Induction of the Estrogen Specific Mitogenic Response of MCF-7 Cells by Selected Analogues of Estradiol-17β: A 3D QSAR Study

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Received May 19, 1997[⊗]

Analogues of estradiol-17β (E_2) have been evaluated for estrogen receptor (ER) binding affinity and mitogenic potential in the human breast cancer cell line MCF-7. These 42 compounds represent subtle modifications of the natural estrogen structure through the placement of hydroxyl, amino, nitro, or iodo groups around the ring system in addition to, or as replacement of, the 3- and 17β-hydroxyls of E_2. The mitogenic activity of the analogues was found to be related to ER binding only to a limited extent. In order to elucidate structural features that are uniquely responsible for receptor binding affinity or mitogen potential of estrogens, the three-dimensional quantitative structure−activity (QSAR) method Comparative Molecular Field Analysis (CoMFA) was employed. Separate CoMFA models for receptor binding and cell growth stimulation were optimized through the use of various alignment rules and region step size. Whereas the CoMFA contour plots did outline the shared structural requirements for the two measured biological properties, specific topological features in this set of estrogens were delineated that distinguish mitogenic potential from ER binding ability. In particular, steric interference zones which affected growth extend in a band from above the A-ring to position 4 and below, whereas the ER binding steric interference zones are limited to isolated polyhedra in the 1,2 and 4 positions and the α face of the B-ring. In addition, electronegative features located around the A-, B-, or C-rings contribute to receptor affinity. However, growth is dependent only on electronegative and electropositive properties near the 3-position. In a final QSAR model for the mitogenic response, the value of ER binding was included along with structural features as a descriptor in CoMFA. The resulting 3D-QSAR has the most predictive potential of the models in this study and can be considered a prototype model for the general evaluation of a steroidal estrogen's growth stimulating ability in MCF-7 cells. For example, the location of D-ring contours illustrate the model's preference for 17β-hydroxy steroids over the less mitogenic 17α- and 16α-hydroxy compounds. In addition, the enhanced mitogenic effect of steric bulk in the 11α-position is also evident. The QSAR studies in this report illustrate the fact that while ER binding may be a required factor of the estrogen dependent growth response in MCF-7 cells, particular structural characteristics, in addition to those responsible for tight receptor binding, must be present to induce an optimal mitogenic response. Therefore, this report demonstrates that the CoMFA QSAR method can be utilized to characterize structural features of test compounds that account for different types of estrogenic responses.

Introduction

In an effort to develop endocrine treatment for breast cancer, a variety of antiestrogenic compounds have been synthesized throughout the last 50 years.[1−3] The majority of these compounds, including the clinically important Tamoxifen, are nonsteroids[4,5] that exhibit various nonspecific pharmacological interactions.[6−8] More recently, a series of 7α-substituted estradiols such as ICI-164,384 have been identified as pure estrogen antagonists with little cytotoxicity.[9−12] However, all of these compounds represent molecular structures quite deviant from the natural estrogen, estradiol-17β (E_2).

The ability of antiestrogens to block estrogen receptor (ER) regulated cellular events is frequently defined as the capacity to inhibit estrogen specific growth of breast cancer cells.[13−16] In some cases, specific aspects of the antiestrogen-ER complex or the control of estrogen specific genes are also characterized.[4,17−22] With the identification of a transcription activation function (AF-2) in the ligand binding domain of ER by others,[23−25] a hypothesis has been presented by this laboratory which suggests that ligands with subtle structure alterations might effectively control specific ER regulated genes through novel interaction with the AF-2 site.[26,27] Examination of this concept has resulted in the characterization of the 2- and 4-hydroxy isomers of E_2 as high affinity ligands for ER which are capable of stimulating certain genes while being inactive in the regulation of other estrogen responsive genes.[28−32] Structure analysis of these isomers has revealed conformation features as well as electrostatic characteristics that may account for the unique estrogenic properties of these ligands.[28,33,34] However, determinations have yet to be made regarding the potential of these and other estrogen analogues to regulate estrogen specific growth of cells.

In the present study, we utilize the ER positive breast cancer cell line MCF-7 to examine the receptor binding affinity and estrogen specific mitogenic potential of 42 steroidal estrogens. The structural alterations found in

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

S0022-2623(97)00329-4 CCC: $14.00

these compounds include the addition or relocation of hydroxyl, keto, amino, and nitro substituents around the 1,3,5(10)-estratriene ring system. The effect of additional ring unsaturations on estrogenic potential is also examined. This data is then evaluated by means of the three-dimensional quantitative structure−activity (QSAR) paradigm Comparative Molecular Field Analysis (CoMFA). The resulting CoMFA models of estrogen activity define the particular structural components responsible for high affinity ER binding which were nevertheless found to be complementary but not sufficient to define an estrogen's capacity to stimulate growth of MCF-7 cells.

Materials and Methods

Steroids. The steroids used in this study are listed in Table 1. Compounds **1**, **5**, **22**−**24**, **31**−**38**, and **40**−**42** were purchased as ultrapure compounds from Research Plus, Inc. (Bayonne, NJ). Steroids **2**, **8**−**10**, **12**, and **14**[35] as well as **25**, **26**, and **27**[36] were synthesized according to published procedures. The synthesis of **3**, **4**, **6**, **7**, **15**, **16**, and **17**[33,37,38] as well as the substituted estradiols **18**, **19**, **20**, and **21**[39] have been reported by this laboratory and others. A description of the synthesis of compounds **28**−**30** and **39** has been recently been reported by this laboratory.[40] Estratrienes **11** and **13** were prepared for the present study (see below). The "pure" estrogen antagonist ICI 164,384 (ICI) was obtained from A. E. Wakeling, Imperial Chemical Industries plc, Pharmaceuticals Division (Alderly Park, Macclesfield SK10 4TG, Cheshire, England). All estrogens were checked for purity by thin layer chromatography and were found to be devoid of contaminating estrogens at levels greater than 1 part in 10^4 parts.[28] Stock solutions (2.4 mM) of each compound used in biological assays were made in ethanol and/or dimethyl sulfoxide.

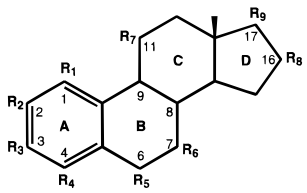
Synthesis of 2- and 4-Nitroestratrien-17*β*-ols. Compounds **11** and **13** were prepared by oxidation of the known amino precursors via *m*-chloroperbenzoic acid oxidation. **Physical Properties. 11**: mp 168−170 °C, MS expct 301.1677, found 301.1668; H-NMR (DMSO) 7.50 (d, 1H), 7.35 (d, 1H), 7.23 (t, 1H), 4.90 (d, 1H), 0.635 (s, 3H). **13**: mp 123−125 °C, MS expct 301.1677, found 301.1682; H-NMR (DMSO) 7.91 (d, 1H), 7.87 (d, 1H), 7.49 (t, 1H), 4.51 (d, 1H), 0.620 (s, 3H).

Biological Assays. Receptor Binding. Binding affinity of the steroids and estrogen analogues for the ER was obtained from the cytosol of confluent MCF-7 cells. The methods, previously reported by this laboratory,[26,31,33] involved classical Scatchard and competitive binding procedures utilizing dextran-coated charcoal.

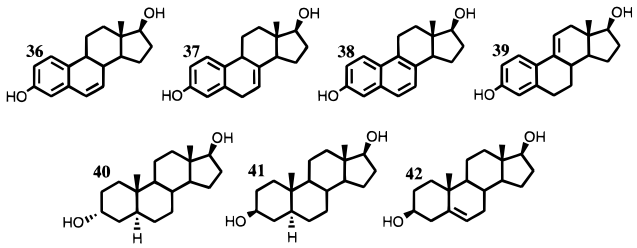
Estrogen Specific Growth Response. The estrogen specific mitogenic effect of each steroid or estrogen on MCF-7 cells was assayed using culture conditions published recently by this laboratory.[41] Stock cells of the MCF-7 clone E3[42] in the passage range of 160 to 190 were maintained in estrogen "rich" media and then withdrawn into an estrogen "free" media 7 days prior to seeding for growth response experiments.[41] The mitogenic activity of each compound was evaluated at concentrations of 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M. All cell growth determinations included positive controls treated with 10^{-11} M E_2 as well as negative controls devoid of estrogen treatment. In addition, a check of estrogen contamination of culture conditions for each experiment was made by including cells treated only with 10^{-7} M ICI. Treatments with this "pure" antiestrogen served to validate the negative control flasks since only experiments with negative control cell counts within 10% of ICI treated flasks at the end of the treatment period (indicating no significant estrogen contamination) were considered valid assays of each compound's mitogenic potential.

