is the lack of a correlation between their RBA's and their estrogenic potencies. The RBA's of all complexes are very low and similar, between 0.30 and 0.46 (see Figure 2). Since we used [^{3}H]E2 as competitor for the estimation of the relative binding affinity to ER, the found RBA's demonstrate nothing more than a weak interaction of the complexes with the binding site I of ER. By use of radiolabeled complexes (direct estimation method) or of [^{3}H]-*Z*-BAPB as competitor (indirect estimation method), much higher RBA's and a correlation between receptor affinity and estrogenic potency should be obtained like in the related ligand series. These experiments are still to be made.

The estrogenic activity of *meso*-**1**−$PtCl_2$, *erythro*-**2**−$PtCl_2$, and *meso*-**3**−$PtCl_2$ could also arise from the diamine ligand liberated from a hormonally inactive complex by bionucleophils. If this is true, the complex should have a lower activity than its ligand in the mouse uterine weight test. This is not the case. To solve this question we studied the stability of tritiated *meso*-1−$PtCl_2$ in cell culture medium as well as its hormonal effects (i.e., triggering of ER processing and progesterone receptor synthesis) in human breast cancer cells (MCF7) under identical conditions. The comparison of the kinetics of ligand release with the time required by *meso*-**1**−$PtCl_2$ to induce these hormonal effects showed that the biological response of the breast cancer cells can be attributed to the intact complex.[28,29]

For the specific proof of the estrogenic activity of *meso*-**1** and of its complex ER-positive MCF7 breast cancer cells transfected with the EREwtc-CAT reporter system of Klein-Hitpass[30] were used as experimental model.[31] This plasmid contains the ERE sequence (ERE: estrogen responsive element) in front of a thymidine kinase promotor with following CAT gene (CAT: chloramphenicol acetyl transferase). As expected *meso*-**1** and its complex increased markedly the CAT activity. Kinetic experiments using this assay confirm the stability and therefore the estrogenic activity of the complex. Furthermore it was demonstrated in gel shift experiments that a specific binding of the ER−*meso*-**1**−$PtCl_2$ complex to the ERE sequence within an oligonucleotide (produced from the vector EREwtc) took place.[31]

In the *RR/SS* series a synclinal arrangement of both phenyl rings (O−O distance = 9.6 Å) is observed in the diamines as well as in their dichloroplatinum(II) complexes, which should result in a preference of binding site II in the interaction with the ER. However, the mouse uterine weight test revealed, that merely D,L-**3** and its complex D,L-**3**−$PtCl_2$ are weakly active estrogens. An optimal fit of the 1,2-diphenylethane fragment to binding site II is hindered in *RR/SS*-configurated diamines and in their complexes due to (i) the spatial arrangement of the two N atoms, which is different from that in the *R/S* isomers, and (ii) the lack of conformational flexibility of the five-membered chelate ring, which hinders the approach of the two phenyl rings and thus the reduction of the O−O distance as in the *R/S* series. Lipophilic alkyl residues linked to the nitrogen atoms of *RR/SS*-configurated ligands or complexes can compensate these negative effects to some degree. However, the resulting estrogenic potency is markedly lower than that of the corresponding diastereomers.

Our findings with the diastereomeric diamines **1**−**3** and their complexes point to a high flexibility of the ER, a prerequisite for the indispensable tight contact of structurally very different estrogens to essential amino acid residues in the hormone binding domain, from which marked hormonal effects result.

Conclusion

The experiments described in this publication show that the alkylation of the parent complex [*meso*-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]-dichloroplatinum(II) (*meso*-1−$PtCl_2$) on the nitrogen atoms leads to compounds with markedly increased estrogenic potencies. This rise in activity is not accompanied by an elevation of the receptor affinity of these drugs, presumably due to the applied assay in which the drugs compete with 17β-[^{3}H]estradiol for its binding site on the estrogen receptor (i.e., binding site I or estradiol binding site). We explain this unexpected finding with the existance of a further binding site (i.e., binding site II), which accepts ligands with a distance of about 8 Å between their two OH groups as it is present in the complexes *meso*-**1**−$PtCl_2$, *erythro*-**2**−$PtCl_2$, *meso*-**3**−$PtCl_2$, and *meso*-**4**−$PtCl_2$. In accordance with our drug targeting concept, we suppose that the new complexes are enriched in breast cancer cells by binding to the estrogen receptors of the latter, where from strong and specific inhibiting effects should result. The investigation of *meso*-**3**−$PtCl_2$ and further compounds for these properties is subject of a further study.

Experimental Section

General Procedures. Melting points (uncorrected) were recorded with a Büchi 510 instrument; ^{1}H-NMR spectra were recorded on a Varian 360 L at 60 MHz; and ^{1}H-NMR spectra of hydroxy-substituted 1,2-diphenylethylenediamines and platinum complexes were taken with a Bruker FT-NMR spectrometer WM 250 at 250 MHz with TMS as internal standard. Elemental analyses were performed at Mikroanalytisches Laboratorium der Universität Regensburg. IR spectra (KBr pellets) were measured with a Perkin-Elmer 580 spektrophotometer.