In each treatment group, triplicate T-25 flasks (Corning Science Products, Corning, NY) were seeded with 2.0×10^5 cells in estrogen free media on day 0. Media containing the compound to be tested at specified concentrations replaced the seeding media on day 1. Cells were refed the appropriate treatment media every other day thereafter. All additions of estrogens to culture media were made such that the concentration of carrier solvent was ≤ 0.1 μL/mL. Duplicate flasks with carrier alone were also evaluated. On day 6, cell nuclei

Table 1. Steroids in Estrogen CoMFA Study



Compd	R1	R2	R3	R4	R5	R6	R7	R8	R9
1	H	H	H	H	H_2	H_2	H_2	H_2	H_2
2	H	H	H	H	H_2	H_2	H_2	H_2	ß-OH
3	OH	H	H	H	H_2	H_2	H_2	H_2	ß-OH
4	H	OH	H	H	H_2	H_2	H_2	H_2	ß-OH
5	H	H	OH	H	H_2	H_2	H_2	H_2	ß-OH
6	H	H	H	OH	H_2	H_2	H_2	H_2	ß-OH
7	NH_2	H	H	H	H_2	H_2	H_2	H_2	ß-OH
8	H	NH_2	H	H	H_2	H_2	H_2	H_2	ß-OH
9	H	H	NH_2	H	H_2	H_2	H_2	H_2	ß-OH
10	H	H	H	NH_2	H_2	H_2	H_2	H_2	ß-OH
11	NO_2	H	H	H	H_2	H_2	H_2	H_2	ß-OH
12	H	NO_2	H	H	H_2	H_2	H_2	H_2	ß-OH
13	H	H	NO_2	H	H_2	H_2	H_2	H_2	ß-OH
14	H	H	H	NO_2	H_2	H_2	H_2	H_2	ß-OH
15	H	I	H	H	H_2	H_2	H_2	H_2	ß-OH
16	H	H	I	H	H_2	H_2	H_2	H_2	ß-OH
17	H	H	H	I	H_2	H_2	H_2	H_2	ß-OH
18	H	NH_2	OH	H	H_2	H_2	H_2	H_2	ß-OH
19	H	H	OH	NH_2	H_2	H_2	H_2	H_2	ß-OH
20	H	NO_2	OH	H	H_2	H_2	H_2	H_2	ß-OH
21	H	H	OH	NO_2	H_2	H_2	H_2	H_2	ß-OH
22	H	H	OH	H	α-OH	H_2	H_2	H_2	ß-OH
23	H	H	OH	H	ß-OH	H_2	H_2	H_2	ß-OH
24	H	H	OH	H	O	H_2	H_2	H_2	ß-OH
25	H	H	OH	H	H_2	α-OH	H_2	H_2	ß-OH
26	H	H	OH	H	H_2	ß-OH	H_2	H_2	ß-OH
27	H	H	OH	H	H_2	O	H_2	H_2	ß-OH
28	H	H	OH	H	H_2	H_2	α-OH	H_2	ß-OH
29	H	H	OH	H	H_2	H_2	ß-OH	H_2	ß-OH
30	H	H	OH	H	H_2	H_2	O	H_2	ß-OH
31	H	H	OH	H	H_2	H_2	H_2	H_2	H_2
32	H	H	OH	H	H_2	H_2	H_2	H_2	α-OH
33	H	H	OH	H	H_2	H_2	H_2	α-OH	H_2
34	H	H	OH	H	H_2	H_2	H_2	α-OH	ß-OH
35	H	H	OH	H	H_2	H_2	H_2	H_2	O



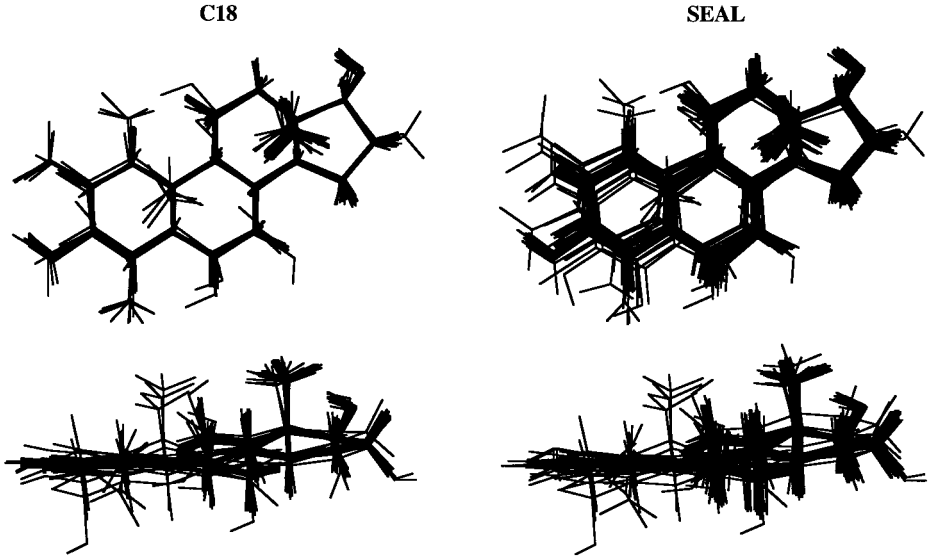


Figure 1. CoMFA alignment rules C18 and SEAL for steroids **2**−**42**. C18 alignment is the RMS fit of carbons 1 through 18 of each steroid with the corresponding atoms of the natural estrogen E_2 (**5**). SEAL alignment is the best superimposition of each steroid with **5** by SEAL calculation (see Computational Methods above). Views are from above, normal to the A-ring plane (top), and from the front, looking across the A-ring plane with substituents on carbons 4 and 6 in the foreground (below).

were counted with a Coulter counter utilizing methods developed in this laboratory and by others.[41,43] Cell counts of experimental cultures were normalized relative to the positive and negative controls of each assay. The values from triplicate flasks at each experimental point were averaged and the standard deviation determined. The EC_{50} for each compound was determined by the Logit method.[44] In order to characterize the mitogenic effect of each steroid tested as an estrogen specific response, a final series of growth experiments were carried out in which cells were treated with both the maximal stimulating concentration of that compound and 10^{-7} M ICI. Such concentrations of ICI are not mitogenic and have been shown to specifically block the effect of potent estrogen agonists.[41]

Computational Methods. The estrogenic potential of all steroids in this study was evaluated by means of the three-dimensional quantitative structure−activity relationship (QSAR) methodology Comparative Molecular Field Analysis (CoMFA[45]). For a given set of compounds, CoMFA identifies structure patterns (steric and electrostatic) which are important to, or responsible for, a measured biological activity. In addition to the precise evaluation of biological parameters for all test compounds, such QSAR analyses utilize corresponding molecular models that represent structural properties as realistically as possible. These models are then aligned in three-dimensional space in an attempt to simulate the relative orientation of each compound in the receptor binding site. In the absence of geometric information regarding the actual binding site, this alignment rule must be based on deductive information such as the structure of the natural ligand or another high affinity analogue. Steric and electrostatic features of compounds are then measured by means of a sampling region which consists of a lattice of points extending throughout the aligned molecules. In this way, each compound in the study is described by the steric and electrostatic interaction energies (Van der Waals and Coulombic) at each region point. Biological data for all test compounds is then related to this set of structure descriptors in order to generate a three-dimensional QSAR which delineates particular structure components responsible for the observed activity.

The statistical relationship between measured biological properties (dependent variables) and physiochemical molecular properties (independent variables) is made by the Partial Least Squares (PLS) method.[46,47] PLS is an improved alternative to multiple regression in that it is specifically designed to address relationships where the predictive physiochemical variables are numerous and may be intercorrelated. In CoMFA, PLS is implemented to find relationships between activities and the numerous lattice points that describe the structure of each compound. When cross-validation is employed in a PLS analysis, the resulting QSAR becomes a predictive model that can be generalized to other types of compounds.[48] The cross-validated PLS produces a "predictive" r^2 which relates the capacity of the QSAR to predict the activity for each of its members while that member is not included in the model. For example, where n is the number of compounds in a study, a cross-validated PLS derives n independent QSARs, each calculated with a different compound omitted. The activity of the missing compound is then predicted from the model derived without it. The differences and standard deviations of all such predictions for all compounds are then used to derive a predictive r^2, called the q^2. Therefore, the cross-validation process (and q^2 value) infers the predictive potential of the CoMFA technique.

Molecular Models. All molecular modeling calculations described herein use default values of the specified software unless indicated. Estrogen models were displayed on a Silicon Graphics Iris 4D/310 workstation with the SYBYL 6.1 and 6.2 molecular modeling software (Tripos Assoc., 1699 S. Hanley Rd., St. Louis, MO 63144). Each steroid model was constructed from SYBYL fragments and then geometry optimized as described previously by this laboratory.[34] Such calculations utilized the PM3 semiempirical molecular orbital method of MOPAC 6.0[49] (QCPE no. 455, The Quantum Chemistry Program Exchange, Creative Arts Building 181, Indiana University, 840 State Highway 46 Bypass, Bloomington, IN 47405) with key word specification for "precise × 100" and a time limit such that convergence was achieved. Hydrogens of hydroxyl and amino groups were added to models such that each rotational position was optimized and compared. Structures with hydroxyl and amino groups in the lowest energy orientation after optimization were used in the QSAR study. In the case of phenolic 3-hydroxyl orientation of E_2 (**5**), the lowest energy conformation was found to be syn to the C3 and C2 bond which is also in agreement with 3-hydroxyl orientation proposed by others.[50] Models of nitro-substituted estrogens were based on X-ray derived conformations[51] and then optimized as described above.

Atomic point charges of models were calculated by the MNDO-ESP method of MOPAC 6.0.[52] All ESP calculations were carried out through SYBYL interface using default values with the inclusion of the keyword specifications 1SCF, ESP, and SLOPE = 1.47 or SLOPE = 1.35 when nitrogen was present.[53]

Alignment Rules. Two separate alignment rules for the steroid models were defined for use in CoMFA (Figure 1). In

the case of the C18 alignment, the 18 mutual carbon atoms of each steroid model were RMS MATCHed by SYBYL to the corresponding carbon atoms on the model of E_2 (**5**). Alternatively, the SEAL alignment was obtained when each steroid model was fit by the SEAL method to the model of E_2. Starting with 100 random orientations, SEAL (QCPE no. 634-SGRW, The Quantum Chemistry Program Exchange) utilizes the steric volumes and the atomic partial charges of two molecular models in a determination of their optimal alignment.[54] SEAL setup parameters for steroid-E_2 alignments included $\alpha = 0.3$, $w_S = 1$, $w_E = 1$, $w_H = 1$, trials = 100, saved = 1 and specification for the Gaussian attenuation function.

CoMFA Parameters. CoMFA and PLS were used as implemented in SYBYL. A CoMFA region was generated manually for each alignment rule such that the field sampling lattice extended 6 Å beyond the steric volume of all models in the *x*, *y*, and *z* directions. All regions utilized a probe atom type of carbon sp^3 with charge of +1. Separate CoMFA regions were defined with *x*, *y*, and *z* lattice spacing of 1 Å as well as 2 Å. All CoMFA models included both steric and electrostatic fields, data scaling set to "CoMFA standard", and a maximum energy cutoff of 30 kcal. For cross-validated runs, the maximum number of components was set to 5, the cross-validation groups set equal to *n* (the total number of compounds in analysis), and the minimum σ value set at 2. For final, non-cross-validated CoMFAs, the number of components was defined in accordance to the optimum determined from cross-validated runs and minimum σ was set to 0. The estrogenic properties of receptor binding and mitogenic potential of each compound were both mathematically transformed for utilization in CoMFA. The ER binding affinity used in CoMFA was the Log 10 of the reported K_a value (log K_a) for compounds **1**−**42**. For all compounds, estrogen specific growth induction was applied to CoMFA as the −Log 10 of the reported EC_{50} value (pEC_{50}).

Results and Discussion

Receptor Binding. The ER affinity of compounds **1**−**6**, **31**−**35**, and **40**−**42** has been recently reported by this laboratory.[28] In the present investigation, additional competitive binding analyses were performed on the remaining compounds in Table 2. In the absence of substituents on the 1,3,5(10)-estratriene nucleus, the ligand bound ER with a K_a too low to measure ($<1.85 \times 10^6$ M^{-1}). Estrogen analogues maintaining the highest affinity for ER were generally found to contain the 3-phenolic and 17β-hydroxyl substituents of the natural E_2 with the 3-phenolic group exerting the greatest influence on the K_a (**2** vs **31**). Repositioning the phenolic hydroxyl to C2 (**4**) yielded an estradiol with a slightly impaired receptor affinity. Moving this hydroxyl to other positions on the A-ring of estratrien-17β-ol produced ligands with diminished ER affinity (**3**, **6**), with estratriene-1,17β-diol (**6**) displaying a K_a of only 1/200th that of E_2. The introduction of other groups (NO_2 or NH_2) on the A-ring of E_2 drastically lowered the K_a of the ligand for ER (**18**−**21**). In the absence of a 3-hydroxyl group, these A-ring substituents, as well as an iodo, decreased the K_a of the estratrien-17β-ol even further (**7**−**17**). An equatorial hydroxyl or keto substitution on carbon 7 of E_2 did not interfere with high affinity receptor binding, whereas estratriene-3,17β-diols with additional hydroxyl (equatorial or axial) groups added to C6 of E_2 were found to have weak (**22**, **23**) affinity for ER. However, a keto group placed on C6 (**24**) was more favorable for the interaction with ER. Estrogens with variations in the D-ring oxygens (**32**−**35**) maintained ER K_a's of 16% that of E_2 (0.6×10^9 M^{-1}) or higher, whereas a hydroxyl group placed on C11 drastically decreased affinity (**28**, **29**). A ketone placed at C11 (**30**) displayed an affinity for receptor which was barely detectable in the competitive binding assay. Additional double bonds in ring-B (**36**−**38**) had a minimal effect on optimal receptor affinity. On the other hand, an unsaturation at C9 in ring C (**39**) reduced affinity appreciably (K_a = 1/5th that of E_2). In the absence of the aromatic A-ring, the 3-hydroxyl group must be axial as in the 5α-androstane-3β,17β-diol to promote measurable binding to ER.

Compiling data on 110 compounds Anstead *et al.*[50] were able to establish the characteristics of the binding of estradiol within its ER site and Gantchev *et al.*[55] have employed QSAR/CoMFA to examine the receptor binding of 40 molecules. Viewing our results in light of these recent studies of the estradiol pharmacophore, we see certain relationships that are evident. As described above, these authors have also found the C3 and 17β hydroxyl groups to be major contributors to ligand affinity, with the phenolic group contributing the greatest energy as a H-donor. The negative influence of

Table 2. Receptor Binding and Mitogenic Effect of Steroids

compd	name[a]	ER K_a[b]	GR EC_{50}[c]	SEAL fit[d]
1	estratriene	<0.00185	201	−0.838
2	estratrien-17β-ol	0.410	0.316	−0.929
3	estratriene-1,17β-diol	0.0180	1.59	−0.845
4	estratriene-2,17β-diol	2.60	0.0330	−0.895
5	estratriene-3,17β-diol (E_2)	3.70	0.00152	−1.00
6	estratriene-4,17β-diol	0.250	4.76	−0.855
7	1-aminoestratrien-17β-ol	<0.00185	28.4	−0.827
8	2-aminoestratrien-17β-ol	0.145	0.127	−0.872
9	3-aminoestratrien-17β-ol	0.278	1.50	−0.959
10	4-aminoestratrien-17β-ol	0.0215	2.00	−0.866
11	1-nitroestratrien-17β-ol	<0.00185	60.4	−0.814
12	2-nitroestratrien-17β-ol	<0.00185	29.3	−0.820
13	3-nitroestratrien-17β-ol	0.00777	2.16	−0.853
14	4-nitroestratrien-17β-ol	<0.00185	34.2	−0.827
15	2-iodoestratrien-17β-ol	0.00333	1.70	−0.890
16	3-iodoestratrien-17β-ol	0.0196	0.174	−0.882
17	4-iodoestratrien-17β-ol	0.00444	1.67	−0.883
18	2-aminoestratriene-3,17β-diol	0.133	0.558	−0.875
19	4-aminoestratriene-3,17β-diol	0.211	0.346	−0.901
20	2-nitroestratriene-3,17β-diol	0.00370	33.5	−0.894
21	4-nitroestratriene-3,17β-diol	0.144	0.900	−0.907
22	estratriene-3,6α,17β-triol	0.0888	0.186	−0.918
23	estratriene-3,6β,17β-triol	0.0700	0.0571	−0.918
24	6-ketoestratriene-3,17β-diol	1.09	0.00338	−0.875
25	estratriene-3,7α,17β-triol	0.0630	0.153	−0.883
26	estratriene-3,7β,17β-triol	3.66	0.0122	−0.881
27	7-ketoestratriene-3,17β-diol	3.39	0.0160	−0.865
28	estratriene-3,11α,17β-triol	0.0115	0.0365	−0.901
29	estratriene-3,11β,17β-triol	0.0622	0.0163	−0.881
30	11-ketoestratriene-3,17β-diol	0.00333	8.97	−0.865
31	estratrien-3-ol	2.93	0.118	−0.909
32	estratriene-3,17α-diol	0.807	0.382	−0.857
33	estratriene-3,16α-diol	2.97	0.237	−0.853
34	estratriene-3,16α,17β-triol (E_3)	0.614	0.0483	−0.917
35	17-oxoestratrien-3-ol (E_1)	0.821	0.0772	−0.900
36	6-estratetraene-3,17β-diol	3.09	0.00419	−0.950
37	7-estratetraene-3,17β-diol	2.85	0.00154	−0.927
38	6,8-estrapentaene-3,17β-diol	1.52	0.0125	−0.915
39	9-estratetraene-3,17β-diol	0.725	0.0990	−0.932
40	5α-androstane-3α,17β-diol	<0.00185	>10000	−0.881
41	5α-androstane-3β,17β-diol	0.0180	4.66	−0.786
42	5-androstene-3β,17β-diol	0.0270	1.41	−0.840

[a] All steroids are 1,3,5 (10) estratrienes unless specified otherwise. [b] K_a ($\times 10^9$ M^{-1}) for estrogen receptor (higher value, tighter association). [c] $EC_{50} \times 10^{-9}$ M in MCF-7 cell growth response assay (lower value, more potent mitogen). [d] SEAL alignment score of steroid with the natural estrogen E_2 (**5**). Value reflects similarity of the overall steric and electrostatic features of fitted steroid relative to template such that a score of −1.0 corresponds to the exact relationship of the template fit with itself.

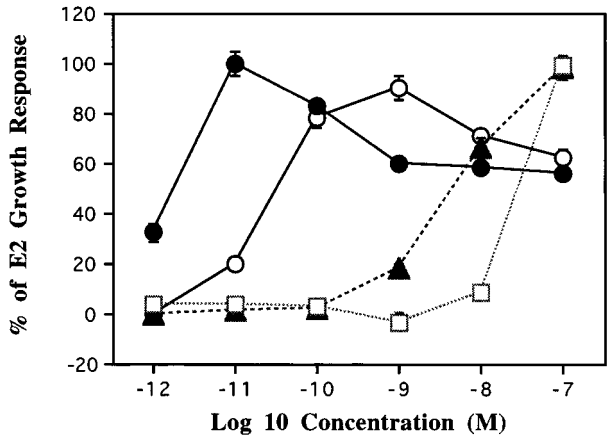


Figure 2. Mitogenic potential of certain A-ring analogues of E_2. Compounds were added to cultures of MCF-7 cells in T-25 flasks as described in Materials and Methods. After 6 days cells were lysed and the nuclei counted. Estrogens were added in ethanol and/or dimethyl sulfoxide. Points represent the average nuclei count of triplicate flasks (±SD) for each concentration of estrogen. Compounds were E_2 (**5**) (●), estratriene-2,17β-diol (**4**) (○), estratriene-4,17β-diol (**6**) (▲) and 4-nitroestratriene-17β-diol (**14**) (□).

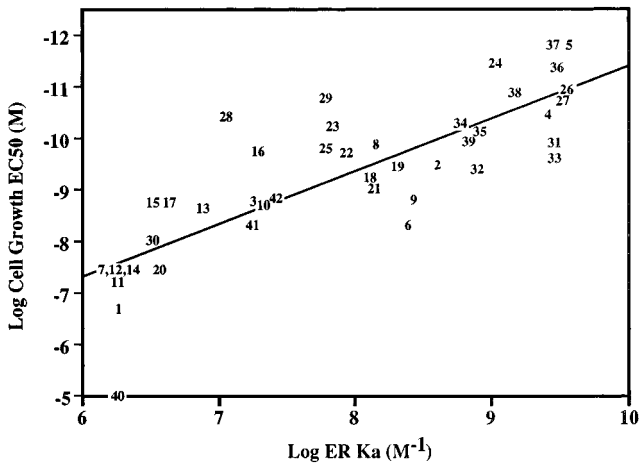


Figure 3. Relationship of the growth response (EC_{50}) induced in cultures of MCF-7 cells by steroids **1**−**42** to the ER binding affinity (K_a) of these ligands. Linear regression (r^2) of line generated by all data was 0.651.