Syntheses. Compounds *meso*- and D,L-**1**, *meso*- and D,L-**1a**, *meso*- and D,L-**3**, and *meso*- and D,L-**4** have been synthesized according to methods reported in the literature.[7]

Method A: *erythro*-*N*-Ethyl-1,2-bis(2,6-dichloro-4-methoxyphenyl)ethylenediamine (*erythro*-2a). To a solution of *meso*-**1a** (10 mmol, 4.10 g) in 60 mL of dry THF was added dropwise *n*-BuLi (15 mmol, 0.96 g), dissolved in 10 mL of dry THF, at a temperature of −65 °C. After the mixture has been stirred for 15 min, a THF solution of diethyl sulfate (10 mmol, 2.18 g) was added. It was allowed to warm to room temperature, and the reaction mixture was stirred additionally for 5 h. Subsequently, 40 mL of ether and 60 mL of 2 N NaOH were added, and the organic layer was separated, washed with water, and dried over $MgSO_4$. Removal of the solvent in vacuo left an oily mixture of *meso*-**1a**, *erythro*-**2a**, and *meso*-**3a**. The compounds were separated by column chromatography (SiO_2, petroleum ether/ether, 2:1) to give 1.62 g of *erythro*-**2a** (37%, colorless crystals, mp 137−138 °C): ^{1}H-NMR ($CDCl_3$) δ 0.87 (t, ^3J = 7 Hz, 3H, CH_3), 1.82 (br, 3H, NH), 2.10−2.60 (m, 2H, CH_2), 3.78 (s, 6H, OCH_3), 5.32 (s, 2H, $CH_{benzylic}$), 6.89 (s, 4H, Ar-H).

***threo*-*N*-Ethyl-1,2-bis(2,6-dichloro-4-methoxyphenyl)-ethylenediamine (*threo*-2a):** yield 1.71 g (38%), colorless oil; ^{1}H-NMR ($CDCl_3$) δ 1.10 (t, ^3J = 7 Hz, 3H, CH_3), 2.18 (br, 3H, NH), 2.46−2.58 (m, 2H, CH_2), 3.71 (s, 6H, OCH_3), 4.94 (d,

^3J = 11 Hz, 1H, $CH_{benzylic}$), 5.13 (d, ^3J = 11 Hz, 1H, $CH_{benzylic}$), 6.60 (d, ^3J = 3 Hz, 2H, Ar-H), 6.79 (d, ^3J = 3 Hz, 2H, Ar-H).

Method B: *erythro*-N-Ethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine (*erythro*-2). A solution of *erythro*-**2a** (3 mmol, 1.31 g) in 60 mL of dry $CHCl_3$ was cooled to −60 °C. At this temperature BBr_3 (13 mmol, 3.01 g) was added. The reaction mixture was brought to room temperature and heated to reflux for 24 h. Subsequently, 20 mL of methanol was added with cooling, and the solvent was evaporated. The residue was dissolved in 5 mL of water, filtrated, and alkalized with 2 N NaOH. Unreacted *erythro*-**2a** was filtered off, and the filtrate was brought to pH 8 with 2 N HCl. The precipitate was collected by suction filtration, washed with water, and dried over P_2O_5: yield 886 mg (72%), colorless powder; mp 161 °C; ^{1}H-NMR ([D$_7$]DMF, D_2O) δ 0.84 (t, ^3J = 7.1 Hz, 3H, CH_3), 2.15−2.28 (m, 1H, CH_2), 2.28−2,38 (m, 1H, CH_2), 5.23 (d, ^3J = 11 Hz, 1H, $CH_{benzylic}$), 5.28 (d, ^3J = 11 Hz, 1H, $CH_{benzylic}$), 6.93 (s, 2H, Ar-H) 6.96 (d, ^3J = 2.5 Hz, 1H, Ar-H), 6.99 (d, ^3J = 2.5 Hz, 1H, Ar-H). Anal. ($C_{16}H_{16}Cl_4N_2O_2$) C, H, N.

***threo*-N-Ethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine (*threo*-2):** yield 910 mg (72%), colorless powder; mp 149−151 °C; ^{1}H-NMR ([D$_7$]DMF) δ 1.08 (t, ^3J = 7.1 Hz, 3H, CH_3), 2.46−2.57 (m, 2H, CH_2), 5.00 (d, ^3J = 10.7 Hz, 1H, $CH_{benzylic}$), 5.13 (d, ^3J = 10.7 Hz, 1H, $CH_{benzylic}$), 6.67 (d, ^4J = 2.5 Hz, 1H, Ar-H), 6.69 (d, ^4J = 2.5 Hz, 1H, Ar-H), 6.86 (d, ^4J = 2.5 Hz, 1H, Ar-H), 6.88 (d, ^4J = 2.5 Hz, 1H, Ar-H). Anal. ($C_{16}H_{16}Cl_4N_2O_2 \cdot 0.5H_2O$) C, H, N.