A-ring substituents is predicted for estradiols (**18**−**21**) and in the case of the 3-deoxy compounds (**3, 4, 6**−**17**), we have shown there to be a greater negative effect on the K_a, except for a 2-hydroxyl group (**4**) which via its proximity to C3 may participate as an H-donor with receptor. Bulky substituents at positions C_2 and C_4 are not well tolerated by the receptor[55] (Table 2). As shown in Table 2 and described for the pharmacophore,[50] small polar groups at the 7α-position are not well tolerated as are α and β substituents at C6 of E_2. On the other hand, we have shown a 6-keto group on E_2 (**24**) to bind relatively well. Small polar groups at C11 create a negative influence on the affinity (Table 2) as predicted.[50] Referring to the A-nor-5α-estrane series, these authors[50] point out the importance of a β-OH to the binding of ligands with nonaromatic A-rings, as we documented in the case of 5α-androstane-3α,17β-diol (**40**) versus 5α-androstane-3β,17β-diol (**41**).

Estrogen Specific Growth Response. With the exception of 5α-androstane-3α,17β-diol (**40**), all steroids examined induced a significant growth response in cultures of MCF-7 cells when they were added to flasks over the concentration range of 10^{-12} to 10^{-6} M (Table 2). Even estratriene (**1**) was found to have a growth response EC_{50} value of 2.10×10^{-7} M (Table 2). The magnitude of the maximum mitogenic effect induced by these compounds was found in every case to be within 20% of the maximum effect observed when cultures were treated with the natural estrogen E_2 (**5**) (selected compounds shown in Figure 2). Noteworthy is the nonsigmoidal nature of these activity curves. Estrogens, which stimulate responsive genes at lower (physiological) concentrations (**4** and **5** in Figure 2). This phenomenon has been reported previously both for growth stimulation[41] and gene induction.[28,29,31,32] It is notable that certain of these mitogenic compounds (**1**, **7**, **11**, **12**, **14**, **41**) were found not to have affinity for the ER within the detection limit of our binding assay (Figure 2, 4-nitroestratrien-17β-ol (**14**)). However, the ability of ICI (10^{-7} M) to block the MCF-7 proliferation of all mitogenic compounds in this study demonstrates that such effects are ER dependent (estrogen specific). Apparently, only a minimal level of ligand−ER complex was required to initiate a growth response in these cells.[41] This assumption is supported by the peak growth stimulatory activity of 10^{-11}M E_2 (Figure 2). The 2.7×10^{-10} K_d of the E_2 complex also indicates that very little of the liganded ER will maximally stimulate growth of MCF-7 cells. Estratriene-2,17β-diol (**4**) and estratriene-4,17β-diol (**6**), which possessed affinities 71% and 7% (respectively) that of E_2 (**5**), required media concentrations 100- and 10000-fold greater than E_2 to match its mitogenic potential (Figure 2). Clearly, for certain estrogen analogues growth stimulation was not directly related to receptor affinity. This observation is the result of gene regulatory differences since cellular (nuclear) uptake of these compounds is equivalent.[28]

When the growth stimulation response of all compounds examined was compared directly to the corresponding affinity of each compound for receptor, a linear relationship was generated with a regression (r^2) of 0.651 (Figure 3). Whereas it appeared that compounds which bind receptor with high affinity tend to induce a strong growth response, the mitogenic potential of a number of steroids was significantly weaker (**6**, **9**, **31**−**33**) or stronger (**16**, **23**, **24**, **28**, **29**, **38**) than would be expected if this response was based solely on the ER K_a values. The data obtained from experiments with 5α-androstane-3α,17β-diol (**40**) do not fit this correlation between K_a and EC_{50} (Figure 2). Since both the K_a and growth stimulation of MCF-7 cells were below the detection limits for this 3α-hydroxy steroid, it appears that measurable activities in both assays are contingent on the 3β orientation of the A-ring hydroxyl group (**41**) (Table 2).

With the exception of steroid **40** (a 3α-hydroxy androstanediol), all 41 estrogen analogues proved to be active in stimulating estrogen specific growth of MCF-7 cells. On the other hand, the induction of transcription of certain estrogen responsive genes is more sensitive to A-ring-substituted estrogens. For example, repositioning the 3-phenolic hydroxyl to the 2 or 4 position (**4**, **6**) yielded ligands which bound ER yet were inactive in stimulating the progesterone receptor or cathepsin D genes (**4**, **6**) and the pS2 or tissue plasminogen activator genes (**4**).[28,29,32] Estratriene-2,17β-diol (**4**)

Table 3. Receptor Binding CoMFA Statistics

align.[a]	lattice, Å[b]	offset[c]	q^2 [d]	S[e]	r^2 [f]	SE[g]	contributions[h]
C18	2		0.395(2)	0.912	0.676	0.667	st = 22, el = 78
C18	2	*X*	0.475(2)	0.849	0.719	0.622	st = 25, el = 75
C18	2	*Y*	0.423(2)	0.890	0.702	0.639	st = 24, el = 76
C18	2	*Z*	0.467(2)	0.856	0.690	0.652	st = 29, el = 71
			average $q^2 = 0.440 \pm 0.080$[i]				
SEAL	2		0.426(2)	0.888	0.703	0.639	st = 26, el = 74
SEAL	2	*X*	0.456(5)	0.901	0.929	0.325	st = 32, el = 68
SEAL	2	*Y*	0.495(2)	0.833	0.752	0.583	st = 29, el = 71
SEAL	2	*Z*	0.482(2)	0.843	0.708	0.633	st = 32, el = 68
			average $q^2 = 0.465 \pm 0.069$[i]				
C18	1		0.529(5)	0.838	0.937	0.306	st = 30, el = 70
C18	1	*X*	0.583(5)	0.788	0.947	0.281	st = 28, el = 72
C18	1	*Y*	0.519(2)	0.813	0.741	0.596	st = 25, el = 75
C18	1	*Z*	0.534(5)	0.833	0.939	0.302	st = 28, el = 72
			average $q^2 = 0.541 \pm 0.064$[i]				
SEAL	1		0.597(5)	0.776	0.958	0.250	st = 34, el = 66
SEAL	1	*X*	0.553(5)	0.816	0.953	0.266	st = 35, el = 65
SEAL	1	*Y*	0.582(5)	0.789	0.957	0.254	st = 32, el = 68
SEAL	1	*Z*	0.593(5)	0.778	0.953	0.264	st = 33, el = 67
			average $q^2 = 0.581 \pm 0.044$[i]				

[a] Alignment rule for CoMFA (see Computational Methods above). [b] Step size in CoMFA field sampling region. [c] Direction of region displacement (one half of lattice step size[b]) relative to default. [d] Cross-validated r^2 (optimal components) with cross-validation groups = *n*. [e] Cross-validated standard error of estimate over the log K_a range of 6.27 to 9.57. [f] Conventional r^2 using optimal components. [g] Conventional standard error of estimate over the log K_a range of 6.27 to 9.57. [h] Relative contributions of the steric and electrostatic explanatory variables of CoMFA (as fractions of 100). [i] Average q^2 of CoMFAs from region displacement experiments ± range.

Table 4. MCF-7 Cell Growth Assay CoMFA Statistics

align.[a]	lattice, Å[b]	offset[c]	q^2 [d]	S[e]	r^2 [f]	SE[g]	contributions[h]
C18	2		0.463(3)	1.075	0.846	0.575	st = 25, el = 75
C18	2	*X*	0.466(4)	1.085	0.913	0.439	st = 30, el = 70
C18	2	*Y*	0.514(3)	1.021	0.863	0.543	st = 29, el = 71
C18	2	*Z*	0.624(3)	0.899	0.862	0.545	st = 36, el = 64
			average $q^2 = 0.517 \pm 0.161$[i]				
SEAL	2		0.424(2)	1.098	0.746	0.729	st = 26, el = 74
SEAL	2	*X*	0.491(3)	1.046	0.874	0.520	st = 31, el = 69
SEAL	2	*Y*	0.481(2)	1.042	0.784	0.673	st = 30, el = 70
SEAL	2	*Z*	0.582(3)	0.948	0.850	0.567	st = 36, el = 64
			average $q^2 = 0.495 \pm 0.158$[i]				
C18	1		0.562(3)	0.970	0.868	0.533	st = 34, el = 66
C18	1	*X*	0.556(2)	0.963	0.789	0.665	st = 29, el = 71
C18	1	*Y*	0.547(2)	0.974	0.796	0.654	st = 28, el = 72
C18	1	*Z*	0.564(2)	0.955	0.799	0.649	st = 31, el = 69
			average $q^2 = 0.557 \pm 0.017$[i]				
SEAL	1		0.542(3)	0.992	0.876	0.516	st = 34, el = 66
SEAL	1	*X*	0.528(2)	0.994	0.790	0.663	st = 31, el = 69
SEAL	1	*Y*	0.529(2)	0.992	0.791	0.660	st = 31, el = 69
SEAL	1	*Z*	0.543(2)	0.997	0.796	0.654	st = 31, el = 69
			average $q^2 = 0.536 \pm 0.015$[i]				

[a] Alignment rule for CoMFA (see Computational Methods above). [b] Step size in CoMFA field sampling region. [c] Direction of region displacement (one half of lattice step size[b]) relative to default. [d] Cross-validated r^2 (optimal components) with cross-validation groups = *n*. [e] Cross-validated standard error of estimate over the pEC_{50} range of 5.00 to 11.82. [f] Conventional r^2 using optimal components. [g] Conventional standard error of estimate over the pEC_{50} range of 5.00 to 11.82. [h] Relative contributions of the steric, electrostatic, and K_a explanatory variables of CoMFA (as fractions of 100). [i] Average q^2 of CoMFAs from region displacement experiments[c] ± range.

activity stimulated growth, whereas estratriene-4,17β-diol (**6**) displayed diminished but measurable mitogenic response. Furthermore, although 4-nitroestratriene-3,-17β-diol (**21**) actively stimulated the pS2 and cathepsin D genes, 2-nitroestratriene-3,17β-diol (**20**) was inactive.[51]

CoMFA. Since ligand structure did not always exert a similar effect on receptor binding and growth stimulation (Figure 3), the CoMFA 3D-QSAR methodology was applied to the data from these experiments as a means of identifying and differentiating the structural features responsible for ER affinity from those which bring about a growth response in MCF-7 cells. Thus, CoMFA models were generated independently for the data sets obtained from the receptor binding and cell growth stimulation experiments (Tables 3 and 4). Furthermore, a cell proliferation QSAR was derived in which receptor K_a values as well as CoMFA fields were employed as the descriptive variables responsible for induction of the growth response (Table 5). This combined model was created as an aid to the understanding of relationships between mitogenic potential and ER K_a values (Figure 3). The validity and precision of each application of CoMFA was evaluated by several means. First, two independent CoMFA models were defined utilizing different alignment rules (Figure 1). CoMFAs made with each alignment rule were then characterized by coarse as well as fine step size field sampling regions (2 Å and 1 Å in Tables 3 and 4). Finally, error introduced solely from these sampling regions (CoMFA error) was systematically evaluated by independent CoMFA determinations made with regions shifted one half of the lattice step size in the *x*, *y*, or *z* direction relative to the aligned test molecules (Tables 3−5). Then, as a means to exhibit a representative assessment of the 3D-QSAR models within a particular alignment and step size definition, the q^2 (predictive r^2) values of lattice shifted, cross-validated CoMFAs were averaged (Tables 3−5). The range of these average q^2 values then served as an indicator of the magnitude of CoMFA error originating from the field sampling lattice.

Compound **1** (estratriene) was omitted from all final CoMFA models since it was poorly predicted by cross-validated determinations (maintained a large residual value, data not shown). This finding might be explained by the fact that each CoMFA model was derived almost entirely from substituted 1,3,5(10)-estratrienes. Therefore, activity of the steroid ring system alone (**1**) would be overestimated by the QSAR models for receptor affinity or mitogenic potential. However, in reality, compound **1** elicits only the weakest of estrogenic responses (Table 2). A compound like **1**, that is devoid of substituents, might better be described in a CoMFA which includes hydrophobic fields as a descriptor variable.[56] Such an analysis is beyond the scope of the present study.

Effect of Region Step Size. In every comparison of sampling region step size, the 1 Å CoMFA models resulted in larger q^2 values and less corresponding deviation among lattice shift experiments (Tables 3 and 4). Thus, error which results exclusively from sampling region placement relative to the sample compounds (CoMFA error) was reduced by using the smaller region step size. Considering the typical range of bond distances found in organic compounds, it is reasonable that 2 Å CoMFA regions may be too coarse to consistently identify subtle structural features in many data sets. Therefore, in general, coarse CoMFA regions may be

Table 5. CoMFA Statistics for Combined Model of Cell Growth

align.[a]	lattice, Å[b]	offset[c]	q^2 [d]	S[e]	r^2 [f]	SE[g]	contributions[h]
C18	1		0.677(4)	0.845	0.936	0.375	st = 25, el = 58, K_a = 17
C18	1	*X*	0.658(3)	0.857	0.903	0.456	st = 23, el = 57, K_a = 20
C18	1	*Y*	0.675(4)	0.847	0.936	0.376	st = 23, el = 60, K_a = 17
C18	1	*Z*	0.666(3)	0.848	0.903	0.449	st = 24, el = 56, K_a = 20
			average $q^2 = 0.667 \pm 019$[i]				
SEAL	1		0.676(4)	0.846	0.942	0.356	st = 26, el = 56, K_a = 18
SEAL	1	*X*	0.651(3)	0.866	0.910	0.440	st = 25, el = 54, K_a = 21
SEAL	1	*Y*	0.654(3)	0.862	0.912	0.436	st = 25, el = 55, K_a = 20
SEAL	1	*Z*	0.671(3)	0.841	0.912	0.435	st = 24, el = 55, K_a = 21
			average $q^2 = 0.663 \pm 0.025$[i]				

[a] Alignment rule for CoMFA (see Computational Methods above). [b] Step size in CoMFA field sampling region. [c] Direction of region displacement (one half of lattice step size[b]) relative to default. [d] Cross-validated r^2 (optimal components) with cross-validation groups = n. [e] Cross-validated standard error of estimate over the pEC_{50} range of 5.00 to 11.82. [f] Conventional r^2 using optimal components. [g] Conventional standard error of estimate over the pEC_{50} range of 5.00 to 11.82. [h] Relative contributions of the steric, electrostatic, and receptor binding explanatory variables of CoMFA (as fractions of 100). [i] Average q^2 of CoMFAs from region displacement experiments[c] ± range.

more prone to error introduced by the relative register of the molecular models in the sampling lattice. Furthermore, use of a fine lattice spacing in CoMFA might be expected to produce more useful contour plots (more continuous) than those derived from a coarse sampling region.[57] This preference for a smaller region step size rather than the SYBYL default of 2 Å concurs with previous 3D-QSAR models made in this laboratory[58,59] and by others.[55,57] It should be noted, however, that when step size was reduced from 2 Å to 1 Å, CoMFA computation time increased 40 fold on average. As a result of the observation that 1 Å CoMFAs of this data set were more precise, 2 Å models were omitted in the combined models shown in Table 5.

Effect of Alignment Rule. CoMFA models of ER binding displayed the highest q^2 when the SEAL alignment method was utilized (average $q^2 = 0.581$ vs 0.541, Table 3). On the other hand, 3D-QSAR models of the MCF-7 cell mitogenic capacity of steroids maintained a larger q^2 when the C18 alignment rule was employed (avg $q^2 = 0.557$ vs 0.536, Table 4). However, since the difference between these averaged q^2 values was minimal, it appeared that even though the C18 and SEAL rules account for different receptor occupancy models (Figure 1), either alignment might be realistic for describing the biological activities measured in this study. Furthermore, when compared to the effect that sampling region step size had on CoMFA results (see above), the alignment rule for these steroids was not as important, so long as it was objective.

Contour Plots. While the q^2 value is the primary means to determine if a CoMFA is significant,[48] the most intuitive means to analyze such models is through the use of contour plots. CoMFA contours delineate specific regions of three-dimension space where steric and electrostatic structure features contribute to activity.[45] Such plots, corresponding to the three applications of CoMFA in this study, are shown in Figures 4−6. Depicted contours are derived from the STDEV*COEFF fields of a representative, 1 Å, final CoMFA (no cross-validation). Actual values of the extracted fields are represented by dark (80%) and light (20%) polyhedra positioned around E_2 (**5**). Therefore, in the case of steric contour results (left panel of each figure), dark polyhedra define areas of the steroid structure where added steric bulk contributes to the observed result (dependent variable). Conversely, light polyhedra in this same figure indicate regions of the steroid where steric bulk detracts from the measured activity. At the same time, electrostatic CoMFA contour plots (right panel of each figure) display dark and light polyhedra which circumscribe regions near the steroid where negative and positive electrostatic fields, respectively, contribute to the experimental results modeled.

CoMFA models of receptor binding or estrogen specific growth, both define QSARs with 30% steric and 70% electrostatic contributions from independent variables (Tables 3 and 4). Thus, structural features that introduce electrostatic character to the steroid are most important to both tight receptor association and mitogenic activity. The steric component of these models might then be considered a constraint to locating functional groups responsible for the required electrostatic features.

Based on the linear regression relationship between ER K_a and cell proliferation potential of the steroids (Figure 3), the contour plots for receptor binding and mitogenic potential would be expected to have similarities. Whereas Figures 4 and 5 do share contour features, significant differences are also apparent. Based on the influence of A-ring substituents of limited steric bulk utilized in these studies, receptor binding and cell growth CoMFAs both define similar locations near the estrogen 2 and 3 positions where steric bulk contributes to activity. However, the regions of the steroid where steric bulk detracts from activity are clearly different between these two models (Figures 4 and 5). For example, the steric inhibiting regions outlined as light polyhedra in the cell growth model (Figure 5) are more expansive than those in the receptor binding model (Figure 4). Specifically, the growth CoMFA defines steric interference zones extending in a band from above the A-ring to the 4 position and then continues below the steroid. At the same time, the ER binding steric interference zones are limited to isolated polyhedra in the 1-, 2-, and 4-positions of the A-ring as well as on the α face (below) of the B-ring. Additionally, the electrostatic contour plots convey models for binding whereby electronegative features located around the A-, B- or C-rings can contribute to receptor affinity. This contrasts with the cell growth model (Figure 4) which is dependent only on electronegative and electropositive characteristics near the 3 position.