Method C: General Procedure. K_2PtCl_4 (208 mg, 0.5 mmol) and 0.5 mmol of DMSO were dissolved in 2.5 mL of water and stirred for 24 h. To this reaction mixture were added 20 mL of water and a solution of the ligand (0.5 mmol) in 0.5 N HCl. Subsequently, the pH was adjusted to 4 by treatment with 0.5 N NaOH, and the mixture was heated to 50−60 °C for 8 h with stirring. During this time the pH was adjusted several times to 4−5. After this 10 mL of 2 N HCl was added, and the mixture was stirred for an additional hour. The precipitate was separated from the hot solution by suction filtration, washed with hot water, and dried over P_2O_5.

***erythro*-N-Ethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl-)ethylenediamine]dichloroplatinum(II) (*erythro*-2−$PtCl_2$):** yield 260 mg (77%), beige powder; ^{1}H-NMR, see Table 1; IR (KBr) ν 320 cm^{-1} (Pt−Cl). Anal. ($C_{16}H_{16}Cl_6N_2O_2Pt$) C, H, N.

***threo*-N-Ethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (*threo*-2−$PtCl_2$):** yield 237 mg (70%), beige powder; ^{1}H-NMR, see Table 1; IR (KBr) ν 320, 330 cm^{-1} (Pt−Cl). Anal. ($C_{16}H_{16}Cl_6N_2O_2Pt$) C, H, N.

***meso*-N,N'-Diethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (*meso*-3−$PtCl_2$):** yield 180 mg (51%), beige powder; ^{1}H-NMR, see Table 1; IR (KBr) ν 325 cm^{-1} (Pt−Cl). Anal. ($C_{18}H_{20}Cl_6N_2O_2Pt$) C, H, N.

D,L-*N,N'*-Diethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl-)ethylenediamine]dichloroplatinum(II) (D,L-3−$PtCl_2$): yield 190 mg (54%), beige powder; ^{1}H-NMR, see Table 1; IR (KBr) ν 320 cm^{-1} (Pt−Cl). Anal. ($C_{18}H_{20}Cl_6N_2O_2Pt$) C, H, N.

***meso*-N,N'-Dimethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (*meso*-4−$PtCl_2$):** yield 200 mg (59%), beige powder; ^{1}H-NMR, see Table 1; IR (KBr) ν 310 cm^{-1} (Pt−Cl). Anal. ($C_{16}H_{16}Cl_6N_2O_2Pt$) C, H, N.

D,L-*N,N'*-Dimethyl-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (D,L-4−$PtCl_2$): yield 304 mg (90%), yellow powder; ^{1}H-NMR, see Table 1; IR (KBr) ν 330 cm^{-1} (Pt−Cl). Anal. ($C_{16}H_{16}Cl_6N_2O_2Pt$) C, H, N.

Biological Methods: Estrogen Receptor Binding Assay. The applied method was described by Hartmann et al.[16] The relative binding affinity (RBA) of the test compounds is determined by the displacement of 17β-[^{3}H]estradiol. At 4 °C the test compounds (used in six to eight appropriate concentrations) are shaken with calf uterine cytosol (100 μL) and 17β-[^{3}H]estradiol (0.5 pmol; specific activity 90−115 Ci/nmol) for 16 h. To stop the incubation, dextran-coated charcoal is added, and after centrifugation the radioactivity of a 100 μL supernatant aliquot (total volume 500 μL) is counted. The binding of 17β-[^{3}H]estradiol (0.5 pmol control) is estimated in the same way. To estimate the nonspecifically bound part of 17β-[^{3}H]-estradiol (0.5 pmol) a surplus of nonradioactive 17β-estradiol (2 nmol) is added to the probe in order to displace the specifically bound part of the 17β-[^{3}H]estradiol. On a semilog plot, the percentage of bound labeled steroid vs concentration of the competitor is plotted. At least six concentrations of each compound are chosen to get a linear graph. From this plot those molar concentrations of unlabeled estradiol and of the competitors are determined which reduce the binding of the radioligand by 50%.

Estrogen Assays. Estrogenic effects are determined by stimulation of the uterine growth as described.[32] On three consecutive days the compounds, dissolved in poly(ethylene glycol) 400/H_2O (1.8% NaCl) 1:1 (0.1 μL/mouse), are daily administered sc to female, immature NMRI mice (age, 20 days at test beginning; body weight, 10−12 g; six mice/group). The uteri are excised 24 h after the last injection, fixed with Bouin's solution, dried, and weighed.

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