Therefore, functional groups that may be involved in hydrogen bonding with ER appear to be most effectively located at the 2 or 3 position of the A-ring. This

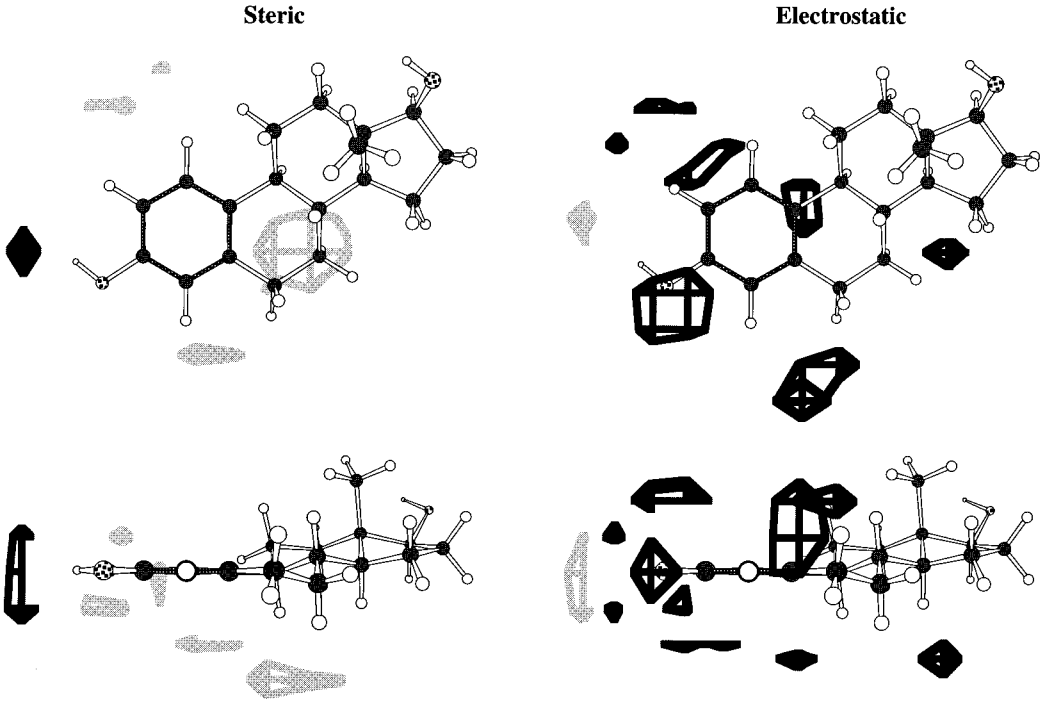


Figure 4. CoMFA contour plots of receptor binding data for compounds **2**−**42**. Steric (left) and electrostatic (right) contours surrounding E_2 (**5**) represent 80% (dark) and 20% (light) of the STDEV*COEFF fields retrieved from the 1 Å CoMFA (SEAL alignment) with $q^2 = 0.597$. Steric model contours indicate the location of steric bulk that enhances (dark) or detracts from (light) the receptor binding in this series of compounds. Electrostatic model contours indicate the location of electronegative (dark) and electropositive (light) character that enhances the receptor binding of these compounds. Views are from above, normal to the A-ring plane (top) and from the front, looking across the A-ring plane with hydrogens of carbons 4 and 6 in the foreground (below).

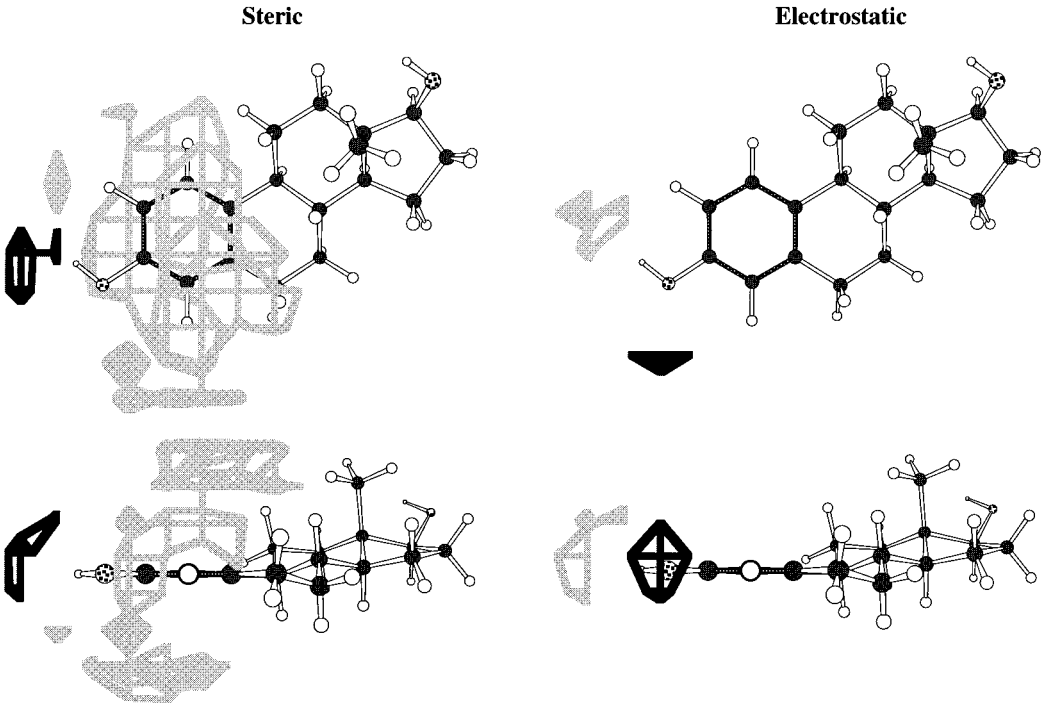


Figure 5. CoMFA contour plots of MCF-7 cell growth assay data for compounds **2**−**42**. Steric (left) and electrostatic (right) contours surrounding E_2 (**5**) represent 80% (dark) and 20% (light) of the STDEV*COEFF fields retrieved from the 1 Å CoMFA (C18 alignment) with $q^2 = 0.564$. Steric model contours indicate the location of steric bulk that enhances (dark) or detracts from (light) the mitogenic effect in this series of compounds. Electrostatic model contours indicate the location of electronegative (dark) and electropositive (light) character that enhances the mitogenic effect of these compounds. Views are from above, normal to the A-ring plane (top) and from the front, looking across the A-ring plane with hydrogens of C4 and C6 in the foreground (below).

interaction site must also be positioned coplanar to the A-ring as is demonstrated by the inability of 5α-androstane-3α,17β-diol (**40**) to elicit an estrogen response while 5α-androstane-3β,17β-diol (**41**) and 5-androstene-3β,17β-diol (**42**) have substantial activity. While the 3β-hydroxyl was required for maximal ER binding and MCF-7 cell proliferation, the estrogenic potential of the androstane derivatives illustrates that an aromatic A-ring is not required for activity. These observations regarding the possible importance of 3-phenolic

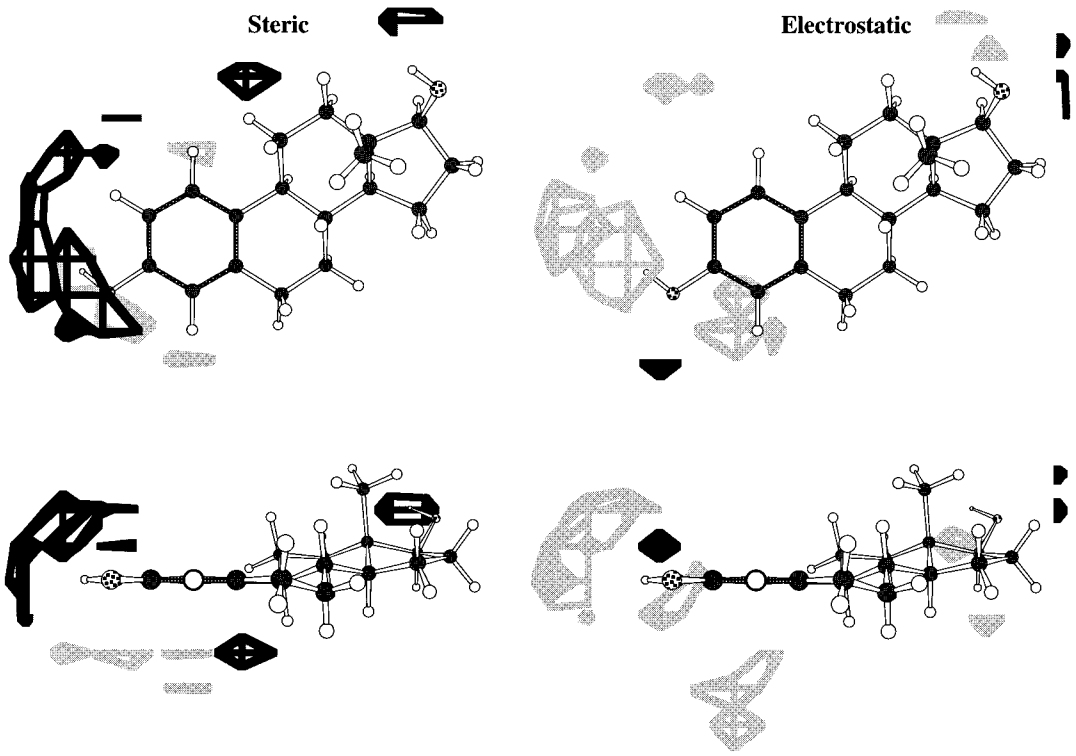


Figure 6. Combined model CoMFA contour plots of MCF-7 cell growth assay data for compounds **2**−**42**. Steric (left) and electrostatic (right) contours surrounding E_2 (**5**) represent 80% (dark) and 20% (light) of the STDEV*COEFF fields retrieved from the 1 Å combined CoMFA (C18 alignment, receptor binding as additional independent variable) with $q^2 = 0.677$. Steric model contours indicate the location of steric bulk that enhances (dark) or detracts from (light) the mitogenic effect in this series of compounds. Electrostatic model contours indicate the location of electronegative (dark) and electropositive (light) character that enhances the mitogenic effect of these compounds. Views are from above, normal to the A-ring plane (top) and from the front, looking across the A-ring plane with hydrogens of C4 and C6 in the foreground (below).

hydrogen bonding for estrogen activity are consistent with a ligand−receptor model presented previously by this laboratory[28] and by others.[50] Although an A-ring hydrogen bond interaction may be important to both receptor binding and mitogenic potential of the ligands, other strategic alterations to the E_2 molecule that retain tight binding may in fact detract from the estrogen specific growth response. For example, removal, oxidation, or repositioning of the D-ring 17β-hydroxyl group results in a decreased growth stimulation which is greater than the effect of these structural changes on the K_a for ER (Figure 3, Table 2, see: estratrien-3-ol (**31**), estratriene-3,17α-diol (**32**), and estratriene-3,16α-diol (**33**)). Unlike this diminution of the growth response, these D-ring altered estrogens stimulated specific responsive genes in MCF-7 cells to a level equal to their receptor binding affinity.[26,28−30,32] On the other hand, certain alterations (e.g. oxidation, hydroxylation, or unsaturation) in the B- or C-ring appear to enhance growth stimulation of MCF-7 cells to a higher level than would be expected from the diminished affinity of the analogue for receptor (see: estratriene-3,6β,17β-triol (**23**), 6-ketoestratriene-3,17β-diol (**24**), estratriene-3,-11α,17β-triol (**28**), estratriene-3,11β,17β-triol (**29**), and 6,8-estrapentaene-3,17β-diol (**38**)). It is notable, however, that estratrien-17β-ol (**2**), which is devoid of functional groups attached to the A-ring, has both relatively high receptor affinity and significant mitogenic potential. Therefore, in the case of some test compounds which possess amino, nitro, or iodo groups on the A-ring (**7**, **10**−**17**, **20**), such substituents may serve more to block activity than contribute to it (Table 2). Alternatively, when considering the biological activity of some of these A-ring substituted estratrien-17β-ols (**6** and **9** in Table 2 and Figure 2), the possibility exists that they may be bound in the receptor site of ER differently than portrayed by the SEAL and C18 alignments utilized in this study. Because this study has demonstrated that steroids with both a saturated A-ring and 19-methyl can be effective ligands for ER (**41** and **42** in Table 2 and Figure 3), it is speculated that certain estratrien-17β-ols may orient themselves "backward" in the steroid binding site such that the D-ring hydroxyl group might interact with residues normally hydrogen bonded to the 3-hydroxyl on the A-ring of E_2.

Combined CoMFA Model. The CoMFA models presented in Table 5 utilize ER K_a values along with steric and electrostatic fields to describe mitogenic potential. By this means, an understanding of the structure requirements of the cell growth effect can be made in terms of an event that must be preceded by receptor binding. These QSAR models that combine ER K_a values with steric and electrostatic CoMFA fields as independent variables, characterize the mitogenic potential of a steroid as a biological response that is only partially the result of initial receptor binding (20% contribution to model). Then, once bound to ER, steric and electrostatic properties of the steroid are responsible for the MCF-7 mitogenic potential (25% and 55% contribution to model respectively, Table 5). Contour plots for this combined CoMFA model are shown in Figure 6. Most notable in these plots are the expanded steric and electropositive tolerance zones around the A-ring. Also, steric and electrostatic contours around the D-ring are depicted in this model. Thus, this QSAR

of the MCF-7 cell estrogen specific growth stimulation addresses some of the nonlinear relationships between ER K_a values and growth EC_{50} illustrated in Figure 3. The location of D-ring contours serve to illustrate the model's preference for 17β-hydroxy steroids over the slightly less mitogenic 17α- and 16α-hydroxy compounds (**32**, **33**). The enhanced mitogenic effect of steric bulk in the 11α position (estratriene-3,11α,17β-triol, **29**) is also evident in the contour displayed in this area in Figure 6. However, the inability of this or any of the other CoMFA models to explain the relatively poor mitogenic potential of the 4-hydroxy isomer of E_2 (**6**), is noteworthy (Figures 3, 5, and 6). In other recently reported observations, this compound has been consistently shown to maintain weak capacity for the induction of estrogenic responses despite its relatively high affinity for the ER.[28,29,32] This characteristic of estratriene-4,17β-diol (**6**) may be due to structure properties that were not highlighted by the CoMFA models herein. For example we have reported[28] that the electronegative isopotential above the aromatic ring-A of E_2 isomers forms a projecting cloud with the unpaired electrons of the phenolic oxygen. Placing the hydroxyl group on each of the four positions on E_2 (C1, C2, C3, or C4) created an electronegative isopotential cloud at distinct geographical locations. Experiments have shown that estratriene-4,17β-diol (**6**), with its uniquely oriented electronegative isopotential above the A-ring, is ineffective in stimulating certain estrogen responsive genes, whereas the other isomers were active.[28,29,31,32]

The most important feature of the CoMFA models resulting from these investigations are their potential as predictive tools for estimating the ER affinity and mitogenic potential of steroidal estrogens. While reasonable predictions of mitogenic potential can be expected for steroids without ER binding data, inclusion of K_a values in predictions made from the combined CoMFA model should provide the most realistic estimates. These 3D-QSAR models might also contribute to the elucidation of the ligand binding site of the estrogen receptor.[60] Considering the structure constraints inherent to the steroid estrogens of this study,[34] CoMFA models derived from these compounds may provide a realistic steric and electrostatic mirror of portions of the estrogen receptors' hormone interaction site.

Acknowledgment. This work was supported in part by grants CA 44771 and CA 37387 from the NIH and institutional grants to the Michigan Cancer Foundation from the United Foundation of Greater Detroit. Computer and software resources used were purchased from the shared equipment grant no. 1 S10 RR04969 awarded to the Michigan Cancer Foundation by the National Center for Research Resources, National Institutes of Health. The authors thank Matthew Clark, Michael Lawless, and Joy Weese of Tripos Assoc. for help and encouragement in the use of the Sybyl software. Irina Massova and Brent H. Bessler of the Wayne State University Department of Chemistry were essential in the modification of SEAL for the SGI and setting up MOPAC 6.0 for ESP charge calculations, respectively. In addition, comments by Dr. Jerome P. Horwitz of the Division of Hematology-Oncology, Department of Internal Medicine, Wayne State University School of Medicine, were invaluable to interpretation of results.

Supporting Information Available: MCF-7 cell proliferation dose response data for E_2 analogues (13 pages). All molecule models used in study, Sybyl mol2 format, available only on the Internet. Ordering information and Internet access instructions are given on any current masthead page.

References

(1) Agarwal, M. K.; Raynaud, J. P. Steroid antagonists. *FEBS Lett.* **1989**, *245*, 1−3.
(2) Katzenellenbogen, B. S.; Montano, M. M.; Ekena, K.; Herman, M. E.; McInerney, E. M. Antiestrogens-mechanisms of action and resistance in breast cancer. *Breast Cancer Res. Treat.* **1997**, *44*, 23−38.
(3) Jordan, V. C. Molecular mechanisms of antiestrogen action in breast cancer. *Breast Cancer Res. Treat.* **1994**, *31*, 41−52.
(4) Murphy, C. S.; Jordan, V. C. Structural components necessary for the antiestrogenic activity of tamoxifen. *J. Steroid. Biochem.* **1989**, *34*, 407−411.
(5) Magarian, R. A.; Overacre, L. B.; Singh, S.; Meyer, K. L. The medicinal chemistry of nonsteroidal antiestrogens: A review. *Curr. Med. Chem.* **1994**, *1*, 61−104.
(6) Raynaud, J. P.; Ojasoo, T.; Bouton, M. M.; Bignon, E.; Pons, M.; Crastes de Paulet, A. Structure activity relationships of steroid estrogens. In *Estrogens in the Environment II: Influences on development*; McLachlan, J. A., Eds.; Elsevier Science Publishing Co., Inc.: New York, 1985; pp 24−42.
(7) Bignon, E.; Pons, M.; De Paulet, A. C.; Doré J-C; Gilbert, J.; Abecassis, J.; Miquel J.-F.; Ojasoo, T.; Raynaud J.-P. Effect of triphenylacrylonitrile derivatives on estradiol−receptor binding and on human breast cancer cell growth. *J. Med. Chem.* **1989**, *32*, 2092−2103.
(8) Etienne, M. C.; Milano, G.; Fischel, J. L.; Frenay, M.; François, E.; Formento, J. L.; Gioanni, J.; Namer, M. Tamoxifen metabolism: Pharmacokinetic and in vitro study. *Br. J. Cancer* **1989**, *60*, 30−35.
(9) Wakeling, A. E.; Bowler, J. Steroidal pure antioestrogens. *J. Endocrinol.* **1987**, *112*, R7−R10.
(10) Wakeling, A. E.; Bowler, J. Novel antioestrogens without partial agonist activity. *J. Steroid Biochem.* **1988**, *31*, 645−653.
(11) Bowler, J.; Lilley, T. J.; Pittam, J. D.; Wakeling, A. E. Novel steroidal pure antiestrogens. *Steroids* **1989**, *54*, 71−99.
(12) Agarwal, M. K. Steroid receptor structure and antihormone drug design. *Biochem. Pharmacol.* **1992**, *43*, 2299−2306.
(13) Gottardis, M. M.; Jiang S.-Y.; Jeng M.-H.; Jordan, V. C. Inhibition of tamoxifen-stimulated growth of an MCF-7 tumor variant in athymic mice by novel steroidal antiestrogens. *Cancer Res.* **1989**, *49*, 4090−4093.
(14) Gottardis, M. M.; Wagner, R. J.; Borden, E. C.; Jordan, V. C. Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. *Cancer Res.* **1989**, *49*, 4765−4769.
(15) Thompson, E. W.; Katz, D.; Shima, T. B.; Wakeling, A. E.; Lippman, M. E.; Dickson, R. B. ICI 164,384, a pure antagonist of estrogen-stimulated MCF-7 cell proliferation and invasiveness. *Cancer Res.* **1989**, *49*, 6929−6934.
(16) Wakeling, A. E. Comparative studies on the effects of steroidal and nonsteroidal oestrogen antagonists on the proliferation of human breast cancer cells. *J. Steroid Biochem.* **1989**, *34*, 183−188.
(17) Pons, M.; Bignon, E.; Crastes de Paulet, A.; Gilbert, J.; Ojasoo, T.; Raynaud, J. P. Hydroxylated triphenylacrylonitriles adopt a unique orientation within the binding site of the estrogen receptor. *J. Steroid Biochem.* **1990**, *36*, 391−397.
(18) Wiseman, L. R.; Wakeling, A. E.; May, F. E. B.; Westley, B. R. Effects of the antioestrogen, ICI 164,384, on oestrogen induced RNAs in MCF-7 cells. *J. Steroid Biochem.* **1989**, *33*, 1−6.
(19) Ratajczak, T.; Wilkinson, S. P.; Brockway, M. J.; Hähnel, R.; Moritz, R. L.; Begg, G. S.; Simpson, R. J. The interaction site for tamoxifen aziridine with the bovine estrogen receptor. *J. Biol. Chem.* **1989**, *264*, 13453−13459.
(20) Castellano-Díaz, E.; González-Quijano, M. I.; Limiñana, J. M.; Díaz-Chico, B. N. Tamoxifen decreases the estradiol induced progesterone receptors by interfering with nuclear estrogen receptor accumulation. *J. Steroid Biochem.* **1989**, *33*, 133−139.
(21) McCague, R.; Leclercq, G.; Legros, N.; Goodman, J.; Blackburn, G. M.; Jarman, M.; Foster, A. B. Derivitives of tamoxifen. Dependence of antiestrogenicity on the 4-substituent. *J. Med. Chem.* **1989**, *32*, 2527−2533.
(22) Gibson, M. K.; Nemmers, L. A.; Beckman, W. J.; Davis, V. L.; Curtis, S. W.; Korach, K. S. The mechanism of ICI 164,384 antiestrogenicity involves rapid loss of estrogen receptor in uterine tissue. *Endocrinology* **1991**, *129*, 2000−2010.
(23) Gronemeyer, H. Transcription activation by estrogen and progesterone receptors. *Annu. Rev. Genet.* **1991**, *25*, 89−123.
(24) Webster, N. J. G.; Green, S.; Jin, J. R.; Chambon, P. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation factor. *Cell* **1988**, *54*, 199−207.

(25) Berry, M.; Metzger, D.; Chambon, P. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *EMBO J.* **1990**, *9*, 2811−2818.
(26) Brooks, S. C.; Wappler, N. L.; Corombos, J. D.; Doherty, L. M.; Horwitz, J. P. Estrogen structure-receptor function relationships. In *Recent Advances in Steroid Hormone Action*; Moudgil, V. K., Eds.; Walter de Gruyter & Co.: Berlin, 1987.
(27) Brooks S. C.; Horwitz, J. P.; Odden, D.; Corbett, T. A-ring substituted estrogens as inhibitors of the MXT transplantable mammary ductal carcinoma. *Cancer Res.* **1987**, *47*, 4623−4629.
(28) VanderKuur, J. A.; Wiese, T.; Brooks, S. C. Influence of estrogen structure on nuclear binding and progesterone receptor induction by the receptor complex. *Biochemistry* **1993**, *32*, 7002−7008.
(29) Pilat, M. J.; Hafner, M. S.; Kral, L. G.; Brooks, S. C. Differential induction of pS2 and cathepsin D mRNAs by structurally altered estrogens. *Biochemistry* **1993**, *32*, 7009−7015.
(30) VanderKuur, J. A.; Hafner, M. S.; Christman, J. K.; Brooks, S. C. Effects of estradiol-17β analogues on activation of estrogen response element regulated chloramphenicol acetyltransferase expression. *Biochemistry* **1993**, *32*, 7016−7021.
(31) VanderKuur, J. A.; Brooks, S. C. Effect of A-ring isomers of estradiol-17β on gene products in MCF-7 cells. *Steroids* **1994**, *59*, 548−553.
(32) Davis, M. D.; Butler, W. B.; Brooks, S. C. Induction of tissue plasminogen activator mRNA and activity by structurally altered estrogens. *J. Steroid Biochem. Mol. Biol.* **1995**, *52*, 421−430.
(33) Palomino, E.; Heeg, M. J.; Horwitz, J. P.; Brooks, S. C. Binding, x-ray and NMR studies of the three A-ring isomers of natural estradiol. *J. Steroid Biochem.* **1990**, *35*, 219−229.
(34) Wiese, T. E.; Brooks, S. C. Molecular modeling of steroidal estrogens: Novel conformations and their role in biological activity. *J. Steroid Biochem. Mol. Biol.* **1994**, *50*, 61−73.
(35) Horwitz, J. P.; Iyer, V. K.; Vardhan, H. B.; Corombos, J.; Brooks, S. C. *In Vitro* inhibition of estrogen sulfoconjugation by some 2- and 4-substituted estra-1,3,5(10)-trien-17β-ols. *J. Med. Chem.* **1986**, *29*, 692−698.
(36) Iriarte, J.; Ringold, H. J.; Djerassi, C. Synthesis of ring B oxygenated estrogens. *J. Am. Chem. Soc.* **1958**, *80*, 6015−6010.
(37) Brooks, S. C.; Horwitz, J. P. Compositions inhibiting murine MXT ductal carcinoma. US Patent 4,636,496, 1987.
(38) Hecker, E. New estrane derivitives with various substituents in the 3-position. *Chem. Ber.* **1962**, *95*, 977−984.
(39) Tomson, A. J.; Horwitz, J. P. Some 2 and 3 substituted estrone-3-methylethers. *J. Org. Chem.* **1959**, *24*, 2056−2063.
(40) Palomino, E.; Heeg, M. J.; Horwitz, J. P.; Polin, L.; Brooks, S. C. Skeletal conformations and receptor binding of some 9,11-modified estradiols. *J. Steroid Biochem. Mol. Biol.* **1994**, *50*, 75−84.
(41) Wiese, T. E.; Kral, L. G.; Dennis, K. E.; Butler, W. B.; Brooks, S. C. Optimization of estrogen growth response in MCF-7 cells. *In Vitro Cell. Dev. Biol.* **1992**, *28A*, 595−602.
(42) Butler, W. B.; Berlinski, P. J.; Hillman, R. M.; Kelsey, W. H.; Toenniges, M. M. Relation of in vitro properties to tumorigenicity for a series of sublines of the human breast cancer cell line MCF-7. *Cancer Res.* **1986**, *46*, 6339−6348.
(43) Butler, W. B. Preparing nuclei from cells in monolayer cultures suitable for counting and for following synchronized cells through the cell cycle. *Anal. Biochem.* **1984**, *141*, 70−73.
(44) Sokal, R. R.; Rohlf, F. J. In *Biometry*, 2nd ed.; W. H. Freeman and Company: San Francisco, 1981; pp 764−765.
(45) Cramer, R. D. I.; Patterson, D. E.; Bunce, J. D. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. *J. Am. Chem. Soc.* **1988**, *110*, 5959−5967.
(46) Wold, S.; Albano, C.; Dunn, W. J., III; Edlund, U.; Esbensen, K.; Geladi, P.; Hellberg, S.; Johansson, E.; Lindberg, W.; Sjostrom. M. Multivariate data analysis in chemistry. In *Chemometrics, Mathematics and Statistics in Chemistry*; B. R. Kowalski, B. R., Ed.; D. Reidel Publishing Company: Dordrecht, 1984; pp 17−95.
(47) Stahle, L.; Wold, S. Multivariate data analysis and experimental design in biomedical research. *Prog. Med. Chem.* **1988**, *25*, 290−338.
(48) Cramer, R. D., III; Bunce, J. D.; Patterson, D. E. Cross-validation, bootstrapping, and partial least squares compaired with multiple regression in conventional QSAR studies. *Quant Struct.-Act. Relat.* **1988**, *7*, 18−25.
(49) Stewart, J. J. P. MOPAC: A semiempirical molecular orbital program. *J. Comput.-Aided Mol. Des.* **1990**, *4*, 1−105.
(50) Anstead, G. M.; Carlson, K. E.; Katzenellenbogen, J. A. The estradiol pharmacophore: Ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. *Steroids* **1997**, *62*, 268−303.
(51) Palomino, E.; Heeg, M. J.; Pilat, M. J.; Hafner, M.; Polin, L.; Brooks, S. C. Crystal structure, receptor binding, and gene regulation of 2- and 4-nitroestradiols. *Steroids* **1996**, *61*, 670−676.
(52) Besler, B. H.; Merz, K. M.; Kollman, P. A. Atomic charges derived from semiempirical methods. *J. Comput. Chem.* **1990**, *11*, 431−439.
(53) Merz, K. M. Analysis of a large data base of electrostatic potential derived atomic charges. *J. Comput. Chem.* **1992**, *13*, 749−767.
(54) Kearsley, S. K.; Smith, G. M. An alternative method for the alignment of molecular structures: Maximizing electrostatic and steric overlap. *Tetrahedron Comput. Methodol.* **1990**, *3*, 615−633.
(55) Gantchev, T. G.; Ali, H.; van Lier, J. E. Quantative structure−activity relationships/comparative molecular field analysis (QSAR/CoMFA) for receptor-binding properties of halogenated estradiol derivatives. *J. Med. Chem.* **1994**, *37*, 4164−4176.
(56) Kellogg, G. E.; Semus, S. F.; Abraham, D. J. HINT: A new method of empirical hydrophobic field calculation for CoMFA. *J. Comput.-Aided Mol. Des.* **1991**, *5*, 545−552.
(57) Calder, J. A.; Wyatt, J. A.; Frenkel, D. A.; Casida, J. E. CoMFA validation of the superposition of six classes of compounds which block GABA receptors non-competitively. *J. Comput.-Aided Mol. Des.* **1993**, *7*, 45−60.
(58) Horwitz, J. P.; Massova, I.; Wiese, T. E.; Wozniak, A. J.; Corbett, T. H.; Sebolt-Leopold, J. S.; Capps, D. B.; Leopold, W. R. Comparative molecular field analysis of in vitro growth inhibition of L1210 and HCT-8 cells by some pyrazoloacridines. *J. Med. Chem.* **1993**, *36*, 3511−3516.
(59) Horwitz, J. P.; Massova, I.; Wiese, T. E.; Besler, B. H.; Corbett, T. H. Comparative molecular field analysis of the antitumor activity of 9H-thioxanthen-9-one derivatives against pancreatic ductal carcinoma 03 [published erratum appears in *J. Med. Chem.* **1994**, *37* (19), 3196]. *J. Med. Chem.* **1994**, *37*, 781−786.
(60) Hahn, M. Receptor surface models. 1. Definition and construction. *J. Med. Chem.* **1995**, *38*, 2080−2090.

JM9703